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Functional polymorphisms of the glutamate receptor N-methyl D-aspartate 2A gene are associated with heroin addiction

H.J. Zhong[1,2], Z.H. Huo[1,2], J. Dang[1], J. Chen[1], Y.S. Zhu[1] and J.H. Liu[3]

[1]Key Laboratory of Fertility Preservation and Maintenance,
Ningxia Medical University, Ministry of Education, Yinchuan, China
[2]Department of Medical Genetics and Cell Biology,
Ningxia Medical University, Yinchuan, China
[3]Physiology, School of Life Science, Ningxia University, Yinchuan, China

Corresponding author: J.H. Liu
E-mail: aiwapeter@163.com

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ABSTRACT. Heroin dependence is a debilitating psychiatric disorder with a complex inheritance mechanism. Genetic polymorphisms in functional regions of the glutamate receptor, N-methyl D-aspartate 2A (GRIN2A) gene, which encodes the 2A subunit of the N-methyl D-aspartate (NMDA) receptor, may modulate the risk of heroin addiction. We investigated the potential association between 8 single nucleotide polymorphisms (SNPs) of the *GRIN2A* gene (SNPs rs3219790, rs1014531, rs8044472, rs8045712, rs9933624, rs9940680, rs1420040, and rs767749) and heroin addiction using the MassARRAY system and GeneScan. A total of 405 heroin-addicted patients and 397 healthy control subjects were recruited for this study. Statistically significant differences were observed for rs3219790 in the promoter region of the *GRIN2A* gene. The frequency of the (GT)26 repeats in the heroin addiction group was significantly higher than that in the control group [$\chi^2 = 5.475$, P = 0.019, odds ratio (OR) = 1.367, 95% confidence

interval (CI) = 1.051-1.776]. Strong linkage disequilibrium was observed in block 1 (D' > 0.9). However, significant evidence of linkage disequilibrium was not observed between the 7 SNPs in our sample population. These data suggest that *GRIN2A* gene polymorphisms confer susceptibility to heroin addiction and support the hypothesis that dysfunction of GRIN2A is involved in the pathophysiological process of heroin addiction.

Key words: Heroin addiction; Glutamate receptor; Ionotropic; N-methyl D-aspartate 2A; Polymorphisms

INTRODUCTION

Heroin addiction is a chronic relapsing disease characterized by compulsive drug seeking, abuse, tolerance, and physical and psychological dependence. Family, twin, and adoption studies have shown that the heritability of alcoholism is 40-60% (Tsuang et al., 1998; van den Bree et al., 1998; Kendler et al., 2003). Other studies found similar heritability values in the context of substance use (Kendler et al., 1994; Tsuang et al., 1998; Kendler et al., 2003). Recent studies have suggested that polymorphisms in the N-methyl D-aspartate 2A (GRIN2A) gene may be associated with drug addiction, including alcohol and heroin addiction (Levran et al., 2009; Domart et al., 2012).

Glutamate is the principal excitatory neurotransmitter in the brain, and genes encoding glutamate receptors are candidate targets for the treatment of neuropsychiatric disorders (Paoletti et al., 2013). The N-methyl-D-aspartate receptor (NMDA receptor or NMDAR), a glutamate receptor, is the predominant molecule controlling synaptic plasticity and memory function. An NMDA receptor heterotetramer forms between 2 GluN1 and 2 GluN2 subunits, 2 obligatory GluN1 subunits, and 2 regionally localized GluN2 subunits. While a single GluN2 subunit is found in invertebrate organisms, 4 distinct isoforms of the GluN2 subunit are expressed in vertebrates and are referred to as GRIN2A-D (McBain and Mayer, 1994). *GRIN2A* knockout mice show increased spontaneous locomotor activity and deficits in contextual fear conditioning and spatial learning, as well as reduced hippocampal long-term potentiation (Sakimura et al., 1995) thought to be involved in addiction (Squire, 1992). Several studies showed that chronic administration of drugs of abuse, such as alcohol (Nagy et al., 2005), methamphetamine (Simoes et al., 2008), cocaine (Ben-Shahar et al., 2009), and nicotine (Wang et al., 2007), alters GRIN2A activity in the brain, suggesting that the *GRIN2A* gene is an excellent candidate target for treating addiction disorders. These studies suggest that GRIN2A is closely associated with drug addiction and is a key mediator of the pathogenesis of drug addiction.

