Molecular Pharmacology Fast Forward. Published on December 16, 2010 as doi: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

MOL #68650

Plasticity of $GABA_A$ Receptors Following Ethanol Pre-exposure in Cultured Hippocampal Neurons

Yi Shen*, A. Kerstin Lindemeyer*, Igor Spigelman, Werner Sieghart,

Richard W. Olsen, and Jing Liang

Department of Molecular & Medical Pharmacology, David Geffen School of Medicine, University of California Los Angeles, California (Y.S., A.K.L., R.W.O., J.L.), Division of Oral Biology & Medicine, School of Dentistry, University of California Los Angeles, California (I.S., J.L.), and Department of Biochemistry and Molecular Biology, Center for Brain Research, Medical University of Vienna, Vienna, Austria (W.S.)

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: EtOH-induced $GABA_AR$ Plasticity in Neurons

Please send all correspondence to:

Richard W. Olsen

Department of Molecular and Medical Pharmacology

David Geffen School of Medicine at UCLA

Room CHS 23-120, 650 Charles E Young Dr. South, Los Angeles, CA 90095-1735

Tel: 310-825-5093

E-mail: rolsen@mednet.ucla.edu

Text pages – 39

Tables –1

Figures – 7

Abstract words – 248

Introduction words – 739

Discussion words –1498

Abbreviations used in the text: CIE, chronic intermittent ethanol; DIV, days *in vitro*; DMSO, dimethyl sulfoxide; EtOH, ethanol; $GABA_AR$, $GABA_A$ receptors; I_{tonic}, tonic current; mIPSCs, miniature inhibitory postsynaptic currents; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Alcohol use causes many physiological changes in brain with behavioral sequelae. We previously observed (Liang et al., 2007) plastic changes in hippocampal slices recordings paralleling behavioral changes in rats treated with a single intoxicating dose of ethanol (EtOH). Here, we were able to reproduce in primary cultured hippocampal neurons many of the effects of *in vivo* EtOH exposure on $GABA_A$ receptors ($GABA_A$Rs). Cells grown 11-15 days *in vitro* (DIV) demonstrated $GABA_A$R δ subunit expression and sensitivity to enhancement by acute EtOH (60 mM) of $GABA_A$R-mediated tonic current (I_{tonic}) using whole-cell patch-clamp techniques. EtOH gave virtually no enhancement of mIPSCs. Cells pre-exposed to EtOH (60 mM) for 30 min showed, 1 h after EtOH withdrawal, a 50% decrease in basal I_{tonic} magnitude and tolerance to acute EtOH enhancement of I_{tonic}, followed by reduced basal mIPSC area at 4 h. At 24 h, we saw considerable recovery in mIPSC area and significant potentiation by acute EtOH; also, $GABA_A$R currents exhibited reduced enhancement by benzodiazepines. These changes paralleled significant decreases in cell-surface expression of normally extrasynaptic δ and α4 $GABA_A$R subunits as early as 20 min after EtOH exposure, reduced α5-containing $GABA_A$Rs at 1 h, followed by a larger reduction of normally synaptic α1 subunit at 4 h, followed by increases in α4γ2-containing cell-surface receptors by 24 h. Measuring internalization of biotinylated $GABA_A$Rs, we showed for the first time that the EtOH-induced loss of I_{tonic} and cell-surface δ/α4 20 min after withdrawal results from increased receptor endocytosis rather than decreased exocytosis.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

Alcohol abuse is a significant problem in our society. Considerable evidence suggests that γ-aminobutyric acid type A receptors ($GABA_AR$s) are the major target of acute low-dose alcohol (ethanol, EtOH) in the central nervous system (Weiner et al., 1994; Olsen et al., 2007). EtOH allosterically enhances $GABA_AR$ function (Wallner et al., 2003; Olsen et al., 2007), and chronic activation produces plastic changes in $GABA_AR$s which likely contribute to EtOH tolerance, dependence and withdrawal symptoms (Kumar et al., 2009; Liang et al., 2006). $GABA_AR$s, the major inhibitory neurotransmitter receptors, are ligand-gated chloride channels assembled into heteropentamers from a family of 19 subunit genes (Olsen and Sieghart, 2008; Rudolph et al., 2001). $GABA_AR$s with different subunit compositions have distinct localization and physiological and pharmacological properties, accounting for variable sensitivity to $GABA_AR$ modulators including EtOH (Olsen and Sieghart, 2008).

Rapid inhibitory synaptic transmission is mediated by γ2 subunit-containing $GABA_AR$ subtypes, while tonic inhibition primarily depends on extrasynaptic δ subunit-containing $GABA_AR$s (Farrant and Nusser, 2005). The δ-containing $GABA_AR$s have unusual properties such as high affinity but low efficacy for GABA, slow desensitization kinetics, benzodiazepine insensitivity, and high sensitivity for EtOH both in recombinant expression cells (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), and in brain slices (Wei et al., 2004; Hanchar et al., 2005; Liang et al., 2006). Several groups have observed enhancement of inhibitory synaptic transmission in brain slices by low-moderate concentrations of EtOH, and interpreted them as being presynaptic, postsynaptic, or both (e.g., Carta et al., 2004; Breese et al., 2006). It is likely that the relative effects on pre- and postsynaptic events depend on cell type, local circuitry, and activity levels.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Previous reports have shown that $GABA_AR$ function and expression are altered after chronic administration of EtOH *in vivo* and *in vitro*, including hippocampus (Kang et al., 1998; Kumar et al., 2009; Mhatre and Ticku, 1992). The hippocampus has been associated with behavioral correlates of EtOH dependence and withdrawal-like hyperactivity, seizure susceptibility and heightened anxiety (Cagetti et al., 2003; Liang et al., 2004), as well as increased electroencephalographic spiking in EtOH withdrawal (Veatch and Gonzalez, 1996). Several studies have characterized $GABA_AR$ subunit expression in hippocampal neurons (Craig et al., 1994; Brooks-Kayal et al., 1998; Mangan et al., 2005) including the α4 and δ subunits, which are abundantly expressed in hippocampus with a higher level in the dentate gyrus than CA1 region (Sperk et al., 1997; Peng et al., 2002). Chronic EtOH exposure decreases $GABA_AR$ α1 and δ subunit expression and increases the α4, γ1, and γ2 subunits (Cagetti et al., 2003; Ravindran et al., 2007; Kumar et al., 2009).

Previous studies in rats demonstrated temporary plastic changes in $GABA_ARs$ after withdrawal from a single intoxicating dose of EtOH, including rapid loss of extrasynaptic $GABA_ARs$, slower decrease of surface synaptic $GABA_ARs$, followed by increased protein expression of hippocampal α4 and γ2 $GABA_AR$ subunits (Liang et al., 2007). However, how EtOH produces the plastic changes in $GABA_ARs$, including synaptic and extrasynaptic components, as well as the time course of these alterations, have not been fully elucidated. Therefore, we examined whether $GABA_AR$ plastic changes could be induced by a single EtOH exposure in primary cultured hippocampal neurons. We found that cultured neurons exhibit many of the same changes seen *in vivo*, validating the idea of modeling that plasticity *in vitro*. In fact, we showed that the same regulatory events involving the same players occurred in the same types of cells *in vitro* that were responding to EtOH exposure in the intact animal, provided that

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

we grew the embryonic cells sufficiently long in culture (E18 primary cultured hippocampal neurons grown 14-15 days *in vitro* (DIV)) to allow differentiation of the adult phenotype normally found *in situ*. We know of no prior reports in which exposure *in vitro* to a drug of abuse to primary cultured neurons produced similar plastic changes that have been described, characterized, and related to behavioral signs of dependence *in vivo*. Here we report alterations in the levels and subcellular localization including synaptic vs. extrasynaptic positioning for $GABA_A$Rs after one brief exposure to an intoxicating dose of EtOH. Further, the cultured cells can be used for more accurate time-course studies, and are amenable to various biochemical manipulations and measurements than *in vivo* studies. For example, we demonstrated for the first time that the rapid loss of cell-surface α4βδ-type $GABA_A$R induced by EtOH exposure was due to increased endocytosis rather than decreased exocytosis, and was evident at 20 min after EtOH exposure.

Materials and Methods

The Institutional Animal Care and Use Committee approved all animal experiments.

Primary hippocampal neuron culture. Hippocampal neurons from embryonic day 18 Sprague-Dawley rats were prepared by papain dissociation (Worthington Biochemical, Lakewood, NJ) and cultured in Neurobasal medium (Invitrogen) and B27 supplement as previously reported (Stowell and Craig, 1999). Briefly, embryos were removed from maternal rats anesthetized with isoflurane and euthanized by decapitation. Hippocampi were dissected and placed in Ca^{2+}- and Mg^{2+}-free HEPES-buffered Hank's buffered salt solution (pH 7.45). Tissues were dissociated by papain digestion followed by trituration through a Pasteur pipette and papain

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

inhibitor treatment. Cells were pelleted and resuspended in Neurobasal medium containing 2% B27 serum-free supplement, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 mM glutamine (all from Invitrogen), and 10 μM glutamate (Sigma).

Dissociated cells were then plated at a density of 0.3 x 10^5 cells/cm^2 onto 12 mm round coverslips in 24-well plates (for patch clamp recording) and/or at a density of 0.5 x 10^5 cells/cm^2 in 6-well plates (for Western blot and biotinylation assays) coated with poly-D-lysine (Sigma, 50 μg/ml). Cultures were kept at 37°C in a 5% CO_2 humidified incubator. Thereafter, one third to half of the medium was replaced twice a week with Neurobasal culture medium containing 2% B27 supplement, and 0.5 mM glutamine.

EtOH or vehicle exposure. At different times before whole-cell patch-clamp recording and/or biochemical experiments (20 min, 1, 4, 12, and 24 h), half of the medium of cultured neurons (DIV13-14) was replaced with Neurobasal culture medium containing 120 mM EtOH (final EtOH concentration was 60 mM) for 30 min and then the entire medium replaced with half fresh Neurobasal culture medium plus half original medium (kept in the dishes, at 37°C). Control neurons were treated with corresponding vehicle using the same procedure as EtOH-exposed neurons. Neurons were maintained in the incubator until use.

