Ethanol Sensitivity of GABAergic Currents in Cerebellar Granule Neurons Is Not Increased by a Single Amino Acid Change (R100Q) in the α₆ GABAₐ Receptor Subunit

Paolo Botta, Manuel Mameli,¹ Kirsten L. Floyd, Richard A. Radcliffe, and C. Fernando Valenzuela

Department of Neurosciences, University of New Mexico Health Sciences Center Albuquerque, New Mexico (P.B., M.M., C.F.V.); Department of Pharmacology, University of Colorado Health Sciences Center, Aurora, Colorado (K.L.F.); Department of Pharmaceutical Sciences, University of Colorado at Denver and Health Sciences Center, Denver, Colorado (R.A.R.); and Institute for Behavioral Genetics, University of Colorado, Boulder, Colorado (R.A.R.)

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ABSTRACT

Cerebellar granule neurons (CGNs) extrasynaptically express GABAₐ receptors containing α₆β₃δ subunits, which mediate tonic inhibitory currents. Although it has been shown that the function of these receptors is potently and directly enhanced by ethanol, this finding has not been reproducible across different laboratories. In outbred Sprague-Dawley rats, a naturally occurring arginine (R) to glutamine (Q) mutation in position 100 of the α₆ subunit was reported to increase the ethanol sensitivity of these receptors. However, we did not detect an action of this mutation in selectively bred rats (alcohol-tolerant and alcohol-nontolerant). Consequently, we reexamined the effect of the mutation on ethanol sensitivity in Sprague-Dawley rats. Using patch-clamp electrophysiological techniques in cerebellar vermis parasagittal slices, we found that 25 mM ethanol increases the tonic current amplitude, tonic current noise, and spontaneous inhibitory postsynaptic current (sIPSC) frequency to a similar extent in α₆-100R/100R and α₆-100Q/100Q CGNs. Exposure to 80 mM ethanol increased the tonic current amplitude to a significantly greater extent in α₆-100R/100R than in α₆-100Q/100Q CGNs; however, the effects of 80 mM ethanol on the tonic current noise and sIPSC frequency were not significantly different between these groups. In the presence of tetrodotoxin, a non-N-methyl-D-aspartate receptor antagonist, exogenous GABA, and a GABA transporter inhibitor, neither 8 nor 40 mM ethanol consistently affected tonic current amplitude or noise in α₆-100R/100R or α₆-100Q/100Q CGNs. Thus, the α₆- R100Q GABAₐ receptor subunit polymorphism does not increase the acute ethanol sensitivity of extrasynaptic receptors, lending further support to the hypothesis that ethanol modulates these currents indirectly via a presynaptic mechanism.

Studies indicate that ethanol enhances GABAergic transmission in several brain regions via presynaptic and postsynaptic mechanisms (reviewed in Siggins et al., 2005; Breese et al., 2006; Weiner and Valenzuela, 2006). Recently, ethanol has also been shown to enhance the function of δ subunit-containing extrasynaptic GABAₐ receptors (GABAₐ-Rs). Sundstrom-Poromaa et al. (2002) reported that 1 to 3 mM ethanol increases currents mediated by recombinant α₄β₂δ GABAₐ-Rs in Xenopus oocytes and native α₄β₂δ GABAₐ-Rs in acutely dissociated CA1 hippocampal neurons from a rat model of premenstrual syndrome. Subsequently, ethanol was shown to potentiate Xenopus oocyte-expressed α₄β₂δ and α₆β₂δ recombinant receptors at concentrations ≥3 mM and the imidazobenzodiazepine Ro 15-4513 competitively blocked this effect (Wallner et al., 2003, 2006; Hanchar et al., 2006; Olsen et al., 2007). Tonic currents mediated by α₆β₂δ GABAₐ-Rs in dentate gyrus granule cells and ventrobasal thalamic neurons were shown to be significantly potentiated by ethanol (≥30 mM) in a protein kinase Cδ-dependent manner (Wei et al., 2004; Liang et al., 2006; Fleming et al., 2007; Jia et al., 2007; Messing et al., 2007; Mody et al., 2007). Glykys et al. (2007) reported that ethanol (≥20 mM) enhances α₆β₂δ GABAₐ-R-dependent currents in hippocampal GABAₐ-Rs in Xenopus oocytes and native α₄β₂δ GABAₐ-Rs in acutely dissociated CA1 hippocampal neurons from a rat model of premenstrual syndrome. Subsequently, ethanol was shown to potentiate Xenopus oocyte-expressed α₄β₂δ and α₆β₂δ recombinant receptors at concentrations ≥3 mM and the imidazobenzodiazepine Ro 15-4513 competitively blocked this effect (Wallner et al., 2003, 2006; Hanchar et al., 2006; Olsen et al., 2007). Tonic currents mediated by α₆β₂δ GABAₐ-Rs in dentate gyrus granule cells and ventrobasal thalamic neurons were shown to be significantly potentiated by ethanol (≥30 mM) in a protein kinase Cδ-dependent manner (Wei et al., 2004; Liang et al., 2006; Fleming et al., 2007; Jia et al., 2007; Messing et al., 2007; Mody et al., 2007). Glykys et al. (2007) reported that ethanol (≥20 mM) enhances α₆β₂δ GABAₐ-R-dependent currents in hippocampal

