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Selective changes of $GABA_A$ channel subunit mRNAs in the hippocampus and orbitofrontal cortex but not in prefrontal cortex of human alcoholics

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Alcohol dependence is a common chronic relapsing disorder. The development of alcohol dependence has been associated with changes in brain $GABA_A$ channel-mediated neurotransmission and plasticity. We have examined mRNA expression of the $GABA_A$ channel subunit genes in three brain regions in individuals with or without alcohol dependence using quantitative real-time PCR assay. The levels of selective $GABA_A$ channel subunit mRNAs were altered in specific brain regions in alcoholic subjects. Significant increase in the α1, α4, α5, β1, and γ1 subunit mRNAs in the hippocampal dentate gyrus region, and decrease in the β2 and δ subunit mRNAs in the orbitofrontal cortex were identified whereas no changes in the dorsolateral prefrontal cortex were detected. The data increase our understanding of the role of $GABA_A$ channels in the development of alcohol dependence.

Keywords: alcohol dependence, brain, $GABA_A$ channel, post-mortem

INTRODUCTION

Beverages containing alcohol are commonly consumed in today's societies and often abused. The brain is one of the main targets of alcohol. Long-term excessive consumption of alcohol can change the brain and lead to a variety of behavioral changes such as addiction and cognitive dysfunction (Harper, 1998). Magnetic resonance imaging studies have showed reduced hippocampal and prefrontal cortex volume of individuals suffering from alcohol dependence that may contribute to the cognitive deficit associated with chronic alcohol exposure (Jernigan et al., 1991; Sullivan et al., 1995). These aversive effects may be associated with direct and indirect actions of alcohol on various neurotransmitter and neuropeptide systems within the central nervous system (CNS; Harris et al., 2008; Vengeliene et al., 2008; Spanagel, 2009). Among those neurotransmitter receptors, a special focus has been on the association of alcohol action and alcoholism with γ-aminobutyric acid type A ($GABA_A$) ion channels during the last 30 years. Many $GABA_A$ channel subunit genes have been suggested to be associated with human alcoholism (Korpi and Sinkkonen, 2006), but detailed mechanisms remain poorly known and are inconsistent between studies.

The $GABA_A$ channels are GABA-gated anion channels that predominantly mediate inhibitory neurotransmission within CNS. Each $GABA_A$ channel complex is formed by five homologous protein subunits and to date 19 $GABA_A$ channel subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π, and ρ1–3) have been identified in mammals (Olsen and Sieghart, 2008). The various combinations of different subunits and associated proteins account for the diverse pharmacological and biophysical properties of the $GABA_A$ channel complex in the plasma membrane (Birnir and Korpi, 2007; Uusi-Oukari and Korpi, 2010). $GABA_A$ channels in neurons are present at synapses and extrasynaptic sites and mediate fast phasic inhibition and persistent tonic inhibition, respectively (Mody and Pearce, 2004; Semyanov et al., 2004; Lindquist and Birnir, 2006; Jin et al., 2011). Although many previous studies have shown that alcohol potentiates $GABA_A$ channels, how alcohol directly acts on $GABA_A$ channels remains unclear (Korpi et al., 2007). Alcohol action on $GABA_A$ channels depends on the concentration of alcohol and the channel subunits composition (Olsen et al., 2007). Some reports have demonstrated that low concentrations of alcohol (3–30 mM) can enhance the tonic inhibition mediated by extrasynaptic α4/6βδ $GABA_A$ channels but not the phasic inhibition mediated by γ2-containing synaptic $GABA_A$ channels (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Borghese et al., 2006; Korpi et al., 2007; Baur et al., 2009). In contrast, high concentration of alcohol (>60 mM) directly modulates $GABA_A$ channels associated with phasic inhibition and the putative alcohol-binding site was identified in-between transmembrane domains 2 and 3 (Mihic et al., 1997). In addition to the acute alcohol effects on $GABA_A$ channels, chronic exposure to alcohol can affect $GABA_A$ channel functions via alterations in subunit expression, post-translational modification, localization, intracellular signaling, and neurosteroid response (Kumar et al., 2009). In rodent models, chronic alcohol administration differentially changes the expression levels of $GABA_A$ channel subunit mRNAs and proteins across various brain regions (Grobin et al., 2000; Liang et al., 2006; Sarviharju et al., 2006). However, alcohol-dependent disorders in humans are not fully mimicked

by rodent models. Therefore, studies conducted on samples from post-mortem brains of individuals suffering from alcohol dependence may add important information and aid in understanding the mechanisms underlying human alcohol-dependent disorders.

In the present study, we performed quantitative real-time PCR (RT-qPCR) to investigate the expression of $GABA_A$ channel subunit mRNAs in the post-mortem hippocampal dentate gyrus, orbitofrontal, and dorsolateral prefrontal cortex (DL-PFC) of individuals suffering from alcohol dependence and have compared the results to brain samples from individuals without alcohol dependence.

MATERIALS AND METHODS

HUMAN SAMPLES

Twenty-one human controls and 19 individuals suffering from chronic alcohol dependence were included in the study. All individuals were Caucasian males. The individuals suffering from alcoholism consumed ≥80 g alcohol per day during the majority of their adult lives, met the criteria for Diagnostic and Statistical Manual for Mental Disorders, fourth edition and National Health and Medical Research Council/World Health Organization and did not have liver cirrhosis, Wernicke–Korsakoff's syndrome, or multi-drug abuse history. Individuals in the control group had either abstained from alcohol completely or were social drinkers who consumed ≤20 g of alcohol per day on average. Individuals in the control group were matched to individuals suffering from alcoholism by age and post-mortem interval (PMI). Post-mortem brain samples from hippocampal dentate gyrus (including both granule and molecular layer), orbitofrontal cortex (OFC; Brodmann's area 47), and DL-PFC (Brodmann's area 9) were collected at the New South Wales Tissue Resource Center (TRC), University of Sydney, Australia (http://sydney.edu.au/medicine/pathology/trc/index.php). The samples from all three brain regions were collected from the same donor in seven controls and ten individuals suffering from alcoholism. All samples were collected by qualified pathologists under full ethical clearance and with informed, written consent from the next of kin. The detailed demographic data for all subjects are given in **Table A1** in Appendix.