The concentration of 60 mM EtOH used to treat cultured neurons was chosen to match blood levels measured in adult rats after intoxication with gavage of 5 g/kg, which produced ~60 mM blood peak plasma [EtOH] lasting for 2-3 h, and induced significant plasticity in $GABA_A$Rs and drug tolerance (Liang et al., 2007). This dose models levels observed in human binge drinking, and also utilized in our long-term studies on a rat model of alcoholism, chronic intermittent ethanol (CIE), where rats receive a daily intermittent regimen of intoxication and

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

withdrawal for 60 doses (Cagetti et al., 2003). The animals are sedated but not anesthetized by this dose. This concentration also gives significant enhancement of $GABA_AR$-mediated tonic inhibitory currents (I_{tonic}) in hippocampal neurons (Liang et al., 2006). Human lethal blood alcohol concentrations are reported to range from 0.22 to 0.5% wt/vol (50-110 mM), with a mean minimum lethal concentration of around 80 mM (Liang et al., 2007). It is reasonable to assume that blood alcohol concentrations are similar to alcohol concentrations at molecular targets in the brain (Olsen et al., 2007).

Cell death assay. To quantify the amount of cell death, propidium iodide (Sigma) was added to the culture medium at a 5 µg/ml concentration for 20 min. Then the neurons cultured on coverslips were rinsed carefully with phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde for 10 min. After washing 3 times with PBS, the coverslips were mounted on slides with ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole dilactate (DAPI) (Invitrogen) to estimate total cell number. Propidium iodide- and DAPI-positive cells were counted and their ratio in percent of control was determined. Cells were counted in randomly chosen areas (0.4 mm^2) of the respective coverslips (10 areas for each cover slip, 5 coverslips of two independent experiments) using a 40X objective (with numerical aperture 0.75) of fluorescent microscope Olympus BX60**.**

Immunocytochemistry. Cultured hippocampal neurons (DIV14) were fixed with 4% paraformaldehyde and 4% sucrose in PBS (pH 7.4) for 10 min at room temperature and washed three times with PBS. For labeling of surface $GABA_AR$ δ subunit, unspecific binding was blocked under non-permeabilizing conditions with PBS containing 4% normal goat serum

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

(Vector Laboratories) before the cells were incubated with rabbit anti-$GABA_AR$ δ subunit antibody (aa 1-44), 5 µg/ml (from Dr. W. Sieghart), at 4°C overnight. After the primary antibody was removed the cultures were washed three times with PBS and then treated for 20 min with PBS containing 0.1% (v/v) Triton X-100 and 4% normal goat serum. Mouse anti-CaMKII antibody (1:1000) (Affinity Bio Reagents) was applied at 4°C overnight. Neurons were washed three times with PBS, and Alexa fluor 568 goat anti-mouse IgG and Alexa fluor 488 goat anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. Olympus fluorescent microscope BX60 with Hamamatsu digital camera was used for image and Wasabi (version 1.5) software was used for data acquisition.

Whole-cell patch-clamp recording. Immediately before electrophysiological recording, neurons grown on coverslips were transferred to a perfusion chamber (Warner Instruments) and visualized with an inverted microscope (TE200, Nikon). Whole-cell patch-clamp recordings were obtained under voltage clamp mode at room temperature (22-23°C), at a holding potential of -70 mV. Neurons were perfused with an extracellular solution containing (in mM): 137 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 20 glucose and 10 HEPES (310-320 mOsm, pH adjusted to 7.40 with NaOH). Glass pipettes were filled with internal solution containing (in mM): 137 CsCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 10 HEPES and 3 ATP (290-300 mOsm, pH adjusted to 7.30 with CsOH), with an input resistance of 4-7 MΩ. All reagents were from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. $GABA_AR$-mediated miniature inhibitory postsynaptic currents (mIPSCs) were pharmacologically isolated by adding tetrodotoxin (TTX, 0.5 µM), D(-)-2-amino-5-phosphonopentanoate (APV, 40 µM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 µM), and CGP54626 (1 µM, $GABA_B$ receptor antagonist) to the extracellular solution from stock solutions. Stock solutions of CGP54626 were made with pure dimethyl sulfoxide (DMSO).

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Final concentrations of DMSO did not exceed 0.01% in the recording chamber. Control and drug-containing solutions were delivered to the cultured neurons through a removable tip that were positioned close to the soma of target neurons with a Valvelink 8.02 fast-exchange perfusion system (AutoMate Scientific, USA). The flow rate of the perfusion system was around 0.3-0.5 ml/min, and the total volume of the recording chamber was 3 ml. Electrical signals were amplified using an Axonpatch 200B amplifier (Molecular Devices, USA). After establishing the whole cell configuration, at least 5-10 min were allowed to elapse before drug application to allow the membrane patch to stabilize and exchange of ions between the recording electrode and the cytosol to occur. Data were acquired with pClamp software (Version 10.2, Molecular Devices, USA), digitized at 20 kHz (Digidata 1440A, Molecular Devices, USA), and analyzed using the Clampfit software (Version 10.2, Molecular Devices, USA) and the Mini Analysis Program (versions 6.0.7, Synaptosoft, Decatur, GA) (Liang et al., 2007).

Detection and analysis of mIPSCs and I_{tonic}. The method of detection and analysis has been explained previously (Liang et al., 2004). Briefly, the recordings were low-pass filtered off-line (Clampfit software, version 10.1) at 2 kHz. The mIPSCs were detected off-line using the Mini Analysis Program (versions 6.0.7, Synaptosoft, Decatur, GA) with detection threshold criteria of 11 pA amplitude and 35 fC charge transfer. The frequency of mIPSCs was determined from all automatically detected events in a given 100 s recording period. For kinetic analysis, the mIPSCs were automatically detected by the program initially and then manually analyzed based on the criteria that only single event mIPSCs with a stable baseline, sharp rising phase (10 to 90% rise time), and exponential decay were chosen during visual inspection of the recording trace. Double and multiple peak mIPSCs were excluded. For each neuron, mIPSC kinetics and

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

total charge transfer (mIPSC area) were analyzed on average events that were aligned by half rise time. At least 100 individual mIPSC events were recorded under each experimental condition. The decay time constants were obtained by fitting a double exponential to the falling phase of the averaged mIPSCs. The I_{tonic} magnitudes were obtained from the averaged baseline current of a given recording period. The amplitude of the I_{tonic} was calculated by the outward shift of the baseline holding currents after the application of bicuculline (10 μM), a competitive inhibitor of $GABA_A$Rs, which can diminish both synaptic and I_{tonic} magnitude (Mangan et al., 2005; Olsen and Sieghart, 2008). Only current recordings that exhibited a stable baseline were included in the analysis.

Western blot. Cultured hippocampal neurons (DIV14) and hippocampi from $GABA_A$R δ subunit knockout mice (UCLA colony, following Mihalek et al., 1999) were lysed in lysis buffer containing 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 50 mM Na_3PO_4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete protease inhibitor cocktail (Roche). The lysate was centrifuged for 15 min (14,000 x *g*, 4°C) and the supernatant was collected for Western blot analysis. Protein concentrations were determined with BCA Protein Assay Kit (Pierce) according to the manufacturer instructions.

Biotinylation assay for cell-surface receptors. Cultured hippocampal neurons (DIV14) were used. The culture dishes were placed on ice and rinsed twice with ice-cold PBS. Then, the neurons were incubated for 30 min on ice with PBS that contained 1 mg/ml sulfo-NHS-LC-biotin (ProteoChem). After rinsing with Tris-buffered saline (TBS) to quench the biotin reaction,

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

neurons were lysed in 150 µl of modified lysis buffer (see above). The homogenates were centrifuged for 15 min (14,000 x *g*, 4°C). An aliquot (10%) of the supernatant was removed to measure β-actin. The remaining supernatant was incubated with 60 µl of 50% neutravidin agarose (Pierce Chemical Company) for 4 h at 4°C and washed four times with lysis buffer. Agarose-bound proteins were taken up in 40 µl of SDS sample buffer and boiled. Western blots were performed using rabbit anti-$GABA_AR$ α1 (Novus Biologicals), α4 (aa 379-421), α5 (aa 337-388), γ2 (aa 319-366), 1 µg/ml, δ (aa 1-44) subunit antibody, 2 µg/ml (all from Dr. W. Sieghart) and mouse anti-β-actin (Sigma) followed by HRP-conjugated secondary antibodies. Bands were detected using ECL detection kit (Amersham) and analyzed by densitometric measurements using ImageQuant 5.2 (Molecular Dynamics). Bands were stripped with buffer containing 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol and 2% SDS (pH 6.7) and reprobed several times.

Biotinylation assay for internalized receptors. Cell-surface proteins were labeled with 1.5 mg/ml sulfo-NHS-SS-Biotin (Pierce Chemical Company, Rockford, IL) in ice-cold PBS for 20 min at 4°C. Then, cultures were washed carefully with warm culture medium and treated with 60 mM EtOH for 30 min following 20 min withdrawal at 37°C (the same treatment protocol as mentioned above). Next, cultures were placed on ice and biotin coupled to surface proteins was removed using glutathione-containing stripping-buffer (50 mM glutathione, 75 mM NaCl, 75 mM NaOH, 10% FCS, pH 8.5-9.0). After washing with PBS cells were lysed with PBS containing Complete protease inhibitor (Roche), 0.5 mM PMSF, 0.1% SDS and 1% Triton X-100. The amount of biotinylated internalized proteins was quantified with Western blot analysis after separation with NeutrAvidin agarose (Pierce Chemical Company, Rockford, IL) as

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

described above. Rabbit anti-$GABA_A$ receptor α1 (Novus Biologicals), α4 (aa 379-421), α5 (aa 337-388), δ (aa 1-44) subunit antibody, 2 μg/ml (all from Dr. W. Sieghart) and mouse anti-β-actin (Sigma) were used as primary antibodies followed by HRP-conjugated secondary antibodies (Rockland).

Statistical analysis. Data were from three to five independent preparations of neuron cultures. Sigmaplot (Windows version 10.1), SigmaStat (Windows version 3.5) and SAS (version 9.2) were used for data display and statistical analysis. Data were expressed as mean ± SEM, and all values included in the statistics represent data from individul cells. Statistical comparisons were made with the use of Two-way Repeated Measures ANOVA and/or one-way ANOVA ($p < 0.05$ was taken as significant).

Results

To determine if EtOH exposure and withdrawal induces cell death, we compared the proportion of dead neurons found in control cultures compared to those exposed with EtOH 60 mM for 30 min and withdrawn for 24 h using propidium iodide fluorescence. No significant change could be found in the number of dead cells (5.9 ± 1.0% and 7.7 ± 0.6% in control and EtOH-exposed neurons, respectively, $p > 0.05$), indicating neither EtOH exposure for 30 min nor subsequent EtOH withdrawal induced an increase in cell death.

