Altering the Relative Abundance of $GABA_A$ Receptor Subunits Changes GABA- and Ethanol-Responses in *Xenopus* Oocytes

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Background: Variations in *GABRA2* and *GABRG3*, genes encoding the α2 and γ3 subunits of the pentameric $GABA_A$ receptor, are associated with the risk of developing alcoholism in adults, conduct disorder at younger ages, and with differences in electroencephalographic power in the β frequency range. The SNPs associated with alcoholism did not alter the coding of these genes, and extensive DNA sequencing of *GABRA2* did not find coding changes in the high-risk haplotypes. Therefore, we hypothesize that the associations arise from differences in gene expression.

Methods: Here we report studies in *Xenopus* oocytes to examine the functional effects of altering the relative abundance of these 2 receptor subunits on GABA current and response to ethanol, as a model of potential effects of regulatory differences.

Results: When human α2β2γ3 subunits are co-expressed, increasing the amount of the α2 subunit mRNA increased GABA current; in contrast, increasing the amount of the γ3 subunit decreased GABA currents. Acute ethanol treatment of oocytes injected with a 1:1:1 or 2:2:1 ratio of α2:β2:γ3 subunit mRNAs resulted in significant potentiation of GABA currents, whereas ethanol inhibited GABA currents in cells injected with a 6:2:1 ratio. Overnight treatment with ethanol significantly reduced GABA currents in a manner dependent on the ratio of subunits.

Conclusions: These studies demonstrate that changes in relative expression of $GABA_A$ receptor subunits alter the response of the resulting channels to GABA and to ethanol.

Key Words: Ethanol, GABA Receptors, Voltage Clamp.

ALCOHOLISM AND ALCOHOL abuse are prevalent conditions which present a significant burden to society. Alcoholism is a complex multi-factorial disease which has a strong genetic component. Linkage and association analyses have led to the identification of a number of genes in which variations increase risk for alcoholism (for reviews, see Dick and Foroud, 2003; Edenberg and Foroud, 2006; Kohnke, 2008). Family-based studies demonstrated that *GABRA2*, encoding the α2 subunit of the $GABA_A$ receptor, is associated with alcohol dependence and with electroencephalographic differences in the β frequency range of 13–28 Hz (Edenberg et al., 2004). Alcoholics and their offspring differ from controls by having increased power in that frequency band (Bauer and Hesselbrock, 1993; Costa and Bauer, 1997; Rangaswamy et al., 2002, 2003). Bauer and Hesselbrock (1993) reported that enhanced high-frequency beta activity, originating from deep anterior regions of the frontal brain, was the best predictor of relapse in substance-dependent patients, and may also be related to initial risk for dependence. The association between *GABRA2* and alcoholism has been replicated in several other populations, with the same haplotypes conferring higher risk (Covault et al., 2004; Edenberg and Foroud, 2006; Enoch et al., 2006; Fehr et al., 2006; Lappalainen et al., 2005; Soyka et al., 2008). Further analyses demonstrated that at younger ages, the higher-risk alleles of *GABRA2* are associated with conduct disorder (Dick et al., 2006a). *GABRG3*, encoding the γ3 subunit, has also been associated with alcohol dependence (Dick et al., 2004), as has *GABRA1*, encoding the α1 subunit (Dick et al., 2006b).

Our previous study identified multiple SNPs associated with alcoholism, none of which were within coding regions (Edenberg et al., 2004). In addition, sequencing over 40 individuals with high-risk and low-risk haplotypes revealed no nucleotide changes within the coding sequence of *GABRA2*. Detailed analysis of mRNA from human brain demonstrated alternative splicing and alternative promoter use in the human α2 gene (Tian et al., 2005). Promoter activity of naturally occurring haplotypes also differed (Tian et al., 2005). These observations led us to hypothesize that changes in gene regulation may underlie the alcohol-related phenotypes.

Some evidence suggests that there are innate differences in GABA receptor function between lines of rats selectively bred for alcohol preference (P and NP) (Lumeng et al., 1977). Microarray analysis has identified genes expressed

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differentially in the hippocampus of the P and NP lines including many genes involved in synaptic transmission (Edenberg et al., 2005). For example, the *GABRB1* gene was expressed at a 1.6-fold higher level in the hippocampus of the P rat versus the NP rat and *GABRA1* gene expression was higher as well. An additional microarray study corroborated the observation that the *GABRB1* gene was more highly expressed in multiple brain regions in the P versus NP rats (Kimpel et al., 2007). Our hypothesis is that changes in expression levels of the receptor subunits alter GABA signaling.