TOTAL RNA ISOLATION

Total RNA was isolated by using RNeasy Lipid Tissue Mini Kit (QIAGEN, MD, USA) or GenElute total RNA Miniprep (Sigma) and quantified with Nanodrop (Nanodrop Technologies, Inc.). The quality of RNA was evaluated by measuring RNA Quality Indicator (RQI) using Bio-Rad Experion (Bio-Rad Laboratories, Hercules, CA, USA) with Eukaryote Total RNA StdSens assay following the manufacturer's manual. RQI is equivalent to RNA integrity number (RIN) from Agilent (Denisov et al., 2008). RNA samples with RQI values greater than 5 are generally considered as suitable for RT-qPCR (Fleige and Pfaffl, 2006; Fleige et al., 2006). In this

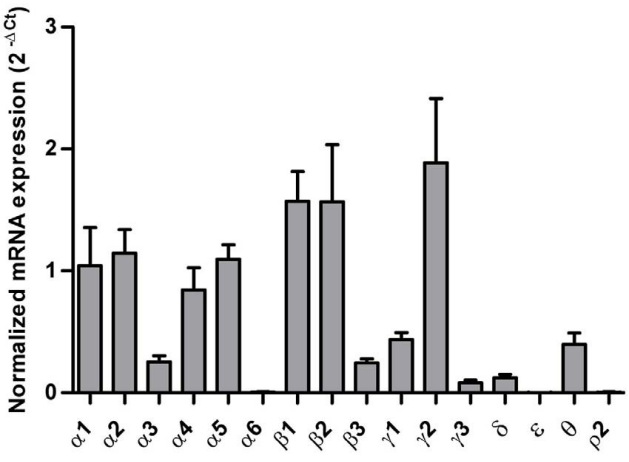

FIGURE 1 | Expression of $GABA_A$ channel subunit mRNAs in the hippocampal dentate gyrus region from control subjects ($n = 15$). The mRNA level of each subunit was normalized to reference genes *ACTB* and *UBC* and presented as mean ± SE.

Table 1 | Sample demographic information.

Characteristics	Hippocampal dentate gyrus			Dorsolateral prefrontal cortex			Orbitofrontral cortex		
	Controls	Alcoholics	*p* Value	Controls	Alcoholics	*p* Values	Controls	Alcoholics	*p* Value
Number	15	13		15	14		14	11	
Age (years)	57 ± 3	56 ± 4	0.909	59 ± 4	59 ± 4	0.981	59 ± 4	58 ± 5	0.774
PMI (h)	30 ± 4.8	30 ± 4.9	0.908	27 ± 4.2	29 ± 4.2	0.777	27 ± 4.6	29 ± 4.7	0.763
Brain pH	6.5 ± 0.05	6.5 ± 0.05	0.599	6.5 ± 0.06	6.5 ± 0.07	0.458	6.6 ± 0.06	6.5 ± 0.13	0.714
RNA quality indicator	6.9 ± 0.23	6.6 ± 0.14	0.23	7.3 ± 0.31	7.8 ± 0.31	0.206	7.7 ± 0.99	7.6 ± 0.96	0.936
Smoking history*	8(67%) S, 4(33%) NS	9(82%) S, 2(12%) NS	0.64	10 (77%) S, 3 (23%) NS	9(82%) S, 2(18%) NS	1.0	10(77%) S, 3(23%) NS	7(78%) S, 2(22%) NS	1.0

PMI, post-mortem interval; S, smoker; NS, non-smoker.

Age, PMI, brain pH, and RNA quality indicator are shown as mean ± SE, and the difference between controls and alcoholics was tested with Student's t-test or Mann–Whitney U-test.

**Smoking histories are not available for all subjects. The proportion of smokers and non-smokers between controls and alcoholics was tested with Fisher's exact test.*

study, samples with RQI less than 5 were not used for experiments. Average RQI of the samples was 7.29 ± 0.12 (mean ± SEM; 83% samples have RQI greater than 6) indicating high quality of isolated total RNA.

QUANTITATIVE REAL-TIME RT-PCR

Total RNA (250 ng) was reverse transcribed into cDNA in a 20 µl reaction mixture using Superscript III reverse transcriptase (Invitrogen). RT negative control was performed by omitting reverse transcriptase in the reaction in order to confirm no genomic DNA contamination in the isolated RNA. Real-time PCRs were performed in a 10 µl reaction mixture containing 4 µl cDNA (1 ng), 1 × PCR reaction buffer, 3 mM $MgCl_2$, 0.3 mM dNTP, 1 × ROX reference dye, 0.8 U JumpStart *Taq* DNA polymerase (Sigma-Aldrich), 5 × SYBR Green I (Invitrogen), and 0.4 µM each of forward and reverse primers. The gene-specific primer pairs (primer sequences shown in **Table A2** in Appendix) were designed using Primer Express Software version 3.0 (Applied Biosystems), synthesized by Invitrogen and further validated using BioBank cDNA from human brain (PrimerDesign). Amplification was performed in 384-well optical plates using the ABI PRISM 7900HT Sequence Detection System (Applied

