

The δ Subunit of γ -Aminobutyric Acid Type A Receptors Does Not Confer Sensitivity to Low Concentrations of Ethanol

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Received July 11, 2005; accepted October 6, 2005

ABSTRACT

GABA_A receptors (GABA_ARs) are usually formed by α , β , and γ or δ subunits. Recently, δ -containing GABA_ARs expressed in *Xenopus* oocytes were found to be sensitive to low concentrations of ethanol (1–3 mM). Our objective was to replicate and extend the study of the effect of ethanol on the function of $\alpha_4\beta_3\delta$ GABA_ARs. We independently conducted three studies in two systems: rat and human GABA_ARs expressed in *Xenopus* oocytes, studied through two-electrode voltage clamp; and human GABA_ARs stably expressed in the fibroblast L(tk⁻) cell line, studied through patch-clamp electrophysiology. In all cases, $\alpha_4\beta_3\delta$ GABA_ARs were only sensitive to high concentrations of ethanol (100 mM in oocytes, 300 mM in the cell line). Expression of the δ subunit in oocytes was assessed through

the magnitude of the maximal GABA currents and sensitivity to zinc. Of the three rat combinations studied, $\alpha_4\beta_3$ was the most sensitive to ethanol, isoflurane, and 5 α -pregnan-3 α ,21-diol-20-one (THDOC); $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ were very similar in most aspects, but $\alpha_4\beta_3\delta$ was more sensitive to GABA, THDOC, and lanthanum than $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs. Ethanol at 30 mM did not affect tonic GABA-mediated currents in dentate gyrus reported to be mediated by GABA_ARs incorporating α_4 and δ subunits. We have not been able to replicate the sensitivity of $\alpha_4\beta_3\delta$ GABA_ARs to low concentrations of ethanol in four different laboratories in independent studies. This suggests that as yet unidentified factors may play a critical role in the ethanol effects on δ -containing GABA_ARs.

The number of possible molecular targets for ethanol action in brain continues to increase, but the number affected by low ethanol concentrations (<20 mM) remains remarkably small (Harris, 1999; Walter and Messing, 1999; Narahashi et al., 2001; Mailliard and Diamond, 2004). One of the main candidates as a site of ethanol action is the γ -aminobutyric acid type A receptor (GABA_AR). Five subunits form a chloride ion channel that can be modulated by benzodiazepines, neurosteroids, barbiturates, intravenous and volatile anesthetics, and alcohols (Mehta and Ticku, 1999; Korpi et al., 2002a). The binding site for benzodiazepines is located in the α - γ subunit interface (Sigel, 2002), and a putative binding site for alcohols and volatile anesthetics is in the extra-

cellular side of the transmembrane domains (Harris et al., 1997; Ueno et al., 1999; Mascia et al., 2000). The most common subunit combination in brain is $\alpha_1\beta_2\gamma_2$ (Fritschy et al., 1992). δ -Containing GABA_ARs are not nearly as abundant as γ -containing GABA_ARs, but they are gradually emerging as unique and fundamental players in GABAergic inhibition.

In the case of α_4 and δ subunits, their immunoreactivities often presented patterns of codistribution in discreet areas (Pirker et al., 2000). In rat thalamus, immunoprecipitation studies determined that two thirds of the α_4 -containing GABA_ARs also contained δ subunits, and in hippocampus, GABA_ARs containing both α_4 and δ represented half of each subunit's population (Sur et al., 1999). δ -Deficient mice (Mihalek et al., 1999) showed decreased α_4 subunit expression in forebrain, whereas γ_{2S} subunit levels were increased (Peng et al., 2002). These studies suggested that α_4 and δ subunits coassemble and that the absence of δ subunits allowed γ_2 subunits to replace them (Korpi et al., 2002b).

This study was supported by National Institutes of Health Grants AA06399 and GM47818 and by the Waggoner Center for Alcohol and Addiction Research (R.A.H.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.105.092452.

ABBREVIATIONS: GABA_AR, γ -aminobutyric acid receptor type A; DGC, dentate granule cell; CRC, concentration-response curve; THDOC, 5 α -pregnan-3 α ,21-diol-20-one; ANOVA, analysis of variance; aCSF, artificial cerebrospinal fluid; mIPSC, miniature inhibitory postsynaptic current; RMS, root mean square.

Later studies showed that, in dentate granule cells (DGCs), immunogold-labeled δ -containing GABA_ARs were located in perisynaptic sites (Wei et al., 2003) and <2% of α_4 and δ subunits colocalized with GABAergic synaptic markers (GAD65 and gephyrin) (Sun et al., 2004). In DGCs, the tonic inhibitory current was apparently mediated by δ -containing receptors, most likely $\alpha_4\beta_3\delta$, because zolpidem did not affect the tonic current, which was highly sensitive to the hypnotic gaboxadol; the phasic current mediated by γ -containing receptors present in synaptic sites was enhanced by zolpidem (Nusser and Mody, 2002; Maguire et al., 2005).

The δ -knockout mice presented alterations in some ethanol-induced behaviors compared with wild-type mice (reduced ethanol consumption, withdrawal from chronic ethanol exposure, and the anticonvulsant effect of ethanol); however, the anxiolytic and hypothermic responses and the development of chronic and acute tolerance were normal (Mihalek et al., 2001). In addition, the discriminatory stimulus effect of ethanol was similar in δ -knockout and wild-type mice (Shannon et al., 2004).

Recently, two studies showed that low ethanol concentrations enhanced currents mediated by GABA_ARs expressed in *Xenopus* oocytes (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). In the first study, $\alpha_4\beta_2\delta$ was enhanced by 1 to 3 mM ethanol, but 10 mM ethanol had no effect. In the study of Wallner et al., the receptors most sensitive to ethanol were formed by α_4 or α_6 subunits in conjunction with β_3 and δ subunits. They found that ethanol enhanced submaximal GABA-induced currents in $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ receptors at concentrations as low as 3 mM, with 30 mM producing 75% potentiation. The $\alpha_4\beta_2\delta$ and $\alpha_6\beta_2\delta$ GABA_ARs were not affected by 3 mM ethanol but were potentiated by 30 mM ethanol, albeit to a lesser extent than the equivalent β_3 -containing GABA_ARs (21%). The combinations $\alpha_4\beta_3\gamma_{2S}$ and $\alpha_6\beta_3\gamma_{2S}$ failed to show any enhancement until the ethanol concentration reached 100 mM, and the $\alpha\beta$ combinations studied ($\alpha_4\beta_2$, $\alpha_6\beta_2$, $\alpha_4\beta_3$, and $\alpha_6\beta_3$) did not show any enhancement up to 300 mM ethanol. In DGCs, the tonic inhibition (mediated by δ -containing GABA_ARs) was enhanced by low concentrations of ethanol (30 mM), whereas the tonic conductance present in CA1 pyramidal cells (mediated by $\alpha_5\beta_2\gamma_2$ -containing GABA_ARs) was not (Wei et al., 2004).

