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Sex Differences in the Expression of the α5 Subunit of the $GABA_A$ Receptor in Alcoholics with and without Cirrhosis of the Liver

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Abstract

Introduction: Alcohol exposure alters the expression of a large number of genes, resulting in neuronal adaptions and neuronal loss, but the underlying mechanisms are largely unknown. MiRNAs are gene repressors that are abundant in the brain. A recent study identified ~35 miRNAs that are up-regulated in the prefrontal cortex of human alcoholics and predicted to target genes that are down-regulated in the same region. Although interactions between alcohol-responsive miRNAs and their target genes have been predicted, few studies have validated these predictions.

Methods: We measured the expression of $GABA_A$α5 mRNA in the prefrontal and motor cortices of human alcoholics and matched controls using real time PCR. The expression of miR-203 was measured in a subset of these cases. The predicted interaction of miR-203 and *GABRA5* was validated for miR-203 using a luciferase reporter assay.

Results: In both frontal and motor cortices the expression of $GABA_A$α5 was significantly lower in cirrhotic alcoholics compared with controls. Further, the pattern of expression between the groups was significantly different between males and females. The expression of miR-203 was higher in the prefrontal cortex of cirrhotic alcoholics compared with controls and uncomplicated alcoholics. These differences were particularly marked in female cases. Co-transfection of *GABRA5* with miR-203 in HEK293T cells reduced luciferase reporter activity.

Conclusion: There are sex differences in the expression of $GABA_A$α5 and miR-203 in the brain of human alcoholics which are particularly marked in alcoholics with cirrhosis of the liver. Further, miR-203 may mediate the changes in expression of this $GABA_A$ receptor isoform that is brought about by alcohol exposure.

Keywords

human; prefrontal cortex; expression; cirrhotic alcoholic; gender

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Introduction

γ-aminobutryric acid (GABA), the most abundant inhibitory neurotransmitter in the brain, exerts its actions via three receptor subtypes: $GABA_A$, $GABA_B$ and $GABA_C$ receptors. $GABA_A$ and $GABA_C$ receptors are ligand-gated ion channels while $GABA_B$ receptors are G protein-coupled receptors (Lobo and Harris, 2008). The roles of $GABA_A$ receptors in mediating the acute and chronic effects of alcohol in the brain are well documented (Reviewed in Forstera et al., 2016). Molecular studies have identified 16 subunit isoforms, which are grouped in classes by sequence homology: α(1–6), β(1–3), γ(1–3), θ, δ, ε, and π, with the ρ subunits (ρ1–3) now considered to be subunits of $GABA_C$ receptors. $GABA_A$ receptors are pentameric and the most common composition includes two α subunits, two β subunits, and one γ subunit. $GABA_A$ receptors with different combinations of subunits have different pharmacological properties, functional roles and regional distribution (Myers et al., 2017). GABA receptors containing the α5 subunit, coded by the *GABRA5* gene, constitute only a small percentage of native $GABA_A$ sites (Sur et al., 1999, Uusi-Oukari and Korpi, 2010), but receptors containing this subunit have been implicated in learning, memory, and addiction.

Several studies have suggested that $GABA_A$α5 receptors mediate the reinforcing effects of alcohol and may represent a new therapeutic target for the development of drugs to reduce harmful drinking (Ruedi-Bettschen et al., 2013). In animal models, ligands selective for $GABA_A$α5 receptors attenuate ethanol reinforcement during operant self-administration (Stephens et al., 2005, Bao et al., 1992, Samson et al., 1989, Samson et al., 1987, Glowa et al., 1988, Rassnick et al., 1993) and decrease alcohol self-administration (McKay et al., 2004). Further, pharmacological manipulation of $GABA_A$α5 receptors with the agonist QH-ii-066 alters drinking behaviour in monkeys (Ruedi-Bettschen et al., 2013). The potential role of $GABA_A$α5 receptors in ethanol reinforcement and reward has also been investigated using $GABA_A$α5 knockout animals. Male mice lacking *GABRA5* show reduced alcohol preference and consumption compared to their male wildtype mice, further suggesting a role for this subunit in ethanol reinforcement (Boehm et al., 2004, Stephens et al., 2005).

Long-term alcohol misuse results in altered gene expression in pathologically susceptible regions of the human brain. These changes in gene expression are thought to be a cellular response to the neurotoxicity of alcohol (Nestler and Aghajanian, 1997). In laboratory rodents, repeated alcohol exposure changes the relative abundance of selected $GABA_A$ subunits in regions of the brain such as the cerebral cortex. For example, chronic alcohol exposure decreases the expression of the α1 and α5 subunits whereas the α4 subunit is increased (Devaud et al., 1995, 1997). Further, alcohol exposure alters the expression of the α1 and α5 subunits in a region- and time-dependent manner (Charlton et al., 1997). Acute exposure to ethanol of rats that had previously been chronically exposed to alcohol for more than a month resulted in an increase (8.7 fold) in the expression of the α5 subunit (Osechkina et al., 2016).

In human subjects, selected isoforms of the $GABA_A$ receptor α subunit class (specifically α1 – 3) are differentially expressed at the mRNA (Lewohl et al., 1997) and protein levels (Lewohl et al., 2001) in the prefrontal cortex of alcoholics, particularly those with cirrhosis

of the liver. In contrast to laboratory rodents, the expression of the α1 subunit of the $GABA_A$ receptor is increased in the frontal cortex of alcoholics relative to controls (Lewohl et al., 1997). These differences are likely to yield $GABA_A$ receptors with distinct pharmacological and functional characteristics (Reviewed in Dick et al., 2006). The mechanism by which this occurs is not known, but previous studies have shown that the expression of selected $GABA_A$ receptor subunit can be selectively altered by miRNAs (Bekdash and Harrison, 2015, Zhao et al., 2012).

MiRNAs are short non-coding RNAs, highly abundant in brain, that regulate the transcription of their mRNA targets. A number of alcohol-responsive miRNAs have been identified by recent studies in rodent and cell culture models, as well as in human alcoholic brain (Balaraman et al., 2012, Guo et al., 2012, Lewohl et al., 2011, Sathyan et al., 2007, Van Steenwyk et al., 2013, Wang et al., 2009, Yadav et al., 2011, Tapocik et al., 2013). Some of these miRNAs selectively target isoforms of $GABA_A$ receptors to manipulate their expression. Specifically, miR-181, miR-216 and miR-203 directly target the α1 subunit (Zhao et al., 2012) and miR-155, miR-186, miR-24, miR-27b and miR-375 directly target the α4 subunit (Bekdash and Harrison, 2015). Of these, only one miRNA, miR-203, is up-regulated in the prefrontal cortex of human alcoholics (Lewohl et al., 2011). This miRNA is also predicted to target the α5 subunit of the $GABA_A$ receptor (Betel et al., 2008).

Here we investigated the expression of the α5 subunit of the $GABA_A$ receptor in the human brain of alcoholics with and without cirrhosis of the liver, and also measured the expression of miR-203 in a subset of these same cases. Further, we used luciferase reporter assays to determine if the 3′-UTR of *GABRA5* is targeted by miR-203.

Materials and Methods

Case Collection

Ethical clearance for the project was provided by the Griffith University Human Ethics Committee (MSC/02/06/HREC). The human brain samples were collected by the Queensland Brain Bank, School of Chemistry and Molecular Biosciences, University of Queensland and the NSW Brain Tissue Resource Centre, University of Sydney by qualified pathologists under full ethical clearance and informed, written consent from the next of kin. Two brain regions were selected for analysis. These included the dorsolateral prefrontal cortex (Brodmann areas 6 and 8), which is selectively vulnerable to neuropathological damage, and the primary motor cortex (Brodmann area 4), which is relatively spared. At the time of collection, autopsied tissue samples were immersed in ~10 vol of 0.32 M sucrose and slowly frozen to best preserve the tissue (Dodd et al., 1986). The samples were stored at –80°C until the RNA was isolated as outlined below.

Case selection

$GABA_A$α5 expression was measured on a cohort of 54 subjects of European origin: 21 controls and 33 alcoholic cases. The dorsolateral prefrontal cortex was available in all subjects, primary motor cortex was available in all but four: two controls and two alcoholic cases. Subjects were classified by average daily ethanol consumption as reported in their

medical records. Individuals classified as controls had consumed < 20 g of ethanol per day or were teetotalers. Alcoholics were defined on National Health & Medical Research Council/World Health Organization criteria as individuals who had consumed more than 80 g of ethanol per day for the majority of their adult lives; many had consumed in excess of 200 g of ethanol per day for over 20 years. Alcoholics were further divided at autopsy into uncomplicated alcoholics, i.e. individuals without co-morbid liver cirrhosis (23 cases), and cirrhotic alcoholics, i.e. individuals with confirmed liver cirrhosis (10 cases). Individuals with a history of poly-drug abuse or other co-morbid conditions such as Wernicke-Korsakoff syndrome, hepatic encephalopathy, or any other neurological condition, were excluded from the study.

