Analysis of Variations in the Glutamate Receptor, N-Methyl D-Aspartate 2A (GRIN2A) Gene Reveals Their Relative Importance as Genetic Susceptibility Factors for Heroin Addiction

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Abstract

The glutamate receptor, N-methyl d-aspartate 2A (GRIN2A) gene that encodes the 2A subunit of the N-methyl D-aspartate (NMDA) receptor was recently shown to be involved in the development of opiate addiction. Genetic polymorphisms in GRIN2A have a plausible role in modulating the risk of heroin addiction. An association of GRIN2A single-nucleotide polymorphisms (SNPs) with heroin addiction was found earlier in African Americans. To identify markers that contribute to the genetic susceptibility to heroin addiction, we examined the potential association between heroin addiction and forty polymorphisms of the GRIN2A gene using the MassARRAY system and Genescan in this study. The frequency of the (GT)26 repeats (rs321979) in the heroin addiction group was significantly higher than that in the control group ({$P=5.360\times10^{-2}$}). The allele frequencies of three polymorphisms (rs1102972, rs1650420, and rs3104703 in intron 3) were strongly associated with heroin addiction ($P<0.001$, 0.0002, and <0.001, after Bonferroni correction). Three additional SNPs from the same intron (rs1071502, rs6497730, and rs1070487) had nominally significant $P$ values for association ($P<0.05$), but did not pass the threshold value. Haplotype analysis revealed that the G-T-C-T-A-C-T-A (block 6) and T-T (block 10) haplotypes of the GRIN2A gene displayed a protective effect ($P<0.001$ and 0.003). These findings point to a role for GRIN2A polymorphisms in heroin addiction among the Han Chinese from Shaanxi province, and may be informative for future genetic or neurobiological studies on heroin addiction.

Introduction

Heroin addiction is a chronically relapsing disease characterized by compulsive drug seeking, drug abuse, tolerance, and physical dependence. According to an adoption study, substance dependence, in general, and opioid addiction, in particular, has a genetic component [1]. Family and twin studies have consistently demonstrated a substantial genetic influence on the development of drug addiction, with inherited risk estimates in the range of 40–60% [2,3]. Chronic drug use alters gene expression, which activates or attenuates biochemical pathways and produces neuroadaptive changes in signal transduction functions. Recent studies suggested that polymorphisms in the N-methyl d-aspartate 2A (GRIN2A) gene may be associated with drug addiction, including alcohol and heroin addiction [4,5].

Glutamatergic neurotransmission is the major excitatory system in the human brain, and genes encoding glutamate receptors are candidate targets for treatment of neuropsychiatric disorders. N-methyl D-aspartate (NMDA) receptors are key factors in glutamatergic neurotransmission which are involved in brain development, excitatory neurotransmission, synaptic plasticity, and memory formation [6–10]. The NMDA receptors are composed of multiple subunits, including at least one NR1 subunit and one or more NR2 subunits (GRIN2A-D) [11], and less commonly, a NR3 subunit (GRIN3A-B) [12]. GRIN2A knockout mice show increased spontaneous locomotor activity and deficits in contextual fear conditioning and spatial learning, along with reduced hippocampal long-term potentiation [13,14] that is thought to be involved in addiction [15]. Moreover, GRIN2A knockout mice failed to show evidence of conditioned place preference, suggesting an impairment in learned reward-related responses to ethanol [16]. Several studies have shown that chronic administration of drugs of abuse, such as alcohol [17], methamphetamine [18], cocaine [19], and nicotine [20], alters the activity of GRIN2A in the brain, suggesting that the GRIN2A gene is an excellent candidate target for treatment of addiction disorders. Importantly, these results indicate that glutamatergic transmission, particularly through GRIN2A-containing NMDA receptors in the
nucleus accumbens, probably contributes to the development of opiate addiction and confirms the hypothesis that subtype-selective NMDA receptor antagonists may be beneficial in the treatment of opiate addiction and withdrawal [21].

The human GRIN2A gene is located on chromosome 16p13.2 and consists of twelve exons and thirteen introns which undergo alternative splicing to form a family of GRIN2A isoforms. Itoikawa et al. [22] have identified a variable (GT)n repeat polymorphism (rs3219790) in the promoter region of this gene that elicited repression of transcriptional activity in a length-dependent manner. Recently, a case-control study showed evidence of an association between the repeat polymorphism and alcohol dependence, with longer alleles overly represented in patients with alcoholism [5]. Moreover, screenings of single-nucleotide polymorphisms (SNPs) in 130 candidate genes incriminated in addiction. In this study, we investigated forty loci in a Chinese population from Shaanxi province to verify the putative association between GRIN2A polymorphisms and heroin addiction.