The *GRIN2A* gene, located on chromosome 16p13.2, consists of 12 exons and 13 introns and contains several different alternatively spliced transcripts. Several single nucleotide polymorphisms (SNPs) have been identified in this gene, including rs3219790 in the promoter region (Itokawa et al., 2003a) and rs1014531, rs8044472, rs8045712, rs9933624, rs9940680, rs1420040, and rs767749 in the 3'-untranslated region (UTR) (Zhao et al., 2013). Itokawa et al. (2003a) identified a variable (GT)n repeat (rs3219790) in the 5'-regulatory region. An *in vitro* promoter assay revealed length-dependent inhibition of transcriptional activity of this gene

by the (GT)n repeat (Itokawa et al., 2003a). A study was conducted to examine the association between this polymorphism and alcohol dependence. Average observed repeat numbers and genotype distributions were significantly different in alcohol-dependent subjects compared to control subjects (Domart et al., 2012). Moreover, significantly more C-A-T (rs4587976-rs1071502-rs1366076) haplotypes were identified in African American heroin-addicted patients (Levran et al., 2009). However, it remains unknown whether these functional polymorphisms modulate the risk of heroin addiction.

Given the crucial role of the brain-derived neurotrophic factor (BDNF) gene in *GRIN2A* and drug addiction, we examined the potential association between *GRIN2A* polymorphisms and heroin addiction. Eight polymorphisms (rs3219790, rs1014531, rs8044472, rs8045712, rs9933624, rs9940680, rs1420040, and rs767749) were evaluated in this study.

MATERIAL AND METHODS

Subjects

A total of 405 unrelated patients with heroin addiction (average age of 36.5 ± 6.8 years) were recruited from the Center for Substance Abuse Treatment, Yinchuan. Participants were daily or nearly daily users of heroin for a minimum of one year prior to assessment. All patients were interviewed independently by 2 psychiatrists according to American Psychiatric Association (1994) diagnostic criteria, medical history, urine test results, and interview responses. A case vignette was developed to assist with diagnosis involving a semi-structured interview with questions regarding (a) the age at initiation and duration of heroin use, (b) quantity of drug used over this period, (c) route of administration (nasal inhalation or injection), (d) whether other substances were used or abused, and (e) comorbidity for any other psychiatric disorder. The study complied with the guidelines of our local Medical Ethical Committee and all participants provided written informed consent. Controls subjects included 397 unrelated healthy individuals (average age of 37.25 ± 6.36 years). These subjects were recruited from the medical examination center of the First Affiliated Hospital of Ningxia Medical University. Subjects with a history of substance abuse, who had participated in other studies, or who suffered from chronic brain diseases were excluded from participation. All participants were Han Chinese from the Ningxia Hui Autonomous Region and were not genetically related. Written informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of Ningxia Medical University, Yinchuan, China.

Genotyping

Three to five milliliters peripheral blood was collected in tubes coated with ethylenediaminetetraacetic acid (EDTA). Genomic DNA was extracted from blood leukocytes using the EZNA™ Blood DNA Midi Kit (Omega Bio-Tek; Norcross, GA, USA) according to the manufacturer protocol. Genotyping of 7 SNPs was performed using the MassARRAY system (Sequenom Inc.; San Diego, CA, USA). Probes and primers were designed using the Assay Design Software (Sequenom) (Table 1).

Primers specific for the (GT)n repeat were used to amplify the repeat-containing ge-

nomic fragment and had the following sequences: a 6-carboxy fluorescein (FAM)-labeled upstream primer, 5'-GAAGGAAGCATGTGGGAAATGCAG-3', and a non-labeled downstream primer, 5'-gtttcTTGCTGGGTACAGTTATCCCCCT-3' (Itokawa et al., 2003a). Polymerase chain reaction (PCR) amplification was performed with an initial denaturation at 95°C for 8 min, followed by 10 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s (-1°C per cycle) and extension for 30 s at 72°C, followed by an additional 20 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension for 30 s at 72°C, and a final extension at 72°C for 6 min, using AmpliTaq Gold DNA polymerase (Applied Biosystems; Foster City, CA, USA). PCR products were analyzed using an ABI 3730 sequencer equipped with the GeneScan software (Applied Biosystems).