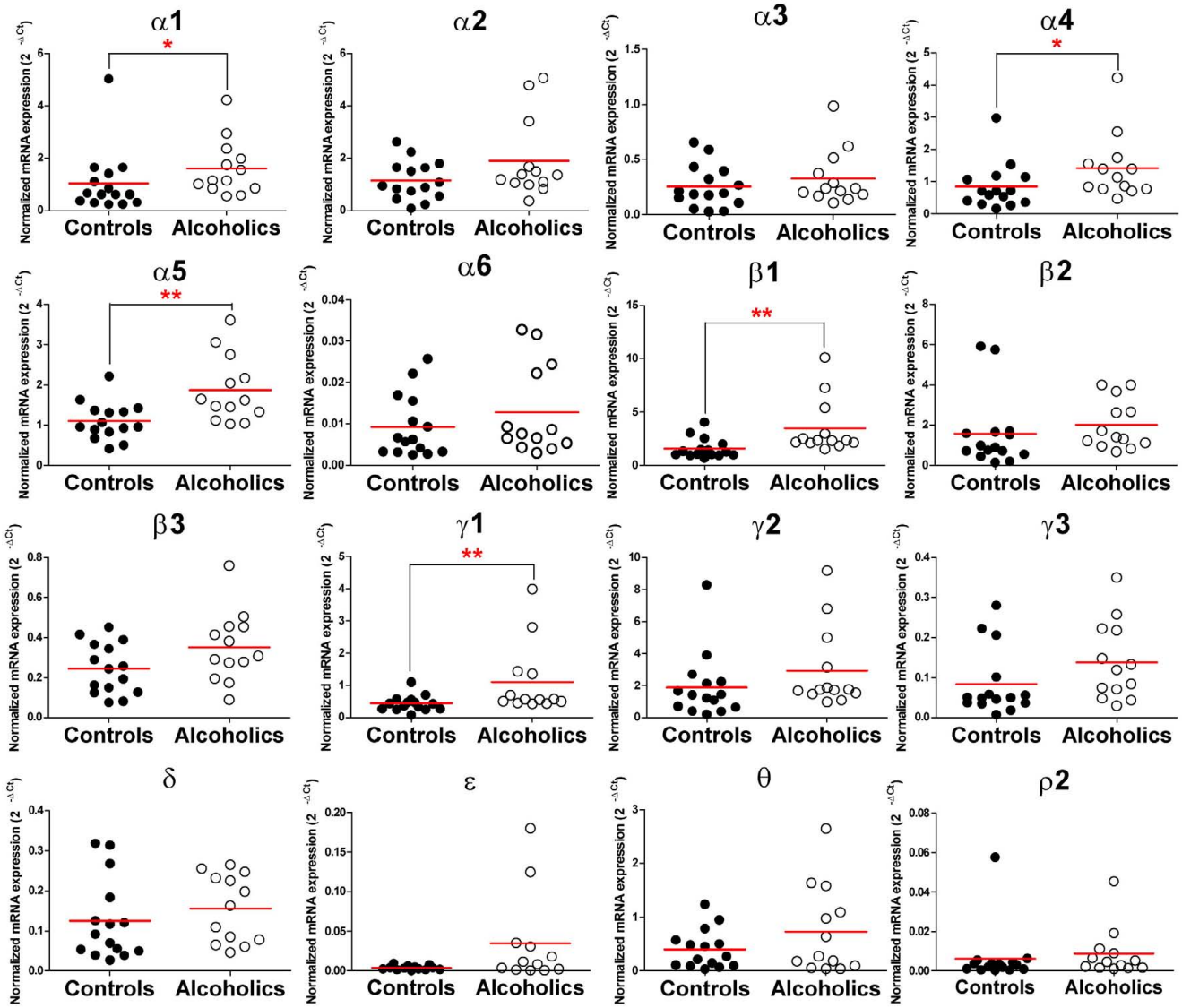

FIGURE 2 | Expression of $GABA_A$ channel subunit mRNAs in the hippocampal dentate gyrus region of controls (●, $n = 15$) and alcoholics (○, $n = 13$). Horizontal lines represented mean levels. Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, α1, $H(1, 28) = 4.39$, $p = 0.036$; α2, $H(1, 28) = 1.72$, $p = 0.19$; α3, $H(1, 28) = 0.73$, $p = 0.39$; α4, $H(1, 28) = 5.41$, $p = 0.02$; α 5, $H(1, 28) = 8.83$, $p = 0.003$; α6, $H(1, 28) = 1.17$, $p = 0.28$; β1, $H(1, 28) = 9.38$, $p = 0.002$; β2, $H(1, 28) = 2.68$, $p = 0.10$; γ1, $H(1, 28) = 7.02$, $p = 0.008$; γ2, $H(1, 28) = 2.83$, $p = 0.09$; γ3, $H(1, 28) = 3.31$, $p = 0.07$; δ, $H(1, 28) = 1.07$, $p = 0.3$; ρ2, $H(1, 28) = 1.97$, $p = 0.16$; θ, $H(1, 28) = 0.38$, $p = 0.53$; ε, $H(1, 28) = 2.32$, $p = 0.13$. One way ANOVA with Bonferroni *post hoc* test, β3, df = 23, $p = 0.06$.

Biosystems) with an initial denaturation of 5 min at 95°C, followed by 45 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. A melting curve was determined at the end of cycling to ensure the amplification of a single PCR product. Cycle threshold values (Ct) were determined with the SDS 2.3 and RQ Manager 1.2 softwares supplied with the instrument. The expression of each target gene relative to a normalization factor (geometric mean of two reference genes) was calculated with DataAssist v2.0 using the $2^{-\Delta Ct}$ method as previously described (Schmittgen and Livak, 2008). Reference genes beta actin (*ACTB*) and ubiquitin C (*UBC*) for hippocampal dentate gyrus (average expression stability value $M = 0.25$), ribosomal large P0 (*RPLP0*) and *ACTB* for prefrontal cortex (average expression stability value $M = 0.22$), and phosphoglycerate kinase 1 (*PGK1*) and peptidylprolyl isomerase A (*PPIA*) for OFC (average expression stability value $M = 0.125$) were selected for normalization according to previously developed approach for analysis of reference genes (Johansson et al., 2007; Kuzmin et al., 2009; Bazov et al., 2011). As the expression of reference genes may vary between different brain regions of human alcoholics, it is of great importance to use validated stable reference genes for normalization.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SigmaPlot and SigmaStat (Systat Software Inc., USA). Normality of data distribution was analyzed using Shapiro–Wilk normality test (see **Table A3** in Appendix). The differences between groups were assessed by one-way ANOVA with Bonferroni *post hoc* test (normally distributed data) or non-parametric Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test (not normally distributed data). A general stepwise linear regression model was used to identify covariates (e.g., age and PMI). Variables with a significant association with group (controls and alcoholics) were included in the final statistical model as covariates. A significant level was set to $p < 0.05$.

RESULTS

The demographic characteristics of individuals in this study are shown in **Table 1** and **Table A1** in Appendix. There was no significant difference in age, PMI, brain pH, RNA quality indicator, and proportions of smokers and non-smokers between individuals with or without alcohol dependence (**Table 1**).

Expression of the 19 $GABA_A$ channel subunit mRNAs (α1–6, β1–3, γ1–3, δ, ε, θ, π, ρ1–3) was quantified by RT-qPCR in the samples collected from the hippocampal dentate gyrus region (HP-DG), the orbitofronral cortex (OFC, Brodmann area 47), and the dorsolateral prefrontal cortex (DL-PFC, Brodmann area 9). Subunit genes that were not detected in any of the three brain regions were the π, ρ1, and ρ3 subunits.

