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ORIGINAL ARTICLE

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Sex differences in $GABA_A$ receptor subunit transcript expression are mediated by genotype in subjects with alcohol-related cirrhosis of the liver

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Abstract

Male and female human subjects show contrasting propensities to misuse drugs of addiction, including alcohol. These differences lead to different psychological and neurological consequences, such as the likelihood of developing dependence. The pattern and extent of brain damage in alcohol-use disorder cases also varies with comorbid disease. To explore mechanisms that might underlie these outcomes, we used autopsy tissue to determine mRNA transcript expression in relation to genotype for two $GABA_A$ receptor subunit genes. We used quantitative Real-Time PCR to measure *GABRA6* and *GABRA2* mRNA concentrations in dorsolateral prefrontal and primary motor cortices of alcohol-use disorder subjects and controls of both sexes with and without liver disease who had been genotyped for these $GABA_A$ receptor subunit genes. Cirrhotic alcohol-use disorder cases had significantly higher expression of *GABRA6* and *GABRA2* transcripts than either controls or non-cirrhotic alcohol-use disorder cases. Differences were observed between sexes, genotypes and brain regions. We show that sex differences in subjects with *GABRA6* and *GABRA2* variants may contribute to differences in susceptibility to alcohol-use disorder and alcohol-induced cirrhosis.

KEYWORDS
alcohol misuse, genotype–phenotype interactions, human brain, qRT-PCR

1 | INTRODUCTION

Alcohol-use disorder (AUD) is common and complex. It has been attributed to both genetic and environmental factors.[1,2] Associations between AUD and consequent societal and economic impacts have been well documented.[3] These can include reduced financial output from loss of productivity, higher healthcare expense for comorbid diseases and accidents from driving under the influence or alcohol-related aggression.[4] Some types of AUD that are reportedly not strongly heritable are found more often in females.[5]

Several neurotransmitters mediate the effects of alcohol. The CNS expression of subunit genes that modulate receptor structure and function is altered in acute and chronic AUD cases.[6] In a

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preliminary study,[7] we found that males and females showed different associations between AUD and a range of markers in genes associated with transmission in tissue available at autopsy, as well as in samples from living subjects who were undergoing treatment for AUD. A primary aim of the present study was to extend this analysis to a larger subset of the treatment group.

At similar levels of consumption, alcohol misuse has differing neurological and physiological outcomes in males and females.[8,9] Genome-wide Association Studies of AUD and other substance-misuse disorders have reported links between $GABA_A$-receptor-associated genes and alcohol misuse.[10,11] However, Song and colleagues using the COGA cohort found no evidence for an association of *GABRA1* or *GABRA6* with alcoholism.[12] Excessive alcohol consumption leads to deleterious neuropathology (neuronal cell loss and reduced dendritic arborisation) and psychological dysfunction[13] in the CNS, particularly in the dorsolateral prefrontal cortex (DPC). Area-specific variations in the expression of $GABA_A$ receptor subunit genes might affect the functionality of the receptor locally, and thereby influence the development of brain damage.[14,15] Comorbid liver cirrhosis exacerbates brain damage because the defective liver fails to remove neurotoxins.[16]

Many single nucleotide polymorphism (SNP) variations in $GABA_A$ receptor subunit genes (*GABR*s) are significantly associated with AUD (see, e.g., Stephens et al., 2017[5] for review) and could provide a link between genotype and AUD phenotype. Associations between *GABRA6* rs3219151, as well as several *GABRA2* SNPs, and AUD were reported in Li et al., 2014.[17] Others have confirmed associations between chromosome 5 *GABR* SNPs and AUD.[18,19] Our own preliminary studies (*v.i.*) suggested that the *GABRA6* association might be sex-linked, and we wanted to confirm this and explore it further with expression studies.[7] Another Chromosome 5 *GABR* locus in our pilot study, *GABRG2* rs211013, showed associations that appeared to be differentially sex-linked to comorbid liver disease. There is evidence from animal studies that $\gamma 2$ and $\alpha 6$ expression may be coordinated; we sought to explore this in our regional human-cortex samples. Local differences in expression might also be the basis of differences between male and female AUD cases. To explore effects found in the genetic analyses, we conducted a study on the expression of $GABA_A$ receptor subunit transcripts in relation to genotype and sex. For this, we measured the levels of *GABRA6* and *GABRA2* transcripts in DPC and Primary Motor Cortex (PMC; control region) tissue obtained at autopsy from AUD cases and non-AUD controls. Contrasts were explored between the sexes in genotype and regional expression.

2 | MATERIALS AND METHODS

2.1 | Subjects

2.1.1 | Cohort of living subjects

Alcohol withdrawal management project (AWMP) subjects, which comprised living AUD cases and non-AUD controls, provided blood and saliva samples. For this study leukocytes were mainly used for DNA extraction; only six AUD subjects and five non-AUD controls chose to offer saliva for DNA, although plasma markers of comorbid disease were also assessed. AUD patients were recruited from the Hospital Alcohol and Drug Service of the Royal Brisbane and Women's Hospital and the Biala Alcohol and Drug Service, Brisbane. The former is a 5-day in-patient treatment program, while the latter is an out-patient treatment program. Both provide withdrawal management for alcohol dependence. All subjects fulfilled DSM-IV criteria for alcohol dependence diagnosed by addiction specialists. Informed written consent was obtained before data collection. Exclusion criteria included concurrent substance abuse or dependence (except nicotine, cannabis and benzodiazepines), and endocrine, neurological or other DSM-IV Axis I and Axis II disorders except major depression and anxiety. Demographics and detailed histories of alcohol and drug use came from a structured interview administrated by a trained researcher.

Non-AUD Controls who had never abused or been dependent on any substance except nicotine by DSM-IV criteria were recruited through poster and staff newsletter advertisements. Substance use was monitored by the Drug Abuse Screening Test.[20] Exclusion criteria for controls were same as those for alcohol-dependent subjects, except for a lower age limit of 25 years to match the age range of the alcohol-dependent subjects. Self-report questionnaires on demographics and history of alcohol and drug use were provided after written informed consent was obtained and were returned to the researcher at the next meeting.

Presumptive indications of cirrhosis of the liver included higher blood plasma levels of the liver enzymes alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP), as well as higher levels of bilirubin and lower levels of albumin and total protein.