γ-Aminobutyric acid$_A$ ($GABA_A$) receptors are ligand-gated ion channels that mediate the majority of fast inhibitory neurotransmission in the central nervous system (Barnard et al., 1998; Mehta and Ticku, 1999; Whiting et al., 1999). They are pentameric integral membrane proteins comprised of different classes of subunits. At least 6 α, 3 β, 3 γ, δ, ε, θ, and π subunit genes and multiple splice variants have been identified. The majority of $GABA_A$ receptors are thought to be composed of 2 α, 2β, and 1 γ subunits (Chang et al., 1996; Farrar et al., 1999) with δ sometimes substituting for the γ subunit (Quirk et al., 1995). $GABA_A$ receptors are targets of many important clinical agents including benzodiazepines, barbiturates, steroids, anesthetics, and anti-convulsants (Barnard et al., 1998; Korpi et al., 2002; Sieghart, 2006). $GABA_A$ receptors are important in many behavioral effects of ethanol consumption including motor in-coordination, sedation, anxiolysis, tolerance, preference, hypothermia, and withdrawal (Buck, 1996; Grobin et al., 1998; Korpi et al., 2002). For example, ethanol mimics some of the effects of $GABA_A$ receptor modulators such as benzodiazepines, neurosteroids, and barbiturates.

Reports about the acute effects of ethanol on $GABA_A$ receptors differ. The sensitivity of $GABA_A$ receptors to ethanol may vary with subunit composition. Ethanol potently (at low mM concentrations) facilitates extrasynaptic $GABA_A$ receptors containing α4 or α6 subunits in combination with δ subunits (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). In contrast, the potency of ethanol is much lower in the more abundant synaptic $GABA_A$ receptors which contain γ subunits instead of δ subunits. However other investigators have been unable to replicate these findings and the inconsistency may be due to species differences, differences in expression levels or GABA concentrations tested, or unknown variables (Borghese and Harris, 2007; Harris and Mihic, 2004). In general, long-term ethanol treatment reduces $GABA_A$ receptor function by several mechanisms including changes in gene expression, posttranslational modification, and subcellular localization of specific subunits (for reviews, see Krystal et al., 2006; Kumar et al., 2004). For example, chronic ethanol administration in rats reduces the level of mRNA and peptides for the α1, α2, and α3 $GABA_A$ receptor subunits in the cerebral cortex (Mhatre and Ticku, 1992) but these effects differ across brain regions. Indirect evidence suggests that phosphorylation, particularly by PKC, may differentially alter $GABA_A$ receptor function in response to ethanol (Kumar et al., 2002, 2004; Morrow et al., 2004). Chronic ethanol treatment selectively increases α1 subunit protein internalization in rat cerebral cortex (Kumar et al., 2003).

In the functional studies presented here, we have mimicked changes in relative gene expression by introducing different ratios of the α2, β2, and γ3 $GABA_A$ receptor subunit mRNAs into *Xenopus* oocytes and have measured $GABA_A$ receptor channel function. In addition, we have examined the effect of acute and chronic ethanol treatment on the electrophysiological characteristics of the channels that result from expression of different subunit ratios.

MATERIALS AND METHODS

Clones

A human $GABA_A$ subunit α2 cDNA (Hadingham et al., 1993a; GenBank accession number NM_000807) from GeneCopoeia (Germantown, MD) was subcloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA) for expression studies. The human β2 subunit cDNA (Hadingham et al., 1993b; NM_000813) in pCDM8 was a kind gift from Peter Wingrove from Merck Laboratories. The human γ3 subunit cDNA (Hadingham et al., 1995; NM_033223) in pCMV6-XL5 was from Origene (Rockville, MD) . The authenticity of all 3 subunits was verified by DNA sequencing before expression studies were conducted.