ABBREVIATIONS: GABAₐ-R, type-A γ-aminobutyric acid receptor; CGN, cerebellar granule neuron; Ro 15–4513, ethyl 8-azido-6-dihydro-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate; IPSC, inhibitory postsynaptic current; sIPSC, spontaneous IPSC; TTX, tetrodotoxin; AT, alcohol-tolerant; ANT, alcohol-nontolerant; ACSF, artificial cerebrospinal fluid; NO-711, 1-(2-[[diphenylmethylen]amino]oxy) ethyl)-1,2,5,6-tetrahydro-3-pyridinecarboxylic acid; ANOVA, analysis of variance.

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¹ Current affiliation: Department of Basic Neurosciences, University of Geneva, Geneva, Switzerland.

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molecular layer interneurons. Hanchar et al. (2005) demonstrated that a single amino acid change (R100Q) in the α6 subunit enhances the potentiating effect of ethanol on currents mediated by recombinant αβ6δ but not αβδ receptors. Extrasynaptic receptors containing αβδ subunits are selectively expressed in cerebellar granule neurons (CGNs). Hanchar et al. (2005) found that 10 mM ethanol directly potentiates these receptors in slices from Sprague-Dawley rats homozygous for α6-100Q, which also display higher sensitivity to ethanol-induced motor impairment.

Although these results suggest that extrasynaptic GABA<sub>A</sub>-Rs are targets of ethanol, the findings of several studies are inconsistent with this notion. Mehta et al. (2007) and Korpi et al. (2007) did not detect effects of ethanol on [<sup>3</sup>H]Ro 15-4513 binding to δ subunit-containing GABA<sub>A</sub>-Rs. Borghese et al. (2006) found that αβδ GABA<sub>A</sub>-Rs expressed in either Xenopus oocytes or fibroblasts are minimally affected by subanesthetic concentrations of ethanol and that 30 mM ethanol does not affect tonic GABAergic currents in dentate gyrus granule cells (reviewed in Borghese and Harris, 2007). Lack of a direct effect of acute ethanol (50–100 mM) exposure on tonic GABAergic currents in dentate gyrus granule cells was also recently reported by another group (Talani et al., 2007). Yamashita et al. (2006) found that ethanol (10, 30, and 100 mM) had either no effect or an inhibitory effect on currents mediated by αβδ, αβδ, or αβδ GABA<sub>A</sub>-R subunits expressed in Chinese hamster ovary cells. Similarly, an inconsistent effect of ethanol (30 mM) on steady-state currents induced by continuous application of low GABA concentrations was observed in cultured CGNs. Casagrande et al. (2007) did not detect a significant effect of 10 and 30 mM ethanol on currents evoked by low GABA concentrations in cultured CGNs. Carta et al. (2004) found that ethanol (≥20 mM) (see Carta et al. for ethanol dose-response data) increases both the frequency of spontaneous inhibitory post synaptic currents (sIPSCs) and tonic current noise and that this effect was not observed when spontaneous action potentials were blocked with tetrodotoxin (TTX). Ethanol also increased the frequency of spontaneous action potentials in Golgi cells, suggesting that it enhances phasic and tonic GABAergic transmission on CGNs via an indirect presynaptic mechanism (Carta et al., 2004). Studies carried out with alcohol-tolerant (AT) (homozygous for α<sub>6</sub>-100R) and alcohol-nontolerant (ANT) (homozygous for α<sub>6</sub>-100Q) rats suggest that the α<sub>6</sub> GABA<sub>A</sub>-R subunit polymorphism does not contribute to ethanol-induced ataxia (Radcliffe et al., 2004; Bottu et al., 2007; Korpi et al., 2007). Electrophysiological experiments with AT and ANT rats indicate that the α<sub>6</sub> GABA<sub>A</sub>-R subunit polymorphism does not modulate ethanol sensitivity of phasic or tonic GABAergic currents in CGNs (Valenzuela et al., 2005; Bottu et al., 2007). We conclude that, although several independent studies have shown that tonic GABAergic currents are modulated by ethanol, it is still uncertain if this is a consequence of direct potentiation of δ subunit-containing extrasynaptic GABA<sub>A</sub>-Rs (Lovingier and Homancik, 2007).