INCREASED LEVELS OF mRNAS FOR $GABA_A$ CHANNEL SUBUNITS α1, α4, α5, β1, AND γ1 IN THE HIPPOCAMPAL DENTATE GYRUS REGION OF ALCOHOLICS

In the hippocampal dentate gyrus region of individuals without alcohol dependence, high expression of α1, α2, α4, α5, β1, β2, and γ2, modest expression of α3, β3, γ1, γ3, δ, and θ, low expression of α6, ε, and ρ2 subunit mRNAs were detected (**Figure 1**).

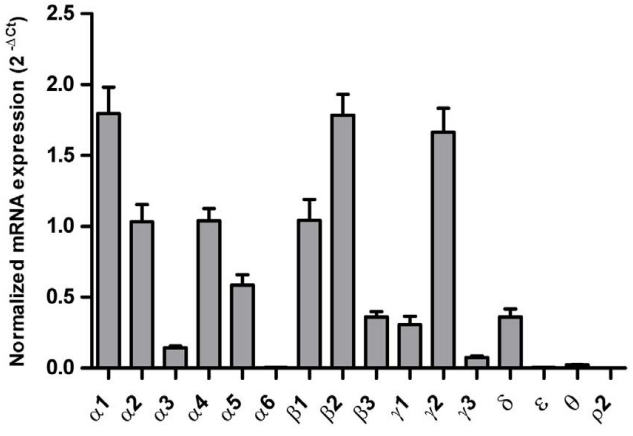

FIGURE 3 | Expression of $GABA_A$ channel subunit mRNAs in the orbitolfrontal cortex of controls ($n = 14$). The mRNA level of each subunit was normalized to reference genes *PPIA* and *PGK1* and presented as mean ± SE.

Interestingly, the mRNA levels of α1, α4, α5, β1, and γ1 subunits were significantly higher in the HP-DG of individuals suffering from alcohol dependence than in the controls. There was a 1.5-fold (α1), 1.6-fold (α4), 1.7-fold (α5), 2.1-fold (β1), and 2.3-fold (γ1) increase of mRNA in the hippocampal dentate gyrus region of individuals suffering from alcoholism as compared to those without alcohol dependence. Stepwise linear regression identified age, PMI, and brain pH as covariates for the α5 expression level. However, inclusion of these covariates in linear regression model did not affect the significant difference in α5 expression level between the two groups. The mRNA levels of other $GABA_A$ channel subunits did not differ between two groups (**Figure 2**).

DECREASED EXPRESSION OF $GABA_A$ CHANNEL SUBUNIT β2 AND δ mRNAS IN THE ORBITOFRONTAL CORTEX OF ALCOHOLICS

In the OFC of individuals without alcohol dependence, high expression of α1, α2, α4, β1, β2, and γ2, modest expression of α3, α5, β3, γ1, γ3, and δ, low expression of α6, ε, θ, and ρ2 subunit mRNAs were detected (**Figure 3**). Of particular interest is the result that the mRNA levels of the β2 and δ subunits were 26 and 47% lower, respectively, in individuals suffering from alcohol dependence as compared to those without alcohol dependence, while the mRNA expression of other subunits did not differ between the two groups (**Figure 4**). Stepwise linear regression identified age as a covariate for the β2 and δ expression level. However, inclusion of age as a covariate in linear regression model failed to affect the significant difference in the β2 and δ expression levels between the two groups.

UNALTERED EXPRESSION OF $GABA_A$ CHANNEL SUBUNIT mRNAS IN THE DORSOLATERAL PREFRONTAL CORTEX OF ALCOHOLICS

The mRNA expression profile of $GABA_A$ channel subunits in the DL-PFC in individuals without alcohol dependence closely resembled that observed in the OFC (**Figures 3** and **5**). Furthermore, no significant differences in the mRNA expression were observed for