In an attempt to replicate and extend the studies discussed above, we expressed the same clones used by Wallner et al. (2003) (rat α_4 , β_3 , and δ) in *X. laevis* oocytes, following their procedures. We compared the effects of several drugs, including ethanol, on the $\alpha_4\beta_3\delta$, $\alpha_4\beta_3$, and $\alpha_4\beta_3\gamma_{2S}$ combinations. Similar studies were performed with equivalent human clones expressed in *X. laevis* oocytes. To compare results in a different expression system, a stable cell line previously shown to express human $\alpha_4\beta_3\delta$ (Brown et al., 2002) was also investigated using patch-clamp analysis. Finally, we determined the effects of ethanol on native extrasynaptic GABA_ARs of DGCs, which are reported to contain α_4 and δ subunits.

Materials and Methods

Rat GABA_A Subunits Expressed in *X. laevis* Oocytes

Materials. Adult female *X. laevis* frogs were obtained from Xenopus Express (Plant City, FL). GABA, ethanol, zinc chloride, and lanthanum chloride were purchased from Sigma Chemical (St.

Louis, MO), 5 α -pregnan-3 α ,21-diol-20-one (THDOC) was purchased from Steraloids (Newport, RI), isoflurane was purchased from Marsam Pharmaceuticals Inc. (Cherry Hill, NJ), and etomidate was purchased from Tocris Cookson Inc. (Ellisville, MO). All other reagents were of reagent grade. GABA, zinc chloride, and lanthanum stocks were prepared in water; etomidate and THDOC were dissolved in dimethyl sulfoxide. The drug stocks were then dissolved in buffer; the final dimethyl sulfoxide concentration was 0.1% v/v or less, which does not affect GABA_A-mediated current.

Clones, Transcription, and Oocyte Injection. The cDNAs encoding the GABA_A subunits were generously provided by Dr. R. W. Olsen (rat α_4 and δ , in a modified pGEM vector), Dr. L. Mahan (rat β_3 , in a modified pGEM vector), and Dr. M. H. Akabas (rat γ_{2S} , in a pGEMHE vector). After linearization, the cDNAs encoding the subunits were used as a template for the synthesis in vitro of 5'-capped RNA (mCAP RNA Capping Kit, Stratagene, La Jolla, CA). *X. laevis* oocytes were manually isolated from a surgically removed portion of ovary. Oocytes were treated with collagenase (type IA, 0.5 mg/ml) for 10 min, and then placed in incubation medium (composition: 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 5 mM HEPES, adjusted to pH 7.5), supplemented with 1000 units of penicillin and 1 mg of streptomycin per liter. Oocytes were then injected into the cytoplasm with 30 nl of diethyl pyrocarbonate-treated water containing cRNA encoding GABA_A subunits. The injected amounts were 0.4:0.4 α/β , 0.4:0.4:1.2 $\alpha/\beta/\gamma$, and 0.4:0.4:4 $\alpha/\beta/\delta$ in nanograms per oocyte. The injected oocytes were kept at 18°C in incubation media for 7 to 10 days.

Electrophysiological Recordings. Recordings were carried out 7 to 10 days after injection. The oocytes were placed in a rectangular chamber (approximately 100 μ l) and continuously perfused with ND96 buffer (2 ml/min) at room temperature (23°C). The perfusion buffer composition was 96 mM NaCl, 1 mM CaCl₂, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES, pH 7.5. The whole-cell voltage clamp at -80 mV was achieved through two glass electrodes (1.5–8 M Ω) filled with 3 M KCl, using a Warner Instruments (Hamden, CT) oocyte clamp (model OC-725C).

All drugs were applied by bath perfusion. All solutions were prepared on the day of the experiment. All modulators were preapplied for 1 min before their coapplication with GABA. The responses were also tested without preapplication using 30 mM ethanol, and no differences were observed.

The concentration-response curves (CRCs) were obtained with increasing GABA concentrations, applied for 20 to 30 s at intervals ranging from 5 to 15 min. From these CRCs, the concentration evoking an EC₅₀ was calculated, along with the Hill coefficient (see *Statistical Analysis*). To study the modulation of GABA-evoked currents by the different drugs, the GABA concentration equivalent to an EC₂₀ was determined after 1 mM GABA produced the maximal current. A washout of 5 min was observed in between all GABA applications, except after 1 mM GABA (15 min). After two applications of EC₂₀ GABA, each of the modulators was preapplied for 1 min and then coapplied with GABA for 30 s. EC₂₀ GABA was applied in between coapplication of GABA and modulator. All experiments shown include data obtained from oocytes taken from at least two different frogs.

Statistical Analysis. Nonlinear regression analysis was performed with Prism (GraphPad Software Inc., San Diego, CA). CRCs were fitted to eq. 1,

$$I = \frac{I_{\max}}{1 + 10^{(\log EC_{50} - \log[GABA]) \times n_H}} \quad (1)$$

where I represents the current, I_{\max} the maximal current, EC₅₀ the agonist concentration for half-maximal response, $[GABA]$ the GABA concentration, and n_H the Hill coefficient. Significant differences were determined by two-tailed unpaired t test, one-way ANOVA, or two-way ANOVA repeated measures. Data are presented as mean \pm S.E.

Human GABA_A Subunits Expressed in *X. laevis* Oocytes

The methods were essentially the same as those used for rat GABA_A subunits. For a detailed description, see S. Stórustovu and B. Ebert (submitted for publication).

Whole-Cell Patch Clamp of Stable L(tk⁻) Cells Expressing Human $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs

Experiments were performed on the stable L(tk⁻) cell lines expressing either $\alpha_4\beta_3\delta$ or $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs after a 24-h induction with 25 nM dexamethasone. Glass coverslips containing a monolayer of cells were placed in a chamber on the stage of a Nikon Diaphot inverted microscope. Cells were perfused continuously with artificial cerebrospinal fluid (aCSF) containing 149 mM NaCl, 3.25 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 11 mM D-glucose, 22 mM D-(+)-sucrose, pH 7.4, and observed with phase-contrast optics. Fire-polished patch pipettes were pulled on a WZ, DMZ-Universal puller using conventional 120TF-10 electrode glass. The pipette tip diameter was approximately 1.5 to 2.5 μ m, with resistances around 4 M Ω . The intracellular solution contained 130 mM CsCl, 10 mM HEPES, 10 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid · Cs, 5 mM ATP · Mg, 1 mM MgCl₂, pH adjusted to 7.3 with CsOH and 320 to 340 mOsm. Cells were voltage-clamped at -60 mV via an Axon 200B amplifier (Axon Instruments, Foster City, CA). Drug solutions were applied to the cells via a multibarrel drug delivery system, which pivoted the barrels into place using a stepping motor. This ensured rapid application and washout of the drug, and measured agonist exchange time using this system was approximately 20 to 30 ms. GABA was applied to the cell for 5 s with a 30-s washout period between applications. Allosteric potentiation of GABA_ARs by ethanol was measured relative to a GABA EC₂₀ individually determined for each cell to account for differences in GABA affinity. Increasing concentrations of ethanol were preapplied for 30 s before coapplication with GABA. Data were recorded using pCLAMP (version 8; Axon Instruments).