$GABA_AR$ δ subunit expression in cultured hippocampal neurons

We previously reported that the δ subunit is a critical component of $GABA_AR$-mediated extrasynaptic inhibition which is sensitive to EtOH (Hanchar et al., 2005; Liang et al., 2006).

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Therefore, documenting and quantifying δ subunit expression in our primary cultured neurons was critical. We first examined whether there is δ subunit expression in mature (DIV $\geq$ 13) cultured hippocampal neurons. Both immunostaining and Western blot results showed $GABA_AR$ δ subunit expression in cultured hippocampal neurons at DIV14 (Fig. 1). The δ subunit was mainly found in α-CaMKII-positive neurons, a marker for glutamatergic, pyramidal principal cells, and the major cells being studied in these cultures by electrophysiology. Immunostaining and Western blot revealed the cells in these cultures expressed the $GABA_AR$ subunits α1, α2, α4, α5, β2, β3, γ2, and δ (data not all shown), as previously reported for cultured hippocampal neurons (Brooks-Kayal et al., 1998; Mangan et al., 2005), which are the same subunits seen in the hippocampal formation *in situ* (Laurie et al., 1992; Sperk et al., 1997; Brooks-Kayal et al., 1998; Peng et al., 2002; Cagetti et al., 2003).

EtOH sensitivity of $GABA_AR$-mediated I_{tonic} and δ subunit expression increase in parallel during neuronal development

Next, we studied whether extrasynaptic sensitivity to EtOH is correlated to δ subunit expression from E18 neurons cultured for various times. Whole-cell voltage-clamp recordings were performed on cultured hippocampal neurons at DIV11 to 15 in the presence of TTX (0.5 μM), APV (40 μM), CNQX (10 μM), and CGP54626 (1 μM), with holding potential at -70 mV. Under these conditions (in the absence of applied GABA), we were able to record both $GABA_AR$-mediated mIPSCs and persistent tonic currents before and during application of EtOH (Fig. 2B). We conclude that GABAergic neurons are present in sufficient quantities to provide both synaptic input to pyramidal cells plus extracellular concentrations of GABA sufficient to support inhibitory tonic currents. We used bicuculline (10 μM) application to evaluate the

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

magnitudes of I_{tonic} at the end of these recordings, which caused a reduction of noise and shift in baseline current and also abolished mIPSCs, demonstrating that both synaptic and tonic inhibition observed were mediated by $GABA_A$Rs. At DIV11, neurons showed very small total charge transfer of mIPSCs, I_{tonic} magnitudes, and no significant enhancement by acute EtOH (60 mM) of I_{tonic} (potentiated by 10.8 ± 10.7%; there was hardly to see any cell responses to acute EtOH, for example, only one neuron in nine neurons total showed potentiation by EtOH). At DIV15, mature neurons showed much larger total charge transfer of mIPSCs and I_{tonic} magnitudes, and significant enhancement by acute EtOH on I_{tonic} (potentiated 116.5 ± 25.5%, more than two-fold times the control current, with 7/8 neurons sensitive to EtOH). Between DIV11 to 15, I_{tonic} from these neurons showed sensitivity to EtOH gradually increasing with age, while total charge transfer of mIPSCs in those neurons was not significantly potentiated by acute EtOH. The δ subunit total expression measured by Western blot over time was also increased during neuronal development (Fig. 2A, left panel). Also, biotinylation results showed clearly that δ subunit expression occurs at the cell surface in mature neurons (DIV14) (Fig. 2A, right panel). Linear regression curve fitting gave slopes of EtOH sensitivity and δ subunit expression of 26.65 and 30.79, respectively (Fig. 2C). These two slopes show no statistically significant difference between them, i.e., they are identical (P = 0.863, linear regression), suggesting that there is a positive relationship between I_{tonic} sensitivity to EtOH and δ expression. These results showed that δ subunit expression and considerable sensitivity to acute EtOH are increased in parallel with the development of cultured hippocampal neurons, which show higher δ subunit expression and higher acute EtOH sensitivity as mature neurons at DIV14-15.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Altered EtOH responsiveness of mIPSCs and I_{tonic} after withdrawal from a 30 min EtOH exposure

We examined the mIPSCs and I_{tonic} responsiveness to an acute EtOH (10-60 mM) challenge in control (vehicle-treated) neurons and neurons at 1 and/or 24 h after withdrawal from EtOH exposure (60 mM, 30 min) (Fig. 3). We observed a significant enhancement (61.0 ± 3.3% and 109.0 ± 12.7% at 30 and 60 mM, respectively, above control) in I_{tonic} by acute EtOH application to untreated neurons (Fig. 3A, C), and virtually no enhancement (30-60 mM) of mIPSCs (Fig. 3A, B) compared to no EtOH. About 3/4 of the neurons recorded (n = 25) showed this EtOH enhancement, consistent with variable gene expression typical of cultured neurons at DIV14-15. However, when acute EtOH was applied to neurons at 1 h withdrawal from EtOH pre-exposure, the potentiation of I_{tonic} was significantly reduced (only increased from 7.7 ± 1.4 to 7.9 ± 1.8 pA at 30 mM and to 8.9 ± 1.5 pA at 60 mM, respectively, Fig. 3A, C); and similar results were also observed in neurons at 24 h after EtOH exposure (only increased from 7.5 ± 1.3 to 7.6 ± 2.1 pA at by 30 mM and to 8.8 ± 1.6 pA at 60 mM, respectively, Fig. 3A, C). The mIPSCs were potentiated even by 30 mM EtOH, in contrast to that observed in control neurons, causing 16.5 ± 6.1% and 33.6 ± 4.6% increases at 30 and 60 mM, respectively (Fig. 3B). Acute EtOH (100 mM) increased I_{tonic} by 155.9 ± 34.1% (n = 13) in control neurons, but only 36.3 ± 9.6% in 24 h withdrawn neurons (n = 6, data not shown, neurons were different from those studied in Fig. 3); however, it caused only slight potentiation of mIPSCs in control neurons but a 46.1 ± 11.8% increase in 24 h EtOH withdrawn neurons (data not shown). These results are similar to previous results in rats treated with EtOH *in vivo* (Liang et al., 2007).

In order to compare the EtOH effects on total charge transfer of mIPSCs and tonic current, we further calculated the changes of total charge transfer of mIPSCs and I_{tonic} in 100-s

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

sections (last 100 s before solution exchange during each period with stable holding currents) of control recording and during 60 mM EtOH and bicuculline application in control and 24 h EtOH withdrawn neurons. In control neurons, mIPSC total charge transfer was not increased by 60 mM EtOH (only from 103.2 ± 14.7 to 105.5 ± 15.1 pC), while the total charge transfer of I_{tonic} was increased by 117.8 ± 21.8% (from 1329 ± 127 to 2589 ± 211 pC) after EtOH application (data not shown). In 24 h EtOH withdrawn neurons, 60 mM EtOH application caused a 35.0 ± 3.4% increase in the mIPSC total charge transfer (from 211.4 ± 13.4 to 286.4 ± 20.0 pC), and only increased the total charge transfer of I_{tonic} by 21.9 ± 5.6% (from 854 ± 173 to 1005 ± 193 pC, data not shown).

To further study the time course of changes in EtOH sensitivity induced by EtOH exposure, we subsequently tested effects of acute EtOH (60 mM) application on recordings from control neurons and neurons obtained at 1, 4, 12, and 24 h after EtOH withdrawal (repeating the observations of EtOH modulation of I_{tonic} altered at 1 and 24 h after EtOH). These results showed that the mIPSC potentiation by acute EtOH (60 mM) was significantly increased 12 and 24 h after EtOH withdrawal (to 32.3 ± 6.9% and to 33.6 ± 4.6%, respectively), but not 4 h or less (Fig. 4C), whereas the loss of acute EtOH (60 mM) enhancement of I_{tonic} was observed already at 1 h (potentiated by only 20.6 ± 10.4%), and similar responses were observed at 12 h (potentiated by 23.3 ± 7.2%) and 24 h (potentiated by 29.4 ± 7.6%) after EtOH exposure as well (Fig. 4D).

Changes in total basal charge transfer of mIPSCs and I_{tonic} magnitude after withdrawal from EtOH exposure

In order to determine EtOH withdrawal-induced changes in mIPSCs and I_{tonic}, we analyzed the total charge transfer of mIPSCs and I_{tonic} magnitude under basal conditions (without

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

acute application of EtOH) in control neurons and neurons obtained at 1, 4, 12, and 24 h after respective EtOH withdrawals. The data revealed that the total charge transfer of mIPSCs was significantly decreased by 36.0 ± 4.5% at 4 h and then recovered considerably at 12 h after EtOH exposure (Fig. 4A), whereas at 1 h after EtOH exposure, a 49.8 ± 10.8% decrease in I_{tonic} magnitude occurred and it remained diminished at 24 h after EtOH exposure (Fig. 4B). We next analyzed mIPSC kinetics and I_{tonic} magnitude from control neurons and neurons after withdrawal for 1, 4, 12, and 24 h after EtOH (Table 1). At 1 h, only I_{tonic} magnitude was significantly reduced compared with control neurons, but no statistically significant changes in kinetics were observed. At 4 h, frequency, area, and I_{tonic} were all decreased without change in rise time and decay time. At 12 h, the frequency and area were considerably restored with persistent diminished I_{tonic}. However, both rise time and decay time of mIPSCs were significantly faster than control neurons. Similar results were observed at 24 h after EtOH withdrawal.