Subjects and Methods

1 Subjects

A total of 210 unrelated subjects with heroin addiction (mean age of 34.82 ± 7.57, 167 males, 43 females) were recruited from the Methadone Maintenance Treatment (MMT) program of Xi’an Mental Health Center. Participants were daily or nearly daily users of heroin for a minimum of one year prior to assessment. The diagnosis of opioid addiction was based on the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) criteria, medical history, urine test results, and interview responses. Participants were excluded if they: met DSM-IV criteria for an additional Axis I disorder; had a history of alcohol, cigarette, amphetamine, or other drug addiction according to DSM-IV; were taking other medications that could affect the central nervous system; had a history of seizures, hematomatological diseases, or severe liver or kidney impairment. In all, 205 healthy blood donors (mean age of 36.13 ± 6.83, 164 males, 41 females) were recruited at the First Hospital Affiliated to the Medical College of Xi’an Jiaotong University. Subjects who had substance abuse, participated in other studies, or suffered from chronic brain diseases were excluded. From 210 subjects, 198 (94%) were tobacco smokers. From 205 controls, 127 (62%) were tobacco smokers. All participants completed a family history questionnaire and were self-identified as Han Chinese from Shaanxi province for three generations. Participants were excluded from the study if they had a relative in this study, or had a mixed ancestry. Written informed consent was obtained from all participants. The study protocol was approved by the Ethical Committee of the Medical College, Xi’an Jiaotong University.

2 Selection of Polymorphisms

Polymorphisms in the promoter region, untranslated regions (UTRs), exons, and introns of the gene for glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A) were systematically screened. Thirty-nine SNPs with minor allele frequencies (MAF) greater than 0.05 were selected from the GRIN2A gene and nearby regions based on a review of published literature (Table S1, Table S2) and a search of HapMap and dbSNP (Han Chinese population). The positions of the polymorphisms in the GRIN2A gene are shown in Fig. 1 and Table 1.

3 Genotyping

Peripheral blood were collected from the enrolled subjects in tubes coated with 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’s protocol. Probes and primers were designed using the Assay Design Software (Sequenom, San Diego, CA, USA). SNP genotyping was performed using matrix assisted laser desorption ionization-time of flight (MALDI-TOF; MassARRAY system, Sequenom Inc., San Diego, CA, USA) mass spectrometry. Briefly, one 5-µl PCR reaction include the following reagents: 1 µl of diluted DNA sample, 0.95 µl of water, 0.625 µl of PCR buffer containing 15 mM MgCl₂, 1 µl of 2.5 mM dNTP, 0.325 µl of 25 mM MgCl₂, 1 µl of PCR primers and 0.1 µl of 5 units/µl HotStar Taq (Qiagen). The reaction was incubated at 94°C for 15 minutes followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and a final incubation at 72°C for 5 minutes. The single base extension reaction was carried out at 94°C for 15 minutes followed by 45 cycles at 94°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, and a final incubation at 72°C for 5 minutes. The single base extension reaction was carried out at 94°C for 30 seconds and then 94°C for 5 seconds, followed by 5 cycles of 52°C for 5 seconds and 80°C for 5 seconds, total 40 cycles, then 72°C for 3 minutes. The reaction mix was desalted by adding 6 mg of cation exchange resin (Sequenom), mixed and resuspended in 25 µl of water. The completed genotyping
## Table 1. Genotype and allele frequencies of the GRIN2A gene SNPs in cases and controls and the results of their associations with risk of heroin addiction.