Table 1. Primer sequences used for genotyping of the *GRIN2A* gene SNPs with the MALDI-TOF Sequenom platform.

Polymorphism sites	Forward primers	Reverse primers	Extension primers
rs1014531	ACGTTGGATGGCCTTCAAAACACGAGGCAC	ACGTTGGATGCTGTATCCACAGTTTGGAGG	CGAGGCACTCTTGGATCTA
rs8044472	ACGTTGGATGGTCCTTCCCTAAACCTTTAAG	ACGTTGGATGATCAACTGCATTTCCGGGAC	CCCTAAACCTTTAAGTAACTC
rs8045712	ACGTTGGATGTGGTCCCGGAAATGCAGTTG	ACGTTGGATGGTTCACCTGGTTAGCAGCTC	cTTTTAATCTTCAAATAATGGACTTA
rs9933624	ACGTTGGATGCCCTAAAGCTTTTTTGTTTGG	ACGTTGGATGTCCCTGATACATTCATACCC	ggTGTTTGGTTTTGTTTTTGAC
rs9940680	ACGTTGGATGCCTCTATGCTTCCCTGATAC	ACGTTGGATGGTTTGGTTTTGTTTTTGAC	CCTGATACATTCATACCCTGGT
rs1420040	ACGTTGGATGTAGCTAGTAAATCCCTAACG	ACGTTGGATGGACTTCTATTCTGACCTCCC	cccccATCCCTAACGTTAGACATATT
rs767749	ACGTTGGATGCATTTACGTAAGTGGTCATGG	ACGTTGGATGTAAATCTTTAGTTTATTAAG	CATGGCCCCAGAGCTT

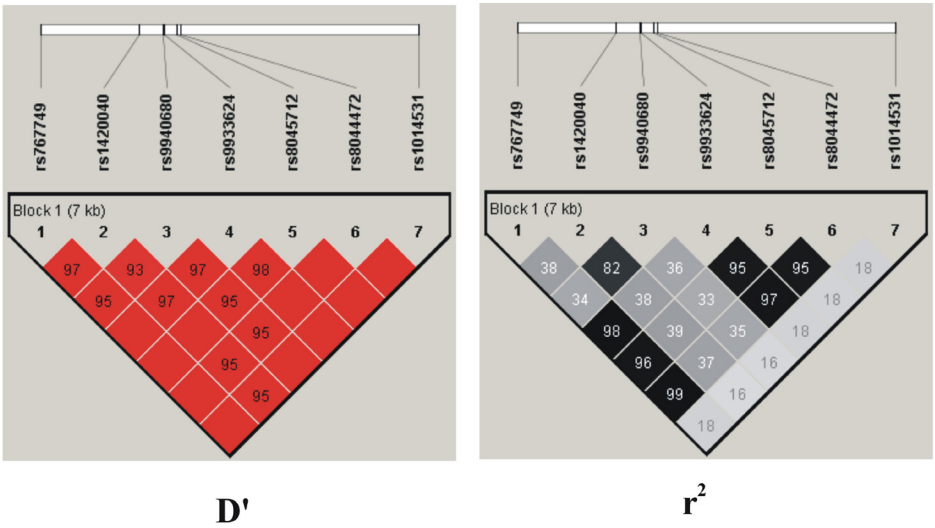
Statistical analysis

Allele and genotype frequencies for each individual polymorphism and deviations from Hardy-Weinberg equilibrium were evaluated using the chi-square test. The potential association between heroin addiction and each polymorphism was analyzed using Fisher's exact test or the Pearson chi-square test. All statistical analyses were carried out using SPSS13.0 (SPSS Inc.; Chicago, IL, USA). Pair-wise linkage disequilibrium (LD) statistics (D' and r^2) and haplotype frequency were computed using Haploview 4.0 to construct haplotype blocks. $P < 0.05$ denoted significant a difference after Bonferroni correction.