The expression of hsa-miR-203a-3p (miR-203) was measured in a subset of the above cases. This analysis was restricted to the dorsolateral prefrontal cortex and was limited to those cases for which total RNA was available. This included 17 controls (8 female, 9 male), 22 uncomplicated alcoholics (9 female, 13 male) and 8 cirrhotic alcoholic cases (3 female, 5 male).

RNA isolation and reverse transcription

Total RNA was extracted using TRIzol™ (Gibco BRL, Invitrogen, Mt Waverly, Vic, Australia) as per the manufacturer's instructions. RNA quality was determined by visual inspection of electropherograms produced by the Agilent 2100 bio-analyzer (Palo Alto, CA, USA). RNA integrity numbers (RINs) for these samples ranged from 2.6 – 7.4 (Supplementary Table 1). RNA concentration was measured by absorbance at 260 nm using a Nanodrop device (Thermo Scientific, Waltham, MA, USA). Total RNA was stored at –80°C until required.

To measure $GABA_A\alpha5$ expression, samples were reverse transcribed using an oligo(dT) 20-mer. A mix consisting of 3.8 μM $oligo(dT)_{20}$ (Sigma-Aldrich), 2 μg total RNA, 0.77 mM dNTPs (Promega, Annandale, NSW, Australia) in a final volume of 13 μL with DEPC-treated water was incubated at 65°C for 5 min and placed on ice for 1 min; 5× first-strand synthesis buffer (4 μL), 50 mM dithiothreitol (DTT), 40 U RNaseOUT™ and 200 U Superscript™ III (Invitrogen) were added and the mix incubated at 50°C for 1 h. The reaction was inactivated by incubation at 70°C for 15 min. Aliquots were stored at –20°C.

To measure the expression of miR-203, samples were reverse transcribed using the miScript II RT Kit (QIAGEN P/L, Chadstone, Vic, Australia). Each reaction consisted of 1 μL template RNA, 4 μL of 5× miScript HiFlex Buffer, 2 μL of 10× miScript Nucleics Mix and 2 μL of miScript Reverse Transcriptase Mix, made up to a final volume of 20 μL with RNase-free water. The mix was incubated for 60 min at 37°C, deactivated by heating to 95°C for 5 min and then placed on ice. The cDNA was stored at –20°C.

Primer design

Primers for $GABA_A\alpha5$ and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* were designed using Primer Express® v1.5 Software (Applied Biosystems) and synthesised by Sigma-Aldrich®. Basic Local Alignment Search Tool (BLAST) was used to verify each primer for specificity and selectivity (Altschul et al., 1997). Primer sequences were:

$GABA_A\alpha5$ Forward 5′–CTT CCC GAT GGA TGC GC–3′, $GABA_A\alpha5$ Reverse 5′–GAG CCG TTG GTC CAG ACG TA–3′, *GAPDH* Forward 5′–TGC ACC ACC AAC TGC TTA GC–3′, *GAPDH* Reverse 5′–GGC ATG GAC TGT GGT CAT GAG–3′.

Real-time PCR

Real-time PCR for $GABA_A\alpha5$ was conducted in the Corbett RotorGene 3000 with SYBR Green® as the detection dye. Each reaction consisted of 5 ng of cDNA, 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems), and 300 nM each of the oligonucleotide primers in a final reaction volume of 25 μL per tube. No-template samples were included with each primer set to act as a negative control, while Universal Human Reference RNA (UHRR; Stratagene, La Jolla, CA, USA) cDNA was used as a positive control to confirm amplification in each experiment for each primer pair. *GAPDH* was used as the endogenous control for determining ΔC_T values. Each sample was amplified in duplicate.

Expression of miR-203 was measured in each sample using the miScript SYBR Green PCR Kit and the Rotor-Gene Q (QIAGEN). Each reaction consisted of 1 μL of template cDNA, 10 μL of 2× QuantiTect SYBR Green PCR Master Mix, 2 μL of 10× miScript Universal Primer and 2 μL of 10× miScript Primer Assay, made up to a final volume of 20 μL with RNase-free water. The amplification cycle consisted of an initial activation step at 95°C for 15 min followed by 40 cycles of 15 s at 94°C, 30 s at 55°C and 30 s at 70°C. Each sample was amplified in duplicate; no-template controls were included for each primer pair. *RNU6B* was used as the reference gene as advised by the Guidelines for miRNA mimic and miRNA inhibitor experiments Handbook (QIAGEN).

Statistical analyses

Using the Rotor-Gene Q Software (QIAGEN), a threshold of 0.03 was set in the exponential phase of the reaction on the amplification plot of fluorescence *vs.* cycle number to determine the C_T for each sample. This was kept constant for all runs. Each sample was normalized to the reference gene (*RNU6B* or *GAPDH*). The difference in C_T values of each sample and the reference gene were expressed as the ΔC_T. These values were then converted to $2^{-\Delta CT}$ values for presentation (Ho et al., 2010, MacKay et al., 2011, Janeczek et al., 2015, Livak and Schmittgen, 2001). Data was analysed using IBM SPSS Statistics 25 software (SPSS Inc. Chicago, IL, USA), by performing *ANOVA* followed by the Tukey *hsd* post hoc test where appropriate. Linear regression was used to determine the effects of age at death, PMI and RIN and covariance analysis (*ANCOVA*) with RIN as the co-variate was used as appropriate. Initially, the expression of $GABA_A\alpha5$ and miR-203 was analysed by Group and then by both Group and Sex. Due to the stratified nature of the analysis, in some instances the groups were insufficiently powered, and results were interpreted with caution.

Transfection of miRNA 3′-UTR target and precursor miRNA clones

HEK293T cells were transfected with a Firefly-luciferase-encoded miTarget™ miRNA 3′-UTR Target Sequence Expression Clone (pEZX-MT01; HmiT054442 *GABRA5*) from GeneCopoeia™ (United BioResearch Products P/L, Dural, NSW, Australia) and a miExpress™ Precursor miRNA Expression Clone (pEZX-MR04; miR-203 (HmiR0249), miR-7 (HmiR0140) or the control precursor (CmiR0001)). Approximately 16 h before

transfection the cells were seeded at 1×10^5 in a 24-well plate with 200 μL of 10% FBS in DMEM (11995, Life Technologies P/L, Mulgrave, Victoria, Australia) without antibiotics and incubated at 37°C in 95% O_2/5% CO_2. DNA-lipid complexes were formed by diluting 400 ng of 3′-UTR plasmid and 500 ng of miRNA precursor in 50 μL of Opti-MEM (Sigma-Aldrich P/L, Castle Hill, NSW, Australia) and combining this with 3 μL of EndoFectin™ Plus Transfection Reagent (GeneCopoeia™) diluted in 50 μL of Opti-MEM. The diluted EndoFectin Plus reagent was added drop-wise to the diluted plasmid solution and mixed gently. The mixture was incubated for 20 min at room temperature to allow the DNA-EndoFectin® Plus complexes to form. Aliquots were then added to each well and the plate gently swirled and incubated at 37°C in 95% O_2/5% CO_2 until the luciferase assay. The eGFP reporter encoded in the miRNA precursor clones was used to determine successful transfection in each of the wells ~23 h later. Post-transfection miRNA levels were measured by real-time PCR; miRNA levels were increased by more than 50-fold post-transfection.

Luciferase Assays

The Luc-Pair™ miR Luciferase Assay Kit (GeneCopoeia) was used to detect Firefly and *Renilla* luciferase expression as per the manufacturer's instructions. It was expected that the 3′-UTR clone with the control precursor (control) in the Luciferase Assay would have a higher luminescence than the cells transfected with the miRNA precursor clones. Firefly and *Renilla* luciferase were measured using the FlexStation 3 Microplate Reader and SoftMax® Pro Microplate Data Acquisition & Analysis software 5.2 (Molecular Devices). *Renilla* luciferase activity was used to normalise the Firefly luciferase signal in the same well. The ratio of luminescence from the Firefly luciferase to *Renilla* was calculated for each well and plotted using GraphPad Prism 8 (GraphPad Software Inc, La Jolla, CA, USA). Two different controls were used; the *GABRA5* clone co-transfected into HEK293T cells with either a scrambled control miRNA precursor clone or the miR-7 precursor clone. The *GABRA5* 3′-UTR does not have a predicted binding site for miR-7. The data was analysed with the independent-samples *t*-test using IBM SPSS Statistics 25 software (SPSS Inc. Chicago, IL, USA).