| SNP   | Variable Location | Group | Genotype (n, %) | Allele (n, %) | P<sub>a</sub> | P<sub>b</sub> | P<sub>c</sub> | OR<sub>d</sub>, 95%CI<sub>e</sub> |
|-------|-------------------|-------|----------------|---------------|--------------|--------------|--------------|-----------------------------|
| 1     | rs767749 3’UTR TT | case  | 37 (17.6)      | 89 (42.4)     | 0.147        | 0.578        | 0.271        | 1.172, 0.884–1.554          |
|       |                   | control| 30 (14.6)      | 84 (41.1)     | 0.144        | 0.578        | 0.271        | 1.172, 0.884–1.554          |
| 2     | rs1420040 3’UTR AG | case  | 70 (33.3)      | 99 (47.1)     | 0.684        | 0.543        | 0.263        | 0.854, 0.647–1.126          |
|       |                   | control| 77 (37.6)      | 95 (46.3)     | 0.684        | 0.543        | 0.263        | 0.854, 0.647–1.126          |
| 3     | rs9940680 3’UTR GG | case  | 70 (33.3)      | 100 (47.6)    | 0.960        | 0.482        | 0.232        | 0.845, 0.640–1.114          |
|       |                   | control| 77 (37.6)      | 97 (47.3)     | 0.960        | 0.482        | 0.232        | 0.845, 0.640–1.114          |
| 4     | rs9933624 3’UTR CC | case  | 70 (33.3)      | 100 (47.6)    | 0.818        | 0.536        | 0.261        | 0.853, 0.647–1.126          |
|       |                   | control| 77 (37.6)      | 96 (46.8)     | 0.818        | 0.536        | 0.261        | 0.853, 0.647–1.126          |
| 5     | rs8045712 3’UTR CC | case  | 40 (19.1)      | 70 (33.3)     | 0.754        | 0.362        | 0.181        | 1.209, 0.916–1.595          |
|       |                   | control| 29 (14.1)      | 99 (48.3)     | 0.754        | 0.362        | 0.181        | 1.209, 0.916–1.595          |
| 6     | rs8044472 3’UTR AA | case  | 22 (10.5)      | 109 (51.9)    | 0.566        | 0.216        | 0.578        | 0.923, 0.697–1.223          |
|       |                   | control| 32 (15.6)      | 93 (45.4)     | 0.566        | 0.216        | 0.578        | 0.923, 0.697–1.223          |
| 7     | rs1014531 3’UTR TT | case  | 8 (3.8)        | 68 (32.4)     | 0.897        | 0.688        | 0.390        | 0.864, 0.600–1.21          |
|       |                   | control| 10 (4.9)       | 72 (35.1)     | 0.897        | 0.688        | 0.390        | 0.864, 0.600–1.21          |
| 8     | rs11866328 Intron13 TT | case  | 8 (3.8)        | 66 (31.5)     | 0.897        | 0.584        | 0.302        | 0.839, 0.619–1.206         |
|       |                   | control| 10 (4.9)       | 72 (35.1)     | 0.897        | 0.584        | 0.302        | 0.839, 0.619–1.206         |
| 9     | rs7191784 Intron12 AG | case  | 30 (14.3)      | 100 (47.6)    | 0.779        | 0.835        | 0.601        | 1.078, 0.813–1.429         |
|       |                   | control| 28 (13.7)      | 93 (45.4)     | 0.779        | 0.835        | 0.601        | 1.078, 0.813–1.429         |
| 10    | rs7191241 Intron12 CC | case  | 30 (14.3)      | 100 (47.6)    | 0.720        | 0.875        | 0.706        | 1.056, 0.797–1.398         |
|       |                   | control| 29 (14.1)      | 93 (45.4)     | 0.720        | 0.875        | 0.706        | 1.056, 0.797–1.398         |
| 11    | rs1362319 Intron12 CC | case  | 57 (27.1)      | 97 (46.2)     | 0.851        | 0.724        | 0.622        | 0.934, 0.711–1.226         |
|       |                   | control| 56 (27.3)      | 101 (49.3)    | 0.851        | 0.724        | 0.622        | 0.934, 0.711–1.226         |
| 12    | rs1362321 Intron12 AG | case  | 51 (24.3)      | 97 (46.2)     | 0.556        | 0.946        | 0.985        | 1.003, 0.763–1.317         |
|       |                   | control| 48 (23.4)      | 98 (47.8)     | 0.556        | 0.946        | 0.985        | 1.003, 0.763–1.317         |
| 13    | rs3104703 Intron3 TT | case  | 43 (20.5)      | 92 (43.8)     | 0.884        | 0.0003       | <0.001       | 0.576, 0.437–0.758         |
|       |                   | control| 64 (31.2)      | 102 (49.8)    | 0.884        | 0.0003       | <0.001       | 0.576, 0.437–0.758         |
| 14    | rs2650432 Intron3 CC | case  | 23 (11.0)      | 80 (38.1)     | 0.827        | 0.524        | 0.760        | 0.955, 0.711–1.283         |
|       |                   | control| 19 (9.3)       | 89 (43.4)     | 0.827        | 0.524        | 0.760        | 0.955, 0.711–1.283         |
| 15    | rs2650431 Intron3 CC | case  | 41 (19.5)      | 95 (45.2)     | 0.670        | 0.341        | 0.289        | 1.162, 0.880–1.533         |
|       |                   | control| 29 (14.1)      | 100 (48.8)    | 0.670        | 0.341        | 0.289        | 1.162, 0.880–1.533         |
| 16    | rs3859125 Intron3 CC | case  | 28 (13.3)      | 90 (42.9)     | 0.616        | 0.513        | 0.479        | 1.110, 0.832–1.481         |
|       |                   | control| 20 (9.8)       | 93 (45.4)     | 0.616        | 0.513        | 0.479        | 1.110, 0.832–1.481         |
Table 1. Cont.

