Genetic variation within *GRIN2B* in adolescents with alcohol use disorder are associated with smaller left orbito-frontal cortex volume

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Abstract

Objective: Both volumes of brain structures and adolescent alcohol dependence show substantial heritability. However, exactly which genes are responsible for brain volume variation in adolescents with substance abuse disorders are currently unknown. The aim of this investigation was to determine whether genetic variants previously implicated in psychiatric disorders are associated with variation in brain volume in adolescents with alcohol use disorder (AUD).

Methods: The cohort consisted of 58 adolescents with DSM-IV AUD and 58 matched controls of mixed ancestry ethnicity. An Illumina Infinium iSelect custom 6000 bead chip was used to genotype 5348 SNPs in 378 candidate genes. Magnetic resonance images were acquired and volumes of global and regional structures were estimated using voxel-based morphometry (VBM). To determine whether any of the genetic variants were associated with brain volume, association analysis was conducted using linear regression in Plink.

Results: Post-hoc t-tests showed that the being homozygous for the A allele for the intronic SNP (rs219927) in GRIN2B was associated with smaller left orbitofrontal cortex volume (uncorrected p-value= 0.001). Additionally, in AUD, as compared to HC, being homozygous for the A allele for the functional 3'UTR SNP (rs890) in GRIN2B was also associated with smaller left orbitofrontal cortex volume (uncorrected p-value< 0.001).

Conclusion: The *GRIN2B* gene is involved in glutamatergic signalling and may be associated with developmental differences in brain regions involved in various processes including reward processing. Such differences may play a role in risk for AUD, and deserve more detailed investigation.

Keywords: alcoholism, GRIN2B receptor, magnetic resonance imaging

Significant outcomes:

1	Variation within the gene GRIN2B may increase risk for AUD
2	Variation within the gene GRIN2B may be associated with differential brain volume

Limitations:

1	Small sample size
2	Two different methods were used in the acquisition of brain images

Introduction

Structural variations in several brain regions have been shown for alcohol use disorder (AUD), in particular, smaller volumes have been found in the prefrontal cortex (Medina et al. 2008), right hippocampus (Agartz et al. 1999, De Bellis et al. 2000), amygdala (Makris et al. 2008) and grey and white matter (Fein et al. 2013, Gazdzinski et al. 2005). In adults with AUD, differential brain volume in the bilateral insular cortex and amygdala was associated with a lack of top-down control over impulsive behaviour (Senatorov et al. 2015). Brain structure has considerable heritability (Baaré et al. 2001, Posthuma et al. 2000, Thompson et al. 2001). Genetic variation within serotonin transporter (5-HTT), gamma-aminobutyric acid A receptor, alpha 2 (GABRA2), brain-derived neurotrophic factor (BDNF), catechol-O-methyltransferase (COMT), dopamine receptor D2 (DRD2), and corticotropin-releasing hormone receptor 1 (CRHR1) have been implicated with alteration in brain structure and function in adolescents with AUD and alcohol-related phenotypes (Glaser et al. 2014, Hill et al. 2009, Hill et al. 2011, Hill et al. 2013). In adults with AUD and alcohol-use related phenotypes, alterations in brain volume were associated with variation in the genes GABRA2, BDNF, glutamate receptor, ionotropic, N-methyl D-aspartate 2B (GRIN2B) and neuregulin 1 (NRG1) (Vergara et al. 2014, Villafuerte et al. 2012). Thus, the genes most implicated in AUD to date (in both adults and adolescents) involve neurotransmission.

While previous studies have examined the association between brain volume and genes in adolescents with AUD, these studies either investigated a select number of candidate genes or adopted a volumetric brain region of interest (ROI) approach, which narrowed the scope of the possible exploration. The aim of this investigation was to determine whether genetic variants previously implicated in psychiatric disorders are associated with variation in brain volume in adolescents with AUD.

Material and methods

Participants

Ethical approval for this study was obtained from the Research Ethics Committees of Stellenbosch University (N06/07/128) and the University of Cape Town (HREC REF 023/2012). The cohort consisted of 58 adolescents with AUD and 58 demographically matched (age, gender, language, education level, and socioeconomic status) control (HC) subjects of mixed ancestry ethnicity, with a lifetime dosage not exceeding 76 units of alcohol. Eligibility was assessed after a detailed medical history was taken by a fully qualified and licensed psychiatrist. Physical and psychiatric examinations were also undertaken by the psychiatrist and each of the participants underwent urine analysis and breathalyser testing to ensure they were not intoxicated during the testing period. The Schedule for Affective Disorders and Schizophrenia for School Aged Children (6–18 years) Lifetime Version (K-SADS-PL) (Kaufman et al. 1997), was administered by a fully qualified and licensed psychiatrist to determine whether any of the participants had current or past psychiatric symptoms (Fein et al. 2013). Additionally, the Timeline Followback (TLFB) procedure was used to determine lifetime history of alcohol use and drinking patterns (Sobell and Sobell 1992). Childhood adversity was measured by the 28-item Childhood Trauma Questionnaire- Short Form (CTQ-SF) (Bernstein et al. 2003).