Recordings from Mouse Hippocampal Slices

Slice Preparation. Hippocampal slices were prepared from mice of either sex (P20–26) and a mixed C57BL/6 and 129/SvJae genetic background, according to standard protocols (Belelli and Herd, 2003). Animals were killed by cervical dislocation in accordance with Schedule 1 of the UK Government Animals (Scientific Procedures) Act 1986. The brain was rapidly removed and placed in oxygenated ice-cold aCSF solution containing 126 mM NaCl, 2.95 mM KCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 0.5 mM CaCl₂, 10 mM MgSO₄, and 10 mM glucose, bubbled with 95% O₂/5% CO₂ to give a pH of 7.4 (315–320 mOsm). The tissue was maintained in ice-cold aCSF while horizontal 300- to 350- μ m slices were cut using a Vibratome (Leica, Nussloch, Germany). The slices were placed on a nylon mesh-covered plastic ring suspended in a beaker filled with circulating, oxygenated, extracellular solution containing 126 mM NaCl, 2.95 mM KCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2 mM CaCl₂, 10 mM D-glucose, and 2 mM MgCl₂ (pH 7.4; 300–310 mOsm). Slices were maintained at room temperature for a minimum of 1 h before being used for recordings.

Electrophysiology. Whole-cell patch-clamp recordings were made at 35°C from hippocampal DGCs visually identified with a Zeiss 2FS (Carl Zeiss, Welwyn Garden City, UK) microscope equipped with differential interference contrast/infrared optics. Patch pipettes were prepared from thick-walled borosilicate glass (Garner Glass Company, Claremont, CA) and had open tip resistances of 3 to 5 M Ω when filled with an intracellular solution that contained 135 mM CsCl, 10 mM HEPES, 10 mM EGTA, 2 mM Mg-ATP, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM lidocaine *N*-ethyl bromide (pH 7.3 with CsOH, 295–305 mOsm). Miniature inhibitory postsynaptic currents (mIPSCs) were recorded using an Axopatch 1D (Axon Instruments) at a holding potential of -60 mV in an extracellular recording solution (see above), additionally containing 2 mM

kynurenic acid (Sigma-Aldrich-RBI, Poole, UK) and 0.5 μ M tetrodotoxin (TCS Biologicals Ltd, Buckingham, UK) to block ionotropic glutamate receptors and action potentials, respectively. Series resistance ranged from 6 to 18 M Ω and was compensated up to 80%. In each case, currents were sampled at 10 kHz and filtered at 2 kHz using an eight-pole low-pass Bessel filter.

Drug Application. Ethanol was prepared on the day of the experiment as a concentrated stock (3 M) and diluted in extracellular solution to a final concentration of 30 mM. Ethanol was applied via the perfusion system (2–4 ml/min) and allowed to infiltrate the slice for a minimum of 10 min while recordings were acquired.

Data Analysis. Data were recorded onto a digital audiotape using a Biologic DTR 1200 recorder and analyzed offline using the Strathclyde Electrophysiology Software, WinEDR/WinWCP (courtesy of Dr. J. Dempster, University of Strathclyde, Glasgow, Scotland). Individual mIPSCs were detected using a -4 pA amplitude threshold detection algorithm and visually inspected for validity. Accepted events were analyzed with respect to peak amplitude, 10 to 90% rise time, and decay time course. A minimum of 50 accepted events were digitally averaged by alignment at the midpoint of the rising phase and the mIPSC was decay-fitted by either monoexponential [$y(t) = Ae^{(-t/\tau)}$] or biexponential [$y(t) = A_1e^{(-t/\tau_1)} + A_2e^{(-t/\tau_2)}$] functions using the least-squares method, where A is amplitude, t is time, and τ is the decay time constant. Analysis of the standard deviation of residuals and use of the F test to compare goodness of fit revealed that the average mIPSC decay was always best fit with the sum of two exponential components. Thus, a weighted decay time constant (τ_w) was also calculated according to the equation: $\tau_w = \tau_1P_1 + \tau_2P_2$, where τ_1 and τ_2 are the decay time constants of the first and second exponential functions and P_1 and P_2 are the proportions of the synaptic current decay described by each component. Tonic current amplitude was calculated as the difference between the holding current before and after application of bicuculline methobromide 30 μ M (Brickley et al., 1996). The root mean square (RMS) noise was determined from sections of record lacking synaptic currents.

All results are reported as the arithmetic mean \pm S.E.M. Statistical significance of mean data were assessed with the unpaired Student's t test or repeated measures ANOVA followed post hoc by the Newman-Keuls test as appropriate, using the Sigma Stat (SPSS Inc., Chicago, IL) software package.

Results

The effect of different concentrations of ethanol on GABA-induced currents through $\alpha_4\beta_3\delta$ GABA_ARs can be observed in the representative tracings in Fig. 1. When $\alpha_4\beta_3\delta$ GABA_ARs were expressed in oocytes (Fig. 1A, rat subunits; Fig. 1B human subunits) and in the stable cell line (human subunits, Fig. 1C), no definite effect could be seen at low ethanol concentrations. The GABA response in $\alpha_4\beta_3\delta$ GABA_ARs showed some desensitization, but it was much less than that in $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs (Fig. 1A). Two protocols were used to evaluate the effects of ethanol: a continuous application of submaximal GABA was applied with increasing concentrations of ethanol coapplied at intervals, as shown in Fig. 1; alternatively, ethanol was preapplied before coapplication of shorter applications of GABA, allowing washout in between, and the results are shown in Figs. 2 and 3.

The ethanol potentiation of EC₂₀ GABA was measured at 30, 100, and 300 mM ethanol in rat $\alpha_4\beta_3$, $\alpha_4\beta_3\gamma_{2S}$, and $\alpha_4\beta_3\delta$ GABA_ARs expressed in oocytes (Figs. 2, A, B, and C, and 3A), following the protocol explained under *Materials and Methods* (1-min ethanol preapplication followed by 30-s GABA and ethanol coapplication and then 5-min washout). Ethanol showed a significant effect with the subunit combination tested ($F_{2,34} = 9.63$, $p < 0.005$) and ethanol concentration