Diazepam and zolpidem tolerance of $GABA_AR$-mediated currents induced by withdrawal from EtOH exposure

It is known that $GABA_ARs$ with different subunit compositions have distinct pharmacological properties, for example, diazepam has no agonist activity at α4-containing $GABA_ARs$ but potentiates other αxβγ2, x = 1,2,3,5 $GABA_ARs$ (Rudolph et al., 2001), while zolpidem is an allosteric positive modulator of $GABA_AR$ function with selectivity for the α1-containing $GABA_ARs$, with intermediate potency on α2/3-containing $GABA_ARs$, and no effect on α5-containing $GABA_ARs$ (Olsen and Sieghart, 2008). To provide additional evidence in support of the hypothesis that $GABA_AR$ subunit alterations mediate the switch in responsiveness of synaptic and extrasynaptic $GABA_ARs$ to acute EtOH after withdrawal from 30 min of 60 mM

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

EtOH exposure, we then applied diazepam and/or zolpidem to test the control neurons and neurons obtained at different times after withdrawal from EtOH. Diazepam significantly potentiated total charge transfer of mIPSCs, by increasing both decay time and amplitude, in control neurons, and neurons obtained at 1 and 4 h after EtOH withdrawal, causing 50.5 ± 3.8%, 47.5 ± 9.4% and 39.0 ± 7.7% increases, respectively; however, such potentiation by diazepam was reduced to 10.5 ± 5.2% at 24 h after EtOH withdrawal (Fig. 5A), as observed in EtOH-exposed rats (Liang et al., 2007). Acute diazepam application also significantly enhanced I_{tonic} in control neurons, causing a 60.4 ± 10.5% increase, but was already reduced dramatically to 26.1 ± 5.1% at 1 h and remained reduced at 24 h after EtOH withdrawal (19.4 ± 4.7%, Fig. 5B). Similarly, zolpidem caused 59.0 ± 9.5%, 55.9 ± 15.2% and 47.4 ± 5.9% increases in mIPSCs total charge transfer recorded from control neurons and neurons obtained at 1 and 4 h after EtOH withdrawal, respectively, but the potentiation was also decreased to 21.8 ± 4.7% at 24 h after EtOH withdrawal (Fig. 5A). No significant effect of zolpidem on I_{tonic} was observed in either control or EtOH-treated neurons (Fig. 5B).

Altered Ro15-4513 effect on total change transfer of mIPSCs after withdrawal from EtOH exposure

Next, we examined the effect caused by withdrawal from EtOH exposure on responsiveness to Ro15-4513, a partial inverse agonist at the benzodiazepine site of α1- and α2-containing $GABA_A$Rs, which was also shown to bind with high affinity at α4-containing $GABA_A$Rs (Liang et al., 2004). Importantly, Ro15-4513 has agonist activity at α4β3γ2 $GABA_A$Rs but does not modulate α4β3δ $GABA_A$Rs. In control neurons, as well as in neurons obtained at 1 and 4 h after EtOH exposure, Ro15-4513 displayed an inhibitory effect on total

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

change transfer of mIPSCs, causing 23.8 ± 5.3%, 25.4 ± 3.7%, 17.0 ± 1.6% decreases, respectively; but at 24 h after EtOH withdrawal, Ro15-4513 slightly but significantly increased mIPSCs by 16.0 ± 6.0% (Fig. 5A). Also, no significant effect of Ro15-4513 on I_{tonic} was observed in both control neurons and EtOH-exposed neurons (Fig. 5B).

Altered cell-surface expression of $GABA_A$Rs after withdrawal from EtOH exposure

Based on the changes in $GABA_A$R function and pharmacological responsiveness to modulators, and the results in our previous study in rats (Liang et al., 2007), the data suggest there are accompanying corresponding alterations in cell-surface expression of $GABA_A$R subunits. Using cell-surface biotinylation following Western blot analysis we were able to show that the cell-surface δ subunit content was significantly reduced by 31.7 ± 9.0% at 1 h after withdrawal compared to control neurons, and it remained diminished at 4 h (reduced by 33.0 ± 6.6%), persisting to 24 h after EtOH (reduced by 27.0 ± 6.9%, Fig. 6A). Because the δ subunit normally co-assembles substantially with the α4 subunit to form extrasynaptic or perisynaptic $GABA_A$Rs (Wei et al., 2004; Liang et al., 2006), we then measured alterations in α4 cell-surface expression at different times. A large decrease (by 77.0 ± 10.5%) in cell-surface α4 was observed at 1 h, and it also remained at 4 h (by 58.7 ± 6.0%), which were consistent with alterations in surface δ subunit; but at 24 h after EtOH, it recovered and significantly increased to 125.3 ± 10.6% of control (Fig. 6A). The γ2 subunit was shown previously to be increased after genetic deletion or seizure-induced decreases in the δ subunit (Peng et al., 2004). We previously showed increased surface γ2 subunit content when δ subunit level was decreased after withdrawal from one dose EtOH *in vivo* (Liang et al., 2007). Therefore, we next measured changes in surface γ2 subunit at corresponding times. At 1 h, no significant change in surface γ2 was detected;

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

however at 4 h, there was a small but significant decrease by 17.7 ± 8.8%, which then increased dramatically to 162.4 ± 13.7% at 24 h compared to control (Fig. 6B). These data are consistent with previous studies (Liang et al., 2004; Liang et al., 2006; Liang et al., 2007), and support the hypothesis that the γ2 subunit is the most likely partner for the up-regulated cell-surface α4 subunit induced by EtOH pre-exposure in cultured hippocampal neurons.

Moreover, we suspected there were also alterations in cell-surface level of α1 subunit, since α1βxγ2 $GABA_AR$ subtypes are typical in synapses (Rudolph et al., 2001) and α1 is known to be down-regulated by EtOH treatment (Liang et al., 2007; Kumar et al., 2009). At 1 h, there was no significant change in α1 subunit, but a large reduction (54.7 ± 14.4%) was observed at 4 h, and remained reduced by 37.0 ± 7.8 % at 24 h after EtOH (Fig. 6B).

In hippocampal pyramidal neurons, especially CA1, α5 subunit-containing $GABA_ARs$ are thought to be critical in tonic inhibition (Caraiscos et al., 2004). Therefore, we measured α5 surface expression at different time points after EtOH withdrawal. A significant reduction in surface α5 subunit was found at 1 h, decreased by 57.7 ± 8.7% of control, which persisted to 4 h (60.3 ± 8.9 %), but then returned to control levels at 24 h after EtOH (Fig. 6C).

Rapidly increased internalization of $GABA_AR$ α4 and δ subunits after EtOH exposure

We hypothesized that the decreased magnitude of I_{tonic} and tolerance to acute EtOH enhancement at 1 h following EtOH exposure are most likely due to α4βδ $GABA_AR$ overactivation followed by internalization. However, a decrease in cell-surface expression of α4βδ $GABA_ARs$ could be contributed by either increased receptor internalization or reduced receptor insertion into the plasma membrane by exocytosis after EtOH exposure/withdrawal, as well as other mechanisms such as cleavage/degradation at the cell surface. Therefore, we next

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

tested whether the alterations in surface $GABA_AR$s are induced by receptor internalization, using a biotinylation assay for internalized receptors. As shown in Fig. 7A, we first labeled cell-surface proteins with cleavable biotin, followed by EtOH exposure/withdrawal, and then cleaved biotin (coupled to surface proteins) carefully. Compared to vehicle-treated controls, at 20 min after EtOH exposure/withdrawal, large increases in the amount of internalized (biotinylated) α4 (increased to 211.3 ± 17.0% of control) and δ (to 169.0 ± 20.4% of control) subunits were observed (Fig. 7 B, C). No significant change in internalization of α5 (to 129.3 ± 17.1%) and α1 (to 86.0 ± 4.5%) of control surface levels of subunits were observed at that time (Fig. 7 B, C). These data show for the first time that the decreases in cell-surface α4 and δ expression at 1 h after EtOH withdrawal are mediated by increased receptor endocytosis rather than reduced membrane insertion of (extrasynaptic) α4 and δ subunit-containing $GABA_AR$s.

Discussion

A single pre-exposure to EtOH alters $GABA_AR$ subunit composition and function in primary cultured hippocampal neurons: an *in vitro* model for alcohol-induced plasticity

A single large dose EtOH exposure induces changes in $GABA_AR$ subunit composition and function in hippocampus of adult rats *in vivo* (Liang et al., 2007). In the current study, we established a primary cultured hippocampal neuron model to measure the direct effects of EtOH exposure and withdrawal. First, we established that under our traditional (Stowell and Craig, 1999) culture conditions, the major cells present, likely pyramidal neurons, exhibit both "synaptic" $GABA_AR$-mediated mIPSCs and extrasynaptic $GABA_AR$-mediated I_{tonic} currents, consistent with previous literature (Craig et al., 1994; Mangan et al., 2005). Such studies showed that there are sufficient GAD+ interneurons in the cultures to not only produce inhibitory

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

synapses on the principal cells but produce sufficient GABA in the extracellular space to allow detection of these $GABA_AR$-mediated currents. Second, our tonic inhibitory currents at DIV14 were significantly enhanced by acute application of modest 30-60 mM EtOH. Further, the results show that within 1 h after withdrawal from EtOH exposure (60 mM, 30 min), the extrasynaptic $GABA_AR$-mediated I_{tonic} and its potentiation by acute EtOH is significantly reduced. At 4 h there is a reduction of synaptic $GABA_AR$-mediated mIPSCs, followed at 24 h after EtOH exposure by restored mIPSCs, but with altered pharmacological responsiveness to modulators including EtOH, diazepam, zolpidem, and Ro15-4513. Importantly, as found in hippocampal slices from EtOH-exposed rats, the rapid reduction in I_{tonic} magnitude, and development of tolerance to acute EtOH, are observed within 1 h withdrawal from EtOH exposure *in vitro*, and the mIPSC kinetic changes show a delayed response detectable at 4 h or longer but not at 1 h withdrawal from EtOH exposure.

Consistent with these findings, biotinylation/Western blot data for $GABA_AR$ subunit cell-surface expression showed that various $GABA_AR$ subunit surface contents were altered, similar to observations in treated rats, showing plastic changes in both extrasynaptic and synaptic $GABA_ARs$. Also, at 24 h, significant up-regulation of total and cell-surface α4 and γ2 subunits is observed, which appears to contribute to the altered modulation of $GABA_AR$ currents by EtOH, diazepam, zolpidem, and Ro15-4513. Thus it is feasible to study EtOH-induced $GABA_AR$ plasticity in mature, δ subunit-expressing cultured neurons.

Preferential EtOH sensitivity of extrasynaptic δ-$GABA_ARs$ determines their rapid down-regulation by intoxicating concentrations of EtOH

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Tonic vs. phasic inhibitory currents, and role of the δ subunit. Tonic inhibitory currents are generated by extrasynaptic $GABA_A$Rs, activated by ~0.2-2.0 μM ambient or 'spillover' extracellular GABA (Semyanov et al., 2004; Farrant and Nusser, 2005; Liang et al., 2006). Tonic currents are mediated primarily by α5βγ2 $GABA_A$Rs in hippocampal CA1 pyramidal neurons (Caraiscos et al., 2004; Mangan et al., 2005) and by α4βδ $GABA_A$Rs in dentate gyrus granule cells (Laurie et al., 1992; Sperk et al., 1997; Peng et al., 2002; Liang et al., 2006), although there is some overlap as well as contribution of other $GABA_A$Rs to the current in each cell type.