Exclusion criteria for study participation included diagnoses of mental retardation, lifetime DSM-IV Axis I other than AUD; lifetime dosages exceeding 30 cannabis joints or 4 methamphetamine doses; current use of sedative or psychotropic medication; signs or history of fetal alcohol syndrome or malnutrition; sensory impairment; history of traumatic brain injury with loss of consciousness exceeding 10 minutes; presence of diseases that may affect the CNS; less than 6 years of formal education; and lack of proficiency in English or Afrikaans. Blood

samples were collected for all of the recruited individuals with the appropriate informed consent.

Genotyping

DNA was extracted from the participants' blood samples using the Maxwell® 16 Blood DNA purification kit (AS1010) (Promega) and the the Maxwell 16 instrument (Promega) at the Centre for Proteomic and Genomic Research (CPGR) (Cape Town, South Africa). An Illumina Infinium iSelect custom 6000 bead chip was used to genotype 5348 SNPs in 378 candidate genes (genes involved in neurotransmitter and neuroendocrine systems) for post-traumatic stress disorder, and SNPs and copy number variation (CNVs) which were "significant hits" from previous psychiatric GWAS studies. The bead chip was run on the Illumina BeadStation 500G System at the University of Michigan DNA Sequencing Core (Michigan, USA). Case and control samples were analysed together. Genotype calls were made using standard clustering algorithms in the GenomeStudio software (Illumina).

MRI Acquisition

MRIs were collected with a 3T Siemens Magnetom Allegra MR Headscanner using Syngo MR software (Siemens Medical Solutions). The scanner is located in the Cape Universities Brain Imaging Center at the Stellenbosch University Health Sciences Campus, South Africa. Images for the first 50 subjects (25 HC and 25 AUD) were acquired using a trans-axial T1-weighted acquisition (TR = 2080 ms, TE = 4.88 mm, acquisition matrix = 256 x 192) at 1.0 mm thickness. The initial review of these images revealed undesirable presence of blood-vessels in the imaging, resulting from the scanner being a head-only model that did allow adequate saturation of the blood to suppress signal before the blood flow enters the head. The use of a sagittal T1 protocol was subsequently implemented in place of the original trans-axial acquisition (TR =

2200 ms, TE = 5.16 ms, acquisition matrix 256 x 256) at 1.0 mm thickness. The remaining 66 subjects (33 HC and 33 AUD) had an MRI using the sagittal protocol. Of the 50 individuals with a transaxial T1-weighted acquisition, 25 individuals (9 HC and 16 AUD) had an additional MRI with the sagittal protocol. In a previous analysis, we demonstrated that the two acquisition protocols produced comparable images that could be combined for analysis (Fein et al. 2013).

MRI Analysis

After manually reorienting and realigning the cross-hair on the AC-PC plane in all our nifti-converted DICOM T1 images, and initial quality control for signal artifacts, morphological changes were calculated in gray matter by segmenting from white matter and cerebrospinal fluid using the voxel-based morphometry (VBM) unified segmentation approach (Ashburner and Friston 2005) in SPM8 (www.fil.ucl.acuk/spm8). Following this segmentation procedure, probability maps of gray matter were "modulated" to account for the effect of spatial normalisation, by multiplying the probability value of each voxel by its relative volume in native space before and after warping. Gray matter images, based on probability maps at each voxel, were spatially normalised using a pediatric template from the Cincinnati Children´s Hospital old children template (www.irc.cchmc.org/software/pedbrain.php) and then co-registered using the same segmented template. Modulated images were smoothed with an 8 mm 'Full Width Half Maximum [FWHM]' Gaussian kernel, in line with other recent VBM studies. This smoothing kernel was applied prior to statistical analysis, to reduce signal noise and to correct for image variability.