Results

Expression of $GABA_A\alpha5$ mRNA in human alcoholic brain

The analysis of relative expression of transcripts of interest using real-time PCR is dependent on using a reference gene that does not differ in expression between individuals. In previous studies (Ho et al., 2010, Janeczek et al., 2015, MacKay et al., 2011) we have used *GAPDH* as a reference gene. Accordingly, the expression of *GAPDH* was measured in each sample by real-time PCR. Analysis of the raw CT values for each case and control showed no significant differences in the expression of *GAPDH* in either the dorsolateral prefrontal cortex (frontal) or the anterior motor cortex (motor) between controls and alcoholics, similar to our previous studies samples using this cohort of individuals (Frontal: $F_{2,50} = 2.530$, $P = 0.090$; Motor: $F_{2,49} = 2.247$, $P = 0.116$).

Case information detailed in Table 1 shows the mean age-at-death, post-mortem interval and RIN for each group. Control and alcoholic case groups were matched for age at death, post-

mortem interval and RIN (*ANOVA*, Age at death: $F_{2,51} = 3.183$, $P = 0.050$; PMI: $F_{2,51} = 1.810$, $P = 0.174$; RIN: $F_{2,51} = 0.190$, $P = 0.827$). Despite efforts to match the case groups, the mean age of the uncomplicated alcoholic group had a tendency to be younger than the control group (Tukey hsd, $P = 0.058$). To determine if the expression of $GABA_A\alpha5$ mRNA was correlated with age at death, PMI or RIN, linear regressions were performed on each parameter. There was no significant correlation between the expression of $GABA_A\alpha5$ mRNA and age-at-death or PMI in either controls or combined alcoholic cases (Table 2; Supplementary Figure 1). However, the expression of $GABA_A\alpha5$ mRNA was significantly correlated with RIN in combined alcoholics (Figure 1). Accordingly, RIN was used as a co-variate in subsequent analyses.

Overall, the expression of $GABA_A\alpha5$ mRNA showed a significant Main Effect for Group in both frontal and motor cortices (*ANCOVA*: Frontal, $F_{2,46} = 5.085$, $P = 0.003$; Motor, $F_{2,43} = 9.364$, $P < 0.001$). The Main Effects for Sex was significant in prefrontal cortex but not motor cortex (*ANCOVA*: Frontal, $F_{1,46} = 5.684$, $P = 0.021$; Motor, $F_{1,43} = 2.176$, $P = 0.148$). The Group × Sex Interaction showed a tendency toward significance in prefrontal cortex but not motor cortex (Frontal, $F_{2,46} = 2.916$, $P = 0.064$; Motor, $F_{2,43} = 2.115$, $P = 0.133$.

Initially, expression differences were analysed across groups regardless of sex. Subsequent analysis addressed potential sex differences in the pattern of expression of $GABA_A\alpha5$ mRNA in both brain regions and across groups. In frontal cortex, the expression of $GABA_A\alpha5$ mRNA was significantly lower in cirrhotic alcoholics compared with both controls and uncomplicated alcoholics (Controls *vs* Cirrhotic alcoholics, $P = 0.002$; Uncomplicated alcoholics *vs* Cirrhotic alcoholics, $P = 0.030$; Figure 2) whereas uncomplicated alcoholics did not differ significantly from controls ($P = 0.225$). In motor cortex, the expression of $GABA_A\alpha5$ mRNA was significantly lower in cirrhotic alcoholics compared with both controls and uncomplicated alcoholics (Controls *vs* Cirrhotic alcoholics, $P < 0.0001$; Uncomplicated Alcoholics *vs* Cirrhotic alcoholics, $P = 0.022$; Figure 2). Further, uncomplicated alcoholics showed lower expression of this subunit than controls (Controls *vs* Uncomplicated alcoholics, $P = 0.045$).

In female cases overall, there was a significant difference in the expression of $GABA_A\alpha5$ mRNA across groups in both the frontal and motor cortices (*ANCOVA*, Frontal: $F_{2,19} = 5.186$, $P = 0.016$; Motor: $F_{2,17} = 5.880$, $P = 0.011$; Figure 3). *Post hoc* analysis in frontal cortex showed that the expression of $GABA_A\alpha5$ mRNA was lower in both uncomplicated alcoholics and cirrhotic alcoholics than in controls (Controls *vs* Uncomplicated alcoholics, $P = 0.006$; Controls *vs* Cirrhotic alcoholics, $P = 0.004$) with no difference between alcoholic groups (Uncomplicated *vs* Cirrhotic alcoholics, $P = 0.593$). In motor cortex, the expression of $GABA_A\alpha5$ mRNA was lower in both uncomplicated and cirrhotic alcoholics than in controls (Controls *vs* Uncomplicated alcoholics, $P = 0.004$; Controls *vs* Cirrhotic alcoholics, $P = 0.001$). There was no difference between the alcoholic groups (Uncomplicated *vs* Cirrhotic alcoholics, $P = 0.358$).

In males, there was a tendency toward a significant difference in the expression of $GABA_A\alpha5$ mRNA across groups in both frontal and motor cortices (*ANCOVA*: Frontal, $F_{2,26} = 2.874$, $P = 0.075$; Motor, $F_{2,25} = 2.921$, $P = 0.072$; Figure 3). *Post hoc* analysis in

frontal cortex showed that the expression of $GABA_A\alpha5$ mRNA was lower in cirrhotic alcoholics than in either controls or uncomplicated alcoholics (Controls *vs* Cirrhotic alcoholics, $P = 0.056$; Uncomplicated *vs* Cirrhotic alcoholics, $P = 0.027$). There was no difference in expression between controls and uncomplicated alcoholics (Controls *vs* Uncomplicated alcoholics, $P = 0.752$). In motor cortex, the expression of $GABA_A\alpha5$ mRNA was significantly lower in cirrhotic alcoholics than in controls (Controls *vs* Cirrhotic alcoholics, $P = 0.022$) and in uncomplicated alcoholics ($P = 0.038$). There was no difference in the expression of $GABA_A\alpha5$ mRNA in the motor cortex of uncomplicated alcoholics compared with controls ($P = 0.677$).

The expression of $GABA_A\alpha5$ mRNA was significantly lower in female uncomplicated alcoholics than in male uncomplicated alcoholic in both cortical regions (*ANCOVA*, Frontal: $F_{1,19} = 6.826$, $P = 0.017$; Motor: $F_{1,18} = 7.246$, $P = 0.015$). The expression of $GABA_A\alpha5$ mRNA did not differ significantly between males and females in either controls or cirrhotic alcoholic cases regardless of brain region (Controls: Frontal: $F_{1,18} = 1.93$, $P = 0.666$; Motor: $F_{1,16} = 0.152$, $P = 0.702$; Cirrhotic alcoholics: $F_{1,7} = 1.60$, $P = 0.317$; Motor: $F_{1,7} = 0.572$, $P = 0.474$.

Expression of miR-203 in human alcoholic brain

To determine if miR-203 was up-regulated in this cohort, the expression of the miRNA was measured by real-time PCR in a subset of cases used for $GABA_A\alpha5$ mRNA analysis. Case information is outlined in Table 1.

There was no significant difference in age at death, PMI or RIN between the groups (*ANOVA*, Age at death: $F_{2,44} = 2.966$, $P = 0.062$; PMI: $F_{2,44} = 1.070$, $P = 0.352$; RIN: $F_{2,44} = 1.388$, $P = 0.260$). There was a tendency for uncomplicated alcoholics to be younger than controls ($P = 0.055$). Linear regression was performed on controls and combined alcoholics to determine if the expression of miR-203 was correlated with either of these factors. There was no significant correlation between the expression of miR-203 and age at death or PMI for either controls or combined alcoholics (Table 2, Supplementary Figure 2). However, the expression of miR-203 was significantly correlated with RIN in combined alcoholic cases (Figure 4). Accordingly, RIN was used as a co-variate in subsequent analyses.

The expression of miR-203 showed a significant Main Effect for Group (*ANCOVA*: $F_{2,40} = 3.689$, $P = 0.034$) but not Sex ($F_{1,40} = 1.982$, $P = 0.167$). The Group × Sex Interaction was also significant ($F_{2,40} = 5.522$, $P = 0.008$). Specifically, the expression of miR-203 was significantly higher in cirrhotic alcoholics compared with controls (Tukey: $P = 0.010$) and uncomplicated alcoholics ($P = 0.045$; Figure 5). There was no difference in expression between controls and uncomplicated alcoholics ($P = 0.318$).