DNA extraction from AWMP leukocytes

Blood samples (10 ml) were drawn from the antecubital vein into an EDTA-coated tube. The samples were centrifuged at 3000*g* for 15 min at 4°C and the buffy coat collected. DNA was extracted from 200 μl buffy coat samples using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

DNA extraction from buccal cells

Mouthwash samples were centrifuged at 2000*g* for 10′ at room temperature. The pellet was resuspended in 3 ml of cell lysis solution (10 m**M** Tris–HCl, 25 m**M** EDTA, 0.5% SDS; pH 8), then 15 μl Protease K (20 mg/ml) added. After incubation at 55°C for 1 h, 1 ml 5 **M** ammonium acetate was added to precipitate protein. The mixture vortexed for 30 s, placed on ice for 20′, then centrifuged at 2000*g* for 10′ at 4°C. The supernatant was transferred into a 15 ml Falcon tube and 3 ml absolute ethanol added. The solution was stored at −20°C overnight, then centrifuged at 2000*g* for 10′ at room temperature. The pellet was washed with 2 ml 75% ethanol and centrifuged at 2000*g* for 3′ at room temperature. The DNA pellet was air-dried for 15′ and resuspended in 200 μl TE buffer (10 m**M** Tris–HCl, 1 m**M** EDTA, pH 8).

All procedures had been approved by the Human Ethics Research Committees of RWBH (ref. 2008/040), The Prince Charles Hospital (ref. HREC/10/QPCH/63) and The University of Queensland (ref. 2009001255).

2.1.2 | Autopsy cohort

DNA and mRNA were prepared from autopsy brain tissue obtained from AUD cases and non-AUD controls. These samples came from the Queensland Brain Bank (QBB) and the NSW Tissue Resource Centre. All subjects were Northern European in origin, age at death 18–93 years; no schizophrenic case was included. Alcohol-use data were obtained from medical records. The mean intake in AUD cases was more than 80 g of ethanol per day for 30 years on average; non-AUD controls had consumed less than 20 g ethanol/day or were teetotal. Samples were also collected from AUD subjects with and without pathologically confirmed cirrhosis of the liver, and from a few subjects with liver disease not caused by AUD. Autopsy subject details are shown in Table S1.

2.2 | Genotyping

2.2.1 | Assay background

In our preliminary study,[7] genotyping was completed on SNPs associated with subjective effects of alcohol ingestion[5,21] that might increase the risk of developing AUD (rs279871, rs279858 and rs279845), and SNPs we identified within functional or structural motifs in the α2 subunit (*v.i.*). We examined genotype–phenotype relationships. We also explored SNPs associated with other neurotransmitters.

Pilot study data were analyzed with the SNP and Variation Suite.[22] Each SNP was first tested separately for its association with AUD (Case vs. Control), levels of alcohol consumption and with two comorbid phenotypes (liver-dependent and -independent). For deeper analysis of associations with AUD, we explored the Chromosome 5q31-q35 *GABR* cluster: *GABRA1*, *GABRA6*, *GABRB2*, and *GABRG2*,[23] and two SNPs in the *RGS4* gene. Of the $GABA_A$ subunit genes, only the *GABRA6* SNP survived in the Validation Cohort.[7] For the *GABRG2* SNP in the Discovery Cohort, the association was only significant ($p < 0.05$) when male and female subjects were analyzed separately, which suggests a sex-linked role for this allele in AUD, but this putative association did not survive validation.

Haplotype blocks were then identified and tested for association using the regression tendency procedure (Golden Helix). We performed regression analysis to identify SNPs that could predict the risk of AUD. The SNP effect estimates were computed[22] under the model previously described[24] and the EMMAX method.[25]

In the present work, we utilised online bioinformatics tools to identify novel *GABRA2* SNPs. All *GABRA2* SNPs were noted at dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).[26] Potentially deleterious SNPs were detected by the SIFT and PolyPhen indices of the Variant Effect Predictor tool (VEP; http://www.ensembl.org/info/docs/tools/vep/index.html).[27]

Literature searches identified sequences in α2 subunit protein domains that are involved in $GABA_A$ receptor functions, such as GABA binding, Cl^- influx, interaction with ethanol, receptor clustering, and localization at synapses (the gephyrin-binding site). Relevant information on these topics was not available for *GABRA2*, only for *GABRA1*. Hence, the sequences of the α1 and α2 isoforms were aligned and compared using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/, discontinued).[28] We then used Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index)[29] and Chimera 1.10.2[30] to build a 3D model of the α2 subunit and identify amino acid residues involved in each function. It was thus possible to select SNPs that correspond to amino acids present in, or close to, coding sequences for relevant protein domains.

Estimation of *GABR* SNP frequencies were performed on both autopsy and AWMP samples using TaqMan® assays as per the manufacturer's instructions (Catalogue ID: 4351379, Thermo Fisher Scientific Australia P/L, Scoresby, VIC, Australia). Examples of assays for the novel SNPs are shown in Figures S1–S3. Genotypes were analyzed by χ^2 tests using an online tool,[31] looking first for overall associations and then partitioning the subjects by sex and the presence of co-morbid liver disease. In living subjects from the AWMP cohort, the presence of cirrhosis was inferred from liver function tests. In tissue obtained from subjects at autopsy, micronodular cirrhosis was confirmed pathologically.

2.3 | Transcript expression

RNA was extracted from PMC and DPC tissues obtained from autopsy brains as outlined previously.[32] Most subjects could act as their own control because tissue from both brain regions was available. RNA Integrity Number (RIN values) were quantified on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc, Santa Clara, CA). RNA was reverse-transcribed to cDNA using GoScript (Promega, Alexandria, NSW, Australia) and aliquoted into a 96-well plate. RIN values are displayed in Table S2. It should be noted that RIN values are quite low in human autopsy samples, but do permit even transcriptome studies, including in AUD cases and controls.[33] Here we compared the expression of targeted transcripts; qRT-PCR was performed with primer pairs designed for each mRNA isomer. We went to great lengths to limit plate-to-plate artefacts by including a pooled standard on every plate. If either target or *GAPDH* housekeeper mRNA in the pooled sample failed to amplify, or their C_T values differed by more than 5% from the average, we discarded all C_T values obtained from that plate.

2.3.1 | Standard curves and gel electrophoresis

GAPDH (housekeeping gene) and *GABRA6* and *GABRA2* (target genes) were used to generate standard curves for qRT-PCR reactions on the QuantStudio 6 Flex 384-well plate instrument with TaqMan® (Applied

Biosystems/Thermo Fisher) as the detection system. Aliquots from these plates (stored at −20°C until required) were used for 2% agarose gel electrophoresis (Figure S4).