Statistical Analysis

As an exploratory analysis, association analysis was conducted using linear regression in Plink (version 1.07) (http://pngu.mgh.harvard.edu/~purcell/plink/). This was to determine whether any of the SNPs (independent variable) had an association with several regions of interest (ROIs) (dependent variable) taken from previous publications examining gray matter volume

in AUD (Brooks et al. 2014, Fein et al. 2013, Vergara et al. 2014). The ROIs, which were tested separately, were: amygdala, caudate, dorsolateral prefrontal cortex (DLPFC), globus pallidus, hippocampus, insula, occipital lobe, posterior cingulate, precuneus, putamen, superior temporal gyrus, and thalamus. The following were included in the regression model as covariates: age, gender, years of education, total matter volume, handedness, and protocol. Additionally total CTQ score was added as a covariate as a previous study on this cohort found an association between CTQ score and brain volume (Brooks et al. 2014) . All samples had a call rate of greater than 99%. Before genotyping and frequency pruning there were 4656 SNPs. A total of 9 SNPs failed the missingness test (i.e. only SNPs with a genotyping rate of 90% were included) and 600 SNPs were excluded because of a minor allele frequency (MAF) of less than 0.05. A total of 4 SNPs were excluded as these were out of Hardy-Weinberg Equilibrium (HWE) (p-value less than 0.00001). All tests were corrected for multiple comparisons (for multiple SNPs) using the Bonferroni correction method.

As a follow-up to the initial association findings, the main effects of group (AUD and HC) and of the identified significant SNP genotypes on brain volume data, 2 x 2 ANCOVA using VBM in the SPM8 package (http:www.fil.ucl.ac.uk/spm8) was implemented. AUD and HC subjects were matched in terms of age, gender, and protocol. Because years of education, handedness and protocol were not significantly associated with any brain volume, only age, gender, and total matter volume were retained as covariates of no interest to control for global differences in head size. All statistical analyses were corrected for multiple comparisons at the peak voxel level using the family-wise error (FWE), although uncorrected but otherwise significant findings are also reported in the table as an indicator for further more statistically powerful studies to examine.

Results

Participants

See table 1 for participant details. The median ages of the HC and AUD groups for the total cohort were 14.77 and 14.98, respectively and were not significantly different. The study participants were predominantly Afrikaans, followed by English speaking and the median number of years of education was 8.0 years for both groups (HC and AUD). The median number of alcohol life dose units for the AUD group in the total cohort was 962.0, and 1.0 for the HC group, where a unit refers to one beer or wine cooler, one glass of wine, or one 43g shot of liquor (on its own, or in a mixed drink). The median total CTQ score was 36.0 and 42.0 for the HC and AUD group, respectively.

Statistical Analysis

Plink 1.07

From the association analysis, only one SNP, rs219927 located in an intron of the gene *GRIN2B* was associated with ROI brain volume in the left posterior cingulate cortex (corrected p-value< 0.05), whereby having a G-allele was associated with a bigger volume. From this, four "functional" variants (two exonic [rs1806201 and rs7301328] and two located in the 3'UTR [rs890 and rs1805502]) within the *GRIN2B* gene were investigated to determine whether these variants were associated with variation in brain volume that was not detected in the earlier association tests due to the multiple testing burden. It was found that the 3'UTR SNP, rs890, was associated with brain volume in the left and right DLPFC (corrected p-value < 0.05), whereby an A-allele was associated with a bigger volume.

Voxel-Based Morphometry

In order to validate the findings from the initial association analyses, ANCOVA and post-hoc t-tests for brain volume and genotype (rs219927 and rs890 in GRIN2B in two separate

ANCOVA analyses) were conducted using VBM. The ANCOVAs showed a significant main effect for rs219927 in the left orbitofrontal cortex (OFC) (x=-21, y=37, z=-16, uncorrected p-value< 0.05) (Table 2). Post-hoc tests indicated that volume in the left OFC was smaller in individuals with the AA genotype compared to those homozygous for the G-allele, although this association was only nominally significant (uncorrected p-value< 0.001) (Table 2). For rs890, a main effect of genotype was observed in the left parahippocampal gyrus (x=-24, y=-30, z=-21, uncorrected p-value< 0.001) and the left OFC (x=-24, y=24, z=-19, uncorrected p-value< 0.001) (Table 3). In addition, a genotype by group interaction was detected for the left OFC (x=-24, y=24, z=-19, uncorrected p-value< 0.001). From the post-hoc analysis it was seen that individuals with AUD and the AA genotype had smaller volumes in the left OFC (x=-34, y=32, z=5, uncorrected p-value< 0.001) (Figure 1) (Table 3).