| SNP    | Variable | Location | Group | Genotype (n, %) | Allele (n, %) | $p^a$ | $p^b$ | $p^c$ | OR, 95%CI |
|--------|----------|----------|-------|-----------------|--------------|------|------|------|-----------|
| 17     | rs844395 | Introns 3| Cases | 56 (26.7)       | C 110 (52.4) | 0.827| 0.383| 0.317| 1.149, 0.875–1.507 |
|        |          |          |       | 44 (20.9)       | T 222 (52.9) |      |      |      |            |
|        |          |          | Cases | 198 (47.1)      |              |      |      |      |            |
|        |          |          | Controls | 53 (25.9)   | 104 (50.7) | 48 (23.4) | 205 (51.2) | 210 (51.2) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
|        |          |          | Controls | 60 (28.6)   | 96 (45.7) | 54 (25.7) | 216 (51.4) | 204 (48.6) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 18     | rs837697 | Introns 3| Cases | 53 (25.9)       | 106 (51.7) | 52 (25.4) | 200 (48.8) | 210 (51.2) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 45 (21.4)   | 109 (51.9) | 56 (26.7) | 199 (47.4) | 221 (52.6) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 19     | rs2650427| Introns 3| Cases | 43 (20.5)       | 104 (49.5) | 63 (30.0) | 190 (45.2) | 230 (54.8) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 20     | rs1969060| Introns 3| Cases | 55 (26.2)       | 97 (47.3) | 55 (26.8) | 203 (49.5) | 207 (50.5) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 21     | rs2937030| Introns 3| Cases | 53 (25.9)       | 97 (47.3) | 55 (26.8) | 203 (49.5) | 207 (50.5) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 22     | rs17682940| Introns 3| Cases | 60 (28.6)       | 96 (45.7) | 54 (25.7) | 216 (51.4) | 204 (48.6) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 47 (22.9)   | 106 (51.7) | 52 (25.4) | 200 (48.8) | 210 (51.2) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 23     | rs10500373| Introns 3| Cases | 53 (25.9)       | 97 (47.3) | 55 (26.8) | 203 (49.5) | 207 (50.5) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 24     | rs11642357| Introns 3| Cases | 43 (20.5)       | 104 (49.5) | 63 (30.0) | 190 (45.2) | 230 (54.8) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 25     | rs7188616 | Introns 3| Cases | 56 (26.7)       | 110 (52.4) | 44 (20.9) | 222 (52.9) | 198 (47.1) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 53 (25.9)   | 104 (50.7) | 48 (23.4) | 205 (51.2) | 210 (51.2) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 26     | rs17683096| Introns 3| Cases | 53 (25.9)       | 97 (47.3) | 55 (26.8) | 203 (49.5) | 207 (50.5) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
| 27     | rs2352748 | Introns 3| Cases | 53 (25.9)       | 97 (47.3) | 55 (26.8) | 203 (49.5) | 207 (50.5) | 0.619, 0.358–0.446 |
|        |          |          | Controls | 46 (22.4)   | 107 (52.2) | 52 (25.4) | 199 (48.5) | 211 (51.5) | 0.619, 0.358–0.446 |
|        |          |          |       | 1.112, 0.847–1.460 |
Table 1. Cont.

| SNP Variable | Location Group | Genotype (n, %) | Allele (n, %) | ρa | ρb | ρc | ORd, 95%CId |
|--------------|----------------|----------------|--------------|----|----|----|------------|
| case         | 6 (2.9)        | 44 (20.9)      | 160 (76.2)   | 56 (13.3) | 364 (86.7) |
| control      | 7 (3.4)        | 56 (27.3)      | 142 (69.3)   | 70 (17.1)  | 340 (82.9)  |
| rs1071502    | Intron 3       | CC C           | TT T         | 0.759 | 0.029 | 0.007 | 1.465, 1.109–1.937 |
| case         | 43 (20.5)      | 102 (48.6)     | 65 (30.9)    | 188 (44.7) | 232 (55.3) |
| control      | 27 (13.2)      | 92 (44.9)      | 86 (41.9)    | 146 (35.6) | 264 (64.4)  |
| rs1366076    | Intron 3       | AA A           | TT T         | 0.506 | 0.336 | 0.153 | 0.819, 0.623–1.077 |
| case         | 35 (16.7)      | 109 (51.9)     | 66 (31.4)    | 179 (42.6) | 241 (57.4) |
| control      | 44 (21.5)      | 107 (52.2)     | 54 (26.3)    | 195 (47.6) | 215 (52.4)  |
| rs1070502    | Intron 3       | GG G           | TT T         | 0.318 | 0.431 | 0.358 | 0.880, 0.670–1.156 |
| case         | 55 (26.2)      | 103 (49.0)     | 52 (24.8)    | 213 (50.7) | 207 (49.3) |
| control      | 56 (27.3)      | 109 (53.2)     | 40 (19.5)    | 221 (53.9) | 189 (46.1)  |
| rs1650420    | Intron 3       | AA A           | GG G         | 0.562 | 0.001 | 0.0002 | 1.671, 1.270–2.198 |
| case         | 68 (32.4)      | 103 (49.0)     | 39 (18.6)    | 239 (56.9) | 181 (43.1) |
| control      | 42 (20.5)      | 97 (47.3)      | 66 (32.2)    | 181 (44.1) | 229 (55.9)  |
| rs1102972    | Intron 3       | CC C           | TT T         | 0.771 <0.001 <0.001 | 2.143, 1.624–2.828 |
| case         | 73 (34.8)      | 99 (47.2)      | 38 (18.0)    | 245 (58.3) | 175 (41.7) |
| control      | 33 (16.1)      | 96 (46.8)      | 76 (37.1)    | 162 (39.5) | 248 (60.5)  |
| rs7499321    | Intron 3       | CC C           | TT T         | 0.396 0.327 0.233 | 1.180, 0.899–1.550 |
| case         | 55 (26.2)      | 102 (48.6)     | 53 (25.2)    | 212 (50.5) | 208 (49.5) |
| control      | 41 (20.0)      | 108 (52.7)     | 56 (27.3)    | 190 (46.3) | 220 (53.7)  |