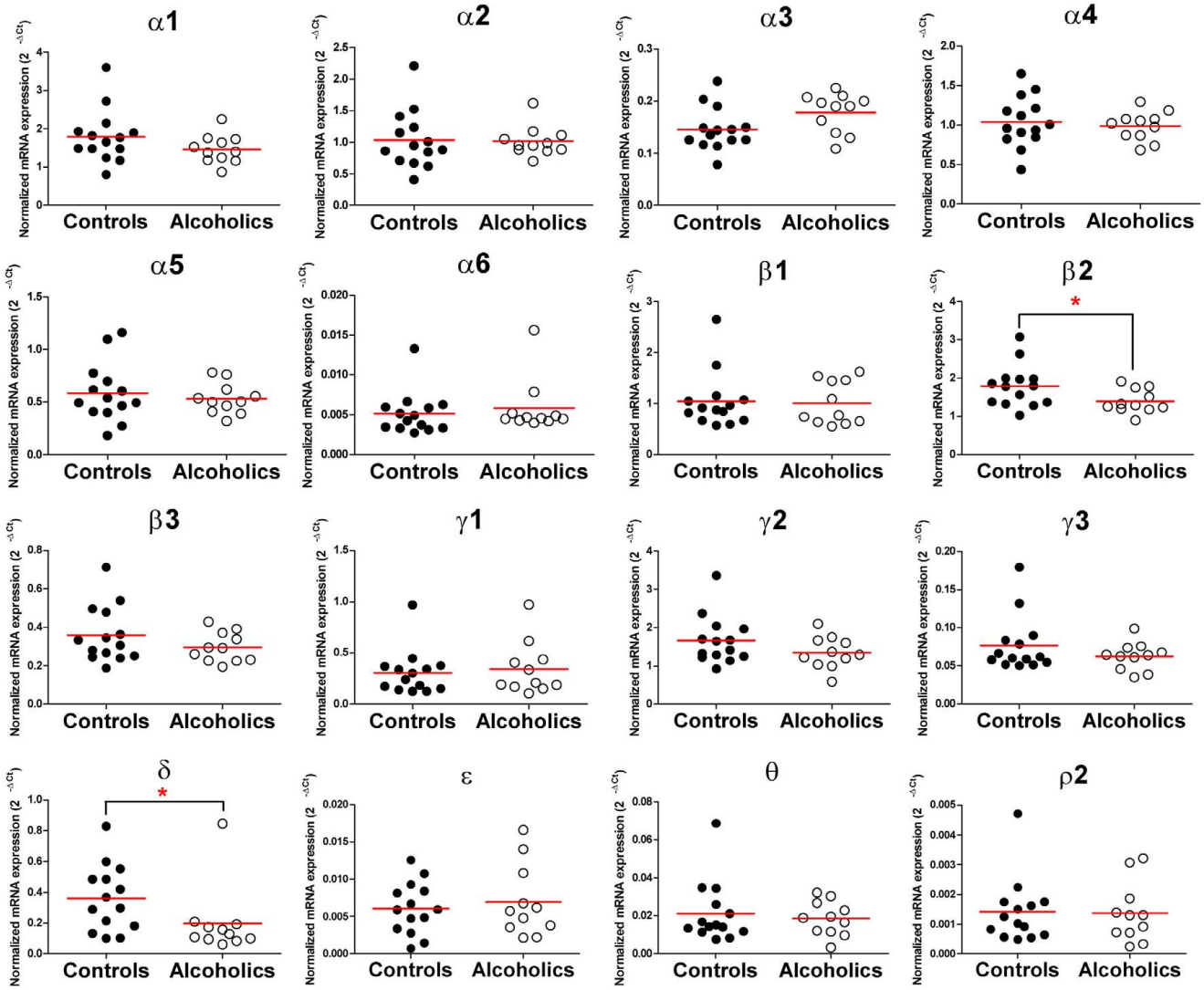

FIGURE 4 | Expression of $GABA_A$ channel subunits mRNA in the orbitofrontal cortex of controls (●, $n = 14$) and alcoholics (○, $n = 11$). Horizontal lines represented mean levels. One way ANOVA with Bonferroni *post hoc* test, α3, df = 23, $p = 0.052$; α4, df = 23, $p = 0.61$; α 5, df = 23, $p = 0.55$; β2, df = 23, $p = 0.045$; ε, df = 23, $p = 0.06$. Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, α1, $H(1, 25) = 2.03$, $p = 0.15$; α2, $H(1, 25) = 0.3$, $p = 0.58$; α6, $H(1, 25) = 0.51$, $p = 0.48$; β1, $H(1, 25) = 0.05$, $p = 0.83$; β3, $H(1, 25) = 1.09$, $p = 0.3$; γ1, $H(1, 25) = 0.15$, $p = 0.70$; γ2, $H(1, 25) = 1.59$, $p = 0.21$; γ3, $H(1, 25) = 0.15$, $p = 0.70$; θ, $H(1, 25) = 0.003$, $p = 0.96$; ρ2, $H(1, 25) = 0.003$, $p = 0.96$; δ, $H(1, 25) = 5.8$, $p = 0.016$.

any of the subunits between alcoholics and non-alcoholic controls (**Figure 6**).

DISCUSSION

The expression of specific $GABA_A$ channel subunit mRNAs was altered in specific brain regions in individuals suffering from alcoholism. In particular, there was a significant increase of the α1, α4, α5, β1, and γ1 subunit mRNAs in the HP-DG; a decrease of the β2 and δ in the OFC, but no change of any subunit expression in the DL-PFC. These data complement previous expression studies in individuals suffering from alcohol dependence providing further evidence for long-term changes in the $GABA_A$ channels in the CNS induced by long-term alcohol consumption (Lewohl et al., 1997, 2001; Mitsyama et al., 1998; Thomas et al., 1998; Buckley and Dodd, 2004).

Gene expression profiling studies by, e.g., quantitative PCR using human autopsy brain tissue can be affected by many pre- and post-mortem factors such as age, gender, ethnicity, and PMI. In this study we have tried to minimize the differences between the two groups of individuals we have studied. All individuals included in the study were Caucasian males and the two groups were matched for parameters such as age, PMI, brain pH, RNA quality indicator value, and proportions of smokers and non-smokers. In addition, the sample size in each group was between 11 and 15 that falls within the group–size range providing rather reliable statistical estimation (Hynd et al., 2003).

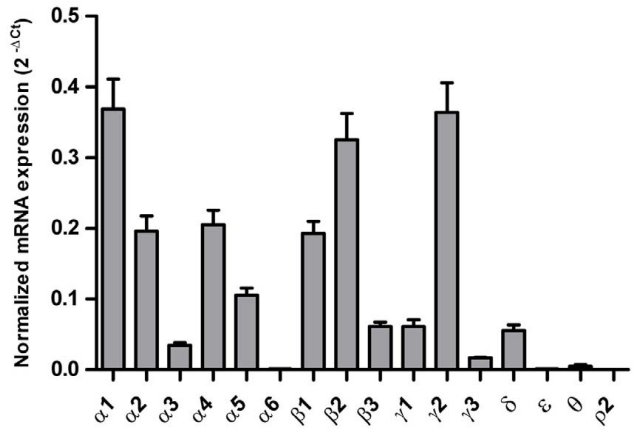

FIGURE 5 | Expression of $GABA_A$ channel subunit mRNAs in the prefrontal cortex of controls ($n = 15$). The mRNA level of each subunit was normalized to reference genes *ACTB* and *RPLP0* and presented as mean ± SE.

$GABA_A$ channels are pentameric GABA-gated chloride channels. A change of subunit composition in the $GABA_A$ channel complex can directly affect its cellular and sub-cellular location as well as physiological and pharmacological properties of the channel, including ethanol sensitivity (Birnir and Korpi, 2007). Several studies have indicated that the expression of $GABA_A$ channel subunits differs at both the mRNA and protein levels between post-mortem brains from non-alcoholic individuals and alcoholics, although the results remain somewhat ambiguous (Lewohl et al., 1997, 2001; Dodd and Lewohl, 1998; Buckley et al., 2000, 2006; Buckley and Dodd, 2004). It has been shown that the expression of the $GABA_A$ channel subunit α1 mRNA but not protein was elevated in the superior frontal cortex of alcoholics (Lewohl et al., 1997; Dodd and Lewohl, 1998). Similarly, in our study α1 mRNA level was increased in the hippocampal dentate gyrus of alcoholic individuals. However, chronic alcohol administration to rodents decreased or did not change the α1 mRNA and protein levels in the cerebral cortex, cerebellum, or hippocampus (Uusi-Oukari and Korpi, 2010). This discrepancy in results between humans and rodents can potentially be attributed to the difference in, e.g., in metabolism or length of alcohol exposure, transcriptional regulation and animal models of alcohol dependence, in addition to the species difference.