In our cultures, immunostaining/Western blot results show abundant δ subunit expression in the primary cultured hippocampal neurons (DIV14-15), as well as the other $GABA_A$R subunits normally expressed in the hippocampus *in situ* (Sperk et al., 1997). Electrophysiology revealed I_{tonic} sensitivity to acute EtOH increased in a parallel manner with increased δ subunit expression in embryonic cultured neurons during neuronal development (DIV11-15). Biotinylation experiments indicate that this $GABA_A$R δ subunit is expressed at the cell surface. Cleavable biotin reveals that the δ subunit is rapidly internalized after exposure to EtOH, accompanied by loss of EtOH enhancement of I_{tonic}. This strongly suggests that δ subunits are involved in EtOH modulation of $GABA_A$Rs. Additional evidence comes from the loss of EtOH potentiation of I_{tonic} in δ subunit knockout mice which show a loss of I_{tonic} potentiation by EtOH in dentate gyrus (Wei et al., 2004; Liang et al., 2006).

Internalization of extrasynaptic α4βδ $GABA_A$Rs. In our recordings from neuronal cultures I_{tonic} is consistently observed without manipulation of [GABA], without any drugs, or with application of acute EtOH and other modulators, as in brain slices (Liang et al., 2007; Liang et al., 2006). Using biotinylation assay, we show significant reduction in cell-surface expression of

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

δ, α4, and α5 subunits at 1 h after EtOH withdrawal, accompanying the drop in I_{tonic}. We also demonstrate internalization of biotinylated subunit protein, indicating that EtOH-induced endocytosis of α4 and δ, but not α5 or α1 subunits at 20 min after EtOH exposure, accounts for the decrease in surface contents, rather than reduced membrane insertion by exocytosis. The very rapid decrease in surface α4 and δ subunits provides strong evidence that extrasynaptic α4βδ receptors are involved in EtOH-induced $GABA_AR$ plasticity. The α4βδ $GABA_ARs$ are particularly sensitive to EtOH (low mM) in recombinant expression cells (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), and in neurons (Olsen et al., 2007). We posit that the first elements to show plastic changes to EtOH exposure are the molecules that act as the first responders.

The rapid loss of α4 subunit after EtOH can be masked by a fairly rapid compensatory increase in α4 readily observed at later times (Mhatre and Ticku, 1992; Kumar et al., 2009), as also seen after chronic BZ (Follesa et al., 2001) and neurosteroid (Gulinello et al., 2001) administration/withdrawal. This striking plasticity of the α4 subunit gene expression has been explained by demonstration that exposure to EtOH (10-60 mM) in cultured cortical neurons for 1-2 h elevates heat shock factor I that binds the promoter region of the Gabra4 gene to enhance transcription (Pignataro et al., 2007). The early but not immediate drop in α1 expression has been observed after EtOH (Kumar et al., 2010), as has a drop in δ subunit (Ravindran et al., 2007), although these reports were not as tightly linked to electrophysiological and behavioral changes as ours (Liang et al., 2006; Liang et al., 2007; Liang et al., 2009).

Basal I_{tonic} is potentiated by diazepam but insensitive to zolpidem, suggesting a contribution from extrasynaptic α5-containing $GABA_ARs$. In addition to tolerance to acute EtOH, the potentiation of I_{tonic} by diazepam is also reduced at 1 h after EtOH exposure,

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

consistent with a decrease of α5, confirmed by biotinylation experiments. This is unlike EtOH-exposed rats, where no significant reduction in α5 subunits was measured, and I_{tonic} remains sensitive to diazepam at 1 h but is reduced at 2 days after EtOH (Liang et al., 2007). This difference in response times may be due to differences in the experimental systems. Nevertheless, assays made possible in this neuron study, such as receptor internalization, provide a more precise time course, showing that α5 internalization induced by EtOH does not occur before 20 min withdrawal from EtOH exposure, but α4 and δ do. These data suggest that the α5βγ2 alterations are downstream from alcohol activation and have a different mechanism of regulation. Also, cell-surface α5 returns to control levels at 24 h after EtOH (Fig. 6C) while I_{tonic} recovers only slightly. Further, there is no recovery of diazepam potentiation of I_{tonic} (Fig. 5B). This might arise from increases in α5β-containing $GABA_A$Rs without γ2 subunit; this combination is expressed at low levels in the extrasynaptic membranes of pyramidal neurons where they can contribute to tonic inhibition (Mortensen and Smart, 2006). To summarize, the refinement of time course of EtOH-induced changes demonstrates rapid increased internalization of α4βδ $GABA_A$Rs and reduced tonic inhibitory currents, but not α1 or α5 subtypes at 20 min, followed by slower down-regulation of surface α1 and α5 subtypes at 1-12 h, and then compensatory increases in newly synthesized α4/γ2-type $GABA_A$Rs to restore the decrease in inhibitory currents.

Plasticity of synaptic $GABA_A$Rs. At 4 h withdrawal from EtOH the function of synaptic $GABA_A$Rs (charge transfer of mIPSCs) is altered (reduced), but not pharmacology and probably not subunit composition. At 12-24 h, mIPSCs have considerably recovered and become modestly enhanced by EtOH. The differences in timing of changes in I_{tonic} (earlier) and synaptic kinetics

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

suggest, not unexpectedly, that the mechanisms of response to EtOH are different. mISPC kinetics were unchanged after withdrawal of 1-4 h, but at 12-24 h, rise time and decay τ1 become faster, accounted for by the switch from α1- to α4-containing $GABA_A$Rs, as seen *in vivo* (Cagetti et al., 2003; Liang et al., 2004; Liang et al., 2006).

"Subunit switches" and importance of receptor subunit localization to function: EtOH-induced $GABA_A$R plasticity involves trafficking as well as protein synthesis

The EtOH-induced alterations of $GABA_A$R functions in cultured neurons, like in rats, result primarily from regulation of the complex trafficking mechanisms that maintain appropriate receptor cell-surface and subcellular localization (Jacob et al., 2008). The mIPSC amplitude (synaptic strength) depends on insertion/removal of synaptic $GABA_A$Rs (Nusser et al., 1998; Kittler et al., 2005). Chronic ethanol (or BZ) exposure increases the internalization of α1-containing $GABA_A$Rs (Tehrani and Barnes, 1991). The mechanisms by which the relative abundance and localization of specific $GABA_A$R subunits are altered by EtOH exposure are not clear, but could involve protein phosphorylation regulation of $GABA_A$R biosynthesis, degradation, or recycling/endocytosis/exocytosis (Kumar et al., 2009).

Acknowledgements

We thank Dr. Xuesi M Shao for comments on statistical analysis.

Authorship Contributions

Participated in research design: Shen, Lindemeyer, Liang, and Olsen.

Conducted experiments: Shen and Lindemeyer.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Contributed new reagents or analytic tools: Sieghart.

Performed data analysis: Shen, Lindemeyer, and Liang.

Wrote or contributed to the writing of the manuscript: Shen, Lindemeyer, Spigelman, Sieghart, Olsen, and Liang.

Others: Olsen, Liang, and Spigelman acquired funding for the research.

References

Breese GR, Criswell HE, Carta M, Dodson PD, Hanchar HJ, Khisti RT, Mameli M, Ming Z, Morrow AL, Olsen RW, Otis TS, Parsons LH, Penland SN, Roberto M, Siggins GR, Valenzuela CF, Wallner M (2006). Basis of the Gabamimetic profile of ethanol. *Alcohol: Clin Exp Res* **30**:731-744.

Brooks-Kayal AR, Jin H, Price M, Dichter MA (1998) Developmental expression of $GABA_A$ receptor subunit mRNAs in individual hippocampal neurons in vitro and in vivo. *J Neurochem* **70**:1017-1028.

Cagetti E, Liang J, Spigelman I, Olsen RW (2003) Withdrawal from chronic intermittent ethanol treatment changes subunit composition, reduces synaptic function, and decreases behavioral responses to positive allosteric modulators of $GABA_A$ receptors. *Mol Pharmacol* **63**:53-64.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing gamma-aminobutyric acid type A receptors. *Proc Natl Acad Sci USA* **101**:3662-3667.

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

Craig AM, Blackstone CD, Huganir RL, Banker G (1994) Selective clustering of glutamate and gamma-aminobutyric acid receptors opposite terminals releasing the corresponding neurotransmitters. *Proc Natl Acad Sci U S A* **91:**12373-12377.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of GABA(A) receptors. *Nat Rev Neurosci* **6**:215-229.

Follesa P, Cagetti E, Mancuso L, Biggio F, Manca A, Maciocco E, Massa F, Desole MS, Carta M, Busonero F, Sanna E, Biggio G (2001) Increase in expression of the $GABA_A$ receptor α4 subunit

gene induced by withdrawal of, but not by long-term treatment with, benzodiazepine full or partial agonists. *Mol Brain Res* **92**:138-148.

Gulinello M, Gong QH, Li X, Smith SS (2001) Short-term exposure to a neuroactive steroid increases α4 $GABA_A$ receptor subunit levels in association with increased anxiety in the female rat. *Brain Res* **910:**55-66.

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

Jacob TC, Moss SJ, Jurd R (2008) GABA(A) receptor trafficking and its role in the dynamic modulation of neuronal inhibition. *Nat Rev Neurosci* **9**:331-343.

Kang MH, Spigelman I and Olsen RW (1998) The sensitivity of $GABA_A$ receptors to allosteric modulatory drugs in rat hippocampus following chronic intermittent ethanol treatment. *Alcohol Clin Exp Res* **22**:2165-2173.

Kittler JT, Chen G, Honing S, Bogdanov Y, McAinsh K, Arancibia-Carcamo IL, Jovanovic JN, Pangalos MN, Haucke V, Yan Z, Moss SJ (2005) Phospho-dependent binding of the clathrin AP2 adaptor complex to $GABA_A$ receptors regulates the efficacy of inhibitory synaptic transmission. *Proc Natl Acad Sci USA* **10**2:14871-14876.

Kumar S, Porcu P, Werner DF, Matthews DB, Diaz-Granados JL, Helfand RS, Morrow AL (2009) The role of GABA(A) receptors in the acute and chronic effects of ethanol: a decade of progress. *Psychopharmacology (Berl)* **205**:529-564.