In females, the expression of miR-203 differed significantly across groups (*ANCOVA*: $F_{2,16} = 5.007$, $P = 0.020$), with higher expression in cirrhotic alcoholics (albeit n = 3) than in either controls ($P = 0.033$) or uncomplicated alcoholics ($P = 0.004$; Figure 6). There was no difference in expression between female uncomplicated alcoholics and female controls ($P = 0.254$). In males the expression of miR-203 showed a tendency to significantly differ across groups (*ANCOVA*: $F_{2,23} = 3.304$, $P = 0.055$; Figure 6) with higher expression in

uncomplicated alcoholics compared with controls ($P = 0.008$). There was no difference in the expression of this miRNA between controls and cirrhotic alcoholics ($P = 0.102$) or between uncomplicated and cirrhotic alcoholics ($P = 0.627$).

The expression of miR-203 was significantly higher in female cirrhotic alcoholics than in male cirrhotic alcoholics (*ANCOVA*: $F_{1,5} = 7.980$, $P = 0.037$) and was also significantly different between male and female uncomplicated alcoholic cases ($F_{1,19} = 7.750$, $P = 0.012$). There was no significant difference between male and female controls ($F_{1,14} = 2.426$, $P = 0.142$).

Experimental validation of the predicted miR-203:GABRA5 interaction

Previous studies have shown that ~35 miRNAs are up-regulated in the frontal cortex of human alcoholics (Lewohl et al., 2011). One of these, miR-203, is predicted by the microRNA.org algorithm (Enright et al., 2003) to target *GABRA5* with a single binding site at position 328 (miRSVR score, −1.2404). To experimentally validate this predicted interaction, HEK293T cells were co-transfected with either a control, miR-203 or miR-7 precursor clone as well as a clone containing the 3′-UTR of the *GABRA5* gene upstream of a luciferase reporter gene. There was decreased luciferase activity when miR-203 was co-transfected with the *GABRA5* 3′-UTR clone ($t_7 = 3.573$, $P = 0.009$) whereas there was no such decrease when co-transfected with either the scrambled control miRNA precursor clone or the miR-7 precursor clone (Figure 7). As a positive control, a miR-7 precursor clone was co-transfected with a plasmid containing the α-synuclein 3′-UTR and luciferase activity was significantly decreased ($t_7 = 7.587$, $P = < 0.001$; data not shown). The interaction between miR-7 and α-synuclein has been validated in previous studies (Junn et al., 2009, Doxakis, 2010).

Discussion

In this study we measured the expression of $GABA_A\alpha5$ in the prefrontal and motor cortices of controls, uncomplicated alcoholics and cirrhotic alcoholics. Overall, the expression of the transcript was reduced in cirrhotic alcoholics in both cortical regions compared to controls and uncomplicated alcoholics. A previous study measured the expression of individual $GABA_A$ receptor mRNAs including the α5 subunit. They found significant increases in the expression of the α1, α4 α5, β1 and γ1 subunits in the hippocampus and decreased expression of the β2 and δ subunits in the orbitofrontal cortex of alcoholics with no differences in expression in the dorsolateral prefrontal cortex (Jin et al., 2011). However, this prior study specifically excluded female cases as well as individuals with cirrhosis of the liver.

We have previously shown that the pattern of gene expression in the frontal cortex of cirrhotic alcoholics is quite different to that seen in alcoholics without cirrhosis of the liver (Liu et al., 2007). Brain damage is also more severe in chronic alcoholics with cirrhosis of the liver (Zahr et al., 2011), which may be due to higher doses of ethanol reaching the brain (Harper, 1998). Chronic alcoholic exposure alters the blood brain barrier such that there is increased diffusion of alcohol into the brain as well as increased exposure to systemic inflammatory molecules, such as cytokines, resulting in neuroinflammation (Hammoud and

Jimenez-Shahed, 2019). Further, hepatic dysfunction results in an increase in neurotoxic metabolites such as ammonia and the development of hepatic encephalopathy (Reviewed in (Hammoud and Jimenez-Shahed, 2019, Davis and Bajaj, 2018). Although individuals with a history of hepatic encephalopathy were excluded from this study, the indirect effects of neurotoxic metabolites on the brain may occur in advance of such a diagnosis (Davis and Bajaj, 2018). It is not possible to determine whether the differences in expression in our study reflect differences in the direct effects of a higher concentration of alcohol reaching the brain, or indirect effects due to systemic inflammation or the presence of other neurotoxic metabolites. As some expression changes are also seen in non-cirrhotic alcoholics, the degree of alcohol exposure might contribute to the extent of the differences.

Studies in animal models have revealed the potential for sex differences in the effect of alcohol exposure on the expression of $GABA_A\alpha5$ (Stephens et al., 2005). Sex differences have also been noted in studies of ethanol reward (Xie et al., 2019) as well as in the physiological and behavioural effects of alcohol in both animal models of addiction (Reviewed in Becker and Koob, 2016) and humans (McCaul et al., 2019). In an effort to address potential sex differences in the expression of $GABA_A\alpha5$, approximately equal numbers of male and female cases were included in each group. In both cortical regions, the expression of $GABA_A\alpha5$ was significantly different between men and women and the pattern of expression was also different when the sexes were analysed separately. In female cases both alcoholic groups had lower expression than the control cases whereas in males, cirrhotic alcoholics had lower expression than both the controls and uncomplicated alcoholics. Thus, the main difference between men and women is in uncomplicated alcoholics. It should be noted that the case groups in this study are small and results should be interpreted with caution.

Although women are more likely to abstain from alcohol and are less likely to engage in problem drinking, females who do drink excessively develop more medical complications than males likely due to differences in alcohol pharmacokinetics (Erol and Karpyak, 2015). Female alcoholics have more severe neuropathological damage (Hommer et al., 2001) and appear to be more vulnerable to damage after shorter periods of alcohol misuse (Mann et al., 1992). The expression differences reported here may reflect the greater brain damage observed in female alcoholics, suggesting either that females are more sensitive to the effects of alcohol or that changes in expression occur at a lower effective dose in women than in men. Further studies are required to address these questions specifically. To date, no other study has specifically addressed the question of sex differences in the expression of $GABA_A\alpha5$ mRNA in response to alcohol exposure.

The expression of the $GABA_A\alpha5$ transcript has not been widely studied in either laboratory rodents or human studies. In laboratory rodents, the expression of $GABA_A\alpha5$ mRNA was decreased in the frontal cortex after 2 weeks and increased in the hippocampus after 12 weeks of ethanol administration (Charlton et al., 1997). Acute intoxication of alcoholised rats enhanced the expression of $GABA_A\alpha5$ mRNA in the hippocampus (Osechkina et al., 2016). In humans, the only previously published study measured the expression of $GABA_A\alpha5$ mRNA in the hippocampus, orbiotofrontal cortex and prefrontal cortex of alcohol-dependent, male, alcoholics without cirrhosis of the liver (Jin et al., 2011).

$GABA_A\alpha5$ was increased in the hippocampus but unchanged in either cortical region which is similar to the results presented here for male, uncomplicated alcoholics. Our previous microarray study of human brain identified ~35 miRNAs up-regulated in the prefrontal cortex of alcoholics (Lewohl et al., 2011). Here we compare the expression levels of an alcohol-responsive miRNA, miR-203, that is predicted to target *GABRA5*, in alcoholics with and without cirrhosis of the liver including both male and female cases. The expression of miR-203 was higher in the prefrontal cortex of alcoholics, with a concomitant lower expression of $GABA_A\alpha5$ mRNA. The difference was particularly marked in female cases. In females, miR-203 was significantly up-regulated in cirrhotic alcoholics only. As predicted, the expression of $GABA_A\alpha5$ mRNA was markedly lower in cirrhotic alcoholics, which suggests a role for this miRNA in the regulation of the transcript. In males, significant up-regulation of miR-203 was seen in both uncomplicated and cirrhotic alcoholics although the magnitude of the up-regulation was somewhat less than that in female cases. In contrast, the expression of $GABA_A\alpha5$ mRNA was lower in both male alcoholic groups and the magnitude of the difference was more marked than that in females. The lack of an exact correlation between the up-regulation of miR-203 and the concomitant down-regulation of $GABA_A\alpha5$ mRNA suggests that other factors or other miRNAs may play a role in the regulation of the transcript. Alternatively, while $GABA_A\alpha5$ is likely to be expressed only by neurons, miR-203 may be expressed by other cell types which may account for the difference. However, since mRNA was only available from a subset of the cases used in the $GABA_A\alpha5$ expression cohort, further studies should be carried out to determine if the expression of miR-203 is inversely correlated with the expression of $GABA_A\alpha5$ mRNA in alcoholics.