$GABA_A$ channels containing the α4, α5, or the δ subunit are of particular interest. These subunits are parts of extrasynaptic $GABA_A$ channels that participate in generating tonic neuronal inhibition that decreases action potential frequency in neurons (Pavlov et al., 2009; Jin et al., 2011). Activation of $GABA_A$ channels containing these subunits is thought to have implications for cognitive function. In a rodent model for excessive alcohol consumption, repeated ethanol withdrawals or longer ethanol exposure increased the α4 subunit protein expression in the hippocampus (Matthews et al., 1998; Cagetti et al., 2003). This is in accordance with our data where the α4 mRNA expression was elevated in alcoholic individuals as compared to the non-alcoholic subjects. In the human hippocampus, the α5 subunit is abundant in the dentate gyrus molecular layer as well as in mid-CA1 regions (Howell et al., 2000; Wainwright et al., 2000; Rissman et al., 2003), whereas in the rodent hippocampus, α5-containing $GABA_A$ channels are only highly expressed in CA1 pyramidal neurons (Sperk et al., 1997). Genetic or pharmacological manipulation of α5-containing $GABA_A$ channels in mice modulates hippocampus-dependent learning (Crestani et al., 2002; Caraiscos et al., 2004; Martin et al., 2010; Prut et al., 2010). It is possible that $GABA_A$ α4 or α5 subunit-selective compounds may potentially be used for the treatment of alcohol-induced cognitive deficit.

Ethanol can induce the release of endogenous GABAergic neurosteroids that further enhance the GABA signaling system in neurons (Biggio et al., 2007). The sensitivity to neurosteroids is higher in γ1 subunit-containing $GABA_A$ channels than in γ2 subunit-containing $GABA_A$ channels (Puia et al., 1993). Chronic ethanol administration in rodents significantly increases the mRNA expression of γ1 subunit in the cerebral cortex (Devaud et al., 1995) and in the hippocampus (Cagetti et al., 2003), and similarly in our study, up-regulation of the γ1 subunit was observed in the HP-DG from alcoholic individuals. Therefore, increased sensitivity to neurosteroids of $GABA_A$ channels may be associated with the alcohol dependence.

Some of the genes encoding the human $GABA_A$ channel subunits are organized into clusters on chromosomes. Chromosome 4 contains four $GABA_A$ channel genes: *GABRA2* (α2), *GABRA4* (α4), *GABRB1* (β1), and *GABRG1* (γ1) (Reich et al., 1998). As the change in the gene regulation of one $GABA_A$ channel subunit may affect the transcription levels of other $GABA_A$ subunit genes in the same cluster (Uusi-Oukari et al., 2000; Steiger and Russek, 2004), it is not surprising to see the up-regulation of three of them (α4, β1, and γ1), in HP-DG of alcoholic individuals in our study. It will be worth using genetic mapping approach to study whether these $GABA_A$ subunit expressions are associated with specific gene polymorphisms and whether the regulation of transcription is similar for these subunit genes (Joyce, 2007). Since the α1, α4, α5, and β1 $GABA_A$ subunits are abundant in the hippocampal dentate gyrus, the increase in the expression of these subunits may have significant functional consequence in alcohol-induced cognitive impairment. Further studies are needed to determine the protein level of these altered subunits and assess their putative functional impact in human alcoholism.

Chronic alcohol consumption in humans has been shown to cause impairment of executive and cognitive functions which require normal prefrontal cortical function (Goldstein et al., 2004; Crego et al., 2010). Here we have examined the expression of $GABA_A$ channel subunits in the sub-regions of cortex, DL-PFC, and OFC from alcoholics. In the OFC of alcoholic individuals, the β2 and δ $GABA_A$ subunits were significantly decreased. Whether this decrease contributes to the impaired GABAergic function in the OFC reported in studies involving alcoholics remains to be determined (Volkow et al., 1993, 1997). In contrast, none of the $GABA_A$ subunits were changed in the DL-OFC of individuals suffering from alcohol dependence as compared to non-alcoholic individuals. This is in agreement with two microarray studies showing no change of any $GABA_A$ subunit mRNAs in the frontal