Xenopus oocyte expression and electrophysiology

All animal procedures were approved by the Animal Care and Use Committee at Indiana University School of Medicine. $GABA_A$ receptor proteins were expressed in *Xenopus laevis* oocytes after injection of *in vitro* transcribed mRNAs. Full-length cDNAs of each of the cloned subunits were used to generate mRNA from the T7 RNA polymerase promoter using an i*n vitro* transcription kit (mMessage Machine; Ambion, Austin, TX). RNA concentration and quality was determined by both gel electrophoresis and UV spectrophotometry. Oocytes were harvested from mature *X. laevis,* and follicle cells were removed with 2 mg/ml collagenase type IA (Sigma, St Louis, MO). Stage V and VI oocytes were injected with 5–30 ng of each $GABA_A$ receptor subunit mRNA as indicated in a total of 50 nL of RNAse-free H_20 using a Drummond automatic microinjector. Seven ratios of $GABA_A$ subunits were tested: (i) $\alpha2\beta2$ only in a 1:1 ratio (10 ng:10 ng); (ii) $\alpha2\beta2\gamma3$ in a 1:1:3 ratio (10 ng:10 ng:30 ng); (iii) $\alpha2\beta2\gamma3$ in a 1:1:1 ratio (10 ng:10 ng:10 ng); (iv) $\alpha2\beta2\gamma3$ in a 3:1:1 ratio (30 ng:10 ng:10 ng); (v) $\alpha2\beta2\gamma3$ in a 2:2:1 ratio (10 ng:10 ng:5 ng); (vi) $\alpha2\beta2\gamma3$ in a 6:2:1 (30 ng:10 ng:5 ng); (vii) $\alpha2\beta2\gamma3$ in a 0.5:2:1 ratio (5 ng:20 ng:10 ng). Since the transcripts and coding regions for all 3 genes are similar in length the molarities will also be similar when 1:1:1 quantities of RNA are injected. Because all 3 genes have optimal Kozak sequences, the ratios of injected subunit mRNAs should be reflected in the amount of specific subunit protein expressed. Oocytes were stored in Oocyte Ringer solution (50% Leibovitz's L15 medium, 15 mM HEPES, 0.8 mM L-glutamine, 0.04 mg/mL gentamycin) at 17°C.

Two-electrode voltage experiments were conducted 3 days after RNA injection. Oocytes were voltage-clamped at −70 mV with 2 glass electrodes filled with 3 M KCl and having a resistance of 0.5–1.5 MΩ using an OC-725 Oocyte Clamp (Warner Instrument Corporation, Hamden, CT). GABA-induced chloride currents were digitized with an Axon Instruments Digidata 1322A (Sunnyvale, CA) and recorded on a computer using pCLAMP software. Oocytes were rapidly superfused (at 5 ml/min) with ND96 (96 mM NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH

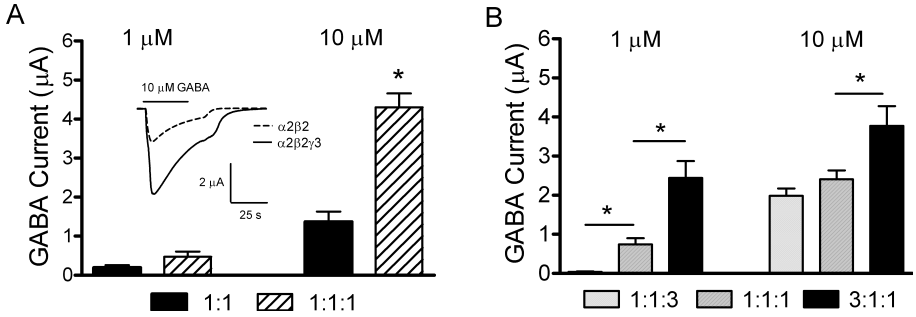

Fig. 1. GABA currents as a function of the ratio of α2, β2, and γ3 subunit mRNAs. (**A**) Peak current amplitudes in response to 1 or 10 μM GABA in oocytes injected with a 1:1 ratio of α2:β2 subunit mRNAs or a 1:1:1 ratio of α2:β2:γ3 subunit mRNAs. Inset depicts representative inward GABA currents in response to 10 μM GABA. The solid line above the traces indicates GABA application. (**B**) Peak current amplitudes in response to 1 or 10 μM GABA in oocytes injected with either 1:1:3 or 1:1:1 or 3:1:1 ratios of α2:β2:γ3 subunit mRNAs. The data are shown as the mean ± SEM. *Indicates a significant difference between groups ($p < 0.05$).