1Values for Hardy-Weinberg equilibrium in controls.
2Values for genotype frequency difference.
3Values for allele frequency difference.
4Values for allele frequency difference.
5Alpha value is adjusted by Bonferroni correction and significant results (P<0.0013).
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Figure 2. Allele frequency distribution of the GRIN2A (GT)n repeat in heroin addiction and controls. Allele size is expressed as the number of GT repeats.
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reactions were spotted onto a 384 well spectroCHIP (Sequenom) using the MassARRAY Nanodispenser (Sequenom) and determined by the matrix-assisted laser desorption ionization time-of-flight mass spectrometer. Genotype calling was performed in real time with the MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom).

The primers specific for the (GT)n repeat used to amplify the repeat-containing genomic fragment were: a 6-carboxyfluorescein (FAM)-labeled upstream primer, 5'-GAAGGAAGCATGTGGGAAATGCAG-3' (the 3' end is 98 bp upstream of the 5' end of the (GT)n repeat; see GenBank accession No.AF443855), and a non-labeled downstream primer, 5'-gtttcttGCTGGTGATCAGTTATCCCCCT-3' (the 3' end is 19 bp downstream of the 3' end of the (GT)n repeat) [22]. Polymerase chain reaction (PCR) amplification was performed with an initial denaturation at 95°C for 8 min, prior to 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 a further 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 GeneScan software (Applied Biosystems).

### 4 Statistical Analysis

The allele and genotype frequencies for each individual polymorphism were compared, and Hardy-Weinberg equilibrium was evaluated using the Chi-square test. Associations between polymorphisms and heroin addiction were assessed by the Fisher’s exact test or the Pearson Chi-square test. The statistically

| (GT)n repeat | Cases (n, %) | Controls (n, %) | $\chi^2$ | $P$ |
|--------------|-------------|----------------|---------|----|
| (GT)13       | 4 (1.0)     | 5 (1.2)        | 0.138   | 0.710 |
| (GT)14       | 0 (0.0)     | 2 (0.5)        | 2.054   | 0.152 |
| (GT)19       | 1 (0.2)     | 3 (0.7)        | 1.054   | 0.305 |
| (GT)20       | 3 (0.7)     | 7 (1.7)        | 1.719   | 0.190 |
| (GT)21       | 26 (6.2)    | 39 (9.5)       | 3.171   | 0.075 |
| (GT)22       | 7 (1.7)     | 17 (4.1)       | 2.543   | 0.105 |
| (GT)23       | 27 (6.4)    | 45 (11.0)      | 3.915   | 0.054 |
| (GT)24       | 54 (12.9)   | 52 (12.7)      | 0.006   | 0.940 |
| (GT)25       | 49 (11.7)   | 41 (10.0)      | 1.330   | 0.249 |
| (GT)26       | 100 (23.8)  | 59 (14.4)      | 5.360   | 0.021 |
| (GT)27       | 48 (11.4)   | 38 (9.3)       | 1.042   | 0.307 |
| (GT)28       | 44 (10.5)   | 40 (9.8)       | 0.118   | 0.731 |
| (GT)29       | 27 (6.4)    | 22 (5.4)       | 0.422   | 0.516 |
| (GT)30       | 10 (2.4)    | 16 (3.9)       | 1.583   | 0.208 |
| (GT)31       | 3 (0.7)     | 6 (1.5)        | 1.086   | 0.297 |
| (GT)32       | 6 (1.4)     | 5 (1.2)        | 0.069   | 0.792 |
| (GT)33       | 7 (1.7)     | 6 (1.5)        | 0.056   | 0.814 |
| (GT)34       | 4 (1.0)     | 6 (1.5)        | 0.455   | 0.500 |
| (GT)36       | 0 (0.0)     | 1 (0.2)        | 1.026   | 0.311 |

![Figure 3. LD plot of the 39 SNPs in GRIN2A gene in controls (n = 205). Values in squares are the pair-wise calculation of $D'$.](https://doi.org/10.1371/journal.pone.0070817.g003)

Table 2. The statistics of the GRIN2A (GT)n repeat in heroin addiction and controls.