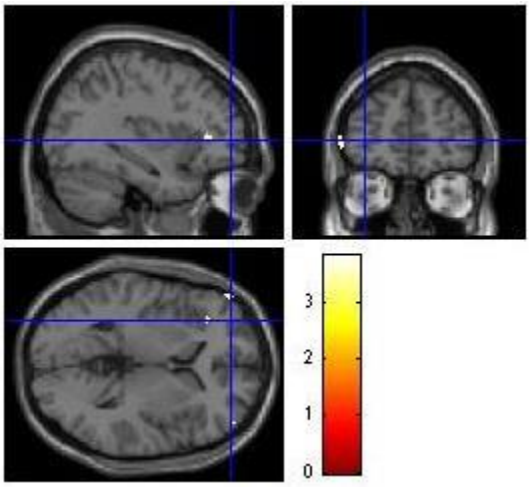

Figure 1: Smaller volume in left OFC for AUD individuals with *GRIN2B* rs890 AA genotype

Discussion

This study is the first to explore which genes, from a large sample of SNPs commonly associated with psychiatric disorders, are associated with brain volume differences in adolescents with AUD versus healthy controls. It was found that an intronic SNP rs219927 and

rs890, a functional 3'UTR SNP, both within the gene *GRIN2B*, are associated with smaller left OFC volume in AUD.

The exploratory analyses identified the SNPs rs219927 and SNP rs890 located within *GRIN2B* as possibly involved in influencing brain volumes in adolescent AUD. *GRIN2B* (12p12) encodes the 2B subunit of the ionotropic *N*-methyl-D-aspartate (NMDA) glutamate receptor (Collingridge et al. 2009) and in brain tissue is primarily expressed in the fronto-parieto-temporal cortex and the hippocampal pyramidal cells (Schito et al. 1997). The NMDA receptor is an ion gated-channel which plays a role in the process of long term potentiation and is thought to be involved in learning and memory (Ishii et al. 1993). Genetic polymorphisms within *GRIN2B* have previously been associated with variation in brain structure. In particular, the *GRIN2B* SNP rs890 was shown to have an association with reduced fractional anisotropy (FA) in several brain areas, including the bilateral frontal region and left cingulate gyrus, in individuals with bipolar disorder (Kuswanto et al. 2013).

In the current investigation, the analysis showed that the *GRIN2B* SNPs rs219927 and SNP rs890 are associated with smaller volume in the OFC, a brain region involved in the process of decision making (Bechara et al. 2000) and reward-related behaviour in response to taste, smell and visual cues (Kringelbach 2005, Rolls 2000). The OFC is implicated in a cortico-striatal-limbic neural circuit accounting for alcohol craving and relapse following a period of abstinence, incorporating the medial prefrontal cortex, anterior cingulate cortex, striatum and amygdala (Sullivan and Pfefferbaum 2014). Damage to, or deficits in this circuitry therefore, likely contribute to impairments in executive functioning, emotion regulation and decision making observed in those with AUD. In line with our observations, a previous study reported that the *GRIN2B* rs1805476 SNP was associated with smaller OFC volume in individuals with obsessive compulsive disorder (Arnold et al. 2009), a disorder associated with a lack of control over anxious symptoms. The intronic *GRIN2B* rs219927 SNP, which we also found to be

associated with brain volume differences in our cohort, has not previously been associated with any brain volume or psychiatric phenotype. This SNP could be in linkage disequilibrium with other causal variants. The OFC has previously been implicated in alcohol abuse and addictive behaviour (Miguel-Hidalgo et al. 2006, Volkow and Fowler 2000), suggesting specific neural pathways, associated with glutamatergic expression and the *GRIN2B* gene in the development of AUD.

This study has some strengths and limitations that must be considered when interpreting our findings. For a gene-imaging study our cohort (AUD=58, HC=58) is relatively small, and future brain imaging studies would benefit from increasing the sample. While, we conducted a powerful and rigorous pre-analysis of the SNPs most associated with previously defined brain volumes of interest implicated in AUD, we cannot rule out false negative findings. Also, the regions identified from the initial exploratory analysis were not found in the ANCOVA analysis, possibly due to a lack of power. In addition, our brain imaging method might have been flawed by the trans-axial to saggital acquisition differences, although our preliminary analyses of potential artifacts between these two approaches revealed no significant effect.

In conclusion, the *GRIN2B* gene, involved in glutamatergic signalling, may be associated with developmental differences in brain regions involved in various processes including reward processing. Such differences may play a role in risk for AUD. These findings deserve further detailed investigation in larger cohorts.

Authors Contributions

Acknowledgements

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Statement of interest

Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional committees on human experimentation and with the Helsinki Declaration of 1975, as revised in 2008.

Figure Legends

Figure 1: Smaller volume in left OFC for AUD individuals with GRIN2B rs890 AA genotype

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