Effects of Ethanol on Tonic GABA Currents in Cerebellar Granule Cells and Mammalian Cells Recombinantly Expressing $GABA_A$ Receptors

Megumi Yamashita, William Marszalec, Jay Z. Yeh, and Toshio Narahashi

Department of Molecular Pharmacology and Biological Chemistry

Northwestern University Medical School

Chicago, IL 60611

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Effects of Ethanol on Tonic GABA Currents

Corresponding author: Dr. Toshio Narahashi

Department of Molecular Pharmacology and Biological Chemistry

Northwestern University Medical School

303 E. Chicago Avenue

Chicago, IL 60611-3008

Tel. 312-503-8284

Fax. 312-503-1700

E-mail: **narahashi@northwestern.edu**

The number of text pages: 39

The number of figures: 8

The number of tables: 1

The number of references: 44

The number of words in the abstract: 248

The number of words in the Introduction: 619

The number of the discussion: 1249

Abbreviations: GABA, γ-aminobutyric acid; HEK cell, human embryonic kidney cell; CHO cell, Chinese hamster ovary cell; DIV, days *in vitro*

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

The effects of ethanol on the $GABA_A$ receptors, which are regarded as one of the most important target sites of ethanol, are very controversial, ranging from potentiation to no effect. The δ subunit-containing $GABA_A$ receptors expressed in *Xenopus* oocytes were recently reported to be potently augmented by ethanol. We performed patch clamp experiments using the cerebellar granule cells and mammalian cells expressing recombinant $GABA_A$ receptors. In granule cells, the sensitivity to GABA increased from 7 days to 11 days *in vitro*. Furosemide, an antagonist of α6-containing $GABA_A$ receptors, inhibited GABA-induced currents more potently at 11-14 days than that at 7 days. Ethanol at 30 mM had either no effect or an inhibitory effect on currents induced by low concentrations of GABA in granule cells. On α4β2δ, α6β2δ or α6β3δ $GABA_A$ receptors expressed in Chinese hamster ovary cells, ethanol at 10, 30 and 100 mM had either no effect or an inhibitory effect on GABA currents. Ethanol inhibition of $GABA_A$ receptor was observed in all the subunit combinations examined. In contrast, the perforated patch clamp method to record the GABA currents revealed ethanol effects on the α6β2δ subunits ranging from slight potentiation to slight inhibition. Ethanol appears to exert a dual action on the $GABA_A$ receptors and the potentiating action may depend on intracellular milieu. Thus, the differences between the $GABA_A$ receptors expressed in mammalian host cells and those in *Xenopus* oocytes in the response to ethanol might be due to changes in intracellular components under patch clamp conditions.

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Ethanol is known to modulate the activity of a variety of neuroreceptors and ion channels. For example, ethanol potentiates the activity of γ-aminobutyric acid$_A$ ($GABA_A$) receptors (Mihic, 1999), 5-HT_3 receptors (Lovinger, 1999), and neuronal nicotinic acetylcholine receptors (Narahashi et al., 1999). On the other hand, ethanol has been reported to inhibit the activity of glutamate receptors (Woodward, 1999), voltage-gated calcium channels (Walter and Messing, 1999), and ATP-gated ion channels (Weight et al., 1999).

Whereas the importance of the $GABA_A$ receptors in the behavioral effects of ethanol is well documented (Pontieri et al., 1996; Koob et al., 1998), the ethanol modulation of $GABA_A$ receptor activity *in vitro* has been very controversial (Aguayo et al., 2002; Sapp and Yeh, 1998). Some investigators found the potentiation of GABA-induced currents (Mihic, 1999), while others did not find any effect of ethanol (White et al., 1990; Marszalec et al., 1994). A variety of explanations have been proposed for the controversial results on the effects of ethanol on the $GABA_A$ receptor. These include receptor subunit combinations (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), cell viability (Aguayo et al., 2002), cell type (Sapp and Yeh, 1998; Mori et al., 2000), and posttranslational processing such as receptor phosphorylation (Aguayo et al., 2002), to mention a few.

Two lines of development have occurred recently with respect to the action of ethanol on $GABA_A$ receptors. One is the subunit dependence of alcohol modulation of the $GABA_A$ receptor, and the other is the significance of tonic GABA currents generated by

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

extrasynaptic $GABA_A$ receptors. When recombinantly expressed in *Xenopus* oocytes, the $\alpha4\beta3\delta$ and $\alpha6\beta3\delta$ combinations of $GABA_A$ receptor subunits are highly sensitive to ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004). These results suggest that ethanol, like many other $GABA_A$ receptor modulators, potentiates GABA currents in a subunit-specific manner. However, a recent consortium study from four laboratories (Borghese et al., 2006) has failed to confirm such claim. Tonic GABA-induced currents generated by low concentrations of GABA in the hippocampal neurons (Bai et al., 2001), the dentate gyrus granule cells (Wei et al., 2004), and cerebellar granule neurons (Hanchar et al., 2005) are potentiated by low concentrations of ethanol. The potentiating action of ethanol on the tonic current may be more important than its action on the phasic synaptic current as the former current contributes more than two-thirds of the total charges in the GABAergic neuron (Hamann et al., 2002). However, 50 mM ethanol failed to potentiate the tonic current in the cerebellar granule neurons (Carta et al., 2004). Thus, the effects of a drug on neuroreceptors could be influenced by the host cells expressing the receptors (Sivilotti et al., 1997).

In the present study, we used $GABA_A$ receptor subunits recombinantly expressed in Chinese hamster ovary (CHO) cells in order to examine the acute effects of ethanol. These host cells are from mammals, and might exhibit pharmacological properties different from *Xenopus* oocytes. Furthermore, we studied the ethanol actions on currents induced by low concentrations of GABA in cerebellar granule cells because tonic GABA currents were detected in these cells contained in slice preparations. Using the whole-cell patch clamp method, we found that ethanol either inhibited or exerted no effect on GABA-induced

currents regardless of subunit combinations of $GABA_A$ receptors. Using the perforated patch clamp method to record the whole-cell GABA currents from the $\alpha6\beta2\delta$ receptors, we found that ethanol had a potentiating, an inhibitory or no effect on the GABA-induced currents. Taking these results together, we propose that ethanol exerts a dual action on the $GABA_A$ receptors, an inhibitory and a potentiating action. The potentiating action is labile, being influenced by intracellular milieus that could be altered depending on the methods to record the GABA currents.

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Methods

Cell Preparations

Culture of cerebellar granule cells

Cerebellar granule cells were cultured as described by Gallo et al. (Gallo et al., 1982) with some modifications. Briefly, the cerebella were isolated from 6-8-day-old Sprague-Dawley rats and cells were dispersed with trypsin and plated at a density of 1 x 10^6cells/cm^2 on 12 mm Nunc cover glasses coated with poly-L-lysine (10 μg/ ml; Sigma-Aldrich, St. Louis, MO). The cultures were maintained up to 2 weeks in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum, 25 mM KCl, 2 mM glutamine, 20 U/ml penicillin and 20 U/ml streptomycin (Invitrogen, Carlsbad, CA) at 37℃ in air + CO_2 (90 + 10% by volume). Under this condition, more than 90 % of the cells in these cultures are glutamatergic granule cells (Gallo et al., 1982).

Transient transfection in Chinese hamster ovary cells

CHO cells were also used as host cells for transfections of $GABA_A$ receptors since HEK 293 cells have been reported to have endogenous β subunits (Kirkness and Fraser, 1993; Fuchs et al., 1995; Ueno et al., 1996). CHO cells were transiently transfected using lipofectamine. Briefly, 5 μg of cDNA per 35 mm culture dish were used in a ratio of 2:2:1 (α:β:δ). Cells were used for patch clamp experiments 24-48 hr after transfection. The cells were also co-transfected with 0.5 μg cDNA encoded with green fluorescence protein (GFP) which could be identified under the fluorescence microscope. Depending on the subunit combinations, the percentage of GFP-positive cells that expressed functional $GABA_A$

receptors varied from 20 to 80%. We found that the β2-containing $GABA_A$ receptors were expressed better than the β3-containing receptors. The rat $GABA_A$ receptor subunit clones were provided by Dr. Steve Moss of the University of Pennsylvania (α6), Dr. Robert Macdonald of Vanderbilt University (α4, δ) and Dr. Robert Pearce of the University of Wisconsin (β2, β3, γ2s). The cDNA was subcloned into a mammalian expression vector. The pCMV vector was used for the expression of α4 and δ subunits, the vector pCEP-4 was used to express β2 and β3 subunits, and the vector pRK5 was used to express α6 subunit.

Whole-Cell Current Recording

Currents were recorded using both the conventional whole-cell and the perforated patch clamp techniques at room temperature (22°C). Pipette electrodes were made from 0.8 mm (I.D.) borosilicate glass capillary tubes and fire-polished. The electrodes had a resistance of 3-8 MΩ for cerebellar granule cells or CHO cells when filled with the pipette solution. The membrane potential was clamped at -80 mV, and a 5–10-min period was allowed following rupture of the membrane to equilibrate the cell interior with the pipette solution. Currents through the electrode were recorded by an Axopatch 200 amplifier (Axon Instruments, Union City, CA), and filtered at 2 kHz. Whole-cell current data are expressed as the mean ± S.D., and *n* represents the number of experiments.

In some experiments, amphotericin B perforated-patch recording was used to minimize intracellular dialysis. Amphotericin B was first dissolved in dimethylsulfoxide (DMSO) at a concentration of 20 mg/ml to make a stock solution which was in turn diluted with pipette solution to a final concentration of 200 μg/ml. The tip of the patch pipette was

filled with the internal solution and then backfilled the rest of the patch pipette with the amphotericin B-containing solution. Even though the DMSO concentration in the patch pipette was around 1%, the final DMSO concentration in the cell should have been very low, because DMSO was delivered from a point source. When the access resistance reached a steady level, recording was started. If the access resistance suddenly changed indicating breaking of the membrane at the tip of the electrode, the cell was discarded.