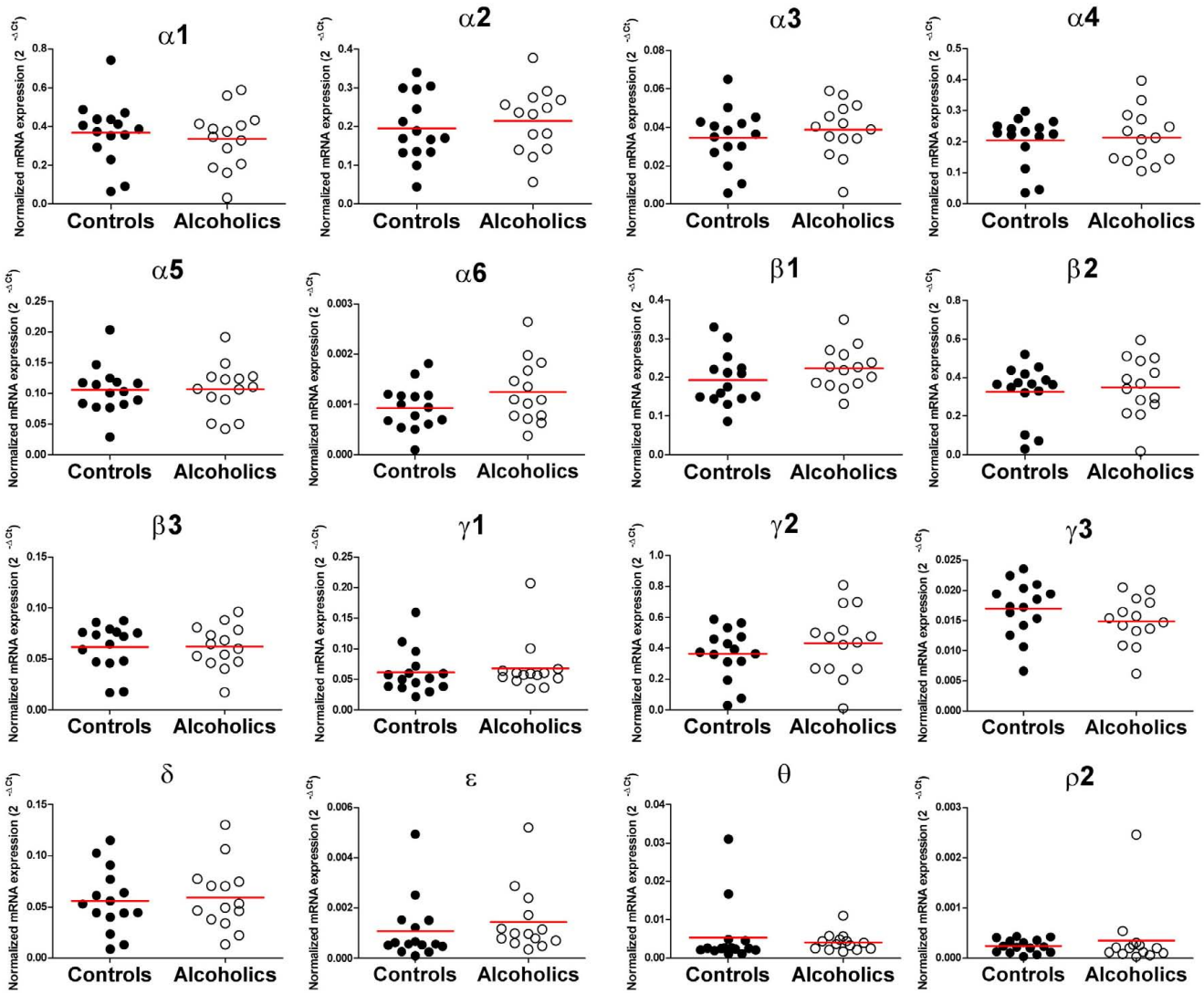

FIGURE 6 | Expression of $GABA_A$ channel subunit mRNAs in the prefrontal cortex of controls (●, $n = 15$) and alcoholics (○, $n = 14$). Horizontal lines represented mean levels. One way ANOVA with Bonferroni *post hoc* test, α1, df = 27, $p = 0.58$; α2, df = 27, $p = 0.55$; α3, df = 27, $p = 0.45$; α4, df = 27, $p = 0.76$; α5, df = 27, $p = 0.93$; α6, df = 27, $p = 0.13$; β1, df = 27, $p = 0.19$; β2, df = 27, $p = 0.66$; β3, df = 27, $p = 0.97$; γ2, df = 27, $p = 0.36$; γ3, df = 27, $p = 0.19$; δ, df = 27, $p = 0.76$; Kruskal–Wallis ANOVA on ranks with Dunn's *post hoc* test, γ1, $H(1, 29) = 0.69$, $p = 0.41$; ε, $H(1, 29) = 0.13$; θ, $H(1, 29) = 1.29$, $p = 0.26$; ρ^2, $H(1, 29) = 0.62$, $p = 0.43$.

cortex of alcoholic subjects (Mayfield et al., 2002; Flatscher-Bader et al., 2005).

In conclusion, we report brain area-specific selective changes in the mRNA expression of $GABA_A$ channel subunits in individuals suffering from alcohol dependence compared to control cases. It is of particular interest that several of the subunits that change with chronic alcohol consumption (e.g., α4, α5, and δ) are present in many extrasynaptic $GABA_A$ channels mediating tonic inhibition. As tonic inhibition has a significant role in determining baseline excitability of neurons this is perhaps not surprising but highlights the importance of $GABA_A$ channels located outside of synapses for drug effects.

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APPENDIX

Table A1 | Demographic data of controls and alcoholics.

Subject no.	Age (years)	PMI (hours)	Brain pH	Brain regions	Smoking history	Cause of death
CONTROLS						
1	34	20.5	6.73	DL-PFC, OFC	Yes	Acute exacerbation of asthma
2	78	6.5	6.2	DL-PFC, OFC	No	Adenocarcinoma
3	63	72	6.9	DL-PFC, OFC, HP-DG	Yes	Coronary artery atherosclerosis
4	82	23.5	6.4	DL-PFC, OFC	NA	Sepsis
5	38	13.5	6.26	DL-PFC, OFC, HP-DG	Yes	Atherosclerotic cardiovascular disease
6	69	16	6.6	DL-PFC, OFC, HP-DG	Yes	Atherosclerotic cardiovascular disease
7	56	24	6.53	DL-PFC, OFC, HP-DG	Yes	Coronary artery atheroma
8	59	20	6.56	DL-PFC, OFC, HP-DG	Yes	Coronary thrombosis
9	56	25	6.1	DL-PFC	NA	Ischemic heart disease
10	56	37	6.76	DL-PFC, OFC, HP-DG	Yes	Left ventricular scarring, hypertension, cardiomegaly
11	82	36	6.24	DL-PFC, OFC, HP-DG	No	Myocardial infarction
12	44	50	6.6	DL-PFC, OFC	Yes	Ischemic heart disease
13	66	22	6.31	HP-DG	NA	Respiratory failure
14	56	48	6.49	HP-DG	Yes	Cardiac failure
15	43	66	6.2	HP-DG	No	Aspiration pneumonia
16	58	12	6.46	HP-DG	Yes	Ischemic heart disease
17	37	24	6.37	HP-DG	NA	Electrocution
18	46	25	6.65	HP-DG	NA	Mitral valve prolapse
19	58	15	6.71	HP-DG	No	Perforated gastric ulcer
20	68	22	6.59	HP-DG	No	Asphyxia
21	53	16	6.84	DL-PFC, OFC	No	Dilated cardiomyopathy
ALCOHOLICS						
1	70	62	6.82	HP-DG	Yes	Cardiomyopathy
2	38	22	6.78	HP-DG	Yes	Ischemic heart disease
3	34	8.5	6.61	DL-PFC, OFC, HP-DG	Yes	Hanging
4	77	20	6.34	DL-PFC, OFC, HP-DG	Yes	Bronchopneumonia
5	65	32	5.66	DL-PFC	NA	Complications of chronic alcoholism
6	50	17	6.3	HP-DG	No	Ischemic heart disease
7	79	48	6.34	DL-PFC, OFC, HP-DG	Yes	Ischemic heart disease
8	39	24	6.56	DL-PFC, OFC, HP-DG	Yes	Aortic stenosis
9	56	22	6.52	DL-PFC, OFC, HP-DG	Yes	Gastro-intestinal hemorrhage
10	59	24	6.57	DL-PFC, OFC	No	Cardiomyopathy
11	56	15	6.66	DL-PFC, OFC, HP-DG	NA	Ischemic heart disease and emphysema
12	56	45	6.51	DL-PFC, OFC, HP-DG	NA	Bleeding esophageal varices
13	44	15	6.48	DL-PFC, OFC, HP-DG	No	Ischemic heart disease
14	81	36	6.44	DL-PFC, OFC, HP-DG	Yes	Sepsis
15	62	49	6.49	DL-PFC	Yes	Ischemic heart disease
16	66	11.5	6.4	DL-PFC	Yes	Pneumonia
17	53	57	6.75	DL-PFC, OFC, HP-DG	Yes	Chronic airflow limitation
18	61	24	6.52	DL-PFC, OFC	Yes	Ischemic heart disease
19	57	18	6.6	DL-PFC, OFC	Yes	Ischemic heart disease

PMI, post-mortem interval; DL-PFC, dorsolateral prefrontal cortex; OFC, orbitofrontal cortex; HP-DG, hippocampal dendate gyrus; NA, not available.