| (GT)n repeat | Cases (n, %) | Controls (n, %) | $\chi^2$ | $P$ |
|--------------|-------------|----------------|---------|----|
| (GT)13       | 4 (1.0)     | 5 (1.2)        | 0.138   | 0.710 |
| (GT)14       | 0 (0.0)     | 2 (0.5)        | 2.054   | 0.152 |
| (GT)19       | 1 (0.2)     | 3 (0.7)        | 1.054   | 0.305 |
| (GT)20       | 3 (0.7)     | 7 (1.7)        | 1.719   | 0.190 |
| (GT)21       | 26 (6.2)    | 39 (9.5)       | 3.171   | 0.075 |
| (GT)22       | 7 (1.7)     | 17 (4.1)       | 2.543   | 0.105 |
| (GT)23       | 27 (6.4)    | 45 (11.0)      | 3.915   | 0.054 |
| (GT)24       | 54 (12.9)   | 52 (12.7)      | 0.006   | 0.940 |
| (GT)25       | 49 (11.7)   | 41 (10.0)      | 1.330   | 0.249 |
| (GT)26       | 100 (23.8)  | 59 (14.4)      | 5.360   | 0.021 |
| (GT)27       | 48 (11.4)   | 38 (9.3)       | 1.042   | 0.307 |
| (GT)28       | 44 (10.5)   | 40 (9.8)       | 0.118   | 0.731 |
| (GT)29       | 27 (6.4)    | 22 (5.4)       | 0.422   | 0.516 |
| (GT)30       | 10 (2.4)    | 16 (3.9)       | 1.583   | 0.208 |
| (GT)31       | 3 (0.7)     | 6 (1.5)        | 1.086   | 0.297 |
| (GT)32       | 6 (1.4)     | 5 (1.2)        | 0.069   | 0.792 |
| (GT)33       | 7 (1.7)     | 6 (1.5)        | 0.056   | 0.814 |
| (GT)34       | 4 (1.0)     | 6 (1.5)        | 0.455   | 0.500 |
| (GT)36       | 0 (0.0)     | 1 (0.2)        | 1.026   | 0.311 |
significance was set at 0.05. Unconditional logistic regression was used to calculate the odds ratio (OR) and 95% confidence interval (CI) in independent association between each locus and the presence of heroin addiction. The Bonferroni correction was used to adjust the test level when multiple comparisons were conducted, and the P value was divided by the total number of loci or haplotypes. All statistical analyses were performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Haplotype blocks were defined according to the criteria of Gabriel et al. [23], as implemented in Haploview 4.0, to examine if some SNPs significant in the single marker association analysis also exist in the haplotype blocks. Pair-wise linkage disequilibrium (LD) statistics (D' and r²) and haplotype frequency were computed, and haplotype blocks were constructed using Haploview 4.0 [24].

Results

We detected repeat numbers ranging from 13 to 36 in the samples in this study. The dominant allele was (GT)26. The allele distribution histogram of heroin-addicted patients was shifted to the right, with longer alleles over-represented in heroin addiction (Fig. 2). The frequency of the (GT)26 allele in the heroin addiction group was significantly higher than that in the control group ($\chi^2=5.360$, $P=0.021$) (Table 2). When we tested for single allelic association of common alleles [frequency >5%: (GT)21 to (GT)29], the Pearson Chi-square test gave the following P values: 0.075 for (GT)21, 0.105 for (GT)22, 0.054 for (GT)23, 0.940 for (GT)24, 0.249 for (GT)25, 0.021 for (GT)26, 0.307 for (GT)27, 0.731 for (GT)28 and 0.516 for (GT)29 (Table 2).

The distribution frequencies of thirty-nine genotyped SNPs were in agreement with the Hardy-Weinberg equilibrium ($P>0.05$, Table 1). Linkage disequilibrium (LD) analyses data revealed 10 haplotypes in controls, and also showed that 8 SNPs (rs767749, rs1420040, rs9935624, rs8045712, rs8044472, rs1014531, and rs11866328) were located in block 1, 2 SNPs (rs7191784 and rs7191241) in block 2, 2 SNPs (rs1362319 and rs1362321) in block 3, 2 SNPs (rs2650432 and rs2650431) in block 4, 2 SNPs (rs3859125 and rs844935) in block 5, 7 SNPs (rs837697, rs2650427, rs1969060, rs2937030, rs17682940, rs10500373, and rs11642357) in block 6, 3 SNPs (rs7188616, rs17683096, and rs2352748) in block 7, 4 SNPs (rs454974, rs11644511, rs1070487, and rs6497730) in block 8, 2 SNPs (rs1071502 and rs1366076) in block 9, and 2 SNPs (rs1102972 and rs7499321) in block 10 (Fig. 3 and Fig. 4). The genotype distributions, allelic frequencies, and haplotypes in the patient and control groups, together with the results of statistical analysis are listed in Tables 1 and 3.

The frequency of the T allele in rs1102972 ($\chi^2=29.408$, $P<0.001$, odds ratio [OR] = 2.143, 95% confidence interval [CI] = 1.624–2.828) and the G allele in rs1650420 ($\chi^2=13.511$, $P=0.0002$, OR = 1.671, 95% CI = 1.270–2.198) in heroin-addicted subjects was significantly lower than that in the controls (Table 1). The rs3104703 G allele frequency in heroin-addicted subjects was significantly higher than that in the controls ($\chi^2=15.618$, $P<0.001$, OR = 0.576, 95% CI = 0.437–0.758) (Table 1). Furthermore, the rs1071502 T, rs6497730 G, and rs1070487 G allele frequency in heroin addicts were higher than that in the controls ($P<0.05$), but did not pass the threshold value. Thirty-three additional GRIN2A SNPs gave negative results (Table 1).