The purpose of this study was to reexamine the influence of the α<sub>6</sub>-R100Q subunit polymorphism on ethanol sensitivity of these receptors in CGNs. We hypothesized that confounding genetic factors present in AT and ANT rats and/or our choice of experimental conditions prevented us from detecting an effect of this polymorphism (Otis et al., 2005; Valenzuela et al., 2005). We tested this hypothesis using similar ethanol concentrations and experimental conditions to those used by Hanchar et al. (2005).

Materials and Methods

Genotyping. Male Sprague-Dawley rats (22–23 days old) were obtained from Charles River Laboratories (area H-41; Hollister CA). The rats were housed at the University of New Mexico Health Sciences Center Animal Resource Facility. All animal procedures were approved by the University of New Mexico Health Sciences Center Institutional Animal Care and Use Committee. Ear punch samples (1–2 mm in diameter) were obtained and shipped frozen to the University of Colorado Health Sciences Center for α<sub>6</sub> subunit single nucleotide polymorphism genotyping, which was accomplished using the Amplification Refractory Mutation System method as detailed in Saba et al. (2001). Briefly, ear punch samples were incubated in digestion buffer (10 mM NaOH and 0.1 mM EDTA) at 95°C for 10 min. DNA was quantified by spectrophotometry and diluted with H<sub>2</sub>O accordingly. Two PCR amplifications were performed on each sample using standard reagents and cycling conditions (12 min at 95°C; 30 cycles: 30 s at 94°C, 30 s at 55°C, 60 s at 72°C; and 7 min at 72°C). One reaction contained a forward primer that was complementary to the sequence of the 3’ to 5′ DNA strand for α<sub>6</sub>-100R (TAAGATCGAGCTCGCGACACATTTTTGCG) and the second reaction contained a forward primer complementary to the sequence of the 3’ to 5′ DNA strand for α<sub>6</sub>-100Q (TAAGATCGAGCTCGCGA-CACATTTCCTGCA); the α<sub>6</sub> single nucleotide polymorphism is at the last position of the primer (note that the last base of the R (CGA) and Q (CGS) subunits is not part of the forward primer sequence). A common reverse primer was included in each reaction to yield a product of 118 base pairs (CATGGTGTACGAGATCGTC). Both reactions also contained a control primer pair to verify that the amplification was successful (forward: TTCTCCCTGGCTTCTCCAG; reverse: AGTTCCTTCATCTTCAAGTTGAG; product = 268 base pairs). The method relies on the tolerance of Taq polymerase for a single mismatch placed at the third position from the 3’ end of the forward primer sequence (C has been replaced with G), but not two mismatches as would be the case, for example, when the 100R primer hybridizes to 100Q template. Thus, amplification takes place only in the presence of a matching combination of forward primer and template. PCR products were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA), which uses a microfluidics based method of electrophoresis. An example of a virtual gel image is shown in Fig. 1, where subjects 15, 6, and 7 are heterozygous (100R/100Q), subject 14, 25%; G/A (100R/100Q; subject 16 is homozygous for glutamine (100Q/100Q), and subject 8 is homozygous for arginine (100R/100R). Genotyping was performed in a total of 56 rats, and these were found to follow an expected 25:50:25 Mendelian distribution (χ<sup>2</sup>, p > 0.8); G/G (100R/100R); n = 14), 25%; G/A (100R/100Q; n = 30), 54%; and A/A (100Q/100Q; n = 12), 21%, in agreement with the report of Hanchar et al. (2005).