2.3.2 | Quantitative real-time PCR

Two qRT-PCR assays were performed; primer concentrations were optimised at 100 ng based on the standard curve data. Each 384-well plate used aliquots diluted from the plate of cDNA samples and a 'supermix' consisting of the TaqMan™ Fast Universal PCR Master Mix (2×) (Applied Biosystems), *GAPDH* (VIC) and either *GABRA2* or *GABRA6* (FAM); the target and housekeeper transcripts were assayed both in combination and separately in different aliquots to test for interference in amplification efficiency. Both reaction sets had a tissue-negative control and two wells with cDNA pooled from eight subjects at a 5× dilution to facilitate comparisons across assays. Samples were amplified in triplicate. The cycle threshold (C_T) was obtained from the point where the PCR product reached a pre-set threshold. ΔC_T was calculated from the difference in median C_T between the target and housekeeper transcripts. Twenty-eight *GABRA2* and 19 *GABRA6* samples were excluded because of a lack of genotype, RIN or ΔC_T data. Exemplar qRT-PCR assays for each transcript are shown in Figures S5 and S6.

2.3.3 | Statistical analysis of transcript expression

Statistica 7.0 (Tulsa, OK) was used to compute analyses of covariance (ANCOVA) for *GABRA6* ΔC_T data, with RIN as the covariate. We previously demonstrated that RIN is a valid covariate for normalising targeted transcript expression values in autopsy cortical tissue.[34] Analysis of variance (ANOVA) was sufficient for *GABRA2* ΔC_T data, because RIN had no significant influence (although we used RIN to normalise both *GABRA2* and *GAPDH* transcript C_T values prior to analysis). Variances across males and females, genotypes, and brain regions were investigated. An ANCOVA was performed on T-allele distribution across male and female subjects for *GABRA6*. Newman–Keuls post hoc tests were used to explore significant differences between ΔC_T least-squares mean values from the ANCOVAs and ANOVAs. ΔC_T was used for primary analyses because unlike $2^{-\Delta CT}$ its distribution did not deviate significantly from Normal (Figure S7). Least-squares ΔC_T mean and *SEM* estimates from the AN(C)OVAs were converted to $2^{-\Delta CT}$ mean and *SEM* values for presentation.

3 | RESULTS

3.1 | 3D modelling of $GABA_A$ receptor α2 subunit

Figure 1 shows the 3D rendition of the α2 subunit of the $GABA_A$ receptor, with the functional domains highlighted in different colours. As noted in Methods, this model is based on the known structure of

FIGURE 1 Functional domains of the $GABA_A$ receptor α2 subunit. Yellow: GABA-binding site. Blue: TM2. Turquoise: TM2-TM3 extracellular loop. Green: TM3. Orange: gephyrin-binding site. Pink: cysteine residues

the α1 subunit, and while the two subunits exhibit slight differences in pharmacology,[35] we have previously shown that both proteins are expressed in human cerebral cortex, especially in the areas sampled in for the present study[36] in AUD cases and non-AUD controls.

Based on the presumption that functional domains conform with those in the α1 subunit, the GABA-binding site for α2, which is located in the interface between α and β subunits, would depend on residues F92 to K96 and R147 to I148.[37] Transmembrane domain 2 (TM2) would be formed by residues V279 to S303 and cover the central pore of the ion channel. Amino acid residues in TM2 and TM3 and in the TM2-TM3 extracellular loop are important for the interaction of the receptor with ethanol and other alcohols.[38] The gephyrin-binding site (important for the clustering of $GABA_A$ receptors at synapses) would correspond to amino acids N364 to A375 located in the intracellular loop between TM3 and TM4.[39]

3.1.1 | SNP selection

Sequence alignment and tridimensional modelling of the α2 subunit allowed us to select deleterious SNPs in regions of the α2 subunit protein that are key for correct receptor functioning. Table 1 contains information about all the SNPs analysed in our study. Table 2 contains

TABLE 1 *GABRA2* SNPs selected for analysis

SNP	Location	Exon/intron	Alleles	AA change (codons)	Protein domain	EUR MAF
rs372811835	4:46250552	Exon 10	–/C	V371Gx (GTT/GGTT)	TM3-TM4 loop (gephyrin-binding site)	Unknown
rs41310789	4:46303462	Exon 8	A/G	F285S (TTT/TCT)	TM2 domain	Unknown
rs279871	4:46303716	Intron 7	T/C	–	–	C: 0.4284
rs279858	4:46312576	Exon 5	C/T	K132 (AAA/AAG)	N-terminal loop	C: 0.4284
rs199725032	4:46312694	Exon 5	A/C	F93C (TTT/TGT)	N-terminal loop (GABA-binding site)	unknown
rs279845	4:46327706	Intron 4	T/A	–	–	A: 0.4443

TABLE 2 Non-synonymous *GABRA2* SNPs and their functional outcomes

SNP	Alleles and AA change (codons)	Protein domain	Functional outcome
rs372811835	–/C V371Gx (GTT/GGTT)	TM3-TM4 loop (gephyrin-binding site)	Truncated receptor. Binding to gephyrin compromised ↓ clustering of receptors at synapses.
rs41310789	A/G F285S (TTT/TCT)	TM2 domain	Change of hydrophobic Phe → polar, neutral Ser in TM2 domain, which lines channel pore. Cl^- influx compromised.
rs199725032	A/C F93C (TTT/TGT)	N-terminal loop (GABA-binding site)	Change of hydrophobic Phe → cysteine in GABA-binding site.

data related to the non-synonymous and potentially deleterious SNPs selected.

3.2 | Genotyping

In a pilot study[7] we found sexually divergent regressions of *GABRA6* rs3219151 genotype on consumption ($\beta = +0.264$, $p = 0.015$ for females dependent on age and liver status, $\beta = -0.101$, $p = 0.043$ for males independent of age). We also found sexually divergent regressions of *GABRG2* rs211013 genotype on consumption and age ($\beta = -0.533$, $p = 0.028$ for females, $\beta = +0.396$, $p = 0.015$ for males) as well as on AUD diagnosis ($\beta = -0.177$, $p = 0.031$ for females, $\beta = +0.136$, $p = 0.014$ for males). We did not assay *GABRG2* transcript expression.

For *GABRA2* SNPs, we detected the same genotype at rs37281135 and rs41310789. The assay for rs199725032 was defective and thus genotype and allele distributions could not be assessed with this SNP. For other *GABRA2* SNPs (rs279871, rs279858 and rs279845), there were no significant differences in genotype and allele distributions between AUD groups, cirrhosis cases, AUD groups by sex or cirrhosis cases by sex (Table 3).

