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Genetic variation within *GRIN2B* in adolescents with alcohol use disorder may be associated with larger left posterior cingulate cortex volume

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Abstract

Objective—Brain structure differences and adolescent alcohol dependence both 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 age and gender-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.

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Authors Contributions

SD co-ordinated the molecular genetic work, performed the statistical analysis, and helped to draft the manuscript. SJB performed the brain imaging and statistical analysis, and helped to draft the manuscript. VC participated in the study design and coordination and helped to draft the manuscript. GF conceived of the study and participated in its design and coordination. RR conceived of the study and participated in its design and coordination. DJS conceived of the study, participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Statement of interest

The authors declare that they have no competing 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.

Results—From the exploratory analysis, the GRIN2B SNP rs219927 was associated with brain volume in the left posterior cingulate cortex (p -value < 0.05), whereby having a G-allele was associated with a bigger volume.

Conclusion—The *GRIN2B* gene is involved in glutamatergic signalling and may be associated with developmental differences in AUD in brain regions such as the posterior cingulate cortex. Such differences may play a role in risk for AUD, and deserve more detailed investigation.

Keywords

alcoholism; GRIN2B receptor; magnetic resonance imaging; adolescent

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 (1), right hippocampus (2,3), amygdala (4) and grey and white matter (5,6). 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 (7). Brain structure has considerable heritability (8–10). Genetic variations 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 in the alteration of brain structure and function in adolescents with AUD and alcohol-related phenotypes (11–14). Furthermore, in adults with AUD and alcohol-use related phenotypes, alterations in brain volume are associated with variation in the genes GABRA2, BDNF, glutamate receptor, ionotropic, N-methyl D-aspartate 2B (GRIN2B) and neuregulin 1 (NRG1) (15,16). 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 a broader range of genetic variants previously implicated across various psychiatric disorders are associated with variation in brain volume specifically 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, with a lifetime dosage not exceeding 76 units of alcohol. Both the AUD and HC groups are of mixed ancestry ethnicity. 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) (17) was administered by a fully qualified and licensed psychiatrist to determine whether any of the participants had current or past psychiatric symptoms (6). Additionally, the Timeline Followback (TLFB) procedure was used to determine lifetime history of alcohol use and drinking patterns (18). Childhood adversity was measured by the 28-item Childhood Trauma Questionnaire- Short Form (CTQ-SF) (19).

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 ms, 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 (6).

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 (20) in SPM8 (www.fil.ion.ucl.ac.uk/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

Exploratory analysis using Plink—As an exploratory step, 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 (6,16,21). 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 (21). All tests were corrected for multiple comparisons (for multiple SNPs) using the Bonferroni correction method.

Voxel-Based Morphometry—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 (primary analysis) as well as post-hoc t-tests were implemented using VBM in the SPM8 package (<http://www.fil.ion.ucl.ac.uk/spm8>). 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 (Mann Whitney U-test p -value= 0.159; Table 1). 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 (combination of wine and fruit juice), one glass of wine, or one 43g shot of liquor (on its own, or in a mixed drink). As expected, adolescents with AUDs had significantly higher lifetime doses of alcohol than the HC group (Mann Whitney U-test p -value<0.001; Table 1). The median total CTQ score was 36.0 and 42.0 for the HC and AUD group, respectively.

Statistical Analysis

Exploratory analysis using Plink—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). From the exploratory 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 (primary analysis) and post-hoc t-tests for brain volume and genotype (rs219927 and rs890 in *GRIN2B* in two separate ANCOVA analyses) were conducted using VBM. For rs890, a main effect (primary analysis) of group was found for the right inferior temporal gyrus (Table 2); however no significant (FWE corrected) main effects for genotype or interactions were obtained (see Table S2 for uncorrected findings). No significant (FWE corrected) associations were found for rs219927 (see Table S1 for uncorrected findings). The uncorrected findings for rs890 and rs219927 are described below.

The ANCOVAs (primary analysis) showed a main effect for rs219927 in the left orbitofrontal cortex (OFC) ($x=-21$, $y=37$, $z=-16$, uncorrected p -value< 0.05) (Table S1). 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 (uncorrected p -value< 0.001).

(Table S1). For rs890, a main effect of genotype (primary analysis) 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 S2). In addition, a genotype by group interaction (primary analysis) 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 S1) (Table S2).