Electrophysiology. Unless indicated, all chemicals were from Sigma-RBI-Fluka (St. Louis, MO). Experiments were performed in parasagittal vermis cerebellar slices that were prepared from the genotyped rats (homozygous only) when they were 27 to 38 days old. Animals were euthanized by rapid decapitation under deep anesthesia with ketamine (250 mg/kg i.p.) and 200- to 250-μm-thick slices were prepared with a Vibratome. Slices were cut in cold solution containing 220 mM sucrose, 26 mM Na<sub>2</sub>HCO<sub>3</sub>, 10 mM glucose, 6 mM MgSO<sub>4</sub>, 3 mM KCl, 1.25 mM Na<sub>2</sub>PO<sub>4</sub>, 0.2 mM Ca<sub>2</sub>O<sub>4</sub>, and 0.43 mM ketamine. This solution was equilibrated with 95% O<sub>2</sub> plus 5% CO<sub>2</sub>. Immediately after the slicing procedure, slices were transferred to a chamber containing artificial cerebrospinal fluid (ACSF) and allowed to recover at 36°C for 45 min, followed by storage at room temperature in the same ACSF (Aitken et al., 1995). ACSF contained 126 mM NaCl, 5 mM KCl, 1.25 mM Na<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 26 mM Na<sub>2</sub>HCO<sub>3</sub>, 2 mM CaCl<sub>2</sub>, and 10 mM glucose equilibrated with 95% O<sub>2</sub> plus 5% CO<sub>2</sub>. After a total recovery time ≥80 min, slices were
transferred to a chamber perfused with ACSF at a rate of 2 to 3 m/min. Whole-cell patch-clamp electrophysiological recordings from CGNs were performed under infrared-differential interference contrast microscopy with Axopatch 200B or Multiclamp 700B amplifiers (Molecular Devices, Sunnyvale, CA). Patch pipettes had resistances of 4 to 6 MΩ. Similarly to the conditions of Hanchar et al. (2005), recordings were obtained in ACSF at 23°C with an internal solution containing 140 mM CsCl, 10 mM HEPES (pH 7.3), 1 mM EGTA, 4 mM magnesium ATP, 0.4 mM GTP, and 4 mM QX-314 (Tocris-Cookson, Ellisville, MO) at a holding membrane potential of −70 mV. When indicated, TTX, bicuculline methiodide, GABA, NO-711, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo([1,2]oxa[4,5]dione (NBQX) (Axxora Life Sciences, Inc., San Diego, CA), and ethanol (190 proof, spectrophotometric grade, Sigma Chemical, St. Louis, MO) were added to the ACSF. CGNs were identified on the basis of their location in the granule layer and their small size (capacitance <5 pF). Access resistance was between 20 and 40 MΩ, and we did not compensate for this; if access resistance changed more than 25%, the recording was discarded.

Data Analysis. Data were acquired and analyzed with pClamp 8 or 9 (Molecular Devices, Sunnyvale, CA); sIPSCs were analyzed with the Mini Analysis program (Synaptosoft, Decatur, GA). The tonic current was calculated by fitting a Gaussian distribution to all-point histograms, constraining the values to 2 bins more negative than the noise. Tonic current amplitude was defined as the mean steady-state current recorded in the absence minus that recorded in the presence of 20 μM of the GABA_A-R antagonist bicuculline. The tonic current noise was defined as the S.D. of the steady-state current recorded in the absence minus that recorded in the presence of bicuculline. The effect of ethanol was calculated with respect to the average of control and washout responses. The Kolmogorov-Smirnov test was used initially to test for significant differences between treatments on sIPSCs in individual cells. Pooled data were statistically analyzed with Prism 4 (GraphPad Software, Inc., San Diego, CA). Data are presented as means ± S.E.M.