For *GABRA6* rs3219151, we detected significant differences in allele and genotype frequencies between AUD and non-AUD males: when male AUD cases were compared with non-AUD male controls, the former had lower frequencies of the C allele and the CC genotype. No such differences were found in females. Genotype and allele distributions did not differ significantly between AUD groups, cirrhosis groups, and cirrhosis groups by sex (Table 4).

It was predicted that $GABA_A$ subunit SNPs would act locally to alter mRNA expression that might differ between males and females. We found evidence for such an effect with the *GABRA6* SNP rs3219151 (*v.i.*).

3.3 | Transcript expression

The *GABRA6* rs3219151 SNP and the *GABRA2* rs279871 SNP were used to partition expression data. The three *GABRA2* SNPs (rs279845, rs279858 and rs279871) are in strong linkage disequilibrium.[40] Because the distribution of $2^{-\Delta CT}$ values deviate significantly from Normal[41] (Figure S7), but quantification was required (i.e., non-parametric statistics were not appropriate), ANOVAs and ANCOVAs were performed directly on C_T values, the distribution of which did not deviate significantly from Normal. Least-squares Mean and *SEM*. C_T values were calculated, and post hoc tests were performed, for each effect before conversion to $2^{-\Delta CT}$ values for presentation. Care was taken when calculating *SEM*. values from the computed ANOVA and ANCOVA output.[42,43] Transcript expression differences by sex, genotype, and brain region were assessed for *GABRA2* (Figures 2 and 5) and *GABRA6* (Figures 3–5). No significant difference ($p > 0.05$) was found between non-AUD controls and AUD cases without liver cirrhosis for either transcript. For the *GABRA6* transcript, significant differences were found for sex, genotype and brain region.

GABRA2 transcript expression trended higher in female AUD cases with cirrhosis of the liver than in the equivalent male AUD cases and was significantly higher ($p < 0.05$) than in non-cirrhotic AUD females and non-AUD control females (Figure 2A). Expression of this transcript did not differ significantly across male subjects. *GABRA2* mRNA expression in cirrhotic AUD CT heterozygotes undifferentiated by sex trended lower than in CC and TT cirrhotic AUD homozygotes but was significantly ($p < 0.05$) higher than in non-cirrhotic AUD CT

TABLE 3 *GABRA2* SNP genotype and allele frequencies

SNP		Non-AUD	AUD	*p*-value (between AUD groups)	*p*-value (between AUD groups by sex)	No Cirrhosis	Cirrhosis	*p*-value (between Cirrhosis groups)	*p*-value (between Cirrhosis groups by sex)
rs279871	C	68 (40.5%)	318 (45.3%)	0.258	Male: 0.382	331 (44.6%)	55 (43.0%)	0.730	Male: 0.783
	T	100 (59.5%	384 (54.7%)		Female: 0.222	411 (55.4%)	73 (57.0%)		Female: 0.352
	CC	13 (15.5%)	67 (19.1%)	0.499	Male: 0.657	70 (18.9%)	10 (15.6%)	0.811	Male: 0.574
	CT	42 (50.0%)	184 (52.4%)		Female: 0.398	191 (51.5%)	35 (54.7%)		Female: 0.502
	TT	29 (34.5%)	100 (28.5%)			110 (29.6%)	19 (29.7%)		
rs279858	C	100 (59.5%)	384 (54.5%)	0.243	Male: 0.359	411 (55.4%)	73 (56.2%)	0.872	Male: 0.611
	T	68 (40.5%)	320 (45.5%)		Female: 0.222	331 (44.6%)	57 (43.8%)		Female: 0.352
	CC	29 (34.5%)	100 (28.4%)	0.481	Male: 0.638	110 (29.6%)	19 (29.2%)	0.916	Male: 0.634
	CT	42 (50.0%)	184 (52.3%)		Female: 0.398	191 (51.5%)	35 (53.8%)		Female: 0.502
	TT	13 (15.5%)	68 (19.3%)			70 (18.9%)	11 (16.9%)		
rs279845	C	68 (40.5%)	318 (45.3%)	0.258	Male: 0.382				Male: 0.783
	T	100 (59.5%	384 (54.7%)		Female: 0.222				Female: 0.352
	CC	14 (16.3%)	78 (22.2%)	0.324	Male: 0.383	80 (21.4%)	12 (18.5%)	0.820	Male: 0.640
	CT	43 (50.0%)	179 (50.9%)		Female: 0.301	189 (50.7%)	33 (50.8%)		Female: 0.174
	TT	29 (33.7%)	98 (27.0%)			104 (27.9%)	20 (30.8%)		

TABLE 4 GABRA6 SNP genotype and allele frequencies

SNP		Non-AUD	AUD	*p*-value (between AUD groups)	*p*-value (between AUD groups by sex)	No Cirrhosis	Cirrhosis	*p*-value (between Cirrhosis groups)	*p*-value (between Cirrhosis groups by sex)
rs3219151	C	67 (50.0%)	265 (41.4%)	0.068	Male: 0.029	279 (43.2%)	53 (41.4%)	0.710	Male: 0.410
	T	67 (50.0%)	375 (58.6%)		Female: 0.668	367 (56.8%)	75 (58.6%)		Female: 0.620
	CC	17 (25.4%)	51 (15.9%)	0.143	Male: 0.033	56 (17.3%)	12 (18.8%)	0.635	Male: 0.230
	CT	33 (49.3%)	163 (50.9%)		Female: 0.734	167 (51.7%)	29 (45.3%)		Female: 0.801
	TT	17 (25.4%)	106 (33.1%)			100 (31.0%)	23 (35.9%)		