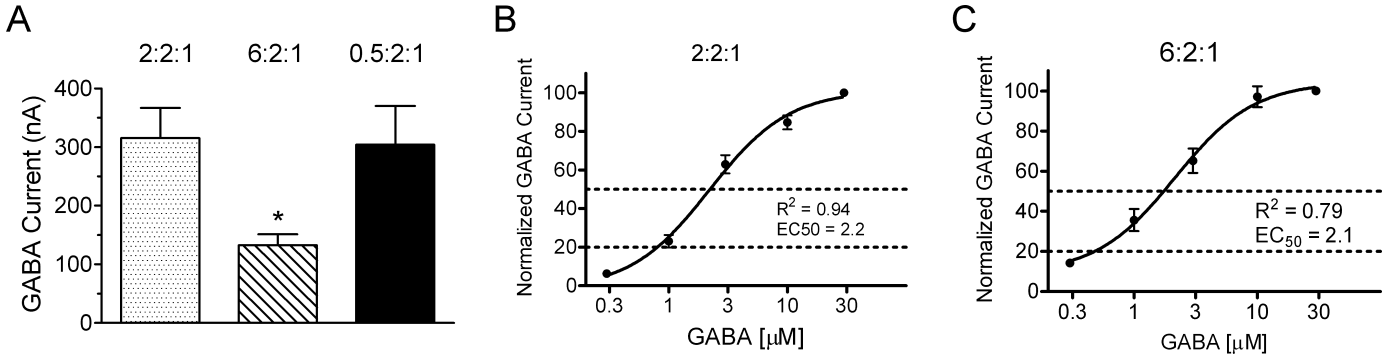

Fig. 2. GABA currents as a function of the ratio of α2, β2, and γ3 subunit mRNAs. (**A**) Peak current amplitudes in response to 10 μM GABA in oocytes injected with either 2:2:1 or 6:2:1 or 0.5:2:1 ratios of α2:β2:γ3 subunit mRNAs. GABA concentration-response curves in oocytes injected with either 2:2:1 (**B**) or 6:2:1 (**C**). Peak currents at each GABA concentration were normalized to the current at 30 μM GABA for each oocyte and averaged within groups. Data are presented as mean ± SE. The solid line through the data indicates the non-linear regression fit with the correlation coefficient (R^2) and calculated EC_{50} values below. Dotted lines indicate the location of EC_{50} and EC_{20} on each graph.

7.6) or ND96 containing the indicated concentrations of GABA, ethanol, or both GABA and ethanol.

In some experiments, oocytes were exposed to GABA (1 or 10 μM) only once for 30 seconds before drug washout (see Figs. 1, 2, and 4). In GABA concentration-response curve experiments (Figs 2*B* and *C*), each oocyte served as its own control, i.e., multiple concentrations of GABA (300 nM–30 μM) where tested on each cell in a random order with a 5 minute wash-out between GABA applications. In acute ethanol experiments, each oocyte served as its own control and the response to 1 μM GABA before and after 44 mM ethanol pretreatment was compared after a 5-minute wash-out. Specifically, GABA responses were tested before ethanol perfusion and after a 60-second pretreatment with ethanol. Immediately after the ethanol pretreatment 1 μM GABA was again superfused for 30 seconds in the continued presence of 44 mM ethanol. In chronic ethanol experiments, half of the oocytes from an injection group were incubated with ethanol (44 or 100 mM) in Oocyte Ringer solution for 16 hours immediately before electrophysiological recording (approximately 2 days after RNA injection).

Data analysis

For each experiment, all treatment and injection groups were completed and replicated in at least 3 individual batches of oocytes from different donors to confirm the reproducibility of the findings. Peak current amplitudes were measured in individual oocytes after GABA treatment and averaged within treatment and injection groups. Data are displayed as the average ± SEM in bar graphs with the number of oocytes tested indicated in the results and/or figure legend. For GABA concentration response experiments, peak current amplitudes for each GABA concentration were normalized to the peak current amplitude from the 30 μM GABA response for each oocyte and averaged within groups. Non-linear regression analysis was used to fit the data and determine GABA EC_{50} values. For acute ethanol treatment experiments where oocytes served as their own controls the data were normalized to peak current amplitude before ethanol and averaged within groups. In chronic ethanol treatment experiments, individual GABA currents were normalized to the average current amplitude of control groups with no ethanol treatment within the same experiment and the mean ratios ± SEM are displayed in bar graphs. Paired *t*-tests or ANOVA with Tukey's posthoc analysis was used to compare groups.

RESULTS

Oocytes were injected with a total of 20–50 ng of mRNA, which is less than the translational capacity of oocytes (Moar et al., 1971). We focused on the α2 and γ3 subunits as their genes are associated with alcohol related phenotypes. The β2 subunit was utilized throughout these studies as it is one of the most abundant of GABA$_A$ β subunits in the brain (Laurie et al., 1992).