Table A2 | Human primers list for quantitative real-time RT-PCR.

Gene	Primer	Product size (bp)	Reference number
α1 (*GABRA1*)	F: GGATTGGGAGAGCGTGTAACC	66	NM_000806
	R: TGAAACGGGTCCGAAACTG		
α2 (*GABRA2*)	F: GTTCAAGCTGAATGCCCAAT	160	NM_000807
	R: ACCTAGAGCCATCAGGAGCA		
α3 (*GABRA3*)	F: CAACTTGTTTCAGTTCATTCATCCTT	102	NM_000808
	R: CTTGTTTGTGTGATTATCATCTTCTTAGG		
α4 (*GABRA4*)	F: TTGGGGGTCCTGTTACAGAAG	105	NM_000809
	R: TCTGCCTGAAGAACACATCCA		
α5 (*GABRA5*)	F: ACGGTGGGCACTGAGAACAT	64	NM_000810
	R: GGAAGTGAGCTGTCATGATTGTG		
α6 (*GABRA6*)	F: ACCCACAGTGACAATATCAAAAGC	67	NM_000811
	R: GGAGTCAGGATGCAAAACAATCT		
β1(GABRB1)	F: GTACAAAATCGAGAGAGTCTGGG	144	NM_000812
	R: GCG AATGTCATATCCTTTGAGCA		
β2(*GABRB2*)	F: GCAGAGTGTCAATGACCCTAGT	137	NM_021911
	R: TGGCAATGTCAATGTTCATCCC		
β3(*GABRB3*)	F: CAAGCTGTTGAAAGGCTACGA	108	NM_000814
	R: ACTTCGGAAACCATGTCGATG		
γ1(*GABRG1*)	F: CCTTTTCTTCTGCGGAGTCAA	91	NM_173536
	R: CATCTGCCTTATCAACACAGTTTCC		
γ2(*GABRG2*)	F: CACAGAAAATGACGGTGTGG	136	NM_000816
	R: TCACCCTCAGGAACTTTTGG		
γ3(*GABRG3*)	F: AACCAACCACCACGAAGAAGA	113	NM_033223
	R: CCTCATGTCCAGGAGGGAAT		
δ (*GABRD*)	F: ACCACGGAGCTGATGAACTT	109	NM_000815
	R: AGGGCATGTAGGATTGGATG		
ε (*GABRE*)	F: TGGATTCTCACTCTTGCCCTCTA	107	NM_004961
	R: GGAGTTCTTCTCATTGATTTCAAGCT		
θ (*GABRQ*)	F: CCAGGGTGACAATTGGCTTAA	63	NM_018558
	R: CCCGCAGATGTGAGTCGAT		
π (*GABRP*)	F: GGCCTTGCTAGAATATGCAGTTG	76	NM_014211
	R: CTTTGTTGTCCCCCTATCTTTGG		
ρ1(*GABRR1*)	Hs00266687_m1 from applied biosystem	94	NM_002042
ρ2 (*GABRR2*)	F: CCTAGAAGAGGGCATAGACATCG	99	NM_002043
	R: TCCAGTAGCTGCTGCATTGTTTG		
ρ3 (*GABRR3*)	F: TGATGCTTTCATGGGTTTCA	111	NM_001105580
	R: CGCTCACAGCAGTGATGATT		
β-actin (*ACTB*)	F: CCTGGCACCCAGCACAAT	144	NM_001101
	R: GGGCCGGACTCGTCATACT		
RPLP0	F: CCTCATATCCGGGGGAATGTG	95	NM_001002
	R: GCAGCAGCTGGCACCTTATTG		
PPIA	F: CCCACCGTGTTCTTCGACAT	116	NM_021130
	R: CCAGTGCTCAGAGCACGAAA		
PKG1	F: AGGGAAAAGATGCTTCTGGG	71	NM_000291
	R: AAGTGAAGCTCGGAAAGCTTCTAT		
UBC	F: CGGTGAACGCCGATGATTAT	124	NM_021009
	R: ATCTGCATTGTCAAGTGACGA		

Table A3 | Analysis of normality of RT-qPCR data distribution by Shapiro–Wilk normality test (*p* values are shown below).

	HP-DG	OFC	DL-PFC
α1	$p < 0.05$	$p < 0.05$	$p = 0.239$
α2	$p < 0.05$	$p < 0.05$	$p = 0.878$
α3	$p < 0.05$	$p = 0.977$	$p = 0.741$
α4	$p < 0.05$	$p = 0.994$	$p = 0.522$
α5	$p < 0.05$	$p = 0.114$	$p = 0.256$
α6	$p < 0.05$	$p < 0.05$	$p = 0.493$
β1	$p < 0.05$	$p < 0.05$	$p = 0.214$
β2	$p < 0.05$	$p = 0.75$	$p = 0.111$
β3	$p = 0.429$	$p < 0.05$	$p = 0.073$
γ1	$p < 0.05$	$p < 0.05$	$p < 0.05$
γ2	$p < 0.05$	$p < 0.05$	$p = 0.821$
γ3	$p < 0.05$	$p < 0.05$	$p = 0.349$
δ	$p < 0.05$	$p < 0.05$	$p = 0.281$
ε	$p < 0.05$	$p = 0.135$	$p < 0.05$
θ	$p < 0.05$	$p < 0.05$	$p < 0.05$
ρ2	$p < 0.05$	$p < 0.05$	$p < 0.05$

$p < 0.05$ indicates the data are not normally distributed. HP-DG, hippocampal dendate gyrus; OFC, orbitofrontal cortex; DL-PFC, dorsolateral prefrontal cortex.