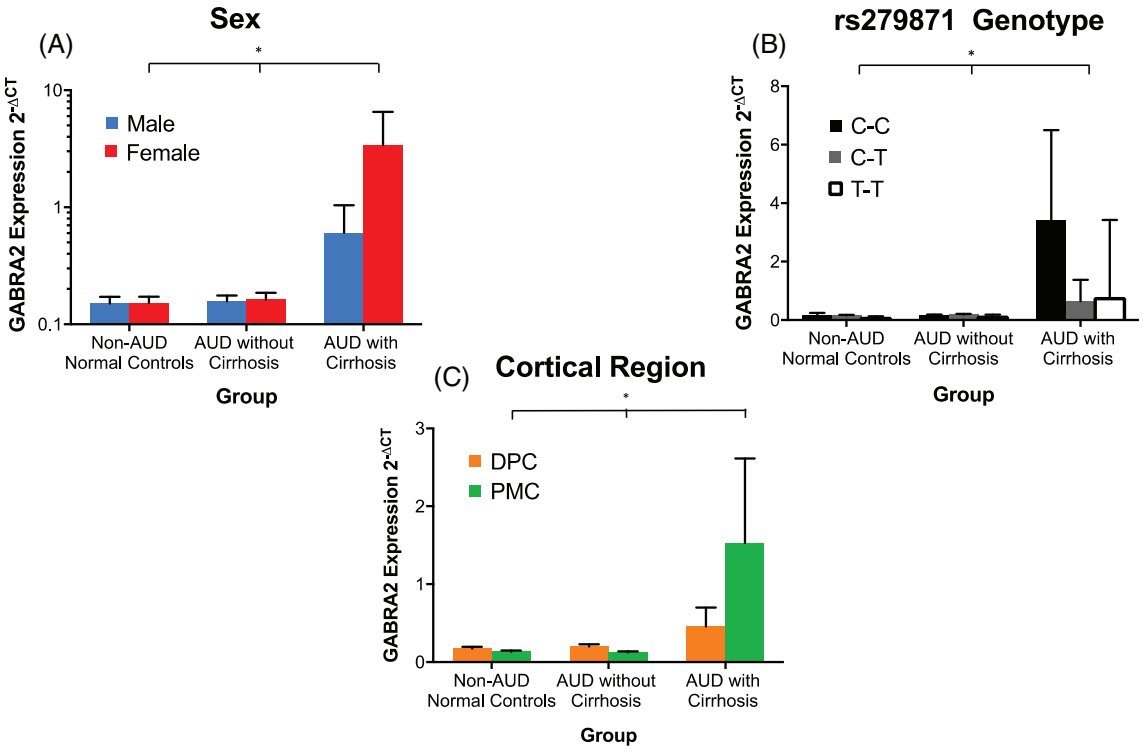

FIGURE 2 Expression of *GABRA2* mRNA transcripts in autopsy tissue. Two-way ANOVAs were performed on C_T measures of *GABRA2* mRNA levels from each qRT-PCR assay normalised to *GAPDH* C_T and corrected for RIN. Factors analyzed were groups, sex and genotype based on the *GABRA2* SNP rs279871. Subjects were divided into the Groups shown below the abcissæ. Least-squares mean ΔC_T and SEM estimates computed in each ANOVA were converted to $2^{-\Delta CT}$ values for presentation. (A) Sex (log_{10} graph): Female AUD cases with cirrhosis showed significantly higher *GABRA2* mRNA expression than the *GABRA2* mRNA expression in females in both other groups. (B) Genotype: significantly higher *GABRA2* mRNA expression in combined (male + female) AUD cirrhotic subjects with CT genotype than in subjects with CT genotype in both other groups. (C) Cortical region: significantly higher *GABRA2* mRNA expression in PMC in combined AUD cirrhotic cases than *GABRA2* mRNA expression in PMC from subjects in both other Groups. Columns are mean ± *SEM GABRA2* mRNA $2^{-\Delta CT}$ values; *, $p < 0.05$ by Newman–Keuls post hoc test on ΔC_T values in ANOVA

heterozygotes and non-AUD control CT heterozygotes (Figure 2B). *GABRA2* mRNA expression was significantly higher ($p < 0.05$) in PMC of AUD cases with cirrhosis of the liver undifferentiated by sex or genotype than in PMC of non-cirrhotic AUD cases and non-AUD controls. Expression of this transcript did not differ significantly across DPC (Figure 2C).

In subjects not differentiated by sex, *GABRA6* CT heterozygote AUD cases with cirrhosis showed higher *GABRA6* mRNA expression than both homozygotes in the same set of AUD cases (Figure 3B). *GABRA6* transcript expression in CT heterozygote AUD cases was higher than in all zygosities of both the other sets of cases (Figure 3B). In subjects not differentiated by sex or genotype, *GABRA6* mRNA expression was higher in the PMC of cirrhotic AUD cases than in the DPC of the same subjects or in the PMC of both other Groups. *GABRA6* mRNA expression in DPC did not vary significantly across Groups (Figure 3C). The magnitude of *GABRA2* expression was markedly higher overall than the magnitude of *GABRA6* expression (Figure 2 cf. Figure 3). For both transcripts, the level of expression was higher in AUD cases with cirrhosis than in AUD cases without comorbid liver disease.

Female AUD cases with at least one T allele showed significantly higher *GABRA6* mRNA expression than female AUD CC homozygotes (Figure 4B). *GABRA6* transcript expression in male AUD cases showed no relationship with the T allele, although it was higher in male AUD cases than in male non-AUD controls (Figure 4A,B).

ANCOVA of ΔC_T expression with RIN as covariate showed a significant Group × Sex interaction solely for *GABRA6* mRNA (Figure 5A vs. Figure 5B). The relative expression differential between males and females was greater for *GABRA6* expression than for *GABRA2* expression in both non-AUD Normal Controls and AUD cases (Figure 5).

4 | DISCUSSION

The present study aimed to investigate the effects of tag SNPs in *GABRA6* and *GABRA2* that are associated with AUD. Overall, the results suggest the rs3219151 mediates expression of *GABRA6* transcripts, and that there were sex differences in expression, while rs279845, rs279858 and rs279871 mediate expression of *GABRA2* transcripts. An understanding of the effects of SNPs in *GABAR*s and