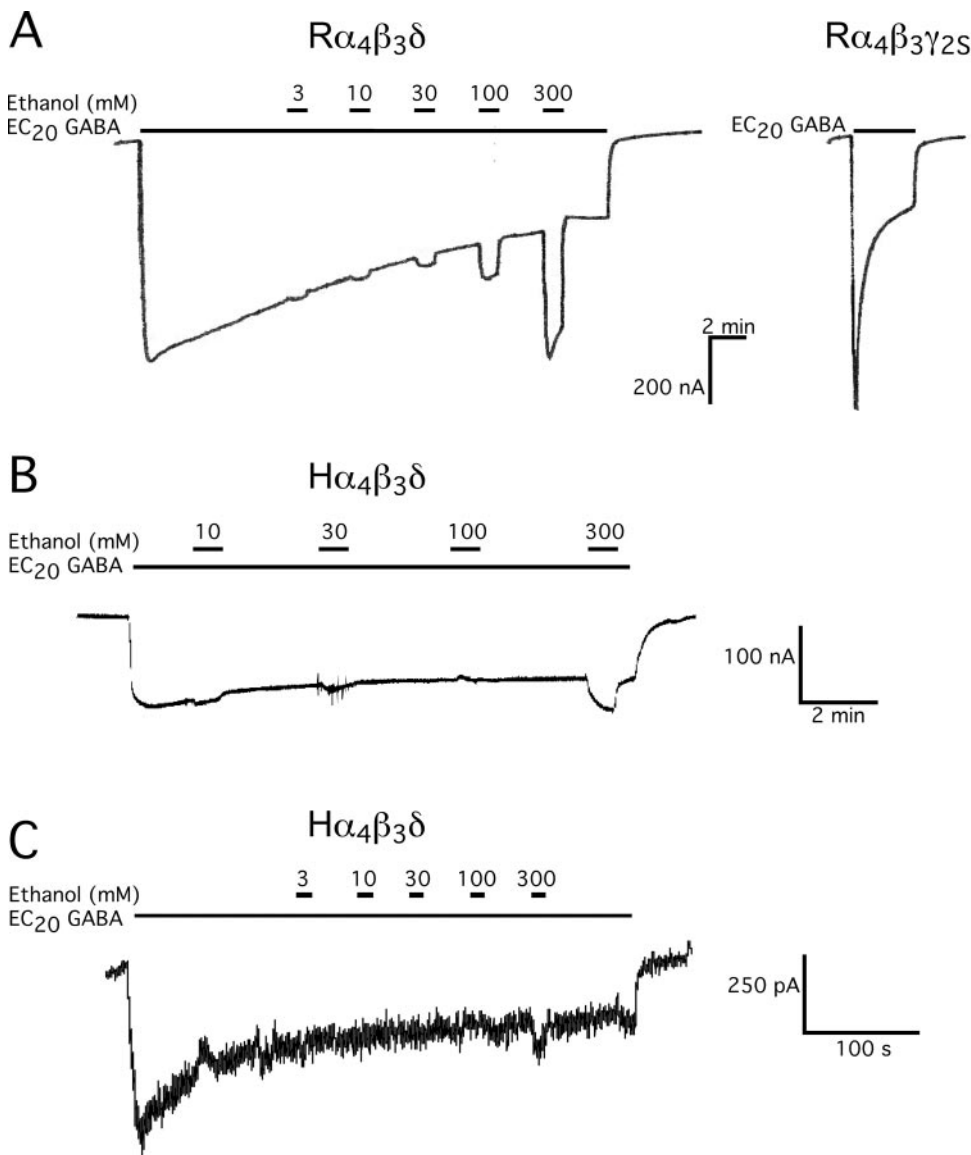


Fig. 1. Tracings showing the ethanol effect on EC₂₀ GABA responses (long application) in $\alpha_4\beta_3$ -containing receptors expressed in oocytes and the stable cell line. A, rat subunits expressed in oocytes. B, human subunits expressed in oocytes. C, human subunits expressed in the L(tk⁻) fibroblast cell line.

($F_{2,34} = 164$, $p < 0.0001$), with a strong interaction ($F_{4,34} = 7.96$, $p < 0.0005$). In all three combinations, the enhancement increased as the ethanol concentration was increased, but none of the subunit combinations showed sensitivity to low ethanol concentrations. Preliminary experiments with 10 mM ethanol also failed to show any effect on $\alpha_4\beta_3\delta$ GABA_ARs (data not shown). At high concentrations, the most sensitive combination was $\alpha_4\beta_3$, and all the combinations showed significant differences in the maximum extent of potentiation. When human $\alpha_4\beta_3\delta$ GABA_ARs were expressed in oocytes, 10 and 30 mM ethanol failed to induce any significant change, whereas 100 and 300 mM ethanol produced a significant enhancement of the GABA-induced current of a magnitude similar to the changes observed with the rat $\alpha_4\beta_3\delta$ GABA_ARs (Figs. 2D and 3B). In the L(tk⁻) cell line, the currents induced by GABA in both $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ were not affected by 1 to 100 mM ethanol, but 300 mM ethanol induced a similar change in both combinations (Fig. 3C).

To verify that all the subunits were being expressed in oocytes, a pharmacological characterization was done. GABA responses obtained in oocytes expressing rat $\alpha_4\beta_3\delta$ GABA_ARs

showed a lower GABA EC₅₀ for $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$ (0.62 and 1.3 μ M, respectively) than for $\alpha_4\beta_3\gamma_{2S}$ (12 μ M) (Fig. 4A). The GABA EC₅₀ values for the human $\alpha_4\beta_3$ and $\alpha_4\beta_3\delta$ subunits expressed in oocytes were 1.6 and 2.3 μ M, respectively (S. Stórustovu and B. Ebert, submitted for publication). Two main differences among the different rat combinations expressed in oocytes were in the maximal GABA-induced current and the inhibition by zinc. The maximal GABA responses 8 days after injection showed a significant effect for the subunit composition tested ($F_{2,72} = 19.4$, $p < 0.0001$). In the oocytes injected with cRNA encoding $\alpha_4\beta_3$, the maximal response to GABA was only 309 ± 63 nA, whereas the oocytes injected with $\alpha_4\beta_3\delta$ showed a response of 3810 ± 360 nA. The oocytes injected with cRNA encoding $\alpha_4\beta_3\gamma_{2S}$ showed a maximal current of 4000 ± 1100 nA (Fig. 4B). The zinc inhibition was markedly greater at $\alpha_4\beta_3$ than at $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs (Fig. 4C). The zinc inhibition showed a significant effect for the subunit composition tested ($F_{2,45} = 606$, $p < 0.0001$). Human $\alpha_4\beta_3\delta$ receptors expressed in oocytes also showed a decreased sensitivity to zinc compared with $\alpha_4\beta_3$