Although interactions between alcohol-responsive miRNAs and their target genes have been predicted, few studies have investigated whether this represents the mechanism by which gene expression is altered in response to alcohol exposure. In this study we validated the predicted interaction between miR-203 and *GABRA5* using a luciferase reporter assay.

Using this technique, we showed that co-transfection of miR-203 and clones containing the *GABRA5* 3′-UTR resulted in a reduction in the 3′-UTR reporter activity in HEK293T cells. Reporter constructs and luciferase assays are powerful tools that are frequently used to investigate miRNA:target interactions. One benefit of these assays is that they reveal interactions between miRNA and target genes irrespective of whether the miRNA works through translational repression or mRNA degradation (Cloonan, 2015). However, results from these type of studies can be variable. The variation can be partially accounted for by differences in reporter constructs, experiment-specific effects of transfection including transfection procedures that may have indirect effects on gene expression and cell physiology (Kozak, 2008), as well as variation as a result of using reporter assays (Jackson and Standart, 2007, Nissan and Parker, 2008). Previous studies have found that the promoter of the reporter construct and transfection procedure can significantly alter the mode or degree of regulation by miRNAs (Lytle et al., 2007, Kong et al., 2008). It is likely that the regulatory mechanisms are more complex *in vivo*, with several miRNA:mRNA interactions occurring simultaneously and involving several regulatory proteins that aid with these interactions (Hendrickson et al., 2009). As such, the results should be interpreted with caution.

GABA receptors containing the α5 subunit constitute only a small percentage of native $GABA_A$ receptors (Sur et al., 1999, Uusi-Oukari and Korpi, 2010) but receptors containing this subunit are proposed to have a role in cognition, learning, memory and addiction. Specifically, a reduction in the expression of $GABA_Aα5$ has been noted in schizophrenia and autism and may be correlated with cognitive deficits in those patients (Bristow et al., 2015). Selected changes in the expression of this subunit of the $GABA_A$ receptor have been noted in the brains of human alcoholics (Jin et al., 2011) as well as in animal models of alcohol misuse (Osechkina et al., 2016). Further, $GABA_Aα5$ receptors are suggested to play a role in mediating some of the behavioural effects of alcohol such as reinforcement and reward (Stephens et al., 2005, Ruedi-Bettschen et al., 2013). Modulation of $GABA_Aα5$ receptors using selective ligands has been shown to alter ethanol self-administration in monkeys (Ruedi-Bettschen et al., 2013) implicating these receptors as potential targets for the development of new medications to reduce harmful drinking. The results presented here indicate a reduction in the expression of $GABA_Aα5$ mRNA in the cortex of alcoholics with cirrhosis of the liver in a sex-dependent manner and may be correlated with an up-regulation of miR-203 which targets this transcript.

MiRNAs act co-ordinately to fine-tune gene expression by simultaneously targeting a large number of transcripts. Thus, an increase in the expression of even a single miRNA will likely have a wide range of cellular effects. MiR-203 is predicted by microRNA.org (http://www.microrna.org/microrna/home.do) to target over 9000 transcripts but few of these predictions have been experimentally validated. One target that has been validated by luciferase reporter assays is the interaction between miR-203 and the α1 subunit of the $GABA_A$ receptor (Zhao et al., 2012). Thus, an up-regulation of miR-203 may have important implications for the expression of α1-subunit containing $GABA_A$ receptors ($GABA_Aα1$ receptors). $GABA_Aα1$ receptors are one of a number of molecular targets for the action of alcohol and changes in expression of this subunit have been identified in rodent models of alcohol exposure (Reviewed in (Olsen and Liang, 2017)). The expression of $GABA_Aα1$ is down-regulated in response to chronic alcohol exposure in rodent models (Devaud et al., 1995, Devaud et al., 1997) although it is not known whether the decrease in expression is mediated by miRNAs such as miR-203. In human studies the expression of $GABA_Aα1$ is increased (Lewohl et al., 1997) or unchanged (Mitsuyama et al., 1998, Jin et al., 2011) in the prefrontal cortex of alcoholics. Thus, the increase in the expression of miR-203 does not appear to correlate with a decrease in the $GABA_Aα1$ transcript. However, since studies involving post mortem human brain are a single time-point, it is not known how the expression of either miR-203 or the $GABA_Aα1$ transcript might change during the earlier stages of the disease.

The expression of highly-related transcripts, such as splice variants of the same gene, can be selectively manipulated through differential miRNA targeting (Pietrzykowski et al., 2008). In cell-culture studies with rat tissue explants an up-regulation of miR-9 in response to alcohol exposure resulted in the selective degradation of selected splice variants of the large-conductance calcium- and voltage-activated potassium (BK) channel, altering the profile of these BK channels, consistent with the development of ethanol tolerance (Pietrzykowski et al., 2008). This suggests a role of miRNAs in the development of tolerance to alcohol that exacerbates the effects of alcohol on the brain. Since miR-203 has the potential to target

both the α1 and α5 subunits of the $GABA_A$ receptor a similar mechanism may alter the subunit composition of $GABA_A$ receptors in the prefrontal cortex in response to alcohol exposure. Differential targeting of $GABA_A$ receptor isoforms by miRNAs may result in selective degradation of some isoforms and not others resulting in a population of receptors with distinct pharmacological properties. Thus, drugs designed to selective target α1 or α5 containing $GABA_A$ receptors may be less efficacious in individuals with reduced expression of these receptor subtypes. The reduction in α5 containing $GABA_A$ receptors in the frontal cortex of female uncomplicated alcoholics as well as both male and female cirrhotic alcoholics has important implications for the development of new drugs which target this receptor subtype. Understanding the mechanisms that underlie the development of alcohol use disorders and associated phenotypes at the molecular level is key to understanding the potential to target these receptors for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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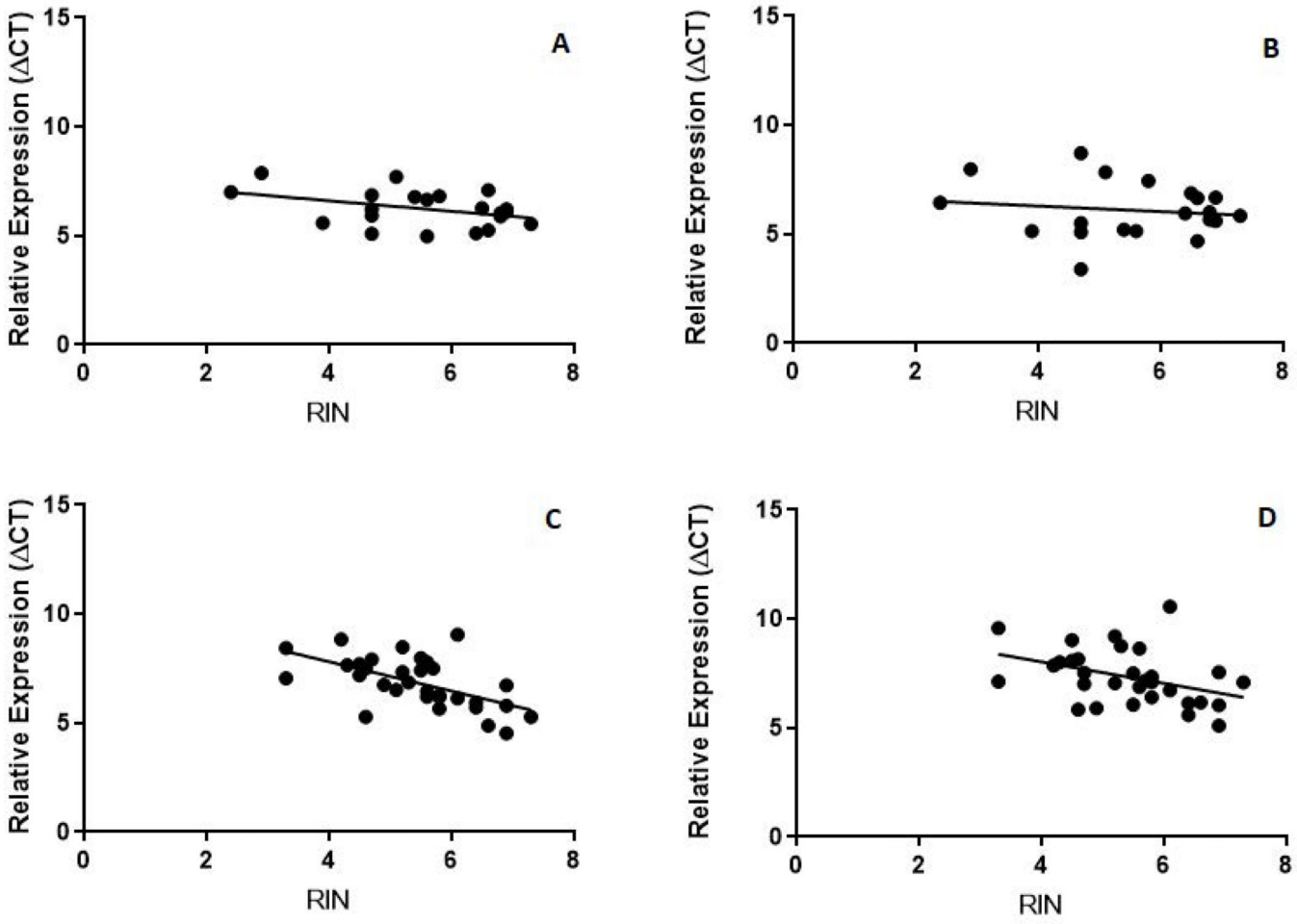