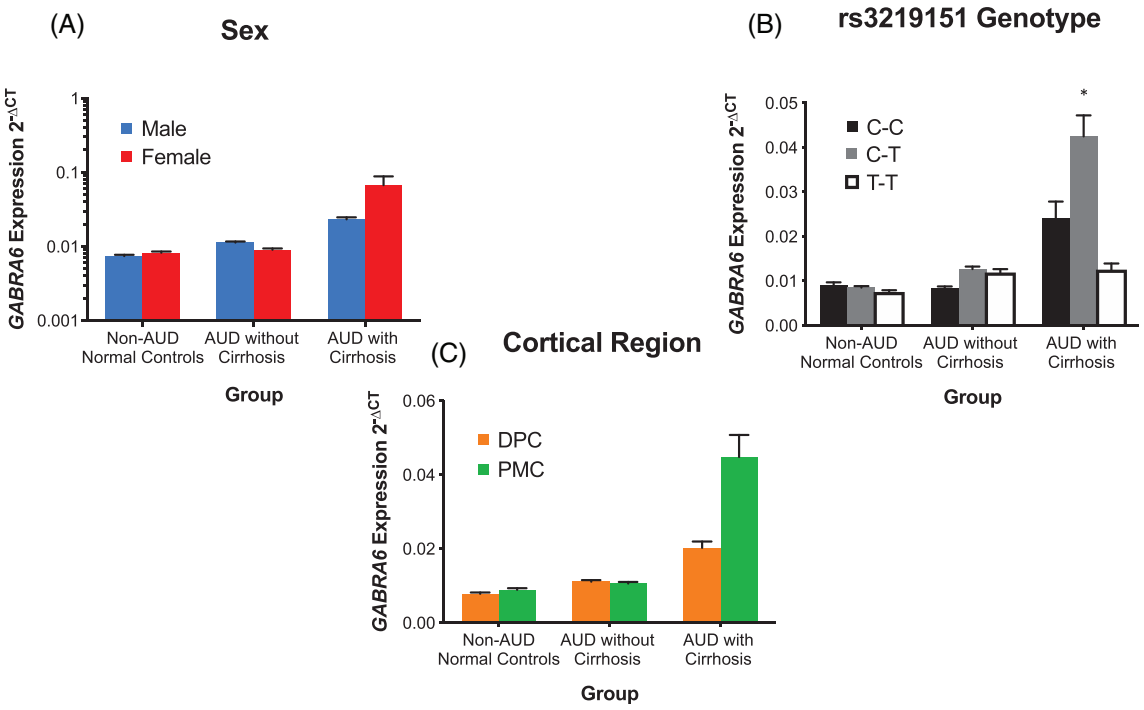

FIGURE 3 Expression of *GABRA6* mRNA transcripts in autopsy tissue. Two-way ANCOVAs were performed with RIN as covariate to estimate *GABRA6* mRNA ΔC_T values (corrected for *GAPDH* C_T) against groups, sex and *GABRA6* SNP rs3219151 genotype. Legend as for Figure 2. (B) *GABRA6* mRNA expression in combined (male + female) C-T heterozygote AUD cases with cirrhosis were significantly higher than *GABRA6* mRNA expression in all other subjects and zygosities. *GABRA6* mRNA ΔC_T values did not differ significantly in either of the other two parts of this analysis. Columns are mean ± *SEM GABRA6* mRNA $2^{-\Delta CT}$ values; *, $p < 0.05$

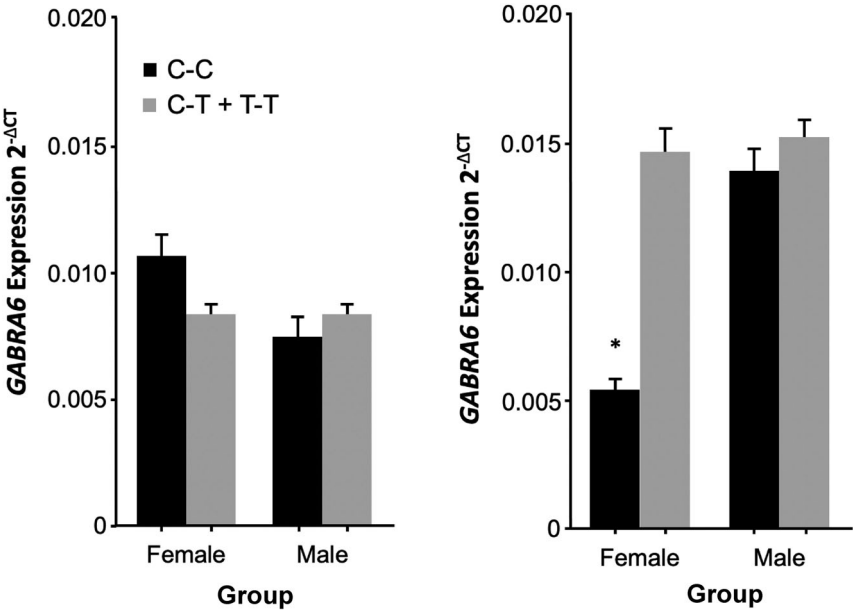

FIGURE 4 Expression of *GABRA6* mRNA transcripts in autopsy tissue from female and male subjects by *GABRA6* SNP rs3219151 genotype. Legend as for Figure 2. (A) non-AUD normal controls. No significant difference across all subjects. (B) Combined AUD cases with and without cirrhosis of the liver. Female *GABRA6* SNP rs3219151 CC homozygotes showed significantly lower *GABRA6* mRNA expression than females with at least one T allele. No significant difference across male cases. Columns are mean ± *SEM GABRA6* mRNA $2^{-\Delta CT}$ values; *, $p < 0.05$

the association of risk alleles with sex differences might yield new approaches to treatments for alcohol addiction.

AUD involves cycles of dysfunctional tolerance to ethanol; withdrawal after abstinence; craving; and persistent drinking despite the subject's awareness of adverse consequences.[17] CNS damage is more severe in AUD cases with a range of comorbid diseases that include cirrhosis of the liver.[44–46] A defective liver is less effective in removing toxins, especially ammonia, from the blood.[47] Excessive brain ammonia is a key driver of brain damage in these patients.[16,48] Other co-morbidities such as severe thiamine deficiency (Wernicke-Korsakoff syndrome) exacerbate brain damage in AUD,[49] but are not considered further here.

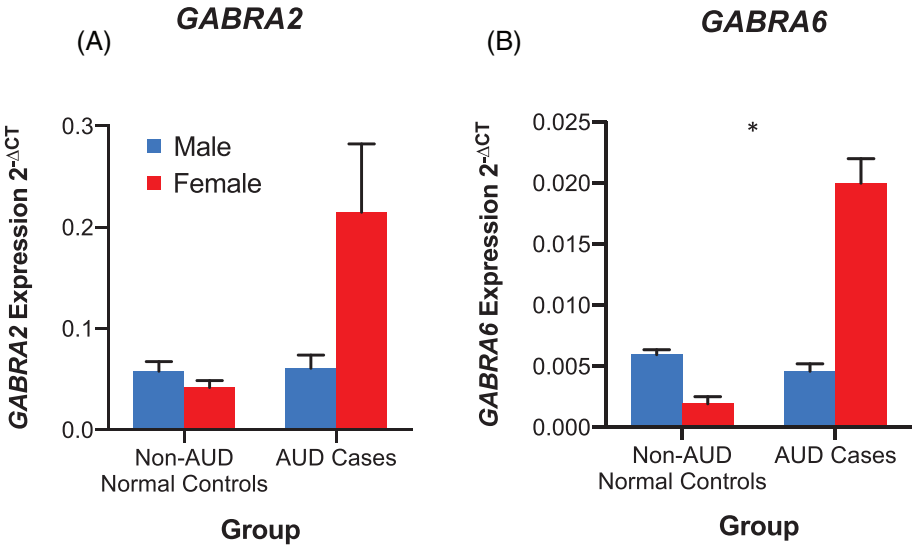

FIGURE 5 Comparison of expression of *GABRA*$_A$ subunit transcripts in AUD and non-AUD cases. Legend as for Figure 2; ANCOVA. (A) *GABRA2* mRNA. Groups Main Effect NS, $p > 0.05$. (B) *GABRA6* mRNA. *, Groups Main Effect significant, $p < 0.05$