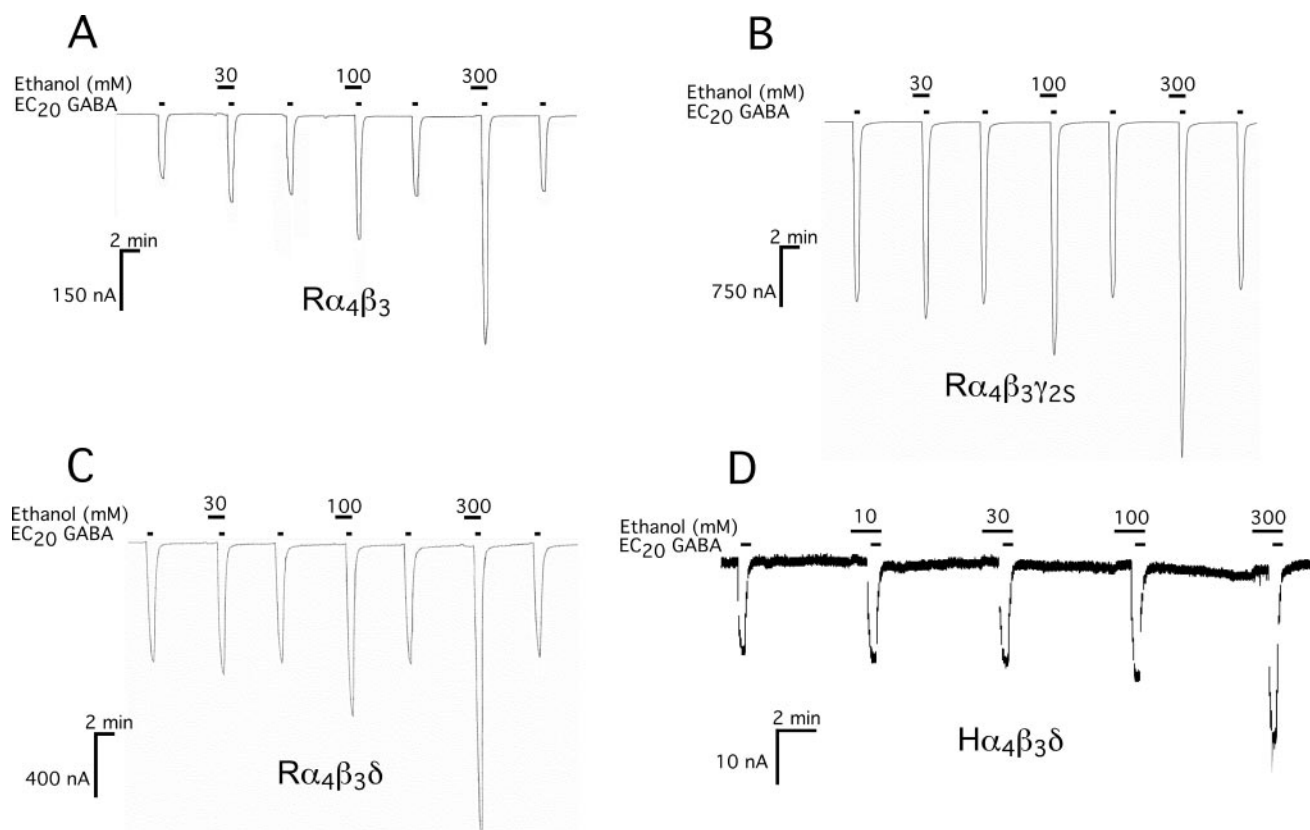


Fig. 2. Tracings showing the ethanol effect on EC₂₀ GABA responses (short application) in $\alpha_4\beta_3$ -containing receptors expressed in oocytes. A, rat $\alpha_4\beta_3$. B, rat $\alpha_4\beta_3\gamma_{2S}$. C, rat $\alpha_4\beta_3\delta$. D, human $\alpha_4\beta_3\delta$.

GABA_ARs (S. Störustovu and B. Ebert, submitted for publication).

Using rat subunits expressed in oocytes, isoflurane potentiation showed significant differences according to the subunit combination tested ($F_{2,14} = 7.14$, $p < 0.01$) and isoflurane concentration ($F_{1,14} = 76.7$, $p < 0.0001$), with a significant interaction ($F_{2,14} = 4.50$, $p < 0.05$). Thus, the same pattern seen with ethanol was observed with the volatile anesthetic isoflurane: no significant differences at low (subanesthetic) concentrations (58 μM , $0.2 \times$ anesthetic EC₅₀) and $\alpha_4\beta_3$ presenting the highest sensitivity at anesthetic concentrations (290 μM , $1 \times$ anesthetic EC₅₀), whereas $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ did not show significant differences (Fig. 5A).

We also tested the effect of 1 μM THDOC on the GABA responses, and THDOC enhancement showed a significant effect for the subunit composition tested ($F_{2,14} = 21.0$, $p < 0.0001$). We found the recurring pattern: $\alpha_4\beta_3$ was the most sensitive combination, and $\alpha_4\beta_3\gamma_{2S}$ exhibited the least potentiation (Fig. 5B). Lanthanum was also used to verify the differential response between $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ (Brown et al., 2002), and we found a significant effect according to the subunit composition tested ($t = 10.7$, $df = 10$, $p < 0.0001$). Lanthanum (100 μM) inhibited GABA-induced responses in $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs, and that inhibition was more pronounced when lanthanum was applied to $\alpha_4\beta_3\delta$ GABA_ARs (Fig. 5C), in a way similar to that previously reported in the stable cell line (Brown et al., 2002).

To record the effects of ethanol on native receptors containing the δ subunit, the tonic current was recorded ($V_H = -60$ mV) from visually identified DGCs in hippocampal slices. In

addition, the effect of ethanol on mIPSCs (i.e., non- δ -containing synaptic GABA_ARs) was determined. The properties of mIPSCs were similar to those previously described (Belelli and Herd, 2003) with a mean peak amplitude of 74 ± 2 pA and a τ_w of 9.7 ± 0.4 ms ($n = 10$). Application of 30 μM bicuculline completely abolished mIPSCs and additionally induced a reduction in membrane noise (i.e., RMS) (Fig. 6, A, B, and C) and an outward current (i.e., tonic current) of 19 ± 3 pA ($n = 8$) (Fig. 6, A, B, and C). Bath application of 30 mM ethanol for a minimum of 10 min had no effect on any of the mIPSCs properties ($p > 0.1$) (Fig. 6D). In addition, ethanol did not affect either the tonic current or the RMS noise calculated from segments of recording lacking mIPSCs ($p > 0.05$) (Fig. 6).

Discussion

The present results extend the pharmacology of α_4 -containing GABA_ARs but also differ from some published results, introducing the possibility of unknown factors that affect the sensitivity of α_4 -containing GABA_ARs to certain drugs, especially ethanol. The GABA EC₅₀ values for $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ in the cell line reported in the literature were lower than the ones observed in oocytes (0.50 and 2.57 μM , respectively) (Brown et al., 2002). Differences between the results from oocytes and from the stable cell lines are likely to be due to the different kinetics in these systems, and they have been observed several times previously (Ebert et al., 1997; Mihic et al., 1997). If we compare our results in the rat subunits expressed in oocytes with those of Wallner et al. (2003), the GABA EC₅₀ values for $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ are