Results

We recorded GABAergic currents from cerebellar granule neurons in ACSF at 23°C and −70 mV using the internal solution described by Hanchar et al. (2005). Under these conditions, we detected phasic sIPSCs that were superimposed on the well characterized tonic current that is present in these neurons (Fig. 2, A and B) (Kaneda et al., 1995; Tia et al., 1996; Rossi and Hamann, 1998; Hamann et al., 2002). Both the sIPSCs and the tonic current were antagonized by bicuculline, confirming that these currents were mediated by GABA_A-Rs. Although there was a trend toward an increase in basal tonic current amplitude in CGNs from α_6-100Q/100Q rats (Fig. 2, A and B; Table 1), the magnitudes of the tonic current amplitude and noise were not significantly different in CGNs from α_6-100R/100R and α_6-100Q/100Q rats (Table 1). Acute application of either a subanesthetic (25 mM; the legal intoxication limit in the United States is 17.4 mM = 0.08 g/dl) or a hypnotic (80 mM = 0.37 g/dl) concentration of ethanol for 5 min increased the tonic current amplitude and noise in a reversible and concentration-dependent manner (Fig. 2; compare with Fig. 3 in Hanchar et al., 2005). The acute effect of 25 mM was not significantly different in slices from α_6-100R/100R and α_6-100Q/100Q rats (p < 0.05 by two-way ANOVA followed by a Bonferroni post hoc test). We did detect a statistically significant difference in sensitivity to 80 mM (p < 0.05 by two-way ANOVA followed by a Bonferroni post hoc test). This concentration increased tonic current amplitude to a greater extent in slices from α_6-100R/100R than α_6-100Q/100Q rats (Fig. 2C). The ethanol-induced increase of tonic current noise was not significantly different in slices from α_6-100R/100R versus α_6-100Q/100Q rats at any of the concentrations tested (Fig. 2D).

We next analyzed the effect of ethanol on phasic GABAergic currents in these neurons. Basal sIPSC frequency was significantly lower in CGNs from α_6-100R/100R than α_6-100Q/100Q rats (Table 1). Basal sIPSC amplitude and decay time (defined as the time to decay from 90 to 37% of the peak) were not significantly different in CGNs from α_6-100R/100R and α_6-100Q/100Q rats (but see Santhakumar et al., 2006, who reported that sIPSCs in CGNs from α_6-100Q/100Q rats have prolonged decay rates with respect to those from α_6-100R/100R rats). Ethanol induced a dose-dependent and reversible increase in sIPSC frequency without consistently affecting amplitude or decay (Fig. 3). These effects were similar in slices from α_6-100R/100R and α_6-100Q/100Q rats (compare with Fig. 3 in Hanchar et al., 2005). Ethanol (25

Fig. 1. Representative results of a genotyping assay for the α_6-R100Q polymorphism. Shown are bands corresponding to PCR products (118 base pairs) obtained with a forward primer that was complementary to the α_6-100R genotype (first reaction) and the α_6-100Q genotype (second reaction) for five different Sprague-Dawley rats. Both reactions also contained a control primer pair to verify that the amplification was successful (product = 268 base pairs). Subjects 15, 6, and 7 are heterozygous (α_6-100R/100Q), subject 16 is homozygous for glutamine (α_6-100R/100R), and subject 8 is homozygous for arginine (α_6-100R/100R). PCR products were analyzed with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) which uses a microfluidics-based method of electrophoresis.
mM) induced a significant ($p < 0.01$ by the Kolmogorov-Smirnov test) leftward shift in the interevent interval cumulative probability distributions in 6 of 13 and 5 of 13 granule cells from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats, respectively. Ethanol (25 mM) induced a significant shift in the amplitude cumulative probability distributions in 2 of 7 and 2 of 8 granule cells from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats, respectively. Ethanol (80 mM) induced a significant leftward shift in the interevent interval cumulative probability distributions in 10 of 17 and 10 of 13 granule cells from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats, respectively. Ethanol (80 mM) induced a significant shift in the amplitude cumulative probability distributions in 4 of 9 and 3 of 9 granule cells from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats, respectively. Pooled data are shown in Fig. 3, C and D, where it is evident that the time course of the effect of ethanol on sIPSC frequency is similar in slices from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats. Figure 3, E through F, shows that ethanol did not differentially affect sIPSC amplitude or decay in slices from $\alpha_6$-100R/100R versus $\alpha_6$-100Q/100Q rats. Figure 4 shows that there was no correlation between the baseline levels of tonic current amplitude and sIPSC frequency and the effect of ethanol on these parameters in $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q CGNs (for average values, see Table 1).