GABA is the primary inhibitory neurotransmitter in mammalian brain. It regulates the excitability of neurones[50] by binding to a range of receptors that are widely distributed in the CNS. Activation of GABA$_A$ trans-membrane ligand-gated ion channels leads to an influx of Cl^- ions that hyperpolarise the target neurone.[12,51] The effects are area-specific and mediated by different GABA$_A$ subunits.[12] GABA$_A$ receptor subunit genes (*GABR*s) have strong associations with AUD and many behavioural effects of alcohol.[12] These include impaired motor coordination and greater sedation, more-severe withdrawal symptoms, and variations in alcohol preference. The *GABRA6* gene may be involved in the actions of ethanol.[52] Alternate splicing is common in *GABR* genes: nine α2 sequences have been found.[53] Although human variants of *GABRA6* have not been found in a key study, the list of alternatively spliced forms of *GABR*s published therein only included forms known to be transcribed in vivo.[53] Variants occur in the 5′UTR and in introns, as well as coding regions. The main function of alternate splicing may be the regulation of subunit expression.[54]

A heritable component is demonstrable with AUD, but the pattern is not clear.[55] Genome-wide Association Studies have highlighted the involvement of *GABR*s.[10,11] However, identifying SNPs that mediate heritability is difficult due to the variable inheritance of haplotype blocks. Previous studies have focused on *GABR* SNPs within blocks associated with AUD. Males and females differ in their responses to alcohol[56]: males are more prone to AUD than females, but females are more susceptible to alcohol-related brain damage despite consuming less and having shorter drinking histories.[57,58] Type I alcoholism[59] is not strongly heritable, but is found more often in females.[5] Sex can affect receptor functionality in human subjects[60]: AUD-related SNPs might change receptor-transcript expression, thereby altering the individuals' likelihood of developing AUD. The present data are consistent with the proposition that the *GABRA6* T allele is potentially protective in females. Li et al.[17] used the same SNP for *GABRA6* as we did, but a different set of *GABRA2* SNPs. They found a significant association of AUD with *GABRA6* rs3219151, as well as with the *GABRA2* rs567926, which is 9.9 kb 3′, and rs279858 as studied here. There is evidence that other SNPs in *GABARs* contribute to susceptibility to AUD and related phenotypes.[61] The SNPs chosen for the present study are associated with AUD[62] or have been thoroughly characterised (see Supplementary material).

There are two main clusters of GABA$_A$ subunit genes. Loci at Chromosome 4p12 comprise *GABRA2*, *GABRA4*, *GABRB1* and *GABRG1*, those at Chromosome 5q31–35 comprise *GABRA6*, *GABRA1*, *GABRB2* and *GABRG2*[17,18,63]; the Chromosome 5 cluster contains prominent examples of variant isomers.[53] *GABRA6* and *GABRA2* have been the focus of previous studies because of their association with drug addiction, including AUD.[18,64–66] There are some recent reports on *GABRG1*,[67,68] but the *GABRA2* gene is reportedly involved with sex-specific gene–environment interactions in the development of addictions,[64] particularly AUD.[17,21,69] In studies that included both male and female subjects, no associations were found between AUD and the Chromosome 5 cluster in the COGA cohort,[62] but were reported for *GABRG2*[19], *GABRA6*, *GABRB2* and *GABRG2*.[70] A study that comprised only males found associations with *GABRA6* and *GABRA1*.[71] SNPs significantly associated with AUD include rs3219151 in *GABRA6* and rs279871, rs279858 and rs279845 in *GABRA2*.[17,18] *GABRA6* rs3219151 T allele carriers not differentiated by sex are reportedly at risk of developing AUD.[19,72] Here, we applied sex-associated analyses to subjects from living and autopsy cohorts. We found a Group × Sex interaction between polymorphisms in *GABRA6* rs3219151 and an AUD phenotype, and with differential expression of *GABRA6* mRNA transcripts.

We found differences between homozygote and heterozygote genotypes for *GABRA6* and *GABRA2*. We have not quantified α6 mRNA previously, but GABA$_A$ receptor subunit genes show both *cis* and *trans* coordinated expression[73]; expression of the γ2 isomer, which shows variation (short S and long L forms), interacts with expression of the α6 isomer in the same cluster in mouse cerebellar granule cells.[74] *GABRA6* rs3219151 CT heterozygotes showed significantly higher *GABRA6* transcript expression than either CC or TT homozygotes (Figure 3). This unusual finding may relate to one or

more of several factors. *GABRA6* rs3219151 is an expression quantitative trait locus (eQTL) of *GABRA6* in cerebellum (CC < CT < TT) and also in cerebral cortex area BA9 (dorsolateral and medial prefrontal cortex), but in the other direction (CC > CT > TT).[75] *GABRA6* is not expressed in any other human brain region.[73] Absolute cortical expression of α6 mRNA here was almost an order of magnitude lower than that of α2 mRNA (Figure 2 cf. Figure 3), which reflects the mainly cerebellar localisation of α6 mRNA.[5] Because of this disparity in scale, any difference appears less pronounced for α2 mRNA in the Figures. *GABRA2* rs279871, also an eQTL, is significantly expressed in cerebral cortex at high probability: by zygosity, CT < CC and CT < TT.[76] We found variant forms of α2 mRNA in human cerebral cortex autopsy tissue and showed that expression of one of the variants was differentially affected by AUD status.[77,78] Here, the effect of genotype on α2 mRNA expression was in the opposite sense to that of α6 mRNA: α2 mRNA levels trended lower in *GABRA2* rs279871 CT heterozygotes than in either CC or TT homozygotes (Figure 2). Whether this might reflect a *trans* effect between genes on Chromosomes 5 and 4 was not determinable by the approach used here. If one allele selectively affected expression of one α2 (or α6) variant, it is conceivable that expression in heterozygotes might lie outside the range between homozygotes.

The Chromosome 15 *GABRAB3* gene is also reportedly associated with AUD.[79] We have found no significant difference in mRNA expression between AUD cases and non-AUD controls in either DPC or PMC for the β1, β2 or β3 isoforms.[80] Both groups in that study contained subjects of both sexes; male and female subjects did not differ in any respect on any of the parameters measured.