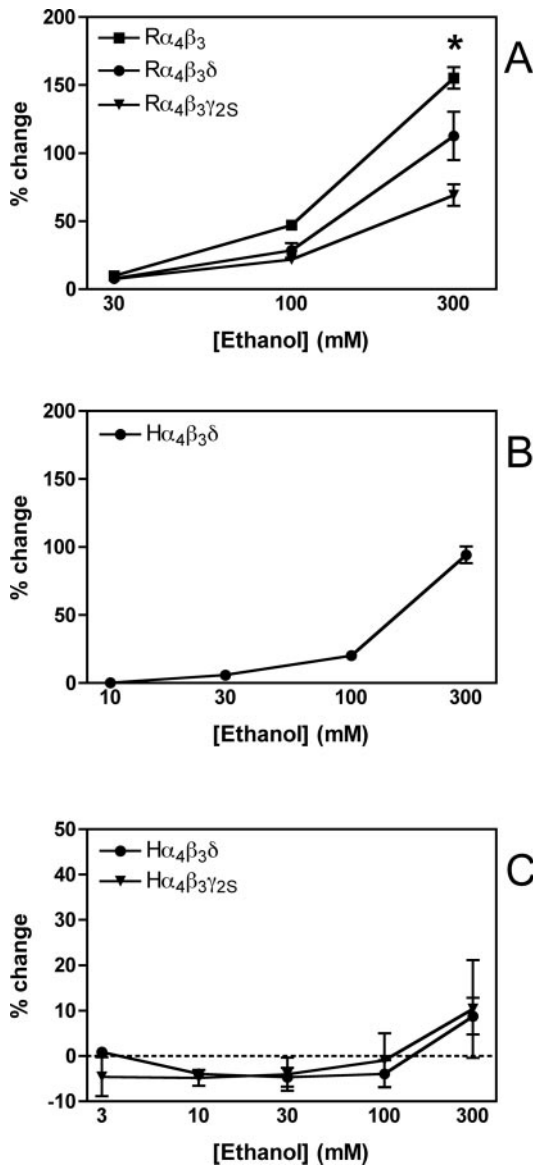


Fig. 3. Modulation of GABA responses by ethanol. A, ethanol (30–300 mM) on EC₂₀ GABA responses of rat subunits expressed in oocytes. *, $p < 0.05$ versus other subunit combinations at 300 mM ethanol ($n = 6-8$). B, ethanol (10–300 mM) on EC₂₀ GABA responses of human subunits expressed in oocytes. ($n = 4$). C, ethanol (1–300 mM) on EC₂₀ GABA responses of human subunits expressed in stable cell line ($n = 3$).

similar, but the GABA EC₅₀ for $\alpha_4\beta_3$ is remarkably different (0.62 versus 22.5 μ M), giving different relationships for our GABA EC₅₀ ($\alpha_4\beta_3 < \alpha_4\beta_3\delta < \alpha_4\beta_3\gamma_{2S}$) and that of Wallner et al. (2003) ($\alpha_4\beta_3\delta < \alpha_4\beta_3\gamma_{2S} < \alpha_4\beta_3$). We are not aware of any other cases reported in the literature in which $\alpha\beta$ GABA_ARs present a higher GABA EC₅₀ than $\alpha\beta\gamma$ GABA_ARs.

The presence of the δ subunit in the GABA_AR was evidenced by higher maximal GABA currents compared with $\alpha_4\beta_3$ GABA_ARs and by a decreased sensitivity to zinc and lanthanum inhibition. α_4 -Containing receptors have been notoriously difficult to express, particularly the $\alpha_4\beta_3$ combination, and the time required to express these receptors and the maximal currents reported here support this fact. For $\alpha_1\beta_1$ - and $\alpha_6\beta_3$ -containing GABA_ARs, the zinc IC₅₀ values indicate a sensitivity of $\alpha\beta > \alpha\beta\delta > \alpha\beta\gamma$ (Thompson et al., 1997; Krishek et al., 1998). For $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ stably expressed

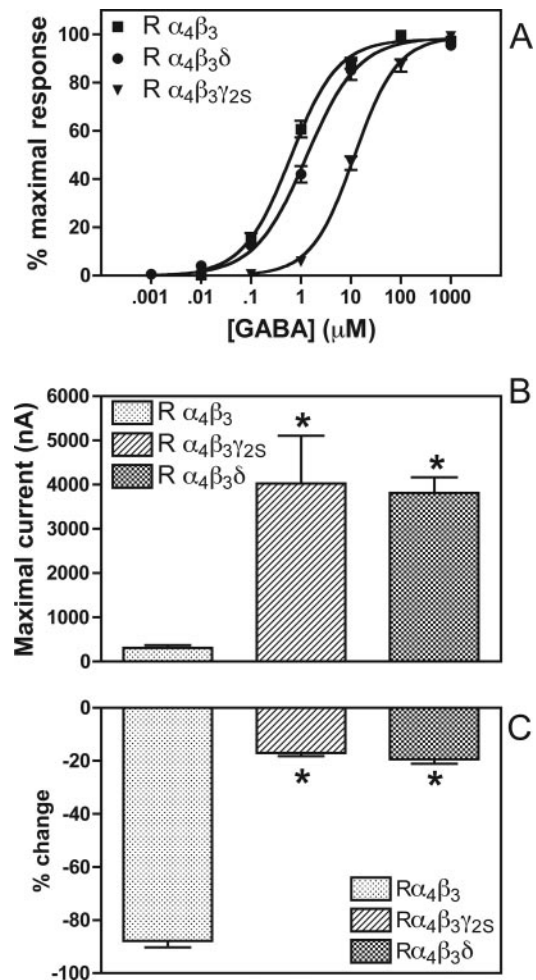


Fig. 4. GABA responses and zinc inhibition of rat subunits expressed in oocytes. A, GABA CRCs. Curve parameters: EC₅₀ (95% confidence interval); Hill slope \pm S.E.M. $\alpha_4\beta_3$: 0.62 (0.51–0.76); 0.92 \pm 0.07 ($n = 9$). $\alpha_4\beta_3\delta$: 1.29 (1.02–1.65); 0.84 \pm 0.07 ($n = 8$). $\alpha_4\beta_3\gamma_{2S}$: 11.8 (9.9–14.1); 1.01 \pm 0.09 ($n = 7$). B, GABA maximal responses 8 days after injection ($n = 17-29$ per subunit combination). *, $p < 0.001$ versus $\alpha_4\beta_3$. C, zinc (1 μ M) on EC₂₀ GABA ($n = 12-19$ per subunit combination). *, $p < 0.001$ versus $\alpha_4\beta_3$.

in a cell line, 1 μ M zinc inhibited 25 and 37% of GABA EC₅₀ responses, respectively (Brown et al., 2002). In our case, rat $\alpha_4\beta_3\delta$ and $\alpha_4\beta_3\gamma_{2S}$ subunits expressed in oocytes were inhibited to the same extent by 1 μ M zinc (approximately 20%), whereas $\alpha_4\beta_3$ showed an almost complete inhibition (90%).