To assess the role of the α2, β2, and γ3 subunits in forming functional $GABA_A$ receptors we compared the amplitudes and characteristics of GABA induced currents produced in oocytes injected with a 1:1 ratio of α2:β2 mRNAs to oocytes injected with a 1:1:1 ratio of α2:β2:γ3 mRNAs. We routinely tested uninjected oocytes for endogenous GABA currents and no currents were detected under these recording conditions. Injecting α2 mRNA alone (up to 30 ng per oocyte) did not produce detectable currents when tested with up to 100 μM GABA. Functional $GABA_A$ receptors were produced by injection of mRNAs for 2 subunits, α2 and β2, however injecting mRNAs for all 3 subunits produced significantly greater currents at 10 μM GABA, 1.38 ± 0.26 μA ($n = 35$) versus 4.30 ± 0.35 μA ($n = 31$, $p < 0.01$) (Fig. 1*A*). Currents induced by 1 μM GABA were slightly larger but not significantly so when all 3 subunits were expressed compared to 2 subunits, 0.19 ± 0.06 μA ($n = 6$) versus 0.47 ± 0.13 μA ($n = 8$). No differences were evident between injection groups in the rates or degree of desensitization in the GABA-induced inward currents (see inset in Fig. 1*A*).

To test the hypothesis that changing the relative abundance of α2, β2, and γ3 subunit mRNAs would alter the characteristics of the expressed channels, we increased the amount of α2 or γ3 mRNA injected relative to the other subunits. Oocytes were injected with a 1:1:3 ratio or a 1:1:1 ratio or a 3:1:1 ratio of α2, β2, and γ3 subunit mRNAs. The peak current amplitudes elicited by 1 μM GABA (Fig. 1*B*) were significantly greater when subunits were injected in a 3:1:1 ratio (2.44 ± 0.43 μA, $n = 17$) than when they were injected in a 1:1:1 ratio (0.74 ± 0.16 μA, $n = 20$). The peak current amplitudes were significantly smaller when subunits were injected in a 1:1:3 ratio (0.06 ± 0.01 μA, $n = 18$) than when they were injected in a 1:1:1 ratio. Likewise greater currents were induced by 10 μM GABA (Fig. 1*B*) in oocytes injected with the 3:1:1 ratio of subunits (3.77 ± 0.50 μA, $n = 48$) compared to the 1:1:1 ratio group (2.41 ± 0.22 μA, $n = 66$). At this higher level of GABA, there was little difference between the 1:1:3 ratio group (1.98 ± 0.19 μA, $n = 79$) and the 1:1:1 group.

To more accurately reflect the most common native structure of $GABA_A$ receptors (2α, 2β, and 1γ subunit), we examined the effects of increasing or decreasing the relative abundance of the α2 subunit compared to a baseline ratio of 2:2:1. As seen in Fig. 2*A*, the 2:2:1 ratio group (316 ± 51 nA, $n = 52$) produced significantly greater currents in response to 10 μM GABA than the 6:2:1 ratio group (133 ± 19 nA, $n = 36$). In these experiments, the 0.5:2:1 group (304 ± 66 nA, $n = 24$) did not significantly differ from the baseline 2:2:1 group.

GABA concentration response curves were determined to compare GABA current characteristics in cells expressing differing subunit ratios (Figs. 2*B* and *C*). EC_{50} values for the GABA response did not differ between oocytes expressing 2:2:1 (95% confidence interval = 1.65–2.93 μM, $n = 9$) and 6:2:1 (95% confidence interval = 1.37–3.36 μM, $n = 12$) ratios of subunits. The GABA concentration of 30 μM was saturating; 100 μM GABA elicited responses equal to or less than the 30 μM concentration (data not shown).

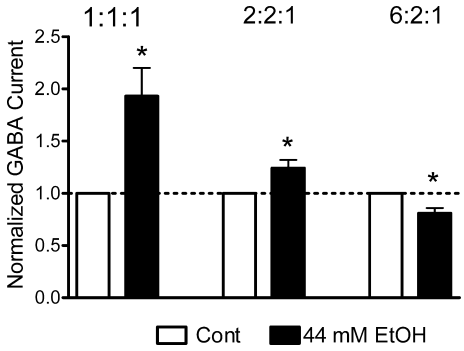

Fig. 3. Effects of acute ethanol treatment on GABA current amplitude. (**A**) Peak current amplitudes in response to 1 μM GABA before and after 44 mM ethanol pretreatment in oocytes expressing either 1:1:1 or 2:2:1 or 6:2:1 ratios of α2:β2:γ3 subunit mRNAs. Data are normalized to peak current amplitudes in response to GABA from each oocyte before ethanol treatment. Ethanol was applied for 60 seconds before and concurrently with GABA administration. *Indicates a significant difference between groups ($p < 0.01$) using paired *t*-tests.