Finally, we investigated whether ethanol affects extrasynaptic receptors directly (Fig. 5). We used the same conditions reported by Hanchar et al. (2005); i.e., the effect of ethanol was assessed in the continuous presence of TTX (0.5 μM), the non-NMDA receptor antagonist NBQX (2 μM), the GABA transporter inhibitor NO-711 (10 μM), and exogenous GABA (300 nM). In the presence of these agents, the tonic current amplitude increased by $92 \pm 19\%$ ($n = 5$) and $85 \pm 18\%$ ($n = 4$) in slices from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q rats, respectively. The tonic current noise increased by $64 \pm 13\%$.

![Fig. 2.](image-url) Comparison of the effect of ethanol on CGN tonic currents in slices from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q Sprague-Dawley rats. A, sample traces of tonic GABAergic currents recorded from $\alpha_6$-100R/100R CGNs in the absence and presence of the indicated ethanol (EtOH) concentrations followed by bicuculline (BIC; 20 μM). Shown on the left are Gaussian distribution fits. B, same as in A but for $\alpha_6$-100Q/100Q CGNs. C, summary of the percent change induced by the indicated concentrations of ethanol on tonic current amplitude. D, same as in C but for tonic current noise. Number of determinations is given above or inside the bars. Bars represent the mean ± S.E.M. *, $p < 0.05$ by two-way ANOVA followed by a Bonferroni post hoc test.

### Table 1

Basal electrophysiological parameters for CGN phasic and tonic GABAergic currents in slices from $\alpha_6$-100R/100R and $\alpha_6$-100Q/100Q Sprague-Dawley rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$\alpha_6$-100R/100R</th>
<th>$\alpha_6$-100Q/100Q</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tonic current amplitude (pA)</td>
<td>$15.4 \pm 1.5$ (26)</td>
<td>$21.2 \pm 3.4$ (27)</td>
<td>0.12</td>
</tr>
<tr>
<td>Tonic current noise (pA)</td>
<td>$2.97 \pm 0.25$ (35)</td>
<td>$3.08 \pm 0.33$ (27)</td>
<td>0.8</td>
</tr>
<tr>
<td>sIPSC frequency (Hz)</td>
<td>$0.43 \pm 0.06$ (28)</td>
<td>$0.74 \pm 0.14$ (26)</td>
<td>0.04</td>
</tr>
<tr>
<td>sIPSC amplitude (pA)</td>
<td>$-34.0 \pm 2.2$ (28)</td>
<td>$-39.0 \pm 2.9$ (26)</td>
<td>0.2</td>
</tr>
<tr>
<td>sIPSC decay time (90 to 37% of peak in ms)</td>
<td>$7.4 \pm 0.4$ (28)</td>
<td>$7.7 \pm 0.4$ (26)</td>
<td>0.6</td>
</tr>
</tbody>
</table>
(n = 5) and 56 ± 7% (n = 4) in slices from α6-100R/100R and α6-100Q/100Q rats, respectively. Under these conditions, neither 8 nor 40 mM ethanol induced consistent changes in tonic current amplitude or noise in slices from either α6-100R/100R or α6-100Q/100Q rats (Fig. 5; compare with Fig. 4 in Hanchar et al., 2005). The effects of ethanol on mIPSCs could not be assessed as these events were not discernible from the tonic current noise background.

Discussion

α6 GABA<sub>A</sub> Receptor Subunit Polymorphism Does Not Increase Ethanol Sensitivity of Tonic Currents. Under our experimental conditions, CGN tonic currents in slices from Sprague-Dawley rats homozygous for α6-100Q did not display higher sensitivity to ethanol than those homozygous for α6-100R. Although we found that a subanesthetic concentration of ethanol (25 mM) produces an increase in tonic current amplitude similar to that reported by Hanchar et al. (2005) but, in contrast to the results of that study, we actually detected a significantly greater effect on tonic current amplitude in α6-100Q/100Q rats. This finding suggests that the sensitivity of CGN tonic GABAergic current amplitude to hypnotic ethanol concentrations is reduced by the α6-R100Q substitution; however, it should be kept in mind that the substitution did not have a significant effect on tonic current noise.