Figure 1:

Effect of GABAAα5 expression on RIN in controls (A, B) and combined alcoholics (C; D) in prefrontal cortex (A, C) and motor cortex (B, D). Regression was carried out on ΔCT values with the statistics reported in Table 2.

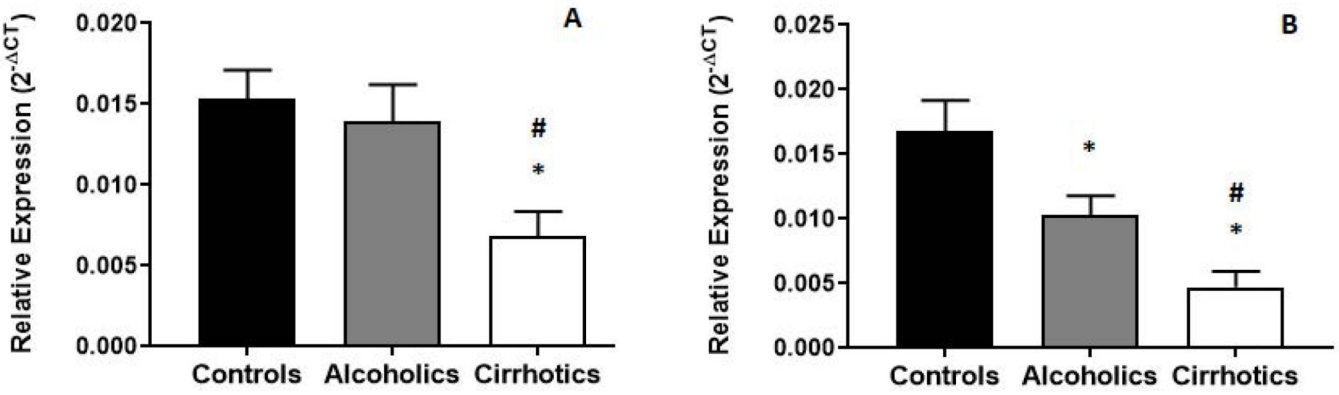

Figure 2:

Relative expression of the GABAAα5 subunit in human prefrontal (A) and motor (B) cortices. Data are presented as mean ΔCT converted to 2–ΔCT ± SEM. Asterisks denote significant changes relative to controls (Tukey hsd $P < 0.05$). Black bars represent controls; grey bars represent uncomplicated alcoholics and white bars represent cirrhotic alcoholics. Significant differences compared with controls are denoted by an asterisk (*); significant differences between uncomplicated and cirrhotic alcoholics are denoted by a hash (#).

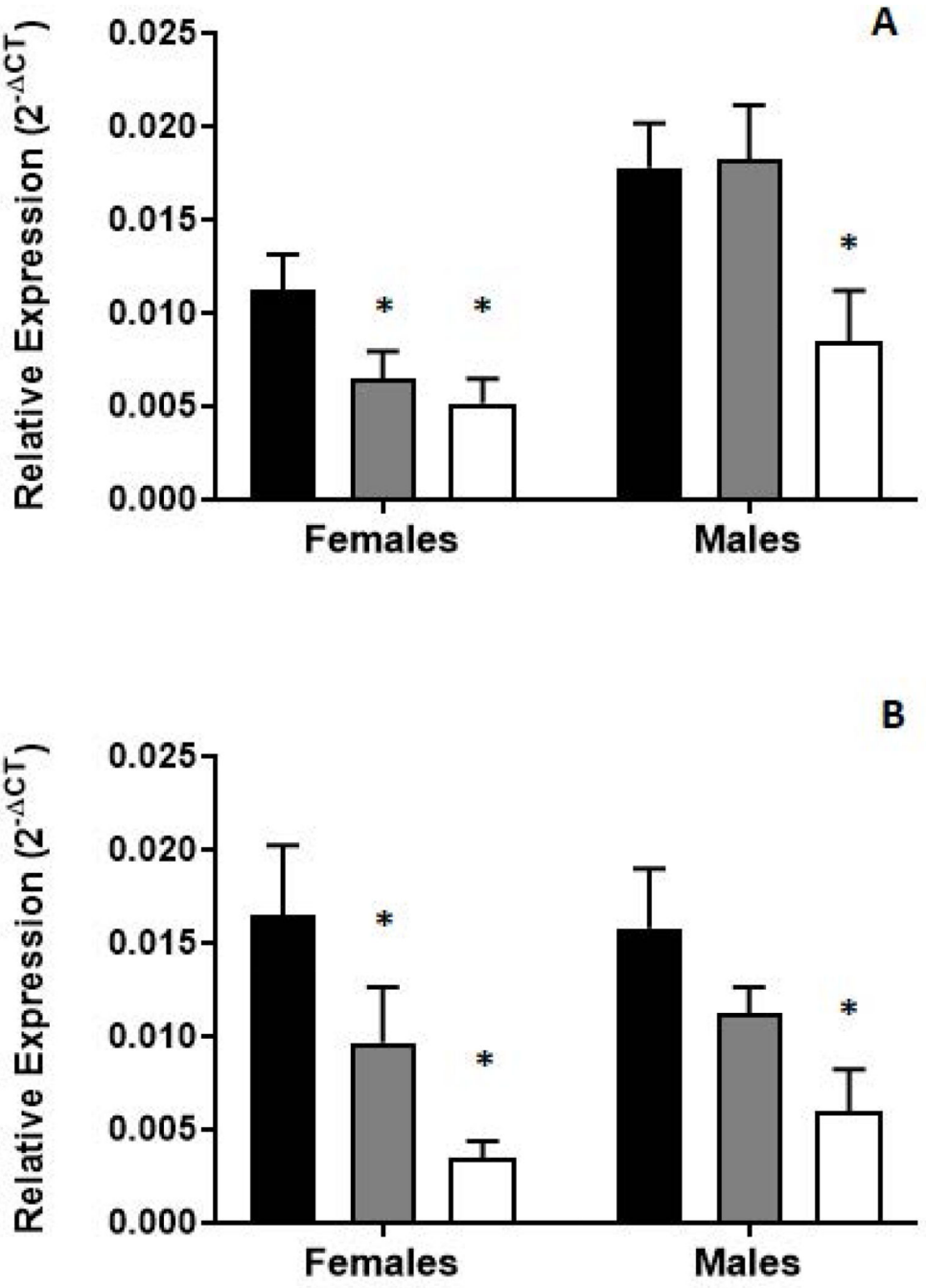

Figure 3:

Relative expression of the GABAAα5 subunit in human prefrontal (A) and motor (B) cortices. Data are presented as mean ΔCT converted to 2–ΔCT ± SEM. Asterisks denote significant changes relative to controls (Tukey hsd $P < 0.05$). Black bars represent controls; grey bars represent uncomplicated alcoholics and white bars represent cirrhotic alcoholics.