We examined the effect of acute ethanol exposure on the GABA response in oocytes injected with 1:1:1, 2:2:1, or 6:2:1 ratios of α2, β2, and γ3 subunit mRNAs (Fig. 3). Each oocyte was challenged with 1 μM GABA while monitoring current size under voltage-clamp conditions before and after ethanol pretreatment. This GABA concentration (1 μM) approximates the EC_{20} for GABA as determined above (see Figs. 2*B* and *C*) and should allow detection of either potentiation or inhibition of GABA response by ethanol. GABA currents were normalized to control currents before ethanol treatment for each oocyte. Ethanol significantly potentiated the amplitudes of GABA-induced currents by 93% in oocytes injected with a 1:1:1 ratio of α2, β2, and γ3 subunit mRNAs ($p < 0.01$, $n = 16$). Ethanol also potentiated the amplitudes of GABA-induced currents by 24% in oocytes injected with a 2:2:1 ratio of α2, β2, and γ3 subunit mRNAs ($p < 0.01$, $n = 15$). In contrast, ethanol inhibited the amplitudes of GABA-induced currents by 19% in oocytes injected with a 6:2:1 ratio of α2, β2, and γ3 subunit mRNAs ($p < 0.01$, $n = 19$).

Because chronic ethanol exposure affects GABA responses, we determined the effect of prolonged ethanol exposure on $GABA_A$ receptors composed of different ratios of subunits. For chronic treatment studies, half of the oocytes from each injection group were incubated in Oocyte Ringer containing ethanol for 16 hours immediately prior to voltage-clamp recording, while the control cells were incubated in Oocyte Ringer alone. Ethanol (44 or 100 mM as indicated) did not alter pH but slightly decreased osmolarity of the Oocyte Ringer solution. Resting membrane potentials and leakage current under initial voltage clamp conditions did not differ between the ethanol-treated groups and controls (data not shown) which suggests that the overall health of the oocytes is similar in controls and ethanol-treated groups. As shown in Fig. 4*A*, chronic ethanol (44 mM) had no significant effect on

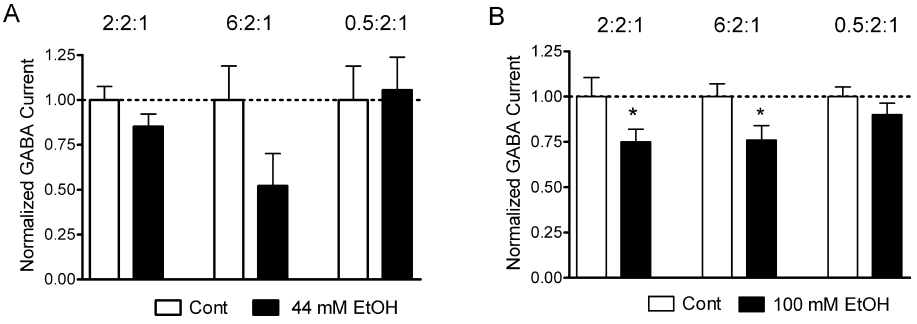

Fig. 4. Effects of chronic ethanol treatment on GABA current amplitude. (**A**) Peak current amplitudes in response to 10 μM GABA in oocytes treated for 16 hours with 44 mM ethanol after injection with 2:2:1 or 6:2:1 or 0.5:2:1 ratios of $\alpha 2$:$\beta 2$:$\gamma 3$ subunit mRNAs. (**B**) Peak current amplitudes in response to 10 μM GABA in oocytes treated for 16 hours with 100 mM ethanol after injection with 2:2:1 or 6:2:1 or 0.5:2:1 ratios of $\alpha 2$:$\beta 2$:$\gamma 3$ subunit mRNAs. Data are normalized to peak current amplitudes of control oocytes; symbols as in Fig. 1.