RESULTS

The genotype distributions of the 7 polymorphisms in controls were consistent with Hardy-Weinberg equilibrium. LD analyses of patient and control data revealed that the 7 SNPs (rs1014531, rs8044472, rs8045712, rs9933624, rs9940680, rs1420040, and rs767749) were located in haplotype block 1 (Figure 1). Genotype distributions, allelic frequencies, and haplotypes in control and patient groups, as well as the results of statistical analyses, are listed in Tables 2 and 3.

Upon testing for single allelic associations between common alleles [frequency > 5%: (GT)21 to (GT)30], the Pearson chi-square test showed the following P values: 0.372 for (GT)21, 0.244 for (GT)22, 0.146 for (GT)23, 0.889 for (GT)24, 0.764 for (GT)25, 0.019 for (GT)26, 0.140 for (GT)27, 0.606 for (GT)28, 0.923 for (GT)29, and 0.545 for (GT)30 (Table 4, Figure 2).



D' r^2

Figure 1. Linkage disequilibrium plot of the 8 SNPs in the *GRIN2A* gene. Values in squares are the pairwise calculation of D' (left) or r^2 (right).

Table 2. Genotype and allele frequencies of the *GRIN2A* gene SNPs in cases (N = 405) and controls (N = 397) and the results of their associations with risk of heroin addiction.

Variant	Location	Group	Genotype (N, %)			Allele (N, %)		P^a	P^b	P^c	OR^d, 95%CI^d
			AA	AG	GG	A	G				
rs1014531	3'UTR	Case	14 (3.457)	132 (32.593)	259 (63.951)	160 (19.753)	650 (80.247)	0.464	0.235	0.864	0.679-1.100
		Control	19 (4.786)	138 (60.453)	240 (60.453)	176 (22.166)	618 (77.834)				
			GG	GA	AA	G	A				
rs8044472	3'UTR	Case	149 (36.790)	213 (52.593)	43 (10.617)	511 (63.086)	299 (36.914)	0.086	0.570	1.060	0.866-1.298
		Control	153 (38.539)	184 (15.113)	60 (15.113)	490 (61.713)	304 (38.287)				
			TT	TC	CC	T	C				
rs8045712	3'UTR	Case	68 (16.790)	195 (48.148)	142 (35.062)	331 (40.864)	479 (59.136)	0.405	0.396	1.090	0.893-1.332
		Control	58 (14.610)	192 (37.028)	147 (37.028)	308 (38.791)	486 (61.209)				
			TT	TC	CC	T	C				
rs9933624	3'UTR	Case	134 (33.086)	195 (48.148)	76 (18.765)	463 (57.160)	347 (42.840)	0.386	0.164	0.868	0.712-1.060
		Control	147 (37.028)	187 (15.869)	63 (15.869)	481 (60.579)	313 (39.421)				
			GG	GC	CC	G	C				
rs9940680	3'UTR	Case	134 (33.086)	195 (48.148)	76 (18.765)	463 (57.160)	347 (42.840)	0.393	0.135	0.859	0.704-1.048
		Control	147 (37.028)	189 (15.365)	61 (15.365)	483 (60.831)	311 (39.169)				
			AA	AG	GG	A	G				
rs1420040	3'UTR	Case	134 (33.086)	192 (47.407)	79 (19.506)	460 (56.790)	350 (43.210)	0.366	0.151	0.864	0.708-1.054
		Control	147 (37.028)	185 (16.373)	65 (16.373)	479 (60.327)	315 (39.673)				
			TT	TG	GG	T	G				
rs767749	3'UTR	Case	52 (12.840)	187 (46.173)	166 (40.988)	291 (35.926)	519 (64.074)	0.752	0.685	0.959	0.782-1.175
		Control	58 (14.610)	177 (40.806)	162 (40.806)	293 (36.902)	501 (63.098)				

[a]Values for Hardy-Weinberg equilibrium in controls. [b]Values for genotype frequency difference. [c]Values for allele frequency difference. [d]Values for allele frequency difference. Alpha value is adjusted by Bonferroni correction and statistically significant results ($P < 0.0071$).