The G-C-T-C-C-T-A haplotype in block 6 occurred significantly more frequently ($\chi^2=37.305$, $P<0.001$, OR = 3.675, 95% CI = 2.180–6.230, protective) and the T-T haplotypes in block 10 occurred more frequently ($\chi^2=8.919$, $P=0.003$, OR = 5.54,
95% CI = 0.375–0.817, protective) in controls (Table 3). These differences were retained even after Bonferroni correction.

**Discussion**

GRIN2A regulates reward-related associative learning, cognition, memory, and structural and behavioral plasticity in the context of drug addiction [13–16,21,25,26], suggesting that GRIN2A acts upon the brain's reward system, which plays a key role in drug addiction. In the past decade, accumulated evidence indicates that NMDA receptors play a pivotal role in the development of tolerance and physical dependence to opiates [27–29]. Our results provide direct evidence that a genetic change in GRIN2A is linked to heroin addiction in humans, and extends the list of variants that may affect the development of heroin addiction [4].

A variable (GT)n repeat in the 5’-regulatory region of the GRIN2A gene has been identified [22]. It was shown that the repeat sequence repressed transcriptional activity in a length-dependent manner, such that the longer the repeat, the lower the promoter activity. In this case-controlled association study, significant differences were found 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 the controls. To our knowledge, our study is the first to identify a significant association between (GT)n repeats in the 5’-regulatory region of the GRIN2A gene and heroin addiction. Indeed, heroin-addicted patients had overall longer alleles than the control subjects. The (GT)n polymorphism in the promoter of GRIN2A has been reported to be associated with schizophrenia and bipolar disorder [22,30,31]. Similarly, a previous study showed that longer alleles of (GT)n repeats were significantly more frequent among alcohol dependence [5]. The average observed repeat number distributions were significantly different in alcohol-dependent subjects (GT repeats: n = 24.5) and the control subjects (GT repeats: n = 23.7) [5]. It is possible that the presence of longer (GT)26 repeat results in decreased GRIN2A receptor function in patients with heroin addiction. This finding represents clinical-genetic evidence pointing toward the role of promoter (GT)n polymorphisms in the GRIN2A gene in the pathophysiology of heroin addiction.

In this study, we evaluated the association between thirty-nine SNPs that efficiently tag the common variation in the GRIN2A gene and heroin addiction. The most intriguing finding of the

| Block | SNP | Haplotype | Case (n, %) | Control(n, %) | χ² | P | OR, 95% CI |
|-------|-----|-----------|------------|--------------|----|---|------------|
| 1     | 1/2/3/4/5/6/7/8 | G-G-C-C-T-A-G-G | 86 (37.1) | 79 (38.5) | 0.253 | 0.615 | 1.106, 0.746–1.639 |
|       |     | T-A-G-T-C-G-G-G | 68 (32.4) | 70 (34.1) | 2.048 | 0.152 | 1.338, 0.898–1.993 |
|       |     | G-A-G-T-C-A-T | 38 (18.1) | 46 (22.4) | 1.212 | 0.271 | 0.764, 0.472–1.235 |
| 2     | 9/10 | G-T | 127 (60.5) | 133 (64.9) | 0.859 | 0.354 | 0.828, 0.556–1.212 |
|       |     | A-C | 78 (37.1) | 76 (37.1) | 0.000 | 0.988 | 1.003, 0.673–1.494 |
| 3     | 11/12 | C-A | 97 (46.2) | 100 (48.8) | 0.279 | 0.597 | 0.901, 0.613–1.325 |
|       |     | A-G | 102 (48.6) | 100 (48.8) | 0.002 | 0.966 | 0.992, 0.675–1.457 |
|       |     | T-C | 86 (41.0) | 81 (39.5) | 0.089 | 0.765 | 1.062, 0.717–1.572 |
| 4     | 14/15 | C-T | 62 (29.5) | 65 (31.7) | 0.233 | 0.629 | 0.902, 0.594–1.370 |
|       |     | T-T | 57 (27.1) | 64 (31.2) | 0.835 | 0.361 | 0.821, 0.537–1.254 |
|       |     | C-C | 68 (32.4) | 67 (32.7) | 0.004 | 0.948 | 0.986, 0.654–1.487 |
| 5     | 16/17 | T-T | 94 (44.8) | 101 (49.3) | 0.846 | 0.359 | 0.834, 0.567–1.227 |
|       |     | T-C | 40 (19.0) | 41 (20.0) | 0.060 | 0.807 | 0.941, 0.579–1.529 |
| 6     | 18/19/20/21/22/23/24 | G-C-T-C-T-G | 69 (32.9) | 67 (32.7) | 0.001 | 0.970 | 1.008, 0.669–1.519 |
|       |     | T-T-C-T-T-A | 97 (46.2) | 101 (49.3) | 0.394 | 0.530 | 0.884, 0.601–1.300 |
|       |     | G-C-T-C-T-A | 137 (65.2) | 185 (80.2) | 37.305 | <0.001 | 0.203, 0.118–0.349 |
| 7     | 25/26/27 | C-A | 93 (44.3) | 92 (44.9) | 0.015 | 0.903 | 0.976, 0.663–1.438 |
|       |     | G-G-G | 106 (50.5) | 113 (55.1) | 0.898 | 0.343 | 0.830, 0.564–1.221 |
| 8     | 28/2930/31 | C-C-G-G | 88 (41.9) | 78 (38.0) | 0.643 | 0.423 | 1.174, 0.793–1.740 |
|       |     | T-A-G-G | 66 (31.4) | 71 (34.6) | 0.482 | 0.488 | 0.865, 0.574–1.303 |
|       |     | T-C-G-G | 31 (14.8) | 32 (15.6) | 0.058 | 0.810 | 0.936, 0.548–1.601 |
|       |     | T-C-A-A | 16 (7.6) | 26 (12.7) | 2.924 | 0.087 | 0.568, 0.295–1.093 |
| 9     | 34/35 | C-T | 91 (43.3) | 73 (35.6) | 2.589 | 0.108 | 1.383, 0.931–2.053 |
|       |     | T-A | 87 (41.4) | 98 (47.8) | 1.707 | 0.191 | 0.772, 0.524–1.138 |
|       |     | T-T | 26 (12.4) | 37 (18.0) | 2.588 | 0.108 | 0.642, 0.373–1.105 |
| 10    | 38/39 | C-C | 103 (49.0) | 83 (40.5) | 3.073 | 0.080 | 1.415, 0.959–2.087 |
|       |     | T-T | 85 (40.5) | 113 (55.1) | 8.919 | 0.003 | 0.554, 0.375–0.817 |
|       |     | T-C | 17 (8.1) | 14 (6.8) | 0.241 | 0.624 | 1.202, 0.576–2.506 |