GABA currents (1.0 ± 0.07, $n = 20$ vs. 0.85 ± 0.07, $n = 18$; $p = 0.16$) in oocytes injected with the presumed native 2:2:1 ratio of $\alpha 2\beta 2\gamma 3$ subunits. Likewise, chronic ethanol (44 mM) did not significantly change, although a trend is apparent, GABA currents in oocytes injected with a 6:2:1 ratio of subunits (1.00 ± 0.19, $n = 14$ vs. 0.52 ± 0.18, $n = 8$; $p = 0.11$), and had no effect on oocytes injected with a 0.5:2:1 ratio (1.00 ± 0.19, $n = 12$ vs. 1.06 ± 0.18, $n = 17$; $p = 0.84$). In contrast, 100 mM ethanol inhibited GABA currents by 25% (1.0 ± 0.11, $n = 23$ vs. $0.75 \pm 0.0.07$, $n = 28$, $p < 0.05$) in oocytes injected with the presumed native 2:2:1 ratio of $\alpha 2\beta 2\gamma 3$ subunits (Fig. 4*B*). Chronic ethanol also reduced GABA currents in oocytes injected with a 6:2:1 ratio of subunits (1.00 ± 0.07, $n = 38$ vs. 0.76 ± 0.08, $n = 43$, $p < 0.05$), but had no effect on oocytes injected with a 0.5:2:1 ratio (1.00 ± 0.06, $n = 23$ vs. 0.92 ± 0.08, $n = 28$; $p = 0.42$).

DISCUSSION

Variations in genes encoding the $\alpha 2$ and $\gamma 3$ subunits of the GABA$_A$ receptor are associated with risk for alcoholism (Dick et al., 2004; Edenberg et al., 2004); the association of *GABRA2* has been replicated in several populations (Covault et al., 2004; Edenberg and Foroud, 2006; Enoch et al., 2006; Fehr et al., 2006; Lappalainen et al., 2005; Soyka et al., 2008). Variations in *GABRA2* have also been associated with power in the beta frequency band (13–28 Hz) of the EEG (Edenberg et al., 2004). Yet, the associated variations did not affect the amino acid sequences, suggesting that expression differences might be involved (Edenberg et al., 2004). Alternative splicing and alternative promoter use have been demonstrated in the human *GABRA2* gene (Tian et al., 2005). To test the hypothesis that variations in the ratios of GABA$_A$ receptor subunits would affect receptor function, we manipulated the subunit ratios in the *Xenopus* oocyte expression system and measured the resulting GABA currents. We modeled changes in gene expression by injecting *Xenopus* oocytes with different ratios of $\alpha 2$, $\beta 2$, and $\gamma 3$ subunits and then compared GABA currents.

Initial studies of newly cloned GABA$_A$ receptors in heterologous expression studies indicated that some combinations of α and β subunits can form functional receptors (Schofield et al., 1987; Ymer et al., 1989) albeit lacking some characteristics of native channels (Pritchett et al., 1989). We found that functional GABA$_A$ channels can be formed from $\alpha 2$ and $\beta 2$ subunits alone, although the currents are smaller than those produced by all 3 subunits (Fig. 1*A*). To test our hypothesis that changing the relative abundance of individual GABA$_A$ receptor subunits could alter functional responses, we modified the amounts of either the $\alpha 2$ or $\gamma 3$ subunit, keeping the concentration of the $\beta 2$ subunit constant. As previous recombinant GABA$_A$ receptor studies had utilized 1:1:1 ratios we initially compared the 1:1:1 ratio with 3-fold increases in either $\alpha 2$ (3:1:1) or $\gamma 3$ subunits (1:1:3). GABA$_A$ receptors formed from different ratios of subunits had different responses to GABA. Increasing the amount of $\alpha 2$ mRNA (3:1:1 ratio of α:β:γ) produced significantly greater GABA currents than a 1:1:1 ratio of subunits (Fig. 1*B*). Increasing the amount of $\gamma 3$ subunit mRNA (1:1:3 ratio) reduced the GABA currents compared to either the 1:1:1 or 3:1:1 ratio of subunits.

As the majority of native GABA$_A$ receptors are believed to be composed of 2α, 2β, and single γ or δ subunits (Chang et al., 1996; Farrar et al., 1999; Quirk et al., 1995) we did additional comparisons in cells expressing 2:2:1 ratios and increased or decreased the $\alpha 2$ abundance However, increasing the ratio of $\alpha 2$ mRNA above the baseline of a 2:2:1 ratio led to decreased GABA currents (Fig. 2*A*). Increasing the $\alpha 2$ or $\gamma 3$ subunits may alter the formation of functional channels bi-directionally by changing receptor assembly or membrane trafficking (Kittler et al., 2002). Overall our study indicates that small changes in the relative abundance of subunit mRNA injected affect GABA$_A$ receptor current amplitude.