Solutions

The external solution for whole-cell patch clamp experiments contained (in mM): NaCl 150, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES acid 5.5, Na^+ HEPES 4.5, and glucose 10. The pH was adjusted to 7.3 with HCl, and osmolality was adjusted to 300 mOsm by D-glucose. The internal solution contained (in mM): CsCl 140, HEPES 10, EGTA 11, Mg^{2+}-ATP 2, Na^+ GTP 0.2, and glucose 5.5. pH was adjusted to 7.3 with CsOH and osmolality was adjusted 300 mOsm by D-glucose. For current recording from granule cells, CsCl 130 mM and TEA-Cl 10 mM were substituted for CsCl 140 mM in internal solution. We have chosen internal solutions without added Ca^{2+} ions since we have been able to obtain stable recordings of GABA currents. We compared two solutions, one with and the other without 1 mM Ca^{2+}, and found no difference between them in terms of recording GABA currents.

Chemicals

GABA (Sigma-Aldrich) was first dissolved in distilled water to make stock solutions. Furosemide and amphotericin B were obtained from Sigma-Aldrich, and 100%

ethanol (USP) was obtained from Pharmco Product, Inc (Brookfield, CT). Isoflurane, sevoflurane and propofol were obtained from Anaquest (Madison, WI) (a division of BOC Inc), Abbott laboratories (North Chicago, IL), and Research Biochemicals International (Natick, MA), respectively. Furosemide and propofol were first dissolved in dimethylsulfoxide before being added to the external solution (0.1% v/v). This concentration of dimethylsulfoxide did not affect the GABA response. Test solutions for inhalation anesthetics were prepared immediately before experiments by diluting the saturated solution and were kept in air-free, closed glass bottles to prevent evaporation of the anesthetics.

Drug Application

GABA and other drugs were applied directly onto the cell by gravity using a fast delivery device consisting of multibarrel tubes connected to a perfusion fast-step switching system. The solution exchange time of this device was 10 to 20 ms. In some experiments, drugs were co-applied with GABA onto the cells.

Data Analysis

EC_{50} values and their slope factors (Hill coefficients) were calculated using the following equation:

$$I=I_{max}\, C^n / (C^n + EC_{50}{}^n) \qquad (1)$$

where I is the amplitude of GABA-induced current, I_{max} the maximum current, C the drug concentration, and n the Hill coefficient. The nonlinear regression analysis was carried out

using the least squares fitting method (Sigmaplot, Version 8.0, SPSS Inc., Chicago, IL) with the aid of a microcomputer. Data are given as the mean ± S.D. with the numbers of experiments, n.

Results

The strategy for studying the effects of ethanol on $GABA_A$ receptors was to compare its effects on native neurons in cultures and on mammalian host cells in which various combinations of subunits were expressed. Cerebellar granule cells were chosen as native preparations for two reasons: One, the $GABA_A$ receptor underwent developmental changes in subunits during maturation in cultures; second, the tonic current was easily discernible in cerebellar granule cells. To mimic the GABA tonic current, 300 nM GABA was used to apply to cells for a period of several minutes. This low GABA concentration was near the ambient level of GABA *in vivo*.

Developmental Changes in GABA-Induced Currents in Granule Cells

The sensitivity to GABA in cerebellar granule cells in primary culture is known to increase during development *in vitro* (Zheng et al., 1994). To characterize the $GABA_A$ receptors in cerebellar granule cells, whole-cell currents were recorded at a holding potential of -80 mV. Various concentrations of GABA were applied every 2 min to generate inward currents, and currents recorded at 7 days *in vitro* (DIV) and 11 DIV are shown in Fig. 1A. The EC_{50} values were estimated from the GABA dose-response curves shown in Fig. 1B, and their values decreased from 12.2 ± 0.16 μM at 7 DIV to 3.1 ± 0.39 μM at 11 DIV ($p<0.05$) with little or no change in the Hill coefficient (1.52 ± 0.28 at 7 DIV, 1.25 ± 0.11 at 11 DIV) (Fig.1B, see Table 1). These results indicate that the apparent affinity of the receptor for GABA increase as granule cells become more matured. Our data are in accord with those reported previously using the cerebellar granule cells (Zheng et al., 1994).

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Furosemide Block of GABA-induced Currents in Granule Cells

To confirm the existence of α6-containing $GABA_A$ receptors in the cerebellar granule cells, the inhibitory effects of furosemide on GABA-induced currents were examined. Furosemide has been reported to be a selective antagonist of the α6-containing $GABA_A$ receptors (Fisher et al., 1997). Furosemide at 100 μM reversibly inhibited steady-state currents to 54.7 ± 5% (n = 5) of the control at 7 DIV and 29.3 ± 7% (n = 5) of the control at 14 DIV (Fig. 2). Since furosemide inhibits the GABA current more effectively at 14 DIV than at 7 DIV ($p<0.05$), the α6 subunits-containing $GABA_A$ receptors increase during maturation *in vitro*.

Modulation by Ro15-4513 of GABA-Induced Currents

The inverse benzodiazepine agonist Ro15-4513 is known to have a high affinity for the α6-subunit containing receptors and to antagonize the ataxic effects of ethanol. Although Ro15-4513 was reported to exert the inhibitory effects on GABA responses mediated by both α1- and α6-containing receptors (Kleingoor et al., 1991), its effects on the α6-subunit containing receptors remain controversial. Ro15-4513 has a very high affinity for the α6-containing $GABA_A$ receptors with a K_d around tens of nanomolar ranges. Thus, at either 100 nM or 1 μM, Ro15-4513 should exert near maximum effects. Ro15-4513 was neither potent nor efficacious in inhibiting the tonic GABA current induced in cerebellar granule cells. Figure 3A shows the inhibitory effects of Ro15-4513 on GABA-induced currents

when Ro15-4513 at 1 μM was co-applied with 300 nM GABA in a cerebellar granule cell (10~14 DIV) (inhibition to 68.8 ± 10.4 % of the control, n = 5). GABA was applied onto the cell at low concentrations and for extended periods of time to mimic the tonic currents observed in slices. The effects of Ro15-4513 on the tonic currents mediated by the α6β2δ and α6β3δ receptors were also examined and compared with those of α6β2γ2s receptor because of the reported existence of δ-containing subunits in the extrasynaptic regions. Although Ro15-4513 at 100 nM potentiated currents induced by 100 nM GABA in the α6β2γ2s receptor (175 ± 10.7% of the control; n = 4), it inhibited GABA-induced currents in both α6β2δ and α6β3δ receptors. Since α6β2δ and α6β3δ receptors behaved similarly, their data were lumped together, resulting a reduction to 57.6 ± 7.1% of the control (n = 6) (Fig. 3). It is concluded that the opposite responses to Ro15-4513 between the α6β2δ and α6β2γ2 receptors are due to the presence of γ2 vs. δ subunits.

The Effects of Ethanol on Currents Induced by a Low Concentration of GABA in Cerebellar Granule Cells

Tonic currents are generated in extrasynaptic receptors by low concentrations of transmitters, and occur in various regions of the brain (Bai et al., 2001; Caraiscos et al., 2004; Semyanov et al., 2004). Recent studies have shown that ethanol potentiates the tonic GABA currents recorded from the cerebellar (Hanchar et al., 2005) and hippocampal slice preparations (Wei et al., 2004).

To examine the effects of ethanol on tonic currents, non-desensitizing whole-cell currents were induced in cerebellar granule cells by prolonged applications of a low

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

concentration of GABA. Figure 4 shows the effects of ethanol on the currents induced by 300 nM GABA in granule cells at 14 DIV. Ethanol at 30 mM inhibited the currents in 2 out of 6 granule cells at 7 DIV, and in 4 out of 7 granule cells at 10-14 DIV. Figure 4A illustrates an example of the lack of the effect of ethanol (panel a) and that of its inhibitory action (panel b) on the current induced by 300 nM GABA. The scattered bar graphs shown in Fig. 4B illustrate that ethanol has either no effect or inhibitory effect on GABA-induced currents. In no case was a potentiating action on GABA tonic currents seen. The mean amplitude of GABA currents in the presence of 30 mM ethanol was decreased to 86.5 ± 18.1% of the control ($n = 6$, $P>0.05$, paired t test) at 7 DIV and 73.1 ± 26.7% ($n = 7$, $P<0.05$, paired t test) at 10~14 DIV (see Table 1).

Effects of Ethanol on Currents Induced by Low Concentrations of GABA in Recombinantly Expressed $GABA_A$ Receptors

Tonic currents are generated by the activation of extrasynaptic δ-containing $GABA_A$ receptors composed mainly of the α6β2/3δ receptors in the cerebellum and the α4β2/3δ receptors in the hippocampus (Nusser et al., 1998). In recent studies (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), the δ-subunit containing $GABA_A$ receptors expressed in *Xenopus* oocytes have been found to be potently augmented by low concentrations of ethanol. Other laboratories failed to reproduce such effects (Borghese et al., 2006). We re-examined the subunit dependency of the ethanol action using the α4-, α6- and δ-containing $GABA_A$ receptors expressed in CHO cells. Concentration-response relationships for GABA activation of the α6β2δ, α6β3δ, and α4β2δ subtypes were constructed. The EC_{50} values

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

and Hill coefficients were estimated to be 0.24 ± 0.11 μM and 0.98 ± 0.11 (n=6), respectively for the α6β2δ combination, 0.41 ± 0.19 μM and 0.86 ± 0.05 (n=4) for the α6β3δ conbination, and 0.67 ± 0.22 μM and 1.02 ± 0.10 (n=4) for the α4β2δ combination (see Table 1).

Ethanol had no effect on GABA-induced currents in the α6β2δ, α6β3δ and α4β2δ receptors at concentrations of 10, 30 mM ($P>0.05$, paired t test), whereas ethanol at 100 mM significantly inhibited GABA-induced currents ($P<0.05$, paired t test) (see Table 1). An example showing the inhibitory action of 100 mM ethanol on the α6β3δ combination is shown in Fig. 5A, with all data on ethanol action are summarized in Fig. 5B. The lack of ethanol potentiation of GABA-induced currents in the α6β2δ, α6β3δ and α4β2δ receptors expressed in CHO cells is surprising in light of the reported potentiating action when some receptors are expressed in *Xenopus* oocytes (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Yet, the CHO cell results are in accord with our data on the cerebellar granule neurons (Fig. 4).

In order to ascertain that there were no technical errors in the experimental procedures, we tested several other drugs that are known to modulate $GABA_A$ receptor currents on α4β2δ, α6β2δ and α6β3δ subtypes expressed in CHO cells. Figure 6A shows a representative experiment obtained from the α6β2δ receptors. Potentiation of GABA-induced currents was consistently observed at clinically relevant concentrations of the inhalational anesthetics isoflurane (280 μM) (229.5 ± 16.0% of the control, n = 12) and sevoflurane (330 μM) (228.2 ± 20.7%, n = 5), and the intravenous anesthetic propofol (3

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

μM) (226.3 ± 26.3%, n = 8). In addition, current inhibition was observed by 100 μM furosemide (24.6 ± 5.2% of the control, n = 7) (Fig. 6B). Similar results were obtained in the α4β2δ and α6β3δ $GABA_A$ receptors as well (data not shown).

Perforated Patch Experiments

As described in the preceding sections, we never observed potentiation of GABA-induced currents by ethanol. This result is at variance with the ethanol potentiation of GABA currents in *Xenopus* oocytes recombinantly expressing α4, α6 and δ GABA receptor subunits (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). One possible reason for the discrepancy is that dialysis of intracellular milieus occurring under the whole-cell patch clamp conditions prevents the ethanol-induced effect (Huang and Narahashi, 1997). In contrast, very little change in the intracellular milieu in oocytes is expected to occur in two-microelectrode voltage clamp conditions. Since the role of phosphatases, protein kinase C and protein kinase A are implicated in the action of ethanol (Morrow et al., 2004), changes in intracellular components under patch clamp conditions may account for the differences in the responses to ethanol between our results and those using oocytes. In order to verify the validity of this hypothesis, we performed perforated patch clamp experiments in which changes in intracellular components would be minimum.

Perforated patch experiments using amphotericin B were performed with the α6β2δ receptor expressed in CHO cells. Figure 7 shows three examples of changes in peak current amplitude during repeated 3 sec applications of 300 nM GABA. These were given every two minutes during a continuous bath perfusion of 100 mM ethanol. Figure 7A depicts a

potentiating action of 100 mM ethanol, and Fig. 7B illustrates an inhibitory action of ethanol. An example of the lack of effect of ethanol is shown in Fig. 7C. Thus, ethanol effects ranged from potentiation to inhibition. As a result, the mean amplitude of GABA currents in the presence of 100 mM ethanol was not significantly different from the control (Fig. 7D) (96.7 ± 10.9% of the control, n = 12, P>0.05, paired t test).

Even though the effects of 100 mM ethanol on the GABA-induced currents of the α6β2δ $GABA_A$ receptor were not significantly different between the whole-cell patch clamp methods (84.5 ± 25.7%) and the perforated patch clamp method (96.7 ± 10.9%), it is instructive to examine the data distribution in detail using the skewness test and the box plot. First, we applied the skewness test to compare the two sets of data. The data from the whole-cell recordings were significantly ($p<0.05$, n=10) skewed toward positive with more values bunched together above the mean and with a long trail below the mean, whereas the data from the perforated patch clamp were not skewed. The box plot is useful to gain an overview of the spread of the data points and identify outliers (Fig. 8). The plot of the data obtained from the whole-cell patch clamp technique also shows that the distribution is significantly skewed since the median (98.5%) is not in the center of the box and differs greatly from the mean value (84.5%). In examining the data further among ten cells exposed to 100 mM ethanol, we have found that the GABA-induced currents in six cells remain essentially unchanged and those in the remaining four cells are reduced to various degrees from few percent to 64%. Despite the large interquartile range, there is no outlier in the data. In contrast to the whole-cell patch clamp data, the perforated patch clamp results are not skewed as the median value (96.1%) is nearly identical to the mean value (96.7%).

Among nine cells exposed to 100 mM ethanol, the GABA-induced currents are enhanced in two cells, not changed in four cells, and slightly decreased in three cells. The degree of the spread of data is reflected in the interquartile range of box plots with the data in whole-cell patch having a large interquartile range than those in the perforated patch. The box plot revealed that the conventional patch clamp technique yields a larger variability of data. The wide spread of data is mostly likely attributed to the varying degree of changes in the intracellular milieu caused by dialysis with the patch pipette solution.

Discussion

Using the whole-cell patch clamp technique to record the GABA-induced currents, we demonstrated that ethanol lacked a potentiating action on GABA-induced currents in cerebellar granule cells and in mammalian cell lines expressing the $GABA_A$ receptors. Ethanol had either no effect on GABA currents or exhibited an inhibitory action (Figs. 4 and 5) in both native neurons and cell lines. Using the perforated patch clamp technique, we showed that ethanol effects ranged from potentiation to inhibition. The extent of inhibition was small. In agreement with previous studies (Feng and Macdonald, 2004), we were able to demonstrate that the volatile anesthetics isoflurane and sevoflurane, and the intravenous anesthetic propofol, consistently potentiated $GABA_A$ responses whereas ethanol did not potentiate GABA currents recorded under the same conditions (Fig. 6). These results suggest that ethanol does not act like either volatile or intravenous anesthetics in potentiating GABA responses. *Xenopus* oocytes, HEK cell line, and other cell lines such as CHO cells have been extensively used as expression hosts to study electrophysiological properties of transfected receptors. Differences in the kinetics and pharmacological profiles of $GABA_A$ receptors were reported to be dependent on host cells. Akk et al. (2004) reported a potent direct activation of the $\alpha4$ containing $GABA_A$ receptors in HEK cells by pentobarbital, whereas Wafford et al. (1996) demonstrated a lack of direct activation of the $\alpha4\beta1\gamma2$ receptors in oocytes. In a recent study, Mercik et al. (2003) reported different desensitization kinetics between the $\alpha1\beta2\gamma2s$ $GABA_A$ receptors expressed in HEK 293 cells and those expressed in QT6 cells derived from fibroblasts. Thus, the lack of

potentiating action of ethanol may depend on differences in intracellular factors of host cells (Harris and Mihic, 2004).

In the present study, cerebellar granule cells were identified morphologically in primary culture and their characteristics were examined. The apparent affinity of the receptor for GABA increased during development *in vitro* (Fig. 1) and furosemide, an antagonist of the α6-subunit containing $GABA_A$ receptor, inhibited GABA-induced currents more potently at 14 DIV than at 7 DIV (Fig. 2). These findings might be related to an increase in the amount of $GABA_A$ receptors containing the α6 subunit during granule cell maturation *in vitro* (Zheng et al., 1994).

The $GABA_A$ receptor system has long been hypothesized to be a target of ethanol action. Despite numerous studies conducted so far, the effects of alcohol on the $GABA_A$ receptor function remain controversial (Aguayo et al., 2002). A recent study has shown that low concentrations of ethanol enhance tonic inhibition mediated by the δ subunit-containing $GABA_A$ receptors in the hippocampal (Wei et al., 2004) and the cerebellar granule cells (Hanchar et al., 2005). In experiments using recombinantly expressed receptors, specific combinations of $GABA_A$ receptors (α4β3δ, α6β3δ) in *Xenopus* oocytes were found to be uniquely sensitive to the potentiating action of ethanol in a concentration-dependent manner (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). Borghese et al. (2006) reported that they had not been able to replicate the sensitivity of α4β3δ $GABA_A$ receptors to low concentrations of ethanol. It was also reported that a single amino acid substitution from arginine (R) to glutamine (Q) at amino acid position 100 in the α6 subunit increased alcohol sensitivity and ethanol-induced motor impairment (Hanchar et al., 2005).

However, another group showed that ethanol did not increase the magnitude of tonic currents in the cerebellar granule cells (Carta et al., 2004). To further explore this issue, Valenzuela et al. (2005) compared the effects of ethanol on the tonic currents of granule cells in cerebellar slices from male inbred alcohol-intolerant rats (100Q genotype) and inbred alcohol-tolerant rats (100R genotype). They reported that there was no difference between them in the effect of ethanol on tonic currents and the frequency of spontaneous inhibitory postsynaptic currents.

Ro15-4513, a benzodiazepine inverse agonist, has been reported to antagonize the ataxic effect of ethanol. Ro15-4513 at 1 μM diminished the GABA response at the benzodiazepine site of non-α6-containing receptors like the α1β2γ2s receptor (Hadingham et al., 1996). However, its effects on α6-containing receptors were variable. Both inhibitory (Knoflach et al., 1996) and potentiating effects on the α6β2γ2s (Hadingham et al., 1996) and α6β2γ2sδ (Hevers et al., 2000) receptor-mediated GABA responses were reported. Brown et al. (2002) showed that the α4β3γ2s receptor exhibited the sensitivity to Ro15-4513 but the α4β3δ receptor did not. Our results also showed that Ro15-4513 enhanced the GABA responses mediated by the α6β2γ2s receptor and inhibited those mediated by the α6β2δ and α6β3δ receptors (Fig. 3). Ro15-4513 was not as potent in inhibiting GABA responses in the cerebellar granule cell, and this might be due to the balance of its inhibitory and potentiatory effects on select GABA receptor subtypes and the proportion of α1βγ, α6βγ and α6βδ receptors in the native neuron.

In our present study, ethanol (100 mM) exhibited an inhibitory effect or no effect on α4, α6, and δ combinations of GABA receptor subunits expressed in CHO cells and on the $GABA_A$ receptors of cerebellar granule cells when the whole-cell patch clamp method was used to record the GABA-induced currents. This inhibitory action of ethanol is not dependent on the subunits tested in the present study.

The 300 nM GABA concentration was chosen because it was comparable to ambient GABA levels to simulate the tonic current. A question is raised as to whether the degree of activation of various $GABA_A$ receptors would influence the ethanol effects since the effects of ethanol on $GABA_A$ receptors are dependent on the concentration of GABA used to activate the receptors. Using the EC_{50}s and Hill coefficients for different receptor subtypes, we have calculated the percentages of receptor activation as shown in Table 1. The ethanol effect does not seem to be related to the degree of receptor activation. For example, 30 mM ethanol gave a similar inhibition of the GABA-induced current in both 7 DIV granule cells and in α4β2δ transfected CHO cells (-13.5% and -15%, respectively) despite the fact that the degree of receptor activation differed greatly (0.4 vs 31%).

The difference in the ethanol effect on the α6β2δ $GABA_A$ receptors between the whole-cell patch clamp and the perforated patch clamp (Fig. 8) suggests that ethanol has a dual action on the receptor: a direct inhibitory action on the receptor and an indirect potentiating action via the second messenger system. In the majority of experiments in which ethanol was continuously present, the overall effects of ethanol might represent the sum of the potentiating and inhibitory actions. Depending on the dose-response relationship

for these two actions, one might expect to see a bell-shape dose-response curve (Sundstrom-Poromaa et al., 2002).

We further propose that the intracellular components are responsible for variable effects of ethanol. Under the whole-cell patch clamp conditions in small mammalian cells, the intracellular milieu will be dialyzed by the patch pipette solution, and the degree of solution exchanges may depend on pipette tip diameter. As a result, there are a wide variety of ethanol effects on $GABA_A$ receptors. In contrast, the two-microelectrode voltage clamp used for *Xenopus* oocyte may minimize such variability. This may provide some explanation for the various results reported on the potentiating action of ethanol on various $GABA_A$ receptors (Sapp and Yeh, 1998). Using the whole-cell patch clamp technique to examine the effects of ethanol on the GABA currents recorded from small cells to large cerebellar Purkinje cells, they found that 100 mM ethanol increased the current by 40% in Purkinje cells whereas it had no effect in cell lines. Further experiments should be designed to tease out the putative dual effect of ethanol.

Acknowledgements

We thank Adam Jones for secretarial assistance and Dan Close for technical assistance.

References

Aguayo LG, Peoples RW, Yeh HH and Yevenes GE (2002) $GABA_A$ receptors as molecular sites of ethanol action. Direct or indirect actions? *Curr Top Med Chem* **2:**869-885.

Akk G, Bracamontes J and Steinbach JH (2004) Activation of $GABA_A$ receptors containing the $\alpha 4$ subunit by GABA and pentobarbital. *J Physiol* **556:**387-399.

Bai D, Zhu G, Pennefather P, Jackson MF, MacDonald JF and Orser BA (2001) Distinct functional and pharmacological properties of tonic and quantal inhibitory postsynaptic currents mediated by γ-aminobutyric $acid_A$ receptors in hippocampal neurons. *Mol Pharmacol* **59:**814-824.

Borghese CM, Storustovu SI, Ebert B, Herd MB, Belelli D, Lambert JJ, Marshall G, Wafford KA, Harris RA (2006) The δ subunit of γ-aminobutyric acid type A receptors does not confer sensitivity to low concentrations of ethanol. *J Pharmacol Exp Ther* **316:**1360-1368.

Brown N, Kerby J, Bonnert TP, Whiting PJ and Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human $\alpha_4\beta_3\delta$ $GABA_A$ receptors. *Br J Pharmacol* **136:** 965-974.

Caraiscos VB, Newell JG, You-Ten KE, Elliott EM, Rosahl TW, Wafford KA, MacDonald JF and Orser BA (2004) Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. *J Neurosci* **24:**8454-8458.

Carta M, Mameli M and Valenzuela CF (2004) Alcohol enhances GABAergic transmission to cerebellar granule cells via an increase in golgi cell excitability. *J Neurosci* **24:**3746-3751.

Feng HJ and Macdonald RL (2004) Multiple action of propofol on αβγ and αβδ $GABA_A$ receptors. *Mol Pharmacol* **66:**1517-1524.

Fisher JL, Zhang J and Macdonald RL (1997) The role of α1 and α6 subtype amino-terminal domains in allosteric regulation of γ-Aminobutyric $acid_a$ receptors. *Mol Pharmacol* **52:**714–724

Fuchs K, Zezula J, Slany A and Sieghart W (1995) Endogenous [3H]flunitrazepam binding in human embryonic kidney cell line 293. *Eur J Pharmacol*. **289**: 87-95.

Gallo V, Ciotti MT, Coletti A, Aloisi F and Levi G (1982) Selective release of glutamate from cerebellar granule cells differentiating in culture. *Proc Natl Acad Sci U SA* **79:**7919-7923.

Hadingham KL, Garrett EM, Wafford KA, Bain C, Heavens RP, Sirinathsinghji DJ and Whiting PJ (1996) Cloning of cDNA encoring the human γ-aminobutyric acid type A receptor α6 subunit and characterization of the pharmacology of α6-containing receptors. *Mol Pharmacol* **49:**253-259.

Hanchar HJ, Dodson PD, Olsen RW, Otis TS and Wallner M (2005) Alcohol-induced motor impairment caused by increased extrasynaptic $GABA_A$ receptor activity. *Nat Neurosci* **8:**339-345.

Harris RA and Mihic SJ (2004) Alcohol and inhibitory receptors: Unexpected specificity from a nonspecific drug. *Proc Natl Acad Sci USA* **101:**2-3.

Hevers W, Korpi ER and Luddens H (2000) Assembly of functional α6β3γ2δ $GABA_A$ receptors in vitro. *Neuroreport* **11:**4103-4106.

Hamann M, Rossi DJ and Attwell D (2002) Tonic and spillover inhibition of granule cells control information flow through cerebellar cortex. *Neuron* **33**: 625-633.

Huang CS and Narahashi T (1997) The role of G proteins in the activity and mercury modulation of GABA-induced currents in rat neurons. *Neuropharmacology* **36:**1623-1630.

Kirkness EF and Fraser CM (1993) A strong promoter element is located between alternative exons of a gene encoding the human gamma-aminobutyric acid-type A receptor beta 3 subunit (GABAB3). *J. Biol Chem* **268**: 4420-4428.

Kleingoor C, Ewert M, von Blankenfeld G, Seeburg PH and Kettenmann H (1991) Inverse but not full benzodiazepine agonists modulate recombinant $\alpha_6\beta_2\gamma_2$ $GABA_A$ receptors in transfected human embryonic kidney cells. *Neurosc Letters* **130:**169-172.

Knoflach F, Benke D, Wang Y, Scheurer L, Luddens H, Hamilton BJ, Carter DB, Mohler H and Benson JA (1996) Pharmacological modulation of the diazepam-insensitive recombinant γ-aminobutyric acidA receptors α4β2γ2 and α6β2γ2. *Mol Pharmacol* **50:**1253-1261.

Koob GF, Roberts AJ, Schulteis G, Parsons LH., Heyser CJ, Hyytia P, Merlo-Pich E and Weiss F (1998) Neurocircuitry targets in ethanol reward and dependence. *Alcohol Clin Exp Res* **22:**3-9.

Lovinger DM (1999) 5-HT_3 receptors and the neural actions of alcohols: an increasingly exciting topic. *Neurochem Int* **35:**125-130.

Marszalec W, Kurata Y, Hamilton BJ, Carter DB and Narahashi T (1994) Selective effects of alcohols on γ-aminobutyric acid A receptor subunits expressed in human embryonic kidney cells. *J Pharmacol Exp Ther* **269:**157-163.

Mercik K, Pytel M and Mozrzymas JW (2003) Recombinant α1β2γ2 $GABA_A$ receptors expressed in HEK293 and in QT6 cells show different kinetics. *Neurosci Lett* **352:**195-198.

Mihic SJ (1999) Acute effects of ethanol on GABAA and glycine receptor function. *Neurochem Int* **35:**115-123.

Mori T, Aistrup GL, Nishikawa K, Marszalec W, Yeh JZ and Narahashi T (2000) Basis of variable sensitivities of $GABA_A$ receptors to ethanol. *Alcohol Clin Exp Res* **24:**965-971.

Morrow AL, Ferrani-Kile K, Davis MI, Shumilla JA, Kumar S, Maldve R and Pandey SC (2004) Ethanol effects on cell signaling mechanisms. *Alcohol Clin Exp Res*. **28:**217-227.

Narahashi T, Aistrup GL, Marszalec W and Nagata K (1999) Neuronal nicotinic acetylcholine receptors: a new target site of ethanol. *Neurochem Int* **35:**131-141.

Nusser Z, Sieghart W and Somogyi P (1998) Segregation of different $GABA_A$ receptors to synaptic and extrasynaptic membranes of cerebellar granule cells. *J Neurosci* **18:**1693-1703.

Pontieri FE, Tanda G, Orzi F and Di Chiara G (1996) Effects of nicotine on the nucleus accumbens and similarity to those of addictive drugs. *Nature* **382:**255-257.

Sapp DW and Yeh HH (1998) Ethanol-GABAA receptor interactions: a comparison between cell lines and cerebellar Purkinje cells. *J Pharmacol Exp Ther* **284:**768-776.

Semyanov A, Walker MC, Kullmann DM and Silver RA (2004) Tonically active $GABA_A$ receptors: modulating gain and maintaining the tone. *Trends Neurosci* **27:**262-269.

Sivilotti LG, McNeil DK, Lewis TM, Nassar MA, Schoepfer R and Colquhoun D (1997) Recombinant nicotinic receptors, expressed in *Xenopus* oocytes, do not resemble native rat sympathetic ganglion receptors in single-channel behaviour. *J Physiol* **500:**123-138.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K and Smith SS (2002) Hormonally regulated $\alpha_4\beta_2\delta$ $GABA_A$ receptors are a target for alcohol. *Nat Neurosci* **5:**721-722.

Ueno S, Zorumski C, Bracamontes, J and Steinbach JH (1996) Endogenous subunits can cause ambiguities in the pharmacology of exogenous aminobutyric acidA receptors expressed in human embryonic kidney 293 cells. *Mol Pharmacol* **50**: 931-938.

Valenzuela CF, Mameli M and Carta M (2005) Single-amino-acid difference in the sequence of alpha6 subunit dramatically increases the ethanol sensitivity of recombinant $GABA_A$ receptors. *Alcohol Clin Exp Res* **29**:1356-1357.

Wafford KA, Thompson SA, Thomas D, Sikela J, Wilcox AS and Whiting PJ (1996) Functional characterization of human gamma-aminobutyric acidA receptors containing the alpha 4 subunit. *Mol Pharmacol* **50**:670-678.

Wallner M, Hanchar HJ, Olsen RW (2003) Ethanol enhances $\alpha_4\beta_3\delta$ and $\alpha_6\beta_3\delta$ γ-aminobutyric acid A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A.* **100:**15218-15223.

Walter HJ and Messing RO (1999) Regulation of neuronal voltage-gated calcium channels by ethanol. *Neurochem Int* **35:**95-101.

Wei W, Faria LC and Mody I (2004) Low ethanol concentrations selectively augment the tonic inhibition mediated by δ subunit-containing $GABA_A$ receptors in hippocampal neurons. *J Neurosci* **24:**8379-8382.

Weight FF, Li C and Peoples RW(1999) Alcohol action on membrane ion channels gated by extracellular ATP (P2X receptors). *Neurochem Int* **35:**143-152.

White G, Lovinger DM and Weight FF (1990) Ethanol inhibits NMDA-activated current but does not alter GABA-activated current in an isolated adult mammalian neuron. *Brain Res* **507:**332-336.

Woodward JJ (1999) Ionotropic glutamate receptors as sites of action for ethanol in the brain. *Neurochem Int* **35:**107-113.

Zheng TM, Zhu WJ, Puia G, Vicini S, Grayson DR, Costa E and Caruncho HJ (1994) Changes in γ-aminobutyrate type A receptor subunit in mRNAs, translation product expression, and receptor function during neuronal maturation *in vitro*. *Proc Natl Acad Sci USA* **91:**10952-10956.

Footnotes

This work was supported by a grant from the National Institutes of Health, R01 AA07836.

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig. 1. Developmental changes in GABA-induced currents in cerebellar granule cells. A. Currents evoked by rapid applications of GABA at concentrations ranging from 0.1 to 100 μM in neurons at 7 DIV (a) and 11 DIV (b). B. Concentration-response relationships for GABA to activate neurons at 7 DIV (○) and 11 DIV (●). The EC_{50} value decreased from 12.2 μM at 7 DIV to 3.1 μM at 11 DIV with little or no change in Hill coefficient (1.5 at 7 DIV, 1.25 at 11 DIV). The data points are the mean peak currents expressed as percentages of the control current, and the error bars are standard deviations (n = 5).

Fig. 2. Furosemide block of tonic currents induced by 300 nM GABA in cerebellar granule cells at 7 DIV and 14 DIV. A. Representative traces showing that furosemide at 100 μM reversibly inhibited GABA-induced currents. B. Furosemide at 100 μM inhibited currents to 54.7 ± 5% (n = 5) of the control at 7 DIV and to 29.3 ± 7% (n = 5) at 14 DIV. The data points are the mean peak currents expressed as percentages of the control, and the error bars are standard deviations (n = 5).

Fig. 3. The effects of Ro15-4513 on GABA-induced currents in cerebellar granule cells and CHO cells ecombinantly expressing $GABA_A$ receptors. A. Ro15-4513 at 1 μM inhibited currents induced by 300 nM GABA. B. Potentiating effects of Ro15-4513 on tonic currents induced by 100 nM GABA in the α6β2γ2s receptors (a) and inhibitory effects in the α6β3δ receptors (b). C. Summary graph. Ro15-4513 at 1 μM inhibited tonic currents induced by 300 nM GABA in granule cells (68.8 ± 10.4% of the control; n = 5). Ro15-4513 at 100 nM

potentiated tonic currents induced by 100 nM GABA in the α6β2γ2s receptor (175 ± 10.7% of the control; n = 4) and inhibited GABA-induced currents in the α6β2δ and α6β3δ receptors (57.6 ± 7.1% of the control; n = 6). The data points are the mean peak currents expressed as percentages of the control current, and the error bars are standard deviations.

Fig. 4. The effects of ethanol on tonic currents induced by 300 nM GABA in cerebellar granule cells. A. Representative traces obtained from cerebellar granule cells at 14 DIV. Ethanol at 30 mM had no effect on GABA-induced current in one cell (a) and inhibitory effect in another cell (b). B. Mean amplitudes of currents in the presence of ethanol as percentage of the control were 86.5 ± 18.1% (n = 6, $P>0.05$, paired t test) in 7 DIV and 73.1 ± 26.7% (n = 7, $P<0.05$, paired t test) in 10-14 DIV. Individual data are plotted as open circle.

Fig. 5. The effects of ethanol on δ subunit-containing $GABA_A$ receptors expressed in CHO cells. A. A representative trace in which ethanol inhibited the current induced by 300 nM GABA in the α6β3δ receptors. B. Ethanol at 10 and 30 mM had no effects on GABA-induced currents in most cases or inhibitory effects in some cases in the α4β2δ, α6β2δ and α6β3δ subunit combinations. Ethanol only at 100 mM significantly inhibited GABA responses ($P < 0.05$) in all combinations. The data points are the mean steady-state currents expressed as percentages of the control current, and the error bars are standard deviations. The number of cells tested is given in parenthesis and individual data are plotted as open circles.

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 6. The effects of anesthetics and furosemide on δ subunit-containing $GABA_A$ receptors expressed in CHO cells. Tonic currents induced by 100 nM GABA in the α6β2δ $GABA_A$ receptors were potentiated by the inhalational anesthetics isoflurane (280 μM, 229 ± 16.0% of the control, n = 12), sevoflurane (330 μM, 228.2 ± 20.7%, n = 5) and intravenous anesthetic propofol (3 μM, 226.3 ± 26.3%, n = 8). Furosemide at 100 μM, an α6 subunit-containing $GABA_A$ receptor antagonist, inhibited GABA-induced current (24.6 ± 5.2% of the control, n = 7). The data points are the mean steady-state currents expressed as percentages of the control current, and the error bars are standard deviations.

Fig. 7. The effect of continuous perfusion of 100 mM ethanol on the currents induced by 300 nM GABA in the α6β2δ receptor expressed in CHO cells by the perforated patch clamp recording. Representative currents evoked by repeated 3-sec applications of GABA at a holding potential of -80 mV before, during and after perfusion of 100 mM ethanol. Ethanol exerts potentiating (A), or inhibitory (B), or no effect (C) on the GABA responses. Based on the average of 12 experiments (96.7 ± 10.9%), 100 mM ethanol had no significant effect on GABA responses (D) ($P>0.05$, paired t-test).

Fig.8. Box plots to compare two patch clamp configurations to monitor the effects of ethanol on the α6β2δ $GABA_A$ receptors. The horizontal line inside the box represents the group median value, the bottom and upper sides of the box represent the 25^{th} percentile and 75^{th} percentile, respectively. The uppermost line and the bottommost line represent the 90^{th}

and 10^{th} percentiles, respectively. The box plot reveals that the whole-cell patch clamp yields more spread data than the perforated patch.

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1

Relationship between effects of ethanol on $GABA_A$ receptors and their degrees of receptor activation

	EC_{50} (μM)	Hill coefficient	% activation**	30 mM Ethanol	100 mM Ethanol
Granule cells, 7 DIV	12.2 ± 0.16§	1.52 ± 0.28	0.4	-13.5 ± 18.1%	
Granule cells, 11-14 DIV	3.10 ± 0.39§	1.25 ± 0.11	5.1	-26.9 ± 20.7%*	
α6β2δ	0.24 ± 0.11	0.98 ± 0.11	55	10.0 ± 10.3%	-20.0 ± 15.7%*
α6β3δ	0.41 ± 0.19	0.86 ± 0.05	43	3.42 ± 6.25%	-17.6 ± 7.00%*
δ4β2δ	0.67 ± 0.22	1.02 ± 0.10	31	-15.0 ± 18.9%	-26.0 ± 21.3%*

§These two values for EC_{50}s are significantly different ($p<0.05$).

*The percentage of inhibition was significant at $p<0.05$ as compared with the control.

**Percentages of receptor activation by 300 nM GABA as calculated by equation (1) using the estimated EC_{50}s and Hill coefficients.

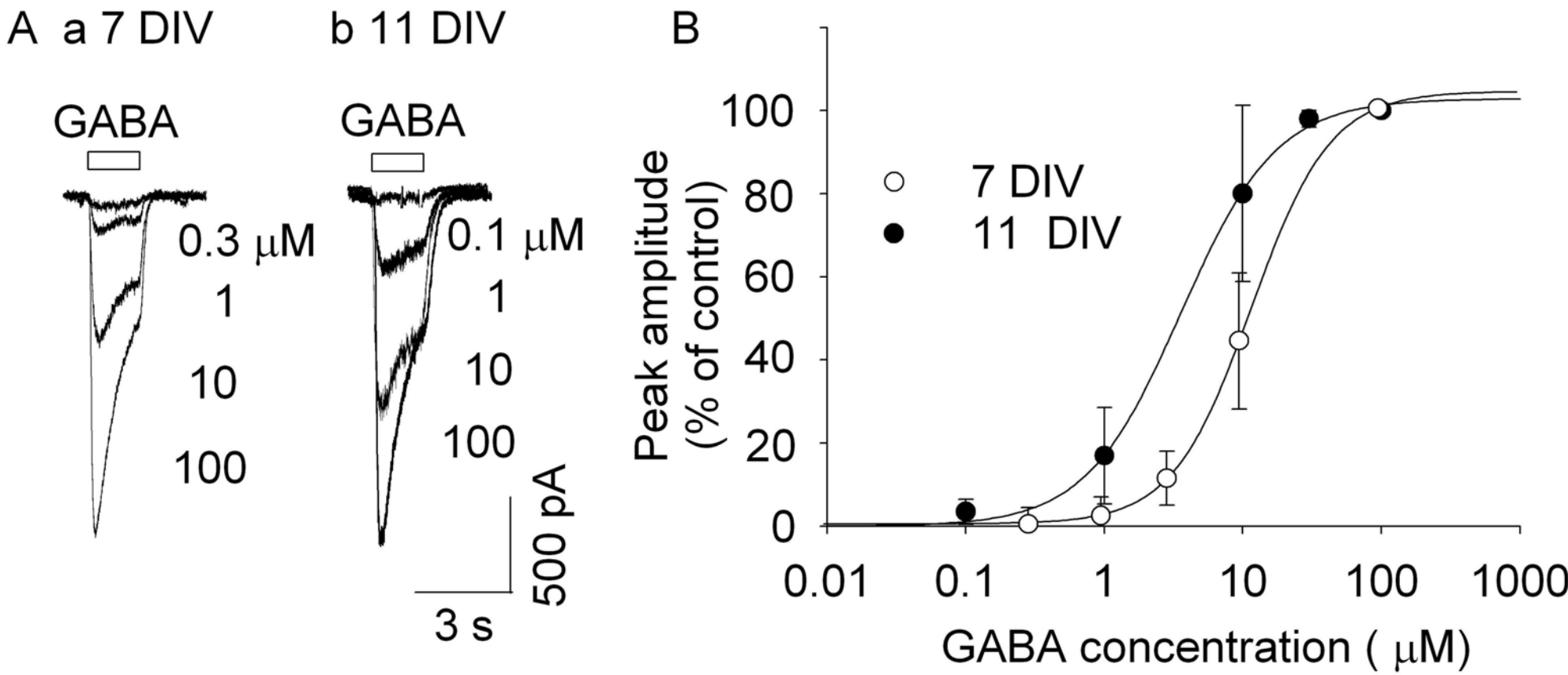
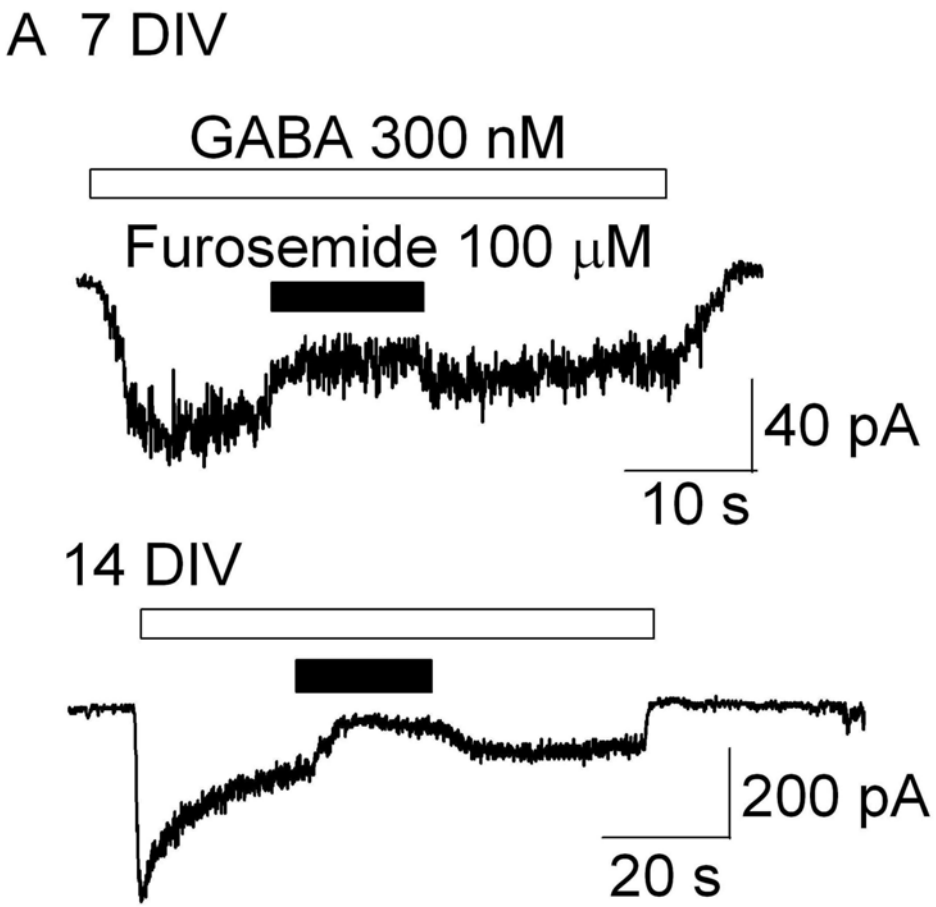


Fig. 1



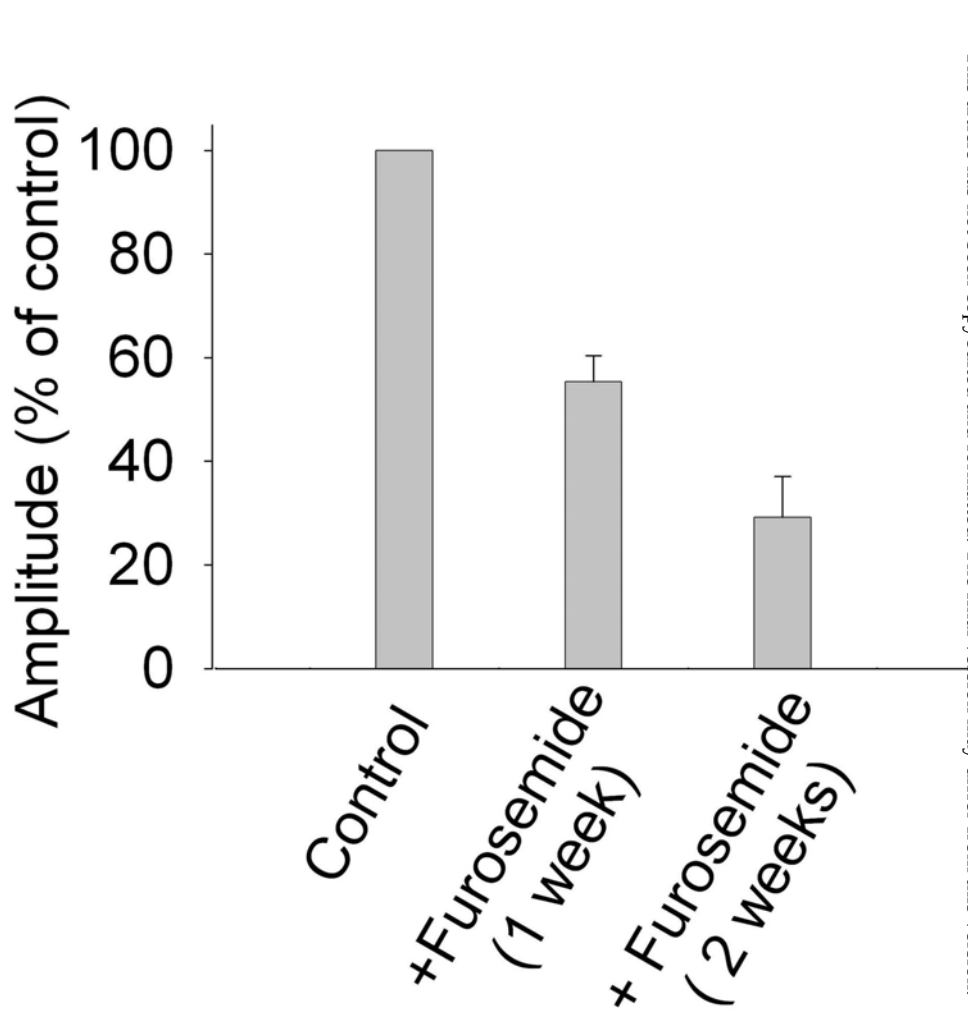


Fig. 2

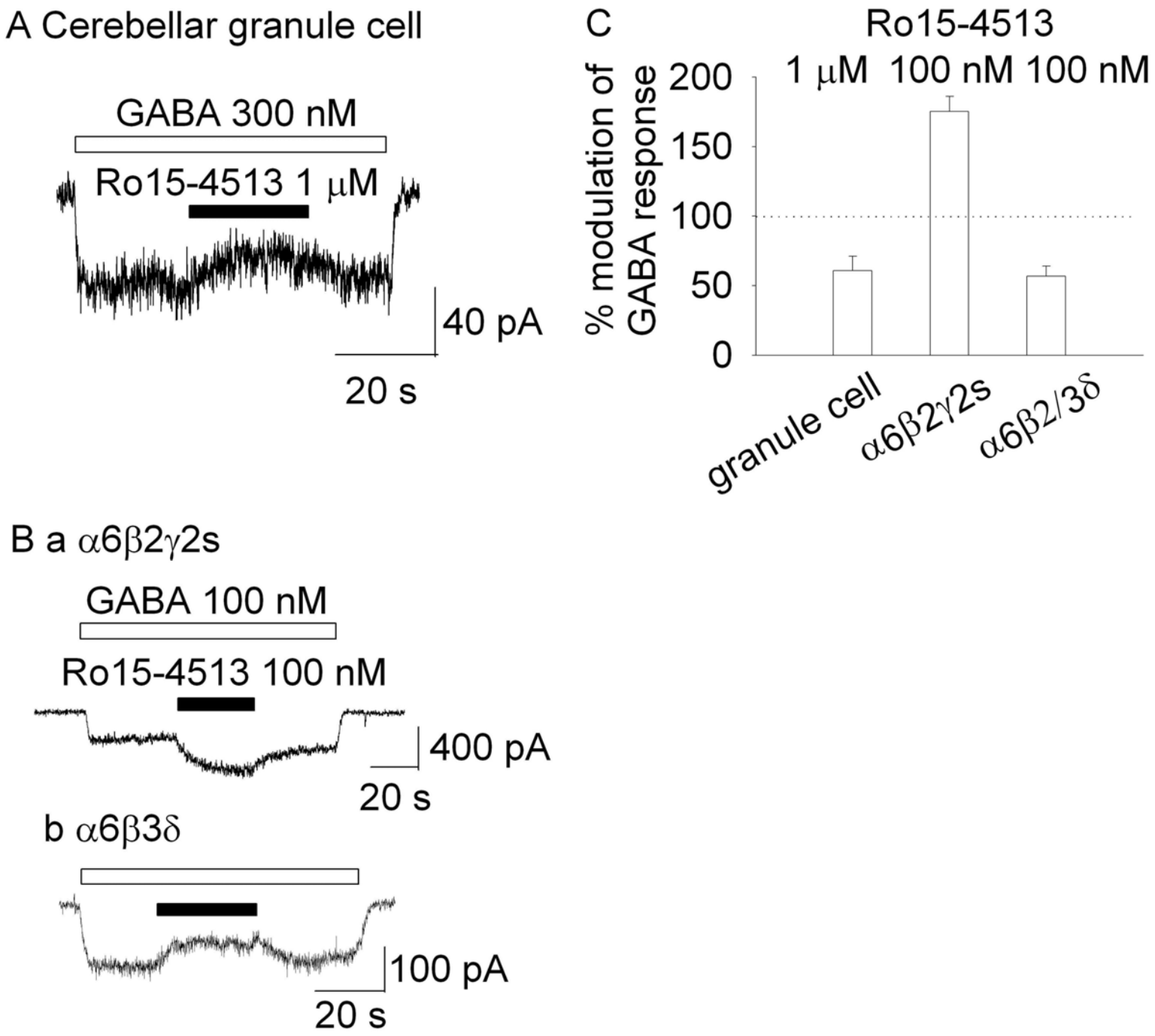


Fig. 3

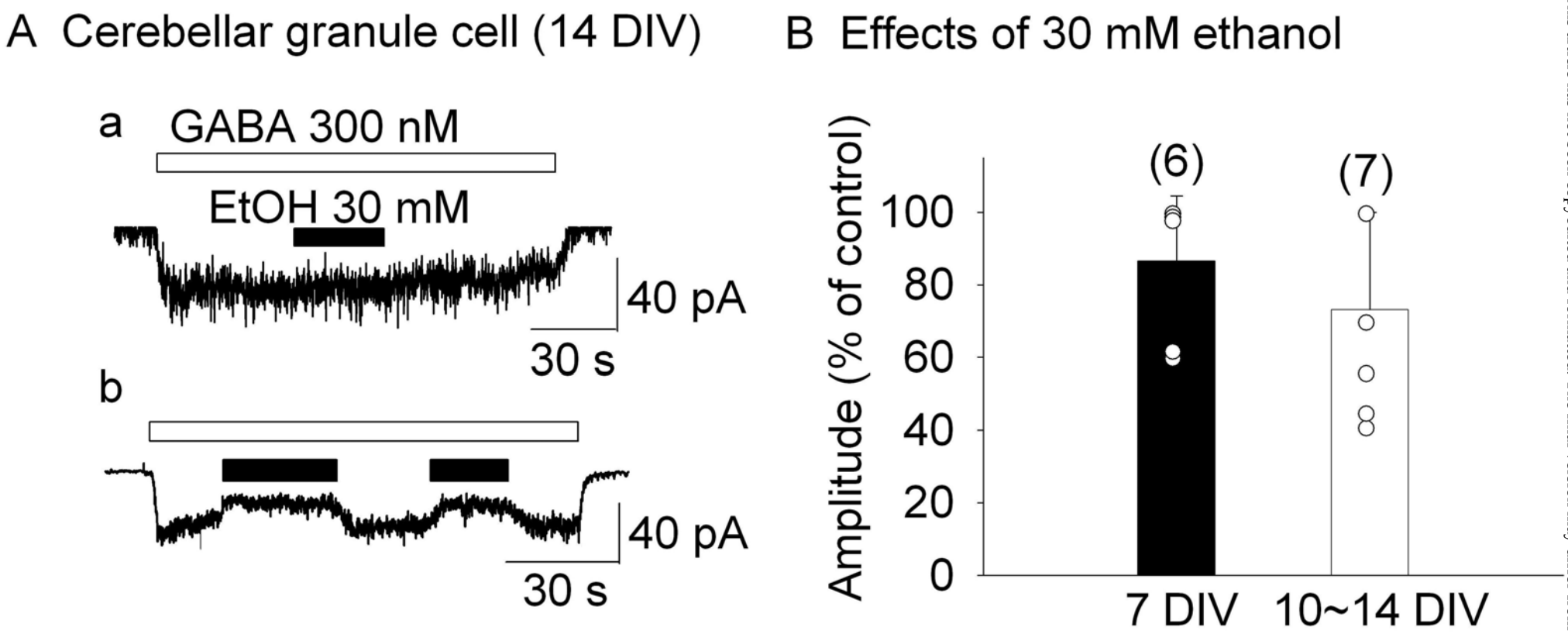


Fig. 4

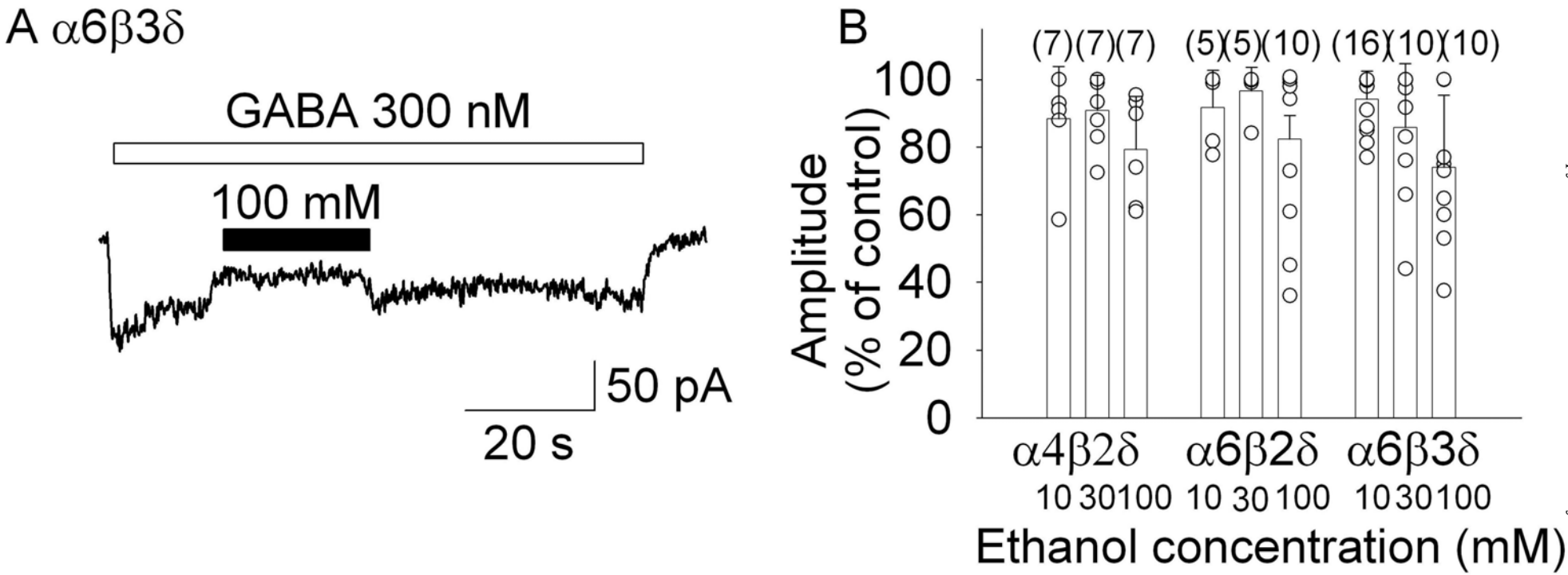


Fig. 5

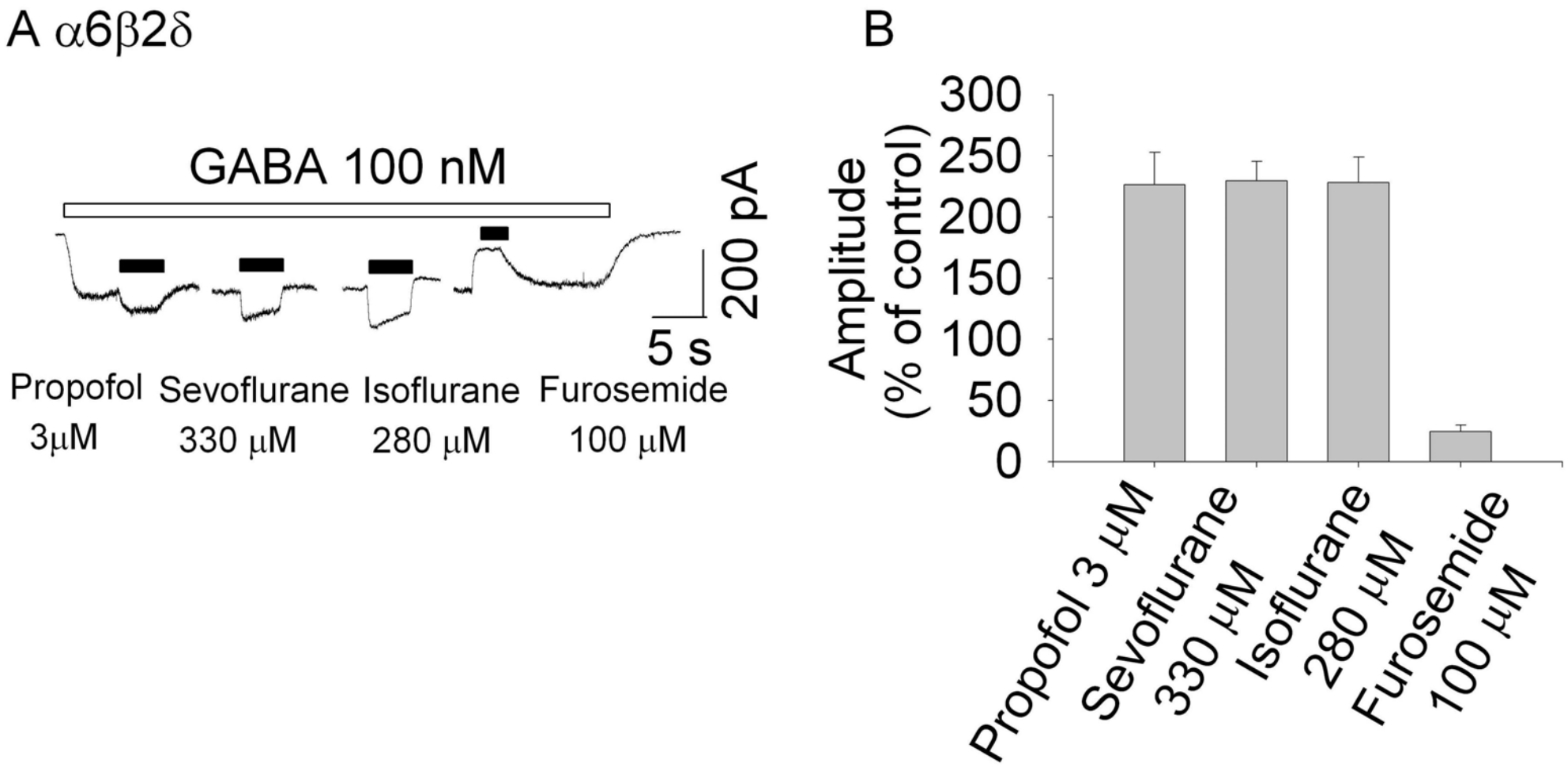
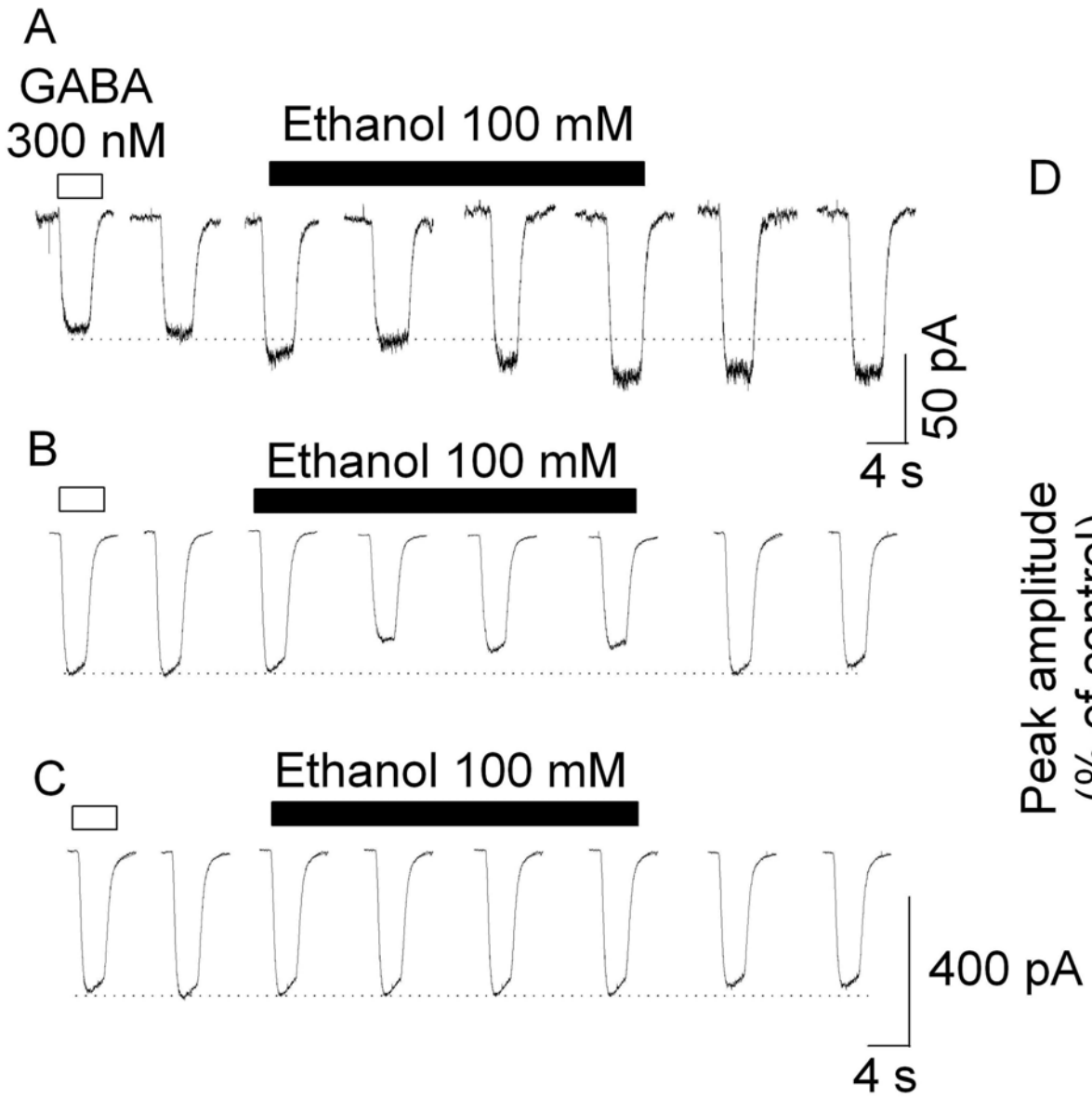


Fig. 6



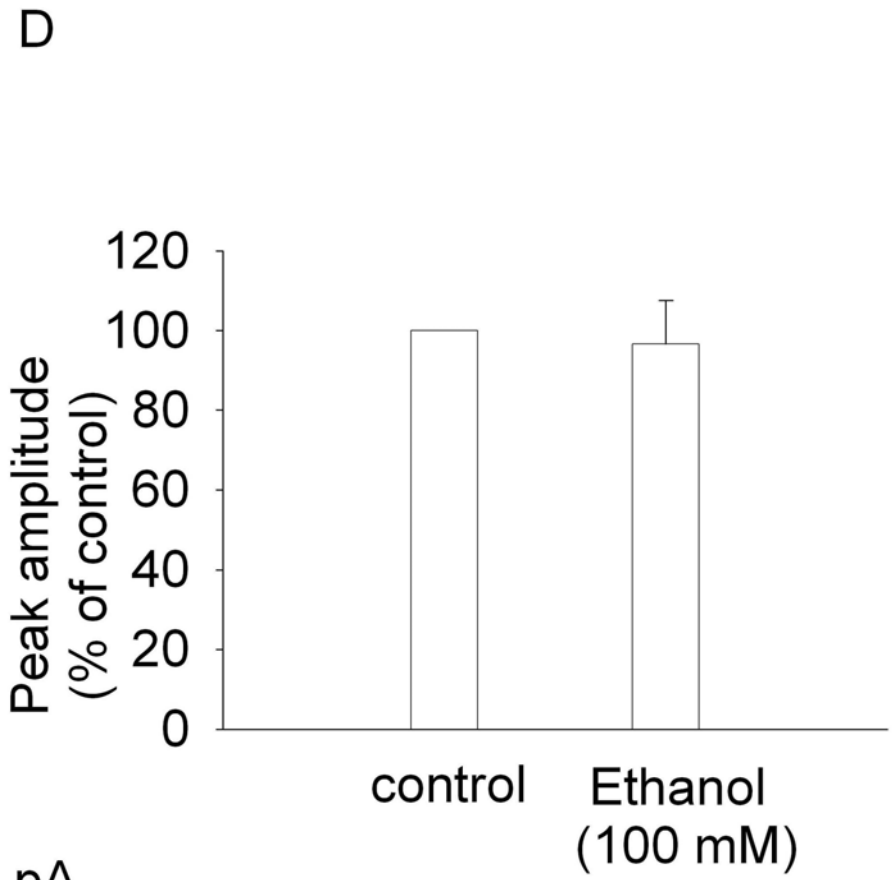


Fig.7

JPET Fast Forward. Published on July 14, 2006 as DOI: 10.1124/jpet.106.106260
This article has not been copyedited and formatted. The final version may differ from this version.

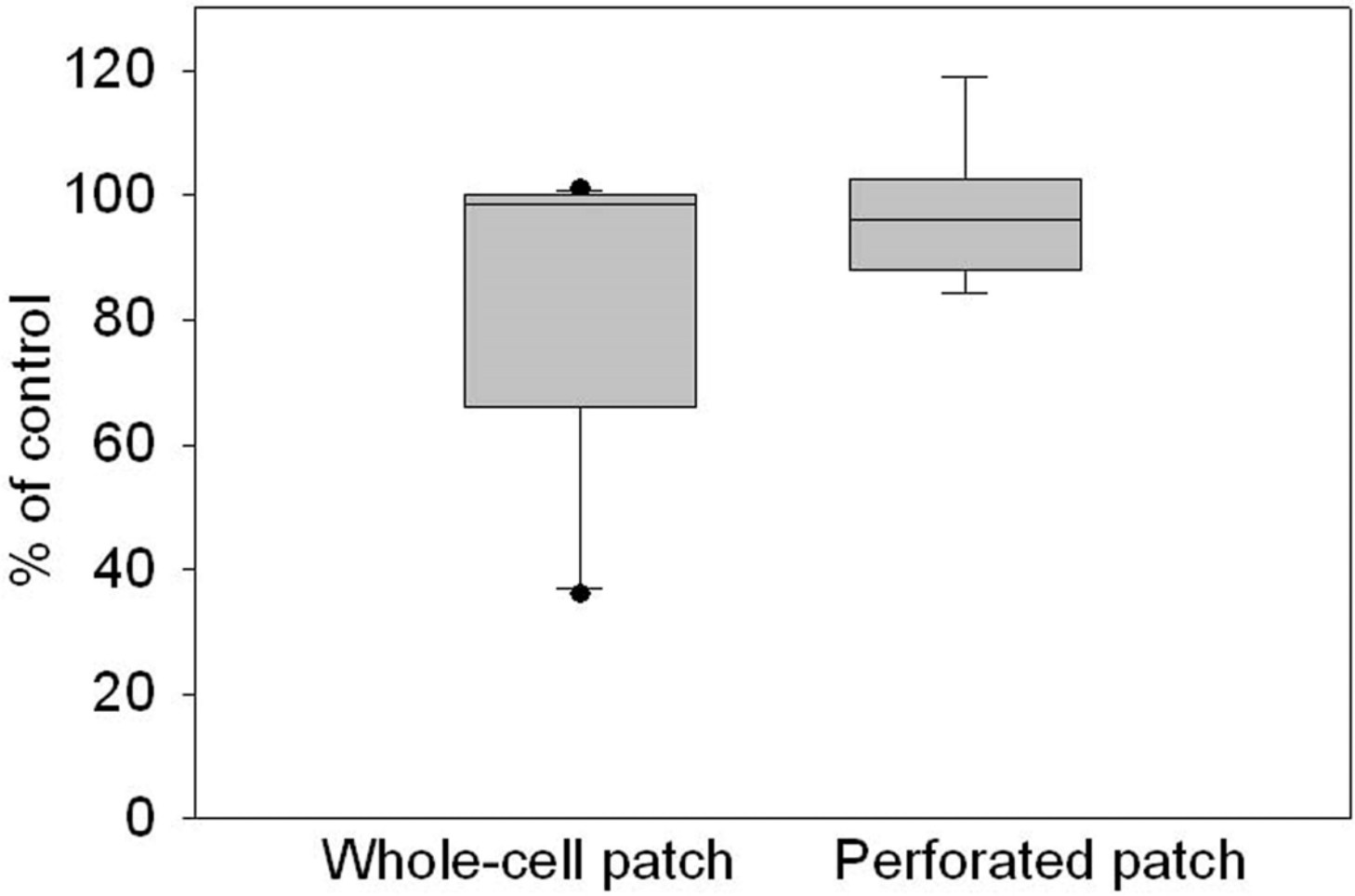


Fig.8