Table 3. *GRIN2A* haplotype in block 1 frequencies and associations with risk of heroin addiction.

Haplotype#	Heroin addiction (N, %)	Controls (N, %)	Statistics			
			χ^2	P	OR	95%CI
CAGCTGG	171 (42.22)	152 (38.29)	1.291	0.256	1.178	0.888-1.562
TGTGCAG	134 (33.09)	140 (35.26)	0.423	0.516	0.908	0.618-1.215
TAGGCAA	86 (21.66)	75 (18.52)	0.686	0.408	1.157	0.819-1.636
TGGGCAG	14 (3.46)	8 (2.02)	1.562	0.211	1.741	0.722-4.197

#Haplotypes with frequency <0.05 were excluded.

Table 4. Statistics of the *GRIN2A* (GT)n repeat in heroin addiction and controls.

(GT)n repeat	Cases (N, %)	Controls (N, %)	χ^2	P	OR	95%CI
(GT)13	8 (0.988)	10 (1.259)	0.267	0.605	0.782	0.307-1.992
(GT)14	2 (0.247)	4 (0.504)	0.710	0.399	0.489	0.089-2.677
(GT)19	2 (0.247)	6 (0.756)	2.091	0.148	0.325	0.065-1.615
(GT)20	6 (0.741)	8 (1.008)	0.330	0.566	0.733	0.253-2.123
(GT)21	52 (6.420)	60 (7.557)	0.798	0.372	0.837	0.571-1.233
(GT)22	24 (2.963)	32 (4.030)	1.355	0.244	0.727	0.424-1.246
(GT)23	74 (9.136)	90 (11.335)	2.113	0.146	0.786	0.569-1.088
(GT)24	108 (13.333)	104 (13.098)	0.019	0.889	1.021	0.764-1.363
(GT)25	80 (9.877)	82 (10.327)	0.090	0.764	0.952	0.688-1.317
(GT)26	156 (19.259)	118 (14.861)	5.475	0.019*	1.367	1.051-1.776
(GT)27	96 (11.852)	76 (9.572)	2.177	0.140	1.270	0.924-1.746
(GT)28	88 (10.864)	80 (10.076)	0.266	0.606	1.088	0.790-1.498
(GT)29	44 (5.432)	44 (5.542)	0.009	0.923	0.979	0.637-1.505
(GT)30	28 (3.457)	32 (4.030)	0.366	0.545	0.853	0.508-1.430
(GT)31	10 (1.235)	12 (1.511)	0.227	0.634	0.815	0.350-1.896
(GT)32	12 (1.481)	10 (1.259)	0.146	0.702	1.179	0.506-2.744
(GT)33	12 (1.481)	12 (1.511)	0.002	0.961	0.980	0.438-2.194
(GT)34	8 (0.988)	12 (1.511)	0.893	0.345	0.650	0.264-1.599
(GT)36	0 (0.000)	2 (0.252)	2.038	0.153	1.003	0.999-1.006

*Statistically significant differences was observed for (GT)26.