Several methodological factors that could have potentially accounted for the discrepancies between our findings and those of Hanchar et al. (2005) can be ruled out. First, both studies used Sprague-Dawley rats from the same colony and age range and used similar slice preparation and recording conditions. There were only a few differences between our experimental procedures and those used in that study. We used ketamine to anesthetize the rats and also added this agent to the cutting solution to decrease NMDA receptor-
mediated excitotoxicity. We stored the slices in ketamine-free ACSF. Hanchar et al. (2005) used the inhalational anesthetic halothane and their slice cutting and storage solutions contained the NMDA receptor antagonist, DL-amino-5-phosphonovaleric acid. Although we recently determined that these technical differences do not significantly modify sensitivity of CGN tonic currents to ethanol (Botta et al., 2007), it is uncertain whether these can influence the impact of the \( \alpha_6 \)-100Q/100Q mutation on ethanol sensitivity. Second, we used the same procedure to analyze the data (i.e., fitting a Gaussian distribution to all-point histograms). Finally, the basal properties of tonic currents were not dramatically different between the two studies. Hanchar et al. (2005) reported that the mean tonic current under control conditions was approximately 10 pA and that it did not differ significantly between the genotypes. Under our recording conditions, the mean basal tonic current amplitudes were 15 and 21 pA for the \( \alpha_6 \)-100R/100R and \( \alpha_6 \)-100Q/100Q Sprague-Dawley rats, respectively, and these were not significantly different between the genotypes. Thus, differences in experimental procedures, quality of the recordings, and data analysis methodology are unlikely to explain the disagreement between our findings. It should be noted, however, that both male and female rats appear to have been used for electrophysiological experiments by Hanchar et al. (2005), whereas we used only male rats. Therefore, we cannot eliminate the possibility that gender modulates the ethanol sensitivity of CGN tonic currents in \( \alpha_6 \)-100Q/100Q and \( \alpha_6 \)-100R/100R Sprague-Dawley rats.

\( \alpha_6 \) GABA\_R Subunit Polymorphism Does Not Affect Ethanol Sensitivity of sIPSCs. We previously reported that ethanol increases the frequency but not the amplitude or decay time of sIPSCs in CGNs (see Carta et al., 2004 for ethanol dose-response data). Hanchar et al. (2005) reproduced these results and also reported that the \( \alpha_6 \)-100Q/100Q mutation approximately doubles the magnitude of the ethanol-induced increase of sIPSC frequency. In our hands, the mutation did not alter sIPSC sensitivity to ethanol; 25 mM increased sIPSC frequency by 1.5- to 2-fold and 80 mM by 2- to 3-fold, both in \( \alpha_6 \)-100Q/100Q and \( \alpha_6 \)-100R/100R Sprague-Dawley rats. These values are similar to those reported by Hanchar et al. (2005) for \( \alpha_6 \)-100R/100R Sprague-Dawley rats and are in general agreement with those reported by Carta et al. (2004). Basal sIPSC frequency, amplitude, and decay time reported here are similar to those observed by Hanchar et al. (2005) and other investigators (Tia et al., 1996; Wall and Usowicz, 1997; Brickley et al., 2001). Thus, differences in the basal characteristics of sIPSCs recorded from CGNs in the two studies are unlikely to be responsible for the discrepancies between the findings of Hanchar et al. (2005) and those of the present study, particularly considering that basal sIPSC frequency is not correlated with the effect of ethanol on this parameter (Fig. 4). The choice of anesthetic, cutting solution, or incubation solution might not be a factor either

![Fig. 4. Lack of correlation between baseline levels of tonic current amplitude and sIPSC frequency and the effect of ethanol on these parameters. Shown are scatter plots of baseline tonic current amplitude versus 80 mM ethanol-induced percent change in this parameter in CGNs from (A) \( \alpha_6 \)-100R/100R (slope = 1.93 ± 2.9; \( r^2 = 0.03 \)) and (B) \( \alpha_6 \)-100Q/100Q (slope = 0.79 ± 0.58; \( r^2 = 0.14 \)) Sprague-Dawley rats. Also shown are scatter plots of basal sIPSC frequency versus 80 mM ethanol-induced percent change in this parameter in CGNs from (C) \( \alpha_6 \)-100R/100R (slope = 96.5 ± 146; \( r^2 = 0.03 \)) and (D) \( \alpha_6 \)-100Q/100Q (slope = -49 ± 51; \( r^2 = 0.07 \)) Sprague-Dawley rats. In all cases, the slopes were not significantly different from 0 (\( p = 0.2–0.5 \)).](https://tjantjapjournals.org)
as it did not significantly affect the action of ethanol on sIPSC frequency (Botta et al., 2007).