Wallner et al. (2003) reported no significant ethanol enhancement on $\alpha_4\beta_3$ GABA_ARs, whereas 3 mM ethanol produced approximately 16% enhancement in $\alpha_4\beta_3\delta$ GABA_ARs, but no ethanol effect was observed in $\alpha_4\beta_3\gamma_{2S}$ GABA_ARs until 100 mM ethanol was applied. In our study, none of the combinations showed a significant effect at low ethanol concentrations (10–30 mM). The three subunit combinations showed different responses at 300 mM ethanol, with a sensitivity order of $\alpha_4\beta_3 > \alpha_4\beta_3\delta > \alpha_4\beta_3\gamma_{2S}$. Human $\alpha_4\beta_3\delta$ expressed in oocytes showed the same sensitivity to ethanol as rat subunits, whereas human $\alpha_4\beta_3\delta$ subunits expressed in the stable cell line were even less sensitive to ethanol, but showed no difference from $\alpha_4\beta_3\gamma_{2S}$ subunits. Furthermore, a volatile anesthetic showed the same pattern as ethanol in the rat subunits expressed in oocytes: no differential sensitivity at low isoflurane concentrations, but at high concentrations the most sensitive combination was $\alpha_4\beta_3$. The only other

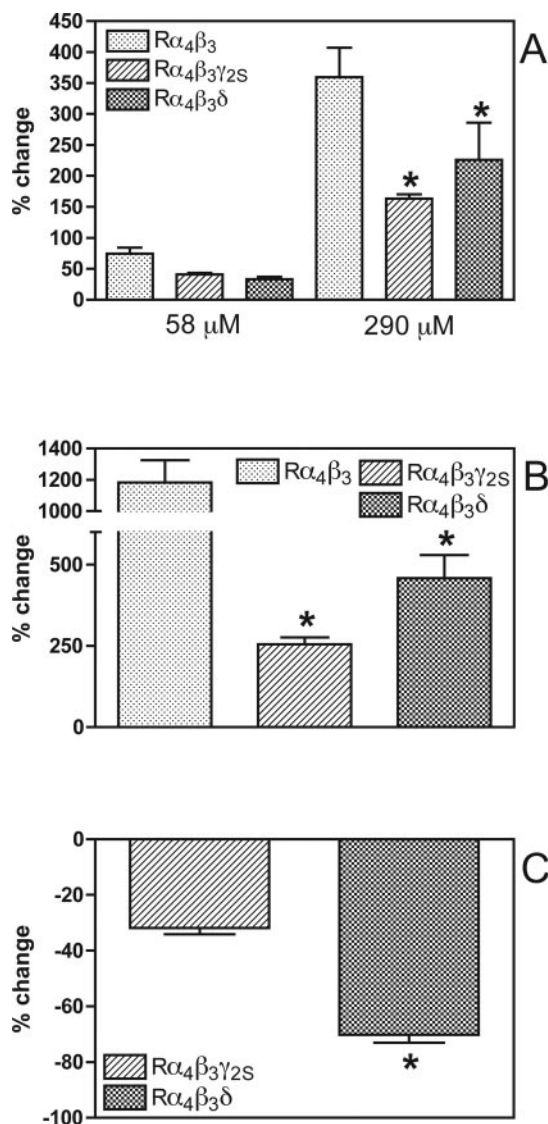


Fig. 5. Modulation of GABA responses by isoflurane, THDOC, and lanthanum of rat subunits expressed in oocytes. A, isoflurane (58 and 290 μM) on EC_{20} GABA responses. *, $p < 0.05$ versus $\alpha_4\beta_3$ ($n = 5-6$). B, THDOC (1 μM) on EC_{20} GABA responses. *, $p < 0.001$ ($n = 6-9$). C, lanthanum (100 μM) on EC_{20} GABA responses. *, $p < 0.0001$ versus $\alpha_4\beta_3\gamma_{2S}$ ($n = 5-7$).

study of the isoflurane effect on δ -containing GABA_ARs was with $\alpha_1\beta_1$ -containing receptors, in which all three combinations ($\alpha_1\beta_1$, $\alpha_1\beta_1\delta$, and $\alpha_1\beta_1\gamma_{2L}$) showed the same potentiation by 300 μM isoflurane (Lees and Edwards, 1998), indicating that the δ subunit does not impact on the sensitivity to volatile anesthetics in these GABA_ARs. This is consistent with the finding that actions of two volatile anesthetics (enflurane and halothane) in the tail-clamp test and the loss of righting reflex assay were not altered significantly in δ -knockout mice (Mihalek et al., 1999). Even though the subunit origin (rat versus human) and the expression system [oocyte versus L(tk⁻) fibroblast cell line] were different, the responses to lanthanum and THDOC, as for the other drugs presented in this study, were similar to those reported by Brown et al. (2002).

The $\alpha_4\beta_3$ combination has not been tested extensively previously, and all allosteric compounds elicited greater maximum modulation at this subtype. The low currents induced

by saturating concentrations of GABA might suggest a very low probability of channel opening, and hence a greater extent of modulation may be possible. Similar to other $\alpha\beta$ combinations, $\alpha_4\beta_3$ is highly sensitive to zinc blockade. Interesting as the combination is, there is no unequivocal evidence of the existence of $\alpha\beta$ GABA_ARs receptors in vivo. Approximately 49% of all α_4 subunits were colocalized with γ_{1-3} or δ subunits, suggesting the possibility that half of the α_4 -containing receptors are composed of α_4 and β_{1-3} subunits only (Bencsits et al., 1999). In addition, single-channel recordings in cerebellar granule cells at postnatal day 7 (when α_6 and δ subunits are not yet significantly expressed) showed both low- and high-conductance receptors, suggesting the presence of $\alpha\beta\gamma$ and $\alpha\beta$ receptors (Brickley et al., 1999). However, the only way to prove the presence of GABA_ARs composed only of α_4 and β subunits would be to eliminate all other possibilities (coexpression of ϵ , π , or an unknown subunit, etc.), so the final proof of their presence in vivo may be elusive.

One discrete location that expresses high levels of $\alpha_4\beta\delta$ GABA_ARs is the hippocampal dentate gyrus. Receptors containing α_4 and δ subunits contribute to a tonic or basal current present in this region (Nusser and Mody, 2002). Here we have recorded from hippocampal dentate granules and shown that neither the synaptic inhibitory currents nor the baseline tonic current is affected by 30 mM ethanol, suggesting that native receptors that contain $\alpha_4\delta$ are also not modulated by low concentrations of ethanol. This finding conflicts with previously published data reporting that 30 mM ethanol increases the tonic current of dentate granule cells (Wei et al., 2004). Possible explanations for this difference may reside in the age of the animals (adult compared with P20–26). Indeed the GABA modulatory effects of neurosteroids in dentate granule neurons change with development (Belelli and Lambert, 2005). Furthermore, in the present investigation the tonic current was determined without the addition of exogenous agonist as was done in the study of Wei et al. (2004). Another difference was the mice used in each study: Wei et al. used C57BL/6J mice, whereas we used mice with a mixed C57BL/6J and 129/SvJae genetic background. Different mouse strains may present different sensitivity to the behavioral effects of ethanol, but we have not found any evidence suggesting that that was the case here. In a study comparing the C57BL/6J and 129/SvJ strains (another substrain of 129/Sv), the latter strain was more sensitive to the ethanol effects on tests such as the plus-maze and the loss of righting reflex (Homanics et al., 1999).