Haplotypes* with frequency <0.05 were excluded.

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Table 3. GRIN2A haplotype frequencies and the results of their associations with risk of heroin addiction.

95% CI = 0.375–0.817, protective) in controls (Table 3). These differences were retained even after Bonferroni correction.
present study is that three GRIN2A variants (rs1102972, rs1650420, and rs1304703), all located within a 32-kb area of intron 3, accounted for some of the strongest signals in the association test. Three additional SNPs from the same intron (rs1071502, rs4697970, and rs1070407) had nominally significant P values for association (P<0.05), but did not pass the threshold value. GRIN2A has also recently been shown to be of the highest relevance in human alcohol dependence, among 10 glutamatergic neurosignaling genes [32], and in heroin addiction, among 6 glutamatergic neurosignaling genes [4]. Previously, Levran et al. [4] confirmed a statistically significant association between 6 polymorphisms located at intron 3 (rs1070487, rs4697970, rs46587976, rs1650420, rs1071502, and rs3660762) and heroin-addiction in African Americans. Differences of this kind may be correlated with alterations in hormone levels, neuronal system adaptations, and the pharmacokinetics of substances of abuse [33].

Population stratification is an important issue to be considered when conducting human genetic surveys [34]. In the present study, the experimental and control groups were matched for ethnicity by enrolling subjects from a homogeneous population. Our analysis also has sufficient statistical power to argue an epidemiologically-relevant impact of hereditary variations among ethnic groups by enrolling subjects from a homogeneous population. In the present study, the experimental and control groups were matched for ethnicity by enrolling subjects from a homogeneous population. Our analysis also has sufficient statistical power to argue an epidemiologically-relevant impact of hereditary variations among ethnic groups.

We further investigated the interaction among polymorphisms and observed strong linkage disequilibrium. Haplotype analysis revealed that the G-C-T-C-C-T-A (block 6) and T-T (block 10) haplotypes of the GRIN2A gene displayed a protective effect. There were significant point-wise associations of these variants with heroin addiction. These results indicated that people with these two haplotypes of the GRIN2A gene were less prone to heroin addiction. A previous haplotype analysis of rs46587976-rs1071502-rs1366076 revealed significant association of the G-A-T (protective) and C-A-T (risk) haplotypes in heroin-dependent patients and healthy controls, respectively [4]. To some extent, this finding further supports a role of GRIN2A polymorphisms in heroin addiction, with differences in the specifics of the association between ethnic groups.

In conclusion, 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. Our results are in line with the glutamatergic hypothesis developed to understand the acute and chronic effects of heroin on the brain. The results of this and similar future studies could help understand the neurobiological mechanisms of heroin addiction better, allowing us to devise better treatment strategies.

Supporting Information

Table S1 Comparison of the MAF of 39 SNPs between African American (AA) and Chinese Han (CH) population. It should list the source of this information.

Author Contributions

Conceived and designed the experiments: BZ YZ HC. Analyzed the data: YW JL. Contributed reagents/materials/analysis tools: WW. Wrote the paper: BZ YZ. Managed the literature search: WW. Contributed to and approved the final manuscript: BZ YZ WW HC YW JL.

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