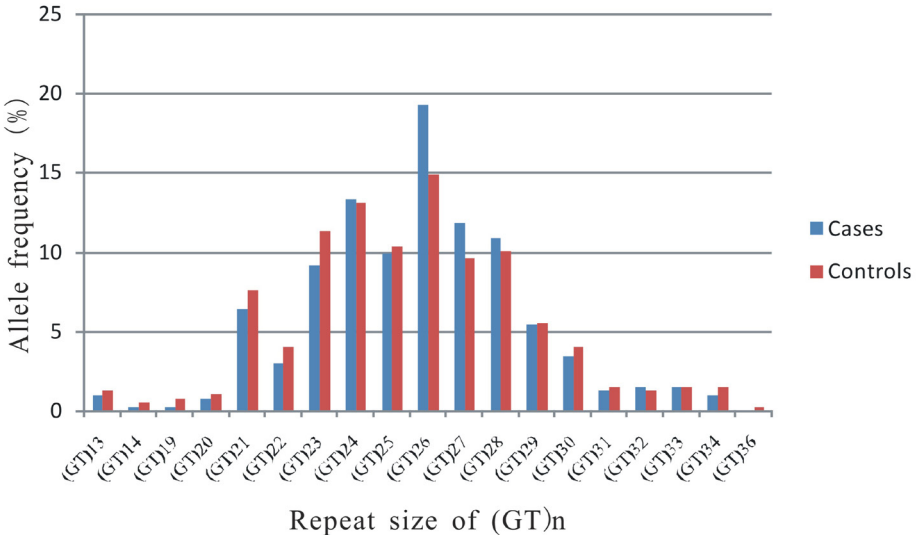


Figure 2. Allele frequency distribution of the *GRIN2A* (GT)n repeat in the heroin addiction and control groups. Allele size is expressed as the number of GT repeats.

We observed a strong linkage of the rs3219790 genotype distribution with heroin addiction ($P < 0.05$). The frequency of the (GT)26 allele was significantly higher in heroin addiction patients than in the healthy controls [$\chi^2 = 5.475$, $P = 0.019$, odds ratio (OR) = 1.367, 95% confidence interval (CI) = 1.051-1.776]. Strong LD was observed in block 1 ($D' > 0.9$). However, significant LD was not observed between the 7 SNPs in our sample population.

DISCUSSION

Most previous studies of the relationship between the *GRIN2A* gene and substance addictive behaviors have involved animal models. *GRIN2A* knockout mice showed altered *GRIN2A* gene expression during alcohol dependence (Squire, 1992). Thus, regulation of *GRIN2A* expression may profoundly affect glutamate homeostasis and restore sensitivity to exogenous agents. The present study examined the association between *GRIN2A* genes and heroin dependence in humans. Our results provide direct evidence that a genetic change in *GRIN2A* is linked to heroin addiction in humans, extending the list of variants that may affect the development of heroin addiction (Levran et al., 2009).

Itokawa et al. (2003b) previously identified a variable (GT)n repeat (rs3219790) in the 5'-regulatory region of GRIN2A, a gene encoding the NR2A subunit of the NMDA receptor complex. They demonstrated that this repeat sequence repressed transcriptional activity in a length-dependent manner, with longer repeats inducing greater promoter repression (Itokawa et al., 2003b). Additional *in vitro* promoter assays revealed that the longer alleles of the (GT)n polymorphism reduced the expression of *GRIN2A*. This finding was supported by a receptor-binding assay in postmortem brains, indicating that reduced *GRIN2A* expression may be a risk factor for schizophrenia (Itokawa et al., 2003b). A case-control study showed evidence of an association between the repeat polymorphism and schizophrenia, with longer alleles overly represented in patients with severe outcome (Tang et al., 2006). In the present case-control association study, significant differences were observed in the distribution of allele frequencies of (GT)n repeats in the *GRIN2A* gene between heroin-addicted subjects and healthy controls. The frequency of the (GT)26 repeat in heroin addicts was significantly higher than that in controls. Indeed, heroin addiction subjects exhibited longer alleles than control subjects overall. Therefore, the presence of longer (GT)n repeat suggests decreased NMDA receptor function in heroin-addicted subjects. Similarly, a previous study showed that longer alleles of (GT)n repeats were significantly more frequent among subjects with alcohol dependence (Domart et al., 2012). The average observed repeat number distributions were significantly different in alcohol-dependent subjects (GT repeats: N = 24.5) *vs* control subjects (GT repeats: N = 23.7) (Domart et al., 2012). These findings further implicate rs3219790 as a genetic regulator of sensitivity to drugs of abuse. The presence of a longer (GT)26 repeat may result in decreased GRIN2A receptor function in patients with heroin addiction. This finding represents clinical-genetic evidence of the role of promoter (GT)n polymorphisms in the *GRIN2A* gene in the pathophysiology of heroin addiction.

In conclusion, the present study identified a strong association between the rs3219790 polymorphism of the *GRIN2A* gene and heroin addiction. Our findings support the glutamatergic hypothesis developed to understand the acute and chronic effects of heroin on the brain. These findings encourage future efforts aimed at identifying functional polymorphisms within and close to the *GRIN2A* gene using a systemic approach in a larger sample set. These data suggest that *GRIN2A* gene polymorphisms confer susceptibility to heroin addiction.

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