However, the consequences of the genetic background on ethanol effects can be decisive, as observed with other δ -containing receptors that have originated conflicting reports. Hanchar et al. (2005) described how the ethanol sensitivity of $\alpha_6\beta_3\delta$ GABA_ARs was increased when the $\alpha_6\text{Q}100$ was replaced by R and that rats from outbred strains of Sprague-Dawley, which were homozygous for R in that position, were more sensitive to ethanol-induced motor impairment. However, Valenzuela et al. (2005) performed similar experiments in inbred alcohol-nontolerant and alcohol-tolerant rats, which were homozygous for the 100Q and 100R genotypes, respectively. They observed an ethanol-induced increase of the GABA_AR-mediated tonic current; however, the enhancement was the same for both genotypes. Other differences detected by Hanchar et al. (2005) were not seen by Valenzu-

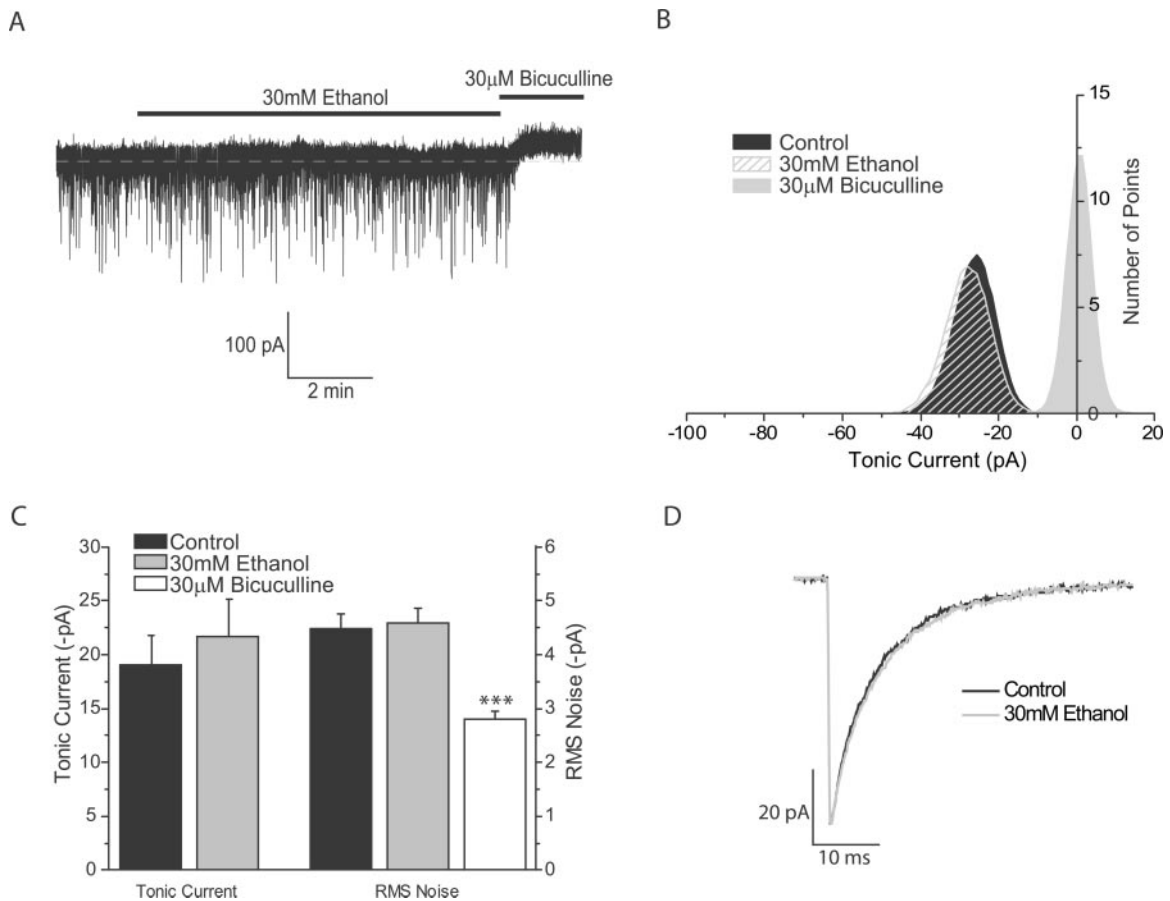


Fig. 6. The effect of ethanol upon GABA_AR-mediated transmission in DGCs. A, bath application of 30 mM ethanol had no effect on the holding current in an exemplar DGC voltage-clamped at $V_H = -60$ mV. The subsequent application of bicuculline (30 μ M) abolished mIPSCs and induced a reduction in membrane noise and an outward current of ~ 20 pA. B, an all-points histogram illustrating the amplitude of the holding current (normalized to the current in the presence of bicuculline) under control conditions (black area), in the presence of 30 mM ethanol (striped area) and after the application of 30 μ M bicuculline (gray area) recorded from an exemplar DGC. Note that ethanol has no effect on the amplitude of the tonic current. C, bar graph summarizing on the left axis the amplitude of the tonic current of eight DGCs in the absence (black bar) and in the presence (gray bar) of 30 mM ethanol and on the right axis the RMS noise under control conditions (black bar), after application of 30 mM ethanol (gray bar) and 30 μ M bicuculline (white bar). D, overlaid averaged mIPSCs recorded from an exemplar DGC under control conditions and after the bath application of 30 mM ethanol. Note that ethanol has no effect on the mIPSCs.

ela et al. (2005), and the latter pointed to the fact that “the mechanism by which ethanol modulates tonic GABAergic currents in cerebellar granule neurons appears to be highly dependent on the experimental conditions”. Valenzuela et al. (2005) also suggested the possibility of a presynaptic site of action for ethanol enhancement of the tonic current. These experiments do not shed light on the differences observed when δ -containing GABA_ARs are expressed in heterologous systems, but they are additional evidence of a complex situation, in which not all factors have been determined for the ethanol mechanism of action at low concentrations.

The results with ethanol presented here are different from those reported by Wallner et al. (2003) in that no effects of low concentrations were observed. Given the fact that we used the same clones and the same expression system and followed the same general procedure, it is difficult to understand the cause of the observed differences. Furthermore, Sundstrom-Poromaa et al. (2002) reported substantially different results with the combination $\alpha_4\beta_2\delta$, lending further support to the complexity of the matter. Wallner et al. (2003) demonstrated a reproducible effect at low ethanol concentrations, but the fact that we have not been able to replicate these results in three different laboratories in independent

studies suggests that the effect is not as robust as supposed and that as yet unidentified factors might play a fundamental role in the ethanol effects on δ -containing GABA_ARs.

Acknowledgments

B.E. thanks Marianne Faber for optimizing the oocyte handling, thereby making these studies possible. K.A.W. thanks Alison Macaulay for technical help. C.M.B. and R.A.H. thank Kathryn L. Carter and Rachel Phelan for the oocyte harvest.

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