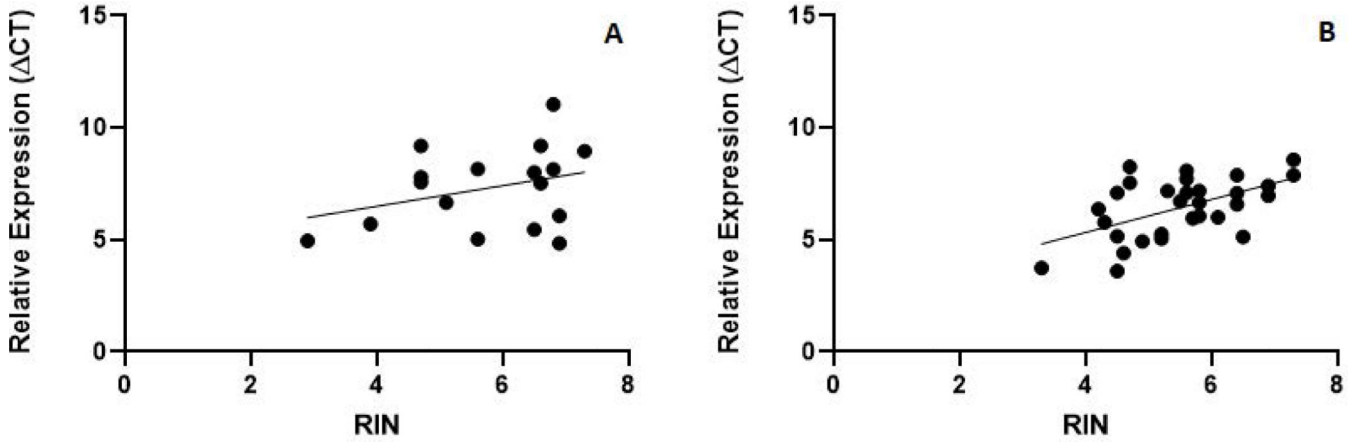

Figure 4:

Effect of miR-203 expression on RIN in controls (A) and combined alcoholics (B) in prefrontal cortex. Regression was carried out on ΔCT values with the statistics reported in Table 2.

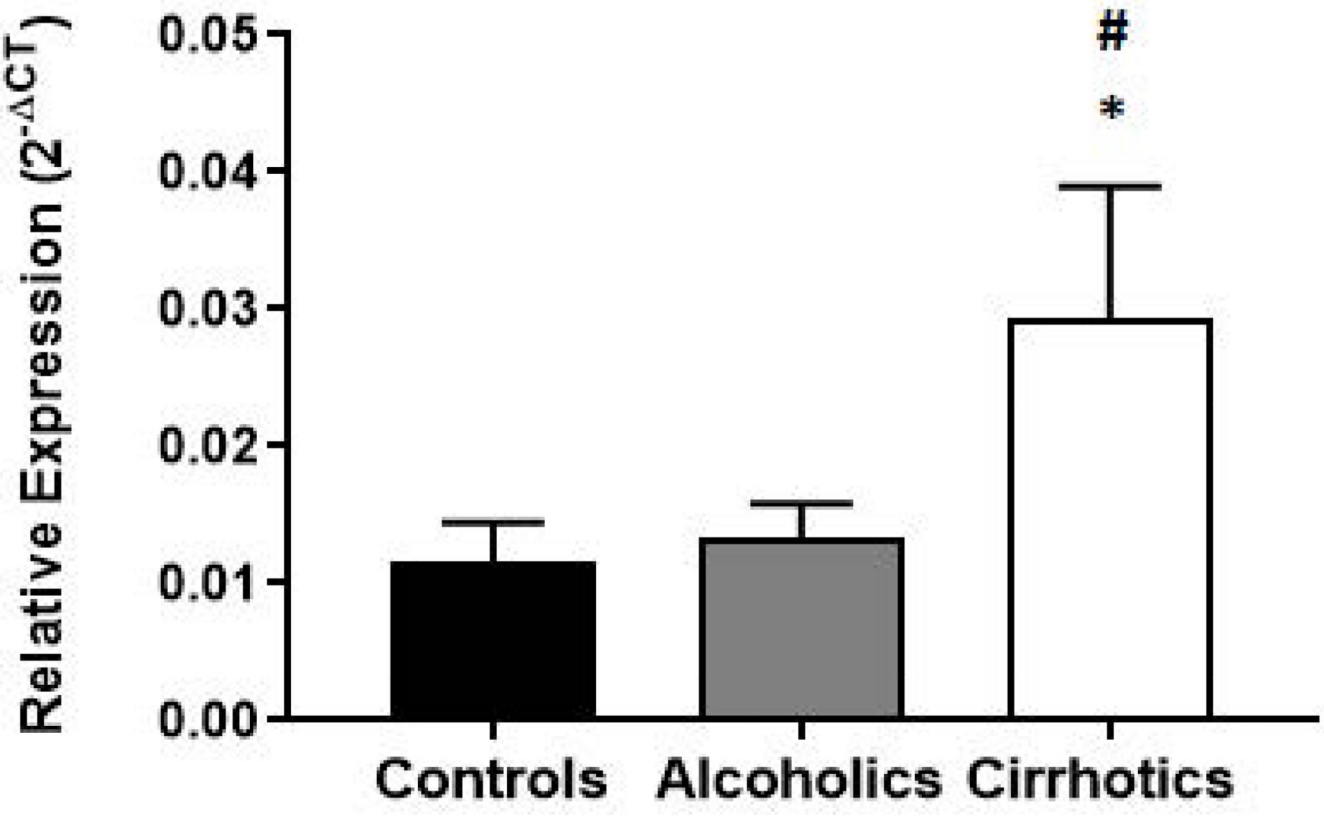

Figure 5:

Relative expression of miR-203 in human prefrontal cortex. Data are presented as mean ΔCT converted to 2–ΔCT ± SEM. Asterisk denotes significant changes relative to controls (Tukey hsd $P < 0.05$). Black bars represent controls; grey bars represent uncomplicated alcoholics and white bars represent cirrhotic alcoholics. Significant differences compared with controls are denoted by an asterisk (*); significant differences between uncomplicated and cirrhotic alcoholics are denoted by a hash (#).

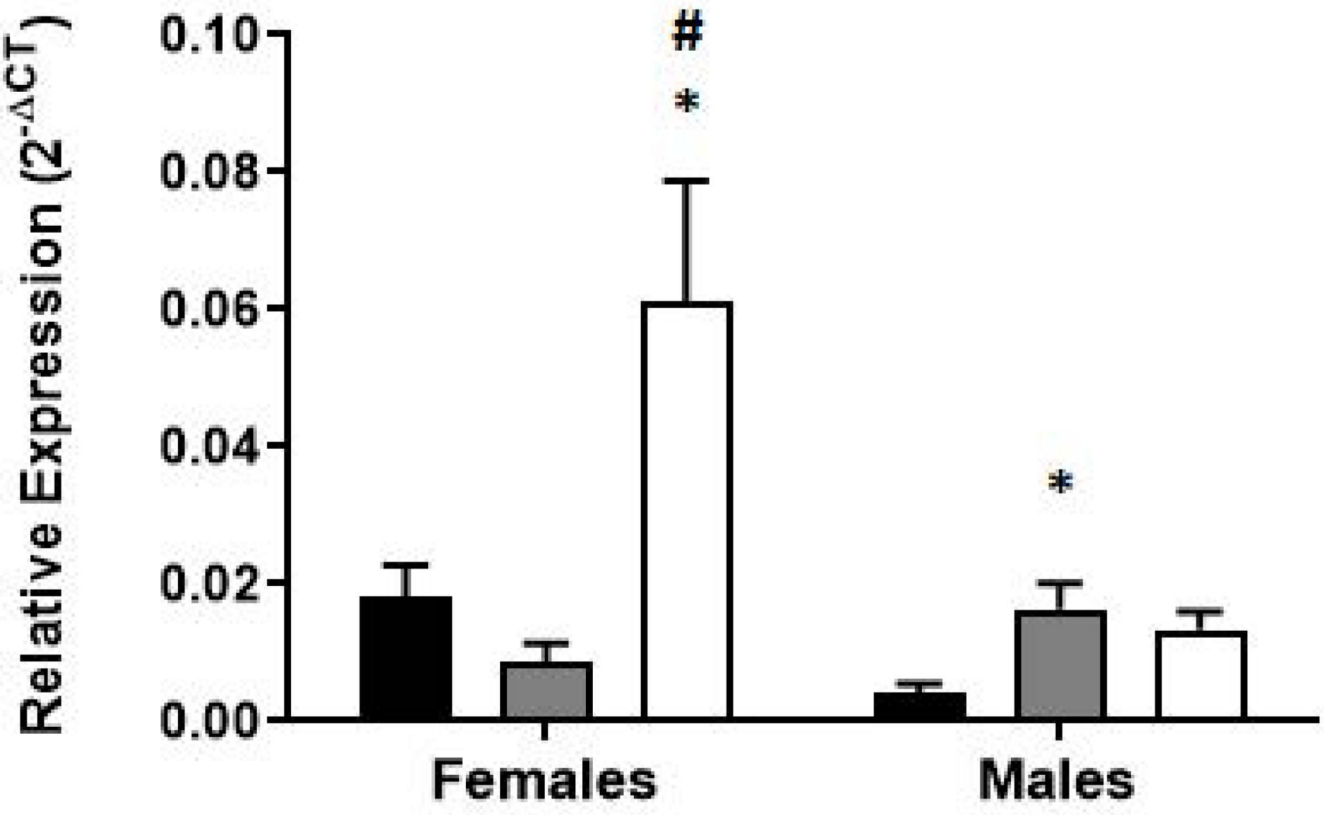

Figure 6:

Relative expression of miR-203 in human prefrontal cortex. Data are presented as mean ΔCT converted to 2–ΔCT ± SEM. Asterisk denotes significant changes relative to controls (Tukey hsd $P < 0.05$). Black bars represent controls; grey bars represent uncomplicated alcoholics and white bars represent cirrhotic alcoholics. Significant differences compared with controls are denoted by an asterisk (*); significant differences between uncomplicated and cirrhotic alcoholics are denoted by a hash (#).