**Ethanol Does Not Directly Affect Tonic Currents in CGNs.** We previously reported that the effects of 50 and 100 mM ethanol on CGN tonic current amplitude and noise were abolished by TTX, suggesting that ethanol acted indirectly via an increase in spillover of GABA released in an action potential-dependent manner (Carta et al., 2004). Hanchar et al. (2005) reported that ethanol (10 mM) increased the CGN tonic current in the presence of TTX and that this effect was significantly greater in slices from 6-100Q/100Q than from 6-100R/100R Sprague-Dawley rats. However, the experimental conditions used by Hanchar et al. (2005) were different from those used by Carta et al. (2004). Specifically, Hanchar et al. (2005) not only exposed the CGNs to TTX but also to exogenous GABA, a GABA transporter inhibitor, and NBQX, which increased tonic current amplitude by approximately 2- to 3-fold. Although we found a similar increase in the tonic current in the presence of these agents, we did not detect an effect of ethanol in slices from 6-100Q/100Q than from 6-100R/100R Sprague-Dawley rats. However, the experimental conditions used by Hanchar et al. (2005) were different from those used by Carta et al. (2004). Specifically, Hanchar et al. (2005) not only exposed the CGNs to TTX but also to exogenous GABA, a GABA transporter inhibitor, and NBQX, which increased tonic current amplitude by approximately 2- to 3-fold. Although we found a similar increase in the tonic current in the presence of these agents, we did not detect an effect of ethanol in slices from either 6-100Q/100Q or 6-100R/100R Sprague-Dawley rats. These findings support our original conclusion that ethanol does not directly modulate 6β2δ GABA<sub>δ</sub>Rs expressed in CGNs (Carta et al., 2004). It is not entirely surprising that these receptors are insensitive to low ethanol concentrations, given that they probably contain the β<sub>2</sub> subunit (reviewed in Wisden et al., 1996) and α<sub>6</sub>β<sub>2</sub>δ recombinant receptors display little sensitivity to subanesthetic ethanol concentrations (Wallner et al., 2003; Hanchar et al., 2005; Yamashita et al., 2006). Moreover, the α<sub>6</sub>-R100Q mutation does not have an impact on ethanol sensitivity in receptors containing α<sub>6</sub>β<sub>2</sub>δ subunits (Hanchar et al., 2005).

In conclusion, the results of the present study, taken together with results from studies performed with AT and ANT rats (reviewed in Botta et al., 2007; Korpi et al., 2007) do not support the hypothesis that the R100Q mutation in the α<sub>6</sub> GABA<sub>δ</sub>R subunit increases ethanol sensitivity of tonic GABAergic currents. Our results with outbred Sprague-Dawley rats do not agree with the argument that confounding genetic differences probably arose during selective breeding of the AT and ANT rats and that these could mask an effect of the α<sub>6</sub> subunit polymorphism on ethanol sensitivity of extrasynaptic GABA<sub>δ</sub>Rs in CGNs (Otis et al., 2005; Santhakumar et al., 2007). The differences in alcohol-induced motor impairment that were observed between 6-100Q/100Q and 6-100R/100R Sprague-Dawley rats are unlikely to be a consequence of this single amino acid substitution. Moreover, our results are in line with those obtained with recombinant and native receptors expressed in cultured cells, indicating that GABA<sub>δ</sub>Rs containing α<sub>6</sub>β<sub>2</sub>δ subunits do not display high sensitivity to ethanol (Yamashita et al., 2006; Casagrande et al., 2007). Our overall conclusion is that extrasynaptic GABA<sub>δ</sub>Rs expressed in CGNs are not directly modulated by acute ethanol exposure under our experimental conditions. Although we cannot eliminate the possibility that different methodological approaches are required to observe...
a direct effect of ethanol on CGN extrasynaptic GABAA-Rs, the results presented here do support the hypothesis that these receptors are, at least in part, indirectly modulated via a presynaptic action of ethanol on Golgi cell excitability (Carta et al., 2004; Botta et al., 2007). We are currently characterizing the mechanism by which ethanol produces this effect.

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References


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Address correspondence to Dr. C. Fernando Valenzuela, Department of Neurosciences, MSC08 4740, 1 University of New Mexico, Albuquerque, NM 87131-0001. E-mail: fvalenzuela@nmld.edu