SNPs in *GABRA2* are associated with a greater risk for alcoholism in both twin-based[81] and family-based[82] studies. However, the functional relationship between those SNPs and AUD has not been determined. SNPs in *GABRA2* have also been associated with illicit drugs use[81] and conduct disorder.[82] This points to a possible role of *GABRA2* in psychiatric conditions linked to deficits in reward learning and impulsive-antisocial behaviour.[83] Key factors in human autopsy brain tissue show no significant correlation with post-mortem intervals or years in storage.[84] We have previously shown, and confirm here, that the RNA Integrity Number (RIN value) may be used to normalise transcript expression, especially with targeted qRT-PCR.[41] RIN values lower than those normally acceptable in studies of laboratory animals do permit transcriptomic studies in autopsy brain.[33]

Genetic variations in $GABA_A$ receptor subunits are associated with individual differences in the development of AUD.[17,51] Acute alcohol exposure increases $GABA_A$ neurotransmission because ethanol binds to the receptor and alters its ligand-gated ion-channel function. In contrast, chronic excessive alcohol use decreases the inhibitory effects of $GABA_A$ and increases the excitatory effects of glutamate. This can lead to neuronal loss in brain regions such as the DPC, possibly from excitotoxicity,[85] whereas the PMC is less vulnerable to the effects of alcohol misuse.[86] Ethanol is a positive allosteric modulator of $GABA_A$ receptors.[87] Animal studies show acute single exposure to ethanol rapidly stimulates GABA-activated Cl^- channels, downregulates extrasynaptically δ-subunit-containing *GABRs*,[88,89] and downregulates postsynaptic α1βγ2 *GABRs* a few hours later.[90,91] Upregulation of *GABRs* containing α4βγ2 and α2βγ1 then occurs 1–2 days later.[90] However, these changes are transient and reversible.[92] When ethanol exposure becomes excessive and chronic, $GABA_A$ plasticity is induced. In particular, downregulated sedation-related postsynaptic α1-containing and upregulated mood-related α4-containing *GABRs* have been observed.[93–98] Glutamate neurotransmission is also inhibited by prolonged intoxication via NMDA glutamate receptors.[92]

Neuronal loss is greater, and can involve more brain regions, if the subject has a comorbid disease such as cirrhosis of the liver (as studied here) and the Wernicke-Korsakoff syndrome.[99,100] Feedback loops are disturbed by overexcitation that increases alcohol craving as the system works to regain neurotransmission balance.[101] Despite this, treatments for AUD based on the GABA-glutamate imbalance are currently not very successful.[92]

AUD with cirrhosis of the liver had more marked effects on both *GABRA6* and *GABRA2* gene expression in males than in females. Transcript expression was significantly lower in male cirrhotic AUD cases than in male non-AUD controls and female cirrhotic AUD cases. The association with AUD may be stronger in males than in females.[5] This suggests that the mechanisms regulating both genes may differ between males and females.[102] The study groups were reasonably balanced for sex. Comorbid diseases such as liver cirrhosis may affect the results by causing further brain damage.[1,47] There was no sex difference between control and non-cirrhotic AUD subjects, which is consistent with our older data.[103] Our pilot study showed sex differences for two Chromosome 5-cluster SNPs, *GABRA6* rs3219151 and *GABRG2* rs211013. There were no sex differences for SNPs in the Chromosome 4 cluster, including the *GABRA2* SNPs studied here.[7] Males and females may differ in susceptibility to brain damage, specifically in the DPC[1].

The three *GABRA2* SNPs studied are in high linkage disequilibrium.[40] Further interactive analyses between males and females, AUD cases and non-AUD cases, and genotype demonstrated no significant allelic distributions in transcript expression, possibly as a result of the limited number of subjects.[104] Overall, the *GABRA6* SNP had a different association to risk of AUD than the *GABRA2* SNP, possibly because of their different locations within their respective genes.[51] The significant differences suggest genotypes in both genes may modulate amino acid neurotransmission in AUD cases with cirrhosis.[103] Certain genotypes may alter the balance of GABA inhibition feedback of glutamate excitation in the cerebral cortex in a region-specific manner.[1]

AUD cases show region-specific neuropathology, which is evident in the differences between PMC and DPC. PMC in non-cirrhotic AUD cases had significantly lower gene expression than PMC in cirrhotic AUD cases. The lower expression in the DPC may correlate with a decrease in *GABRA6* and *GABRA2* receptor transcripts after prolonged consumption—as we showed selectively for the expression of an α2 mRNA variant in cases not differentiated by genotype.[77] Alcohol may alter *GABRA6* and *GABRA2* assembly to affect the influx of chloride ions through the $GABA_A$ channel, or disrupt receptor trafficking to reduce inhibitory neurotransmission.[1] However, the results from this

study are limited to transcript levels and may not infer differences at protein and receptor levels.[5] Future studies could use the mid-frontal cortex instead of the DPC, as AUD-induce damage in this region causes less neurone loss.[47] This might more accurately show whether *GABR* genes are affected by genotype, rather as than a response to AUD. The subject size was sufficient for this mRNA expression study; however, it did restrict the opportunity to explore more-complex interactions. Larger samples from brain banks are available for future studies.

A drawback of this study and the pilot was the number of cases studied, which is insufficient for genetic analysis. The subjects were quite homogeneous, mainly of Northern European descent. They thus comprise a small comparator among the major GWAS and targeted studies in this area, which cover a range of ethnicities. Our primary focus was not genetics per se, but to explore the relationships between genotype, key primary phenotypes—sex, comorbid liver disease—and targeted expression of $GABA_A$ receptor subunit mRNA transcripts. A key limitation to this approach is the availability of autopsy tissue that has been collected from appropriate cases and controls and preserved in a manner that is optimal for molecular biology analysis. Our group has performed many technical studies in this area.[105]

This study demonstrated that SNPs in the *GABRA6* and *GABRA2* genes mediate gene expression in cirrhotic AUD cases and result in differences between males and females. It highlights the concept that genetic variation can affect transcript expression in specific regions of the brain for key genes associated with AUD.

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CONFLICT OF INTEREST

All authors declare they have no conflicts of Interest.

DATA AVAILABILITY STATEMENT

In accord with the Legal and Ethical requirements of Queensland Hospitals and Universities, all data, in both cohorts, was fully anonymized of any identifying information. The Policies of both the QBB and the RBWH Alcohol and Drug Services is that necessary data will be provided to accredited researchers on condition that no personal identification information will appear in any publication. Under these constraints, the services provide demographic, age, medical history, and diagnostic information for every subject, including but not limited to results of clinical tests and histopathological assessments. These policies comply with International Practice.[106]

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