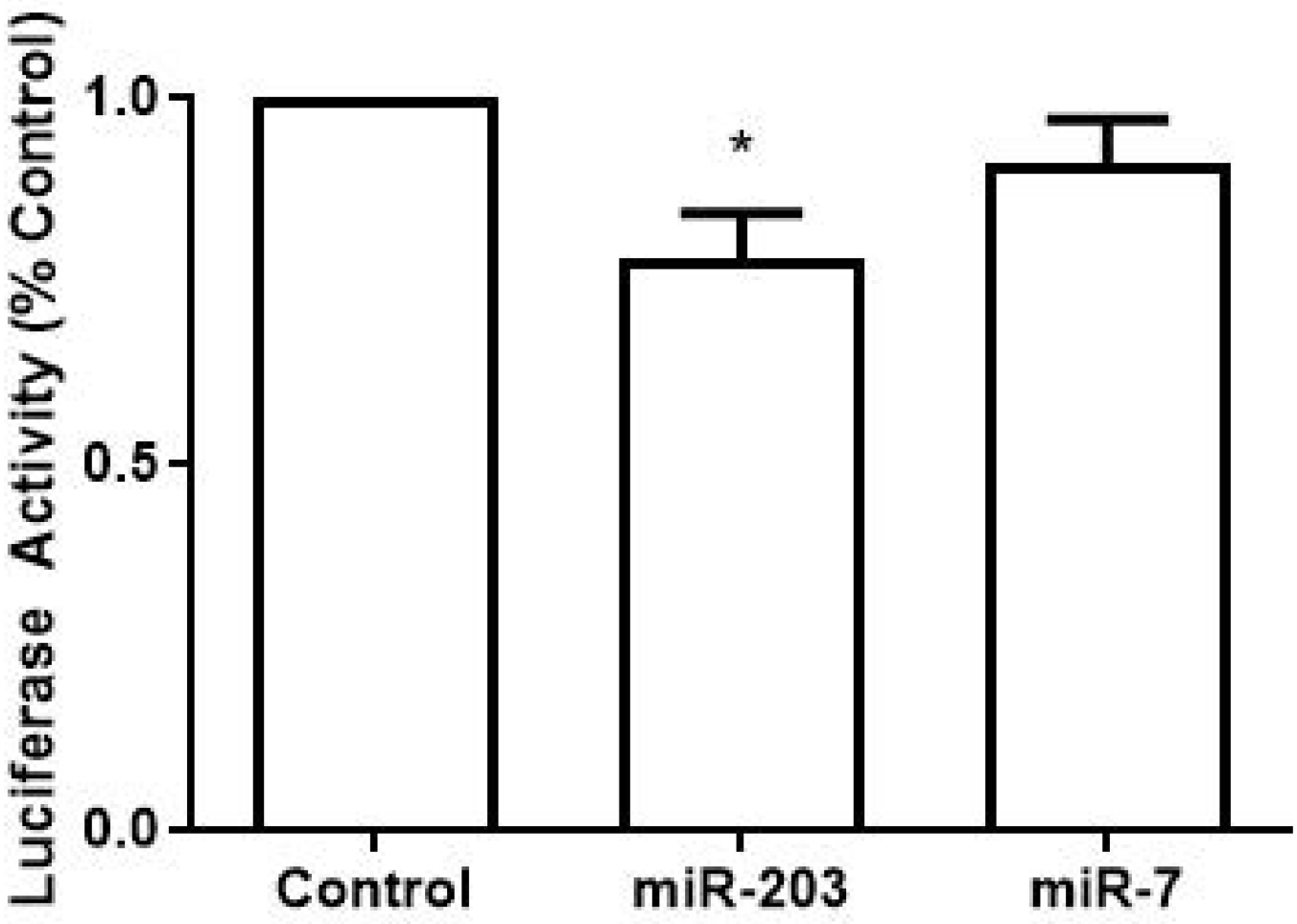

Figure 7:

Effect of miR-203 and miR-7 on GABRA5 3′-UTR luciferase reporter activity. HEK293T cells were co-transfected with clones containing the 3′-UTR of GABRA5 as well as clones containing the miRNA precursor sequences. Firefly and Renilla luciferase activities were measured and the results were graphed as ratios to the control. Independent-samples t-tests were used to analyse the data, compared with the control in which HEK293T cells were co-transfected with the control target 3′-UTR clone and a scrambled miR-precursor clone. Values are means ± SEM; *, $P < 0.05$.

Table 1.

Case information

Group	N	Age (y)	PMI (h)	RIN
GABRA5 Expression Cohort				
Controls (10 female, 11 male)	21	59.5±13.4	33.2±25.2	5.54±1.35
Alcoholics (9 female, 14 male)	23	49.2±15.0	28.9±13.5	5.49±0.98
Cirrhotic alcoholics (5 female, 5 male)	10	58.8±15.3	19.8±6.8	5.27±1.04
miR-203 Expression Cohort				
Controls (8 female, 9 male)	17	59.9±11.8	29.9±22.7	5.77±1.25
Alcoholics (9 female, 13 male)	22	49.6±13.8	29.7±16.8	5.69±0.95
Cirrhotic alcoholics (3 female, 5 male)	8	54.6±14.0	19.5±7.7	5.04±0.97

Table 2:

Regression Analysis

Age at death			
Controls	*GABRA5*	*Frontal*	$F_{1,19} = 0.701$, $P = 0.413$, $R^2 = 0.036$
		Motor	$F_{1,18} = 1.118$, $P = 0.304$, $R^2 = 0.058$
	miR-203	*Frontal*	$F_{1,16} = 0.468$, $P = 0.505$, $R^2 = 0.030$
Combined alcoholics	*GABRA5*	*Frontal*	$F_{1,30} = 0.007$, $P = 0.934$, $R^2 = 0.000$
		Motor	$F_{1,29} = 0.678$, $P = 0.417$, $R^2 = 0.023$
	miR-203	*Frontal*	$F_{1,29} = 0.272$, $P = 0.606$, $R^2 = 0.010$
Post mortem interval			
Controls	*GABRA5*	*Frontal*	$F_{1,19} = 0.223$, $P = 0.642$, $R^2 = 0.012$
		Motor	$F_{1,18} = 0.001$, $P = 0.979$, $R^2 = 0.000$
	miR-203	*Frontal*	$F_{1,16} = 1.215$, $P = 0.288$, $R^2 = 0.075$
Combined alcoholics	*GABRA5*	*Frontal*	$F_{1,30} = 0.823$, $P = 0.372$, $R^2 = 0.027$
		Motor	$F_{1,29} = 0.466$, $P = 0.500$, $R^2 = 0.016$
	miR-203	*Frontal*	$F_{1,29} = 0.321$, $P = 0.576$, $R^2 = 0.011$
RIN			
Controls	*GABRA5*	*Frontal*	$F_{1,19} = 3.471$, $P = 0.078$, $R^2 = 0.154$
		Motor	$F_{1,18} = 1.101$, $P = 0.309$, $R^2 = 0.061$
	miR-203	*Frontal*	$F_{1,16} = 1.758$, $P = 0.205$, $R^2 = 0.045$
Combined alcoholics	*GABRA5*	*Frontal*	$F_{1,30} = 15.25$, $P < 0.001$, $R^2 = 0.337$
		Motor	$F_{1,29} = 5.453$, $P = 0.027$, $R^2 = 0.158$
	miR-203	*Frontal*	$F_{1,29} = 12.002$, $P = 0.002$, $R^2 = 0.300$