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 within the gene *GRIN2B*, is associated with larger left posterior cingulate cortex volume in AUD.

The initial exploratory analysis showed that the *GRIN2B* SNP rs219927 may be associated with larger volume in the left posterior cingulate.. *GRIN2B* (12p12) encodes the 2B subunit of the ionotropic *N*-methyl-D-aspartate (NMDA) glutamate receptor (22) and in brain tissue is primarily expressed in the fronto-parieto-temporal cortex and the hippocampal pyramidal cells (23). 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 (24). 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 (25). Our findings support previous research implicating the *GRIN2B* gene in AUD, and linking it to the posterior cingulate, although in adults the volume is smaller not larger (16). Speculatively, it may be that a relationship exists between age, alcohol exposure and brain volume in the posterior cingulate cortex. In other words, children and adolescents' behaviour are influenced strongly by reward processes, whereas as we age and the prefrontal cortex develops, there is a greater attempt by executive functions to modulate reward and impulsive responses to stimuli such as alcohol. Increased posterior cingulate cortex activation positively correlates with a loss of behavioural control over alcohol in response to craving cues in individuals exposed to alcohol (26). While it is still not entirely clear as to how the posterior cingulate cortex contributes to AUD in adolescents; the volume of this region appears to differ in relation to variation in *GRIN2B*.

In the current investigation, uncorrected findings showed that the *GRIN2B* SNPs rs219927 and SNP rs890 may be associated with smaller volume in the OFC, a brain region involved in the process of decision making (7) and reward-related behaviour in response to taste, smell and visual cues (9). 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 (30). Damage to, or deficits in this circuitry therefore, may contribute to impairments in executive functioning, emotion regulation and decision making observed in those with 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 sagittal 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 such as the posterior cingulate cortex. Such differences may play a role in risk for AUD. These findings deserve further detailed investigation in larger cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 in the left posterior cingulate cortex

Limitations

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

Table 1

Median values and interquartile range for cohort characteristics

Variable	HC			AUD			HC vs AUD p-value (Mann Whitney U-test)
	Females (n= 33)	Males (n= 25)	Total (n=58)	Females (n= 33)	Males (n= 25)	total (n=58)	
Age (years)	14.71(15.14–14.35)	15.07(15.54–14.32)	14.77 (15.33–14.35)	14.89 (15.64–14.06)	15.17 (15.57–14.52)	14.98(15.60–14.41)	0.159
Education (years)	8.0 (8.0–7.0)	8.0 (8.0–7.0)	8.0 (8.0–7.0)	8.0 (9.0–7.0)	8.0 (8.0–7.0)	8.0 (8.0–7.0)	0.577
% Afrikaans-speaking	69.7	84	75.9	60.6	72.0	65.5	0.221 ^a
Alcohol life dose units ^b	1.0 (9.00–0)	1.0 (3.5–0)	1.0 (4.25–0.0)	892.0 (1980.0–442.0)	1012.0 (2076.0–396.0)	962.0 (1987.0–429.0)	<0.001
CTQ-total score	35.0 (46.0–29.0)	39.0 (52.0–30.5)	36.0 (49.0–29.75)	42.0 (48.0–34.5)	42.0 (65.5–33.0)	42.0 (52.0–34.75)	0.062

^a Pearson chi-squared test (d.f. =1).^b Alcohol life dose was measured in units. One unit was defined as one beer or wine cooler (combination of wine and fruit juice), one glass of wine, or one 43g shot of liquor (alone or in a mixed drink).

Table 2

2 x 2 ANCOVA with SNP rs890 (Group x Genotype) and matched for age, gender, group and protocol (with total matter volume, age, gender and total CTQ as covariates of no interest).

Brain region	MNI Coordinates			Brodmann Area	Cluster Size (Voxels)	Z-statistic	P-value (FWE corrected)
	x	y	z				
ANCOVA ANALYSES:							
<i>Main effect of GROUP</i>							
Right inferior temporal gyrus	33	1	-43	20	3519	4.60	0.005
<i>Genotype x Alcohol Group interaction</i>							
Right parahippocampal gyrus	33	1	-43	20	1901	3.88	0.07

MNI=Montreal Neurological Institute Coordinates, FWE=Family Wise Error corrected for multiple comparison (at the peak voxel level); CTQ=Childhood Trauma Questionnaire