While GABA$_A$ receptor gene variants are believed to modulate the predisposition to alcoholism, the effects of ethanol on GABA$_A$ receptor function are not well understood. The effects of acute ethanol treatment on GABA$_A$ receptor function vary widely *in vivo* and *in vitro*. A number of reports suggest that low concentrations of ethanol (< 5 mM) potentiate

GABA responses in some cell types or with specific subunit combinations, notably $\alpha_{4/6}$ and δ (Borghese and Harris, 2007; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003, 2006). In our study, higher but still pharmacologically relevant concentrations of ethanol (44–100 mM) were required to see effects with "less-sensitive" $GABA_A$ subunit combinations. Acute ethanol treatment significantly potentiated GABA induced currents in cells injected with a 1:1:1 and 2:2:1 ratios of human $\alpha 2\beta 2\gamma 3$ subunits, whereas ethanol inhibited GABA responses in cells injected with a 6:2:1 ratio. Our results are notable for 2 reasons as we describe significant ethanol potentiation in $GABA_A$ receptors lacking $\alpha 4/6$ and/or δ subunits and we observed potentiation or inhibition by ethanol which was dependent on subunit ratio. The specificity of the response, i.e., potentiation versus inhibition, is of interest because it demonstrates that changes in subunit abundance can alter response to ethanol.

Long-term ethanol consumption results in the development of tolerance to many of the GABA-mediated effects of ethanol and this adaptation is usually associated with diminished GABA receptor function (reviewed by Kumar et al., 2004). Changes in GABA function after chronic ethanol treatment may be due to changes in gene regulation, assembly, and trafficking or posttranslational modification. The oocyte expression system as used here does not allow us to examine changes in gene regulation as the regulatory sequences are lacking in the injected cRNA and some modulatory proteins aren't present. However, oocytes do contain many common signaling proteins, including various isoforms of PKC, which are hypothesized to regulate GABA function after ethanol treatment, so some aspects of chronic ethanol treatment on specific subunit combinations may be initially studied on oocytes. As seen in Fig. 4, ethanol treatment for 16 hours significantly reduced GABA responses in oocytes injected with a 2:2:1 ratio and a 6:2:1 ratio of subunits, while not affecting current amplitude of oocytes injected with 0.5:2:1 ratios. This result suggests that the effect is specific to particular subunit ratios and not due to non-specific effects of ethanol on the membrane or viability of the cells. In oocytes, either trafficking or post-translational modifications may be occurring and may be mechanistically related. For example, it has been previously shown that $GABA_A$ receptors are internalized in a subunit-specific manner in oocytes after stimulation with PMA, a protein kinase C activator (Chapell et al., 1998; Filippova et al., 2000). Although there is no direct evidence that $GABA_A$ subunits are phosphorylated *in vivo* after ethanol treatment, it has been hypothesized that chronic ethanol may increase phosphorylation of $GABA_A$ subunits and alter expression, function or trafficking (Kumar et al., 2004).

It is unclear whether $GABA_A$ receptors composed of the $\alpha 2\beta 2\gamma 3$ subunit combination exist *in vivo* in areas likely to be involved in alcohol-related phenotypes or predisposition to alcoholism. However, based on *in situ* hybridization and immunocytochemistry studies it is likely that these subunit proteins co-localize in several brain regions which may be important in observed alcohol related phenotypes such as cortex and mesolimbic areas (reward pathways). The $\alpha 2$ subunit is found in 35% of $GABA_A$ receptors and in high concentrations in cortex, striatum, nucleus accumbens, septum, dentate gyrus, and amygdala and hypothalamus. The $\gamma 3$ subunit is less abundant but enriched in some regions which could contribute to risk for alcoholism including cortex, basal nuclei, and hippocampus. The $\beta 2$ subunit has not been linked to alcoholism but is the most abundant β isoform in mature brain. It is also important to point out that although our study has demonstrated differences in response to ethanol based on this subunit combination all 3 subunits may not be required to see similar functional changes.

This report demonstrates that altering the relative abundance of $GABA_A$ receptor subunits results in functional changes in response to both GABA and ethanol. As $GABA_A$ receptor activity is critical for synaptic inhibition throughout the brain, alterations in GABA responsiveness may have wide-reaching impacts on the general state of central nervous system excitability. This study suggests that variations in the relative expression of $GABA_A$ receptor subunit genes may result in differences in response to both GABA and ethanol, and thereby such expression differences may modulate the propensity towards alcoholism.

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