Kumar S, Suryanarayanan A, Boyd K, Commerford C, Lai M, Ren Q, Morrow AL (2010) Ethanol reduces $GABA_A$ α1 subunit receptor surface expression by a PKCγ dependent mechanism in cultured cerebral cortical neurons. *Mol Pharmacol* **77**:793-803.

Laurie DJ, Wisden W, Seeburg PH (1992) The distribution of thirteen $GABA_A$ receptor subunit mRNAs in the rat brain. III. Embryonic and postnatal development. *J Neurosci* **12**:4151-4172.

Liang J, Cagetti E, Olsen RW, Spigelman I (2004) Altered pharmacology of synaptic and extrasynaptic $GABA_A$ receptors on CA1 hippocampal neurons is consistent with subunit changes in a model of alcohol withdrawal and dependence. *J Pharmacol Exp Ther* **310**:1234-1245.

Liang J, Spigelman I, Olsen RW (2009) Tolerance to sedative/hypnotic actions of GABAergic drugs correlates with tolerance to potentiation of extrasynaptic tonic currents of alcohol-dependent rats. *J Neurophysiol* **102**:224-233.

Liang J, Suryanarayanan A, Abriam A, Snyder B, Olsen RW, Spigelman I (2007) Mechanisms of reversible $GABA_A$ receptor plasticity after ethanol intoxication. *J Neurosci* **27**:12367-12377.

Liang J, Zhang N, Cagetti E, Houser CR, Olsen RW, Spigelman I (2006) Chronic intermittent ethanol-induced switch of ethanol actions from extrasynaptic to synaptic hippocampal $GABA_A$ receptors. *J Neurosci* **26**:1749-1758.

Mangan PS, Sun C, Carpenter M, Goodkin HP, Sieghart W, Kapur J (2005) Cultured hippocampal pyramidal neurons express two kinds of $GABA_A$ receptors. *Mol Pharmacol* **67**:775-788.

Mhatre MC, Ticku MK (1992) Chronic ethanol administration alters $GABA_A$ receptor gene expression. *Mol Pharmacol* **42:**415-422.

Mihalek RM, Banerjee PK, Korpi ER, Quinlan JJ, Firestone LL, Mi ZP, Lagenaur C, Tretter V, Sieghart W, Anagnostaras SG, Sage JR, Fanselow MS, Guidotti A, Spigelman I, Li Z, DeLorey TM, Olsen RW and Homanics GE (1999) Attenuated sensitivity to neuroactive steroids in $GABA_A$ receptor δ subunit knockout mice. *Proc Nat Acad Sci USA* **96**:12905-12910.

Mortensen M, Smart TG (2006) Extrasynaptic $\alpha\beta$ subunit $GABA_A$ receptors on rat hippocampal pyramidal neurons. *J Physiol* **577**:841-56.

Nusser Z, Hajos N, Somogyi P, Mody I (1998) Increased number of synaptic GABA(A) receptors underlies potentiation at hippocampal inhibitory synapses. *Nature* **395**:172-177.

Olsen RW, Hanchar HJ, Meera P, Wallner M (2007) $GABA_A$ receptor subtypes: the "one glass of wine" receptors. *Alcohol* **41**:201-209.

Olsen RW, Sieghart W (2008) International Union of Pharmacology. LXX. Subtypes of gamma-aminobutyric acid(A) receptors: classification on the basis of subunit composition, pharmacology, and function. Update. *Pharmacol Rev* **60**:243-260.

Peng Z, Hauer B, Mihalek RM, Homanics GE, Sieghart W, Olsen RW and Houser CR (2002) $GABA_A$ receptor changes in δ subunit-deficient mice:altered expression of α4 and γ2 subunits in the forebrain. *J Comp Neurol* **446**:179-197.

Pignataro L, Miller AN, Ma L, Midha S, Protiva P, Herrera DG, Harrison NL (2007) Alcohol regulates gene expression in neurons via activation of heat shock factor 1. *J Neurosci* **27**:12957-12966.

Ravindran CRM, Mehta AK and Ticku MK (2007) Effect of chronic administration of ethanol on the regulation of the δ-subunit of $GABA_A$ receptors in the rat brain. *Brain Res* **1174**:47-52.

Rudolph U, Crestani F, Möhler H (2001) GABA(A) receptor subtypes: dissecting their pharmacological functions. *Trends Pharmacol Sci* **22**:188-194.

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

Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) GABA(A) receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. *Neuroscience* **80**:987-1000.

Stowell JN, Craig AM (1999) Axon/dendrite targeting of metabotropic glutamate receptors by their cytoplasmic carboxy-terminal domains. *Neuron* **22**:525-536.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, Smith SS (2002) Hormonally regulated α4β2δ GABA(A) receptors are a target for alcohol. *Nat Neurosci* **5**:721-722.

Tehrani MHJ, Barnes, EM Jr (1991) Agonist-dependent internalization of $GABA_A$/ benzodiazepine receptors in chick cortical neurons. *J Neurochem* **57**:1307-1312.

Veatch LM, Gonzalez LP (1996) Repeated ethanol withdrawal produces site-dependent increases in EEG spiking. *Alcohol Clin Exp Res* **20**:262-267.

Wallner M, Hanchar HJ, Olsen RW (2003) Ethanol enhances α4β3δ and α6β3δ gamma-aminobutyric acid type A receptors at low concentrations known to affect humans. *Proc Natl Acad Sci U S A* **100**:15218-15223.

Wei W, Faria LC, 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.

Weiner JL, Zhang L, Carlen PL (1994) Potentiation of $GABA_A$-mediated synaptic current by ethanol in hippocampal CA1 neurons: possible role of protein kinase C. *J Pharmacol Exp Ther* **268**:1388-1395.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

* These authors contributed equally to this work.

This work was supported by National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grants AA07680, AA017991, and AA016100].

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Figure legends

Figure 1. $GABA_AR$ δ subunit is expressed in cultured hippocampal neurons at DIV14. **A**, Immunocytochemistry on non-permeabilized neurons shows δ expression on cell surface (green) of α-CaMKII-positive neurons (red). The merged pictures are shown in the right panel. Scale bar = 10 μm. **B**, Western blot of cell lysate from cultured hippocampal neurons (hip) shows δ expression (40 μg protein/lane (10 μl), 80 μg/lane (20 μl), and 160 μg/lane (40 μl)). Hippocampus of δ knockout (KO) mouse (40 μg protein/lane) is used as a negative control. β-actin is used as a loading control.

Figure 2. Parallel increases in acute EtOH sensitivity and $GABA_AR$ δ subunit expression during development in primary cultured hippocampal neurons (hip DIV11-15). **A,** Left panel: Representative Western blot of δ total expression (tot) in hip neurons during development. Total δ amount is calculated by the optical density of δ subunit signal divided by corresponding optical density of β-actin (% of β-actin). Right panel: biotinylation assay and Western blot of total (tot) and surface (sur) δ expression from neurons at DIV14. **B,** Sample traces of individual cultured hippocampal neuron recordings at DIV11 (top panel, from the same neuron) and DIV15 (bottom panel, from the same neuron). Data are obtained under basal conditions and after acute EtOH (60 mM) application from the same cultures as used in Western blot experiments. Subsequent application of bicuculline (red highlight) reveals the magnitude of I_{tonic} (difference between recorded holding current and green lines). DIV15 cells show larger I_{tonic} and greater EtOH enhancement. **C,** Increased acute EtOH sensitivity of I_{tonic} and δ subunit expression in neurons during development. Total δ expression is normalized as % of DIV11 δ values. The black and gray solid lines are the linear regression fits of all data points in each experiment, respectively.

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

The slopes of fitting curves of I_{tonic} and δ expression are 26.65 and 30.79, respectively ($p > 0.05$ for non-identity, linear regression).

Figure 3. Plasticity of $GABA_AR$-mIPSCs and I_{tonic} in primary cultured hippocampal neurons (DIV14-15) following pre-exposure to EtOH. **A,** Sample traces of individual recordings from a control neuron (top), a neuron obtained at 1 h after 30 min incubation with 60 mM EtOH (EtOH 1 h/w, middle trace), and a neuron obtained 24 h after EtOH exposure (EtOH 24 h/w, bottom trace). Currents are recorded at holding potentials of -70 mV before and after EtOH (10, 30, and 60 mM) application followed with bicuculline (bic, 10 μM). The I_{hold} before and after EtOH application are indicated by dashed lines (basal, red line commencing at left margin. Subsequent application of bic (red dashed line commencing near far right end,) reveals the I_{tonic} amplitude (Control, 22 pA; EtOH 1 h/w, 3 pA; EtOH 24 h/w, 4 pA). EtOH increases I_{tonic} amplitude (30 mM, green dashed line; 60 mM, yellow dashed line). EtOH exposure eliminates EtOH acute effects on I_{tonic}. For each trace, examples of mIPSCs with faster time scales are given above for each [EtOH]. EtOH has no significant effects in control or in 1 h/w, but in 24 h/w, EtOH enhances mIPSCs. **B,** EtOH effects on total charge transfer of mIPSCs (n = 10-25). Open circles are control; open triangles (1 h/w), and closed circles (24 h/w). **C,** EtOH effects on I_{tonic} magnitude from the same recordings as in **B** (n = 10-25). Each point represents a mean ± SEM value. *, $p < 0.05$ vs. control neurons; †, $p < 0.05$ vs. pre-EtOH value (Two-way Repeated Measures ANOVA).

Figure 4. Time course of changes in total charge transfer of mIPSCs and I_{tonic} magnitude as well as acute EtOH sensitivity in primary cultured hippocampal neurons (DIV14-15) at various times

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

after 30 min incubation with 60 mM EtOH. **A,** Changes in total charge transfer of mIPSCs recorded in control neurons and neurons obtained at 1, 4, 12, and 24 h after respective treatments. (n = 10-24). **B,** Changes in I_{tonic} magnitude after EtOH withdrawal from the same recordings as in **A** (n = 10-23). **C,** Acute EtOH sensitivity (60 mM) of total charge transfer of mIPSCs recorded in control neurons and neurons obtained at 1, 4, 12, and 24 h after respective treatments (n = 9-24). **D,** Acute EtOH sensitivity (60 mM) in I_{tonic} magnitude after EtOH withdrawal from the same recordings as in **C** (n = 9-24). Data are mean ± SEM of values. *, $p < 0.05$ vs. control neurons (one-way ANOVA).

Figure 5. Time course of changes in effects of diazepam (DZ, 0.3 μM), zolpidem (ZP, 0.3 μM), and Ro15-4513 (Ro, 0.3 μM) on total charge transfer of mIPSCs and I_{tonic} magnitude in primary cultured hippocampal neurons (DIV14-15) at various times after withdrawal from EtOH exposure. **A,** DZ, ZP, and Ro effects on total charge transfer of mIPSCs (% control, n = 6-13) obtained at 1, 4, and 24 h after respective treatments. **B,** DZ, ZP, and Ro effects on I_{tonic} magnitude from the same recordings as in **A** (n = 6-13). *and † are as described in Fig. 3.

Figure 6. Time course of changes in cell-surface expression of $GABA_AR$ subunits after withdrawal from EtOH exposure measured by biotinylation assay in cultured hippocampal neurons. **A,** Upper panel: Representative Western blot of the biotinylation assay for δ (54 kDa) and α4 (67 kDa) subunit cell-surface expression obtained at 1, 4, and 24 h after respective treatments compared to vehicle-treated controls (ctrl). β-actin (42 kDa) of total cell lysate is used as a loading control. Lower panel: Quantification of changes in cell-surface expressed δ and α4 subunits. Surface protein amount is calculated by the optical density of each cell-surface subunit

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

signal divided by optical density of the corresponding β-actin signal of the total cell lysate (% of β-actin) and compared to control which is set 100%. **B,** Upper panel: Cell-surface expressed γ2 (47 kDa) and α1 (52 kDa) subunits. Lower panel: Quantification of changes in cell-surface expressed γ2 and α1 subunits. **C**, Upper panel: Cell-surface expressed α5 (55 kDa) subunit. Lower panel: Quantification of changes in cell-surface expressed α5 subunit. Data are mean ± SEM of values (n = 3-5). Note that the representative bands of α1, α4, α5, and γ2 are from the same Western blot experiment. *, $p < 0.05$ vs. control neurons (one-way ANOVA).

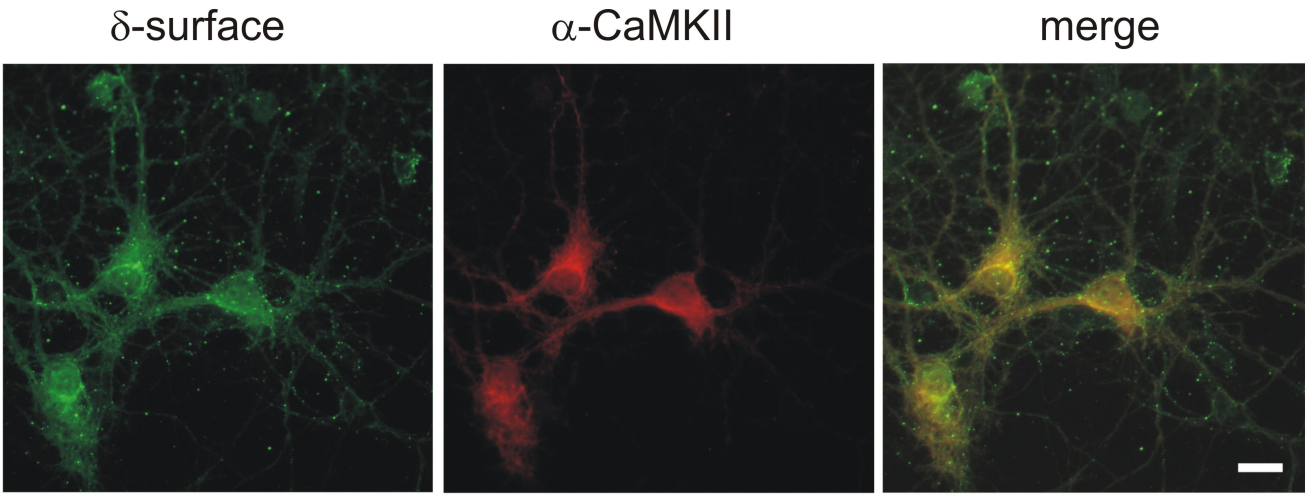
Figure 7. Differential internalization of $GABA_AR$ subunits demonstrated by cleavable biotinylation assay in cultured hippocampal neurons at 20 min after withdrawal from EtOH exposure compared to vehicle-treated controls (ctrl). **A,** The timeline for experimental protocol. **B,** Representative Western blots for total (tot) and internalized (int) $GABA_AR$ α4, δ, α5, and α1 subunits (n = 3-5) obtained at 20 min after EtOH exposure/withdrawal. β-actin was measured to demonstrate cell integrity and was only detectable in the total cell lysate. **C,** Quantification of the changes in the amount of internalized α4, δ, α5, and α1 subunits relative to control neurons (dashed line). Internalized protein amount is calculated by the optical density of each subunit divided by the respective total amount. Data are mean ± SEM of values (n = 3-5). *, $p < 0.05$ vs. control neurons (one-way ANOVA).

Molecular Pharmacology Fast Forward. Published on December 16, 2010 as DOI: 10.1124/mol.110.068650
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1. Time course of changes in mIPSC kinetics and I_{tonic} magnitudes after EtOH exposure/withdrawal. Data (mean ± SEM) are obtained from control neurons and neurons (n = 10-24) at 1, 4, 12, and 24 h after exposure to 30 min of 60 mM EtOH. *, $p < 0.05$ vs. control neurons (one-way ANOVA).

	Frequency (HZ)	mIPSC area (fC)	Rise time (ms)	Decay time (ms)	I_{tonic} (pA)
Control	2.2 ± 0.2	2067 ± 128	2.8 ± 0.2	13.2 ± 0.8	14.0 ± 1.0
EtOH 1 h	2.2 ± 0.2	1974 ± 116	2.3 ± 0.3	13.3 ± 0.5	7.7 ± 1.4*
EtOH 4 h	1.2 ± 0.2*	1322 ± 92*	2.4 ± 0.3	12.7 ± 0.5	5.7 ± 0.9*
EtOH 12 h	1.5 ± 0.3	1786 ± 90	1.3 ± 0.1*	7.0 ± 0.5*	5.8 ± 1.2*
EtOH 24 h	1.9 ± 0.3	1854 ± 180	1.8 ± 0.2*	7.5 ± 0.6*	7.6 ± 1.4*

Figure 1


δ-surface
α-CaMKII
merge

B

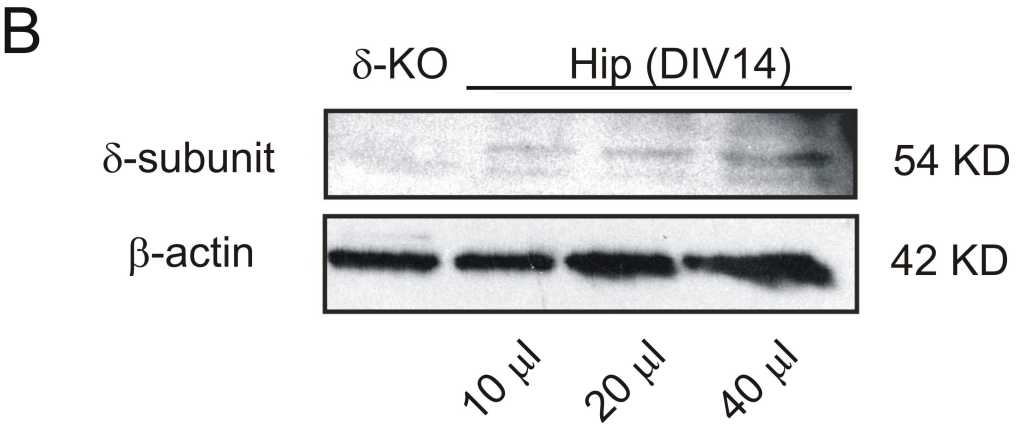

δ-KO
Hip (DIV14)
δ-subunit
β-actin
54 KD
42 KD
10 μl
20 μl
40 μl

Figure 2

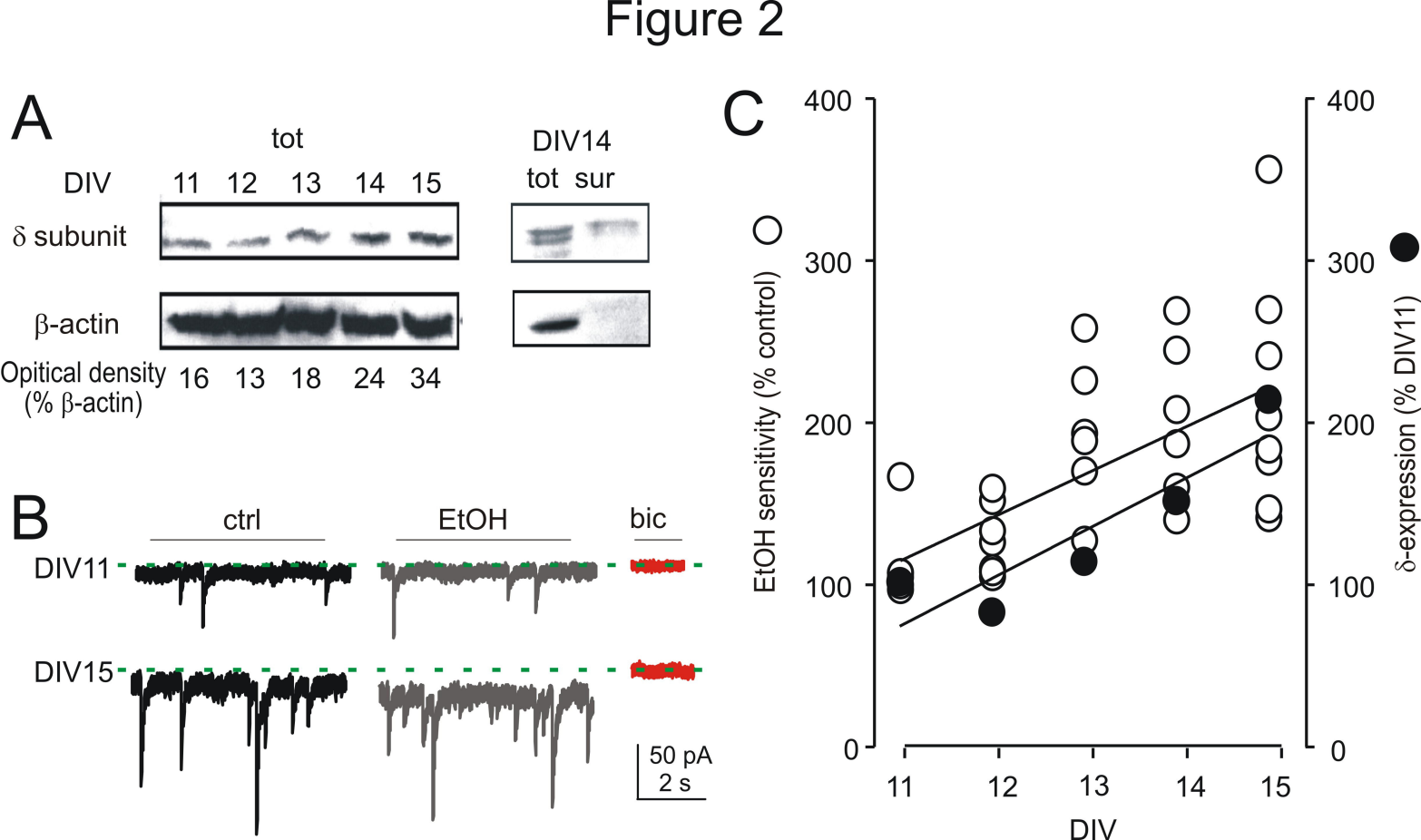

A
tot
DIV14
DIV 11 12 13 14 15
tot sur
δ subunit
β-actin
Optical density (% β-actin) 16 13 18 24 34
B
ctrl
EtOH
bic
DIV11
DIV15
50 pA
2 s
C
EtOH sensitivity (% control)
δ-expression (% DIV11)
400
300
200
100
0
11 12 13 14 15
DIV

Figure 3

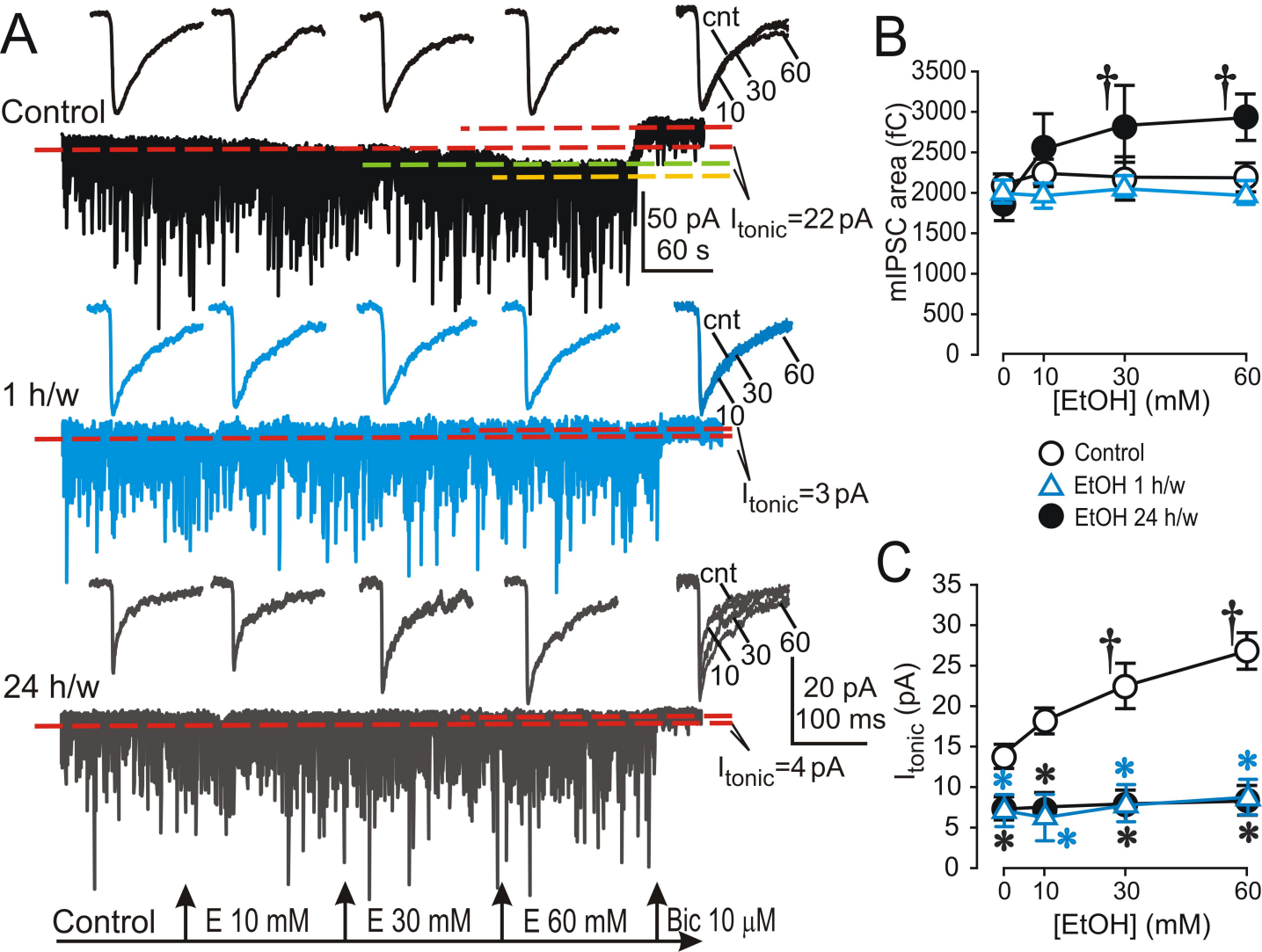

A
Control
cnt
10
30
60
50 pA
60 s
I tonic=22 pA
1 h/w
cnt
10
30
60
I tonic=3 pA
24 h/w
cnt
10
30
60
20 pA
100 ms
I tonic=4 pA
Control
E 10 mM
E 30 mM
E 60 mM
Bic 10 μM
B
mIPSC area (fC)
3500
3000
2500
2000
1500
1000
500
0
0
10
30
60
[EtOH] (mM)
Control
EtOH 1 h/w
EtOH 24 h/w
C
I tonic (pA)
35
30
25
20
15
10
5
0
0
10
30
60
[EtOH] (mM)

Figure 4

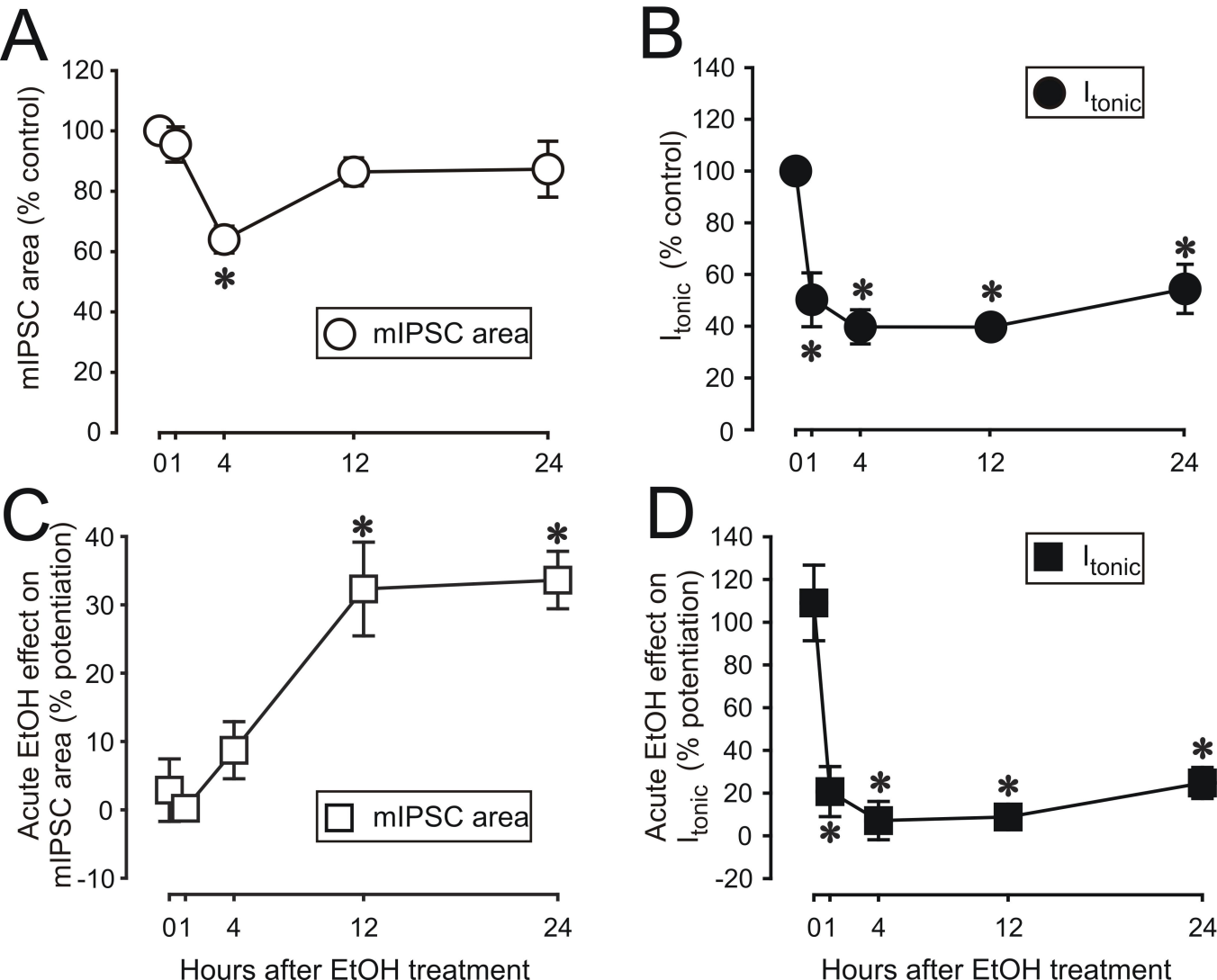

A
mIPSC area (% control)
120
100
80
60
40
20
0
mIPSC area
01
4
12
24
B
I_{tonic} (% control)
140
120
100
80
60
40
20
0
I_{tonic}
01
4
12
24
C
Acute EtOH effect on mIPSC area (% potentiation)
40
30
20
10
0
-10
mIPSC area
01
4
12
24
Hours after EtOH treatment
D
Acute EtOH effect on I_{tonic} (% potentiation)
140
120
100
80
60
40
20
0
-20
I_{tonic}
01
4
12
24
Hours after EtOH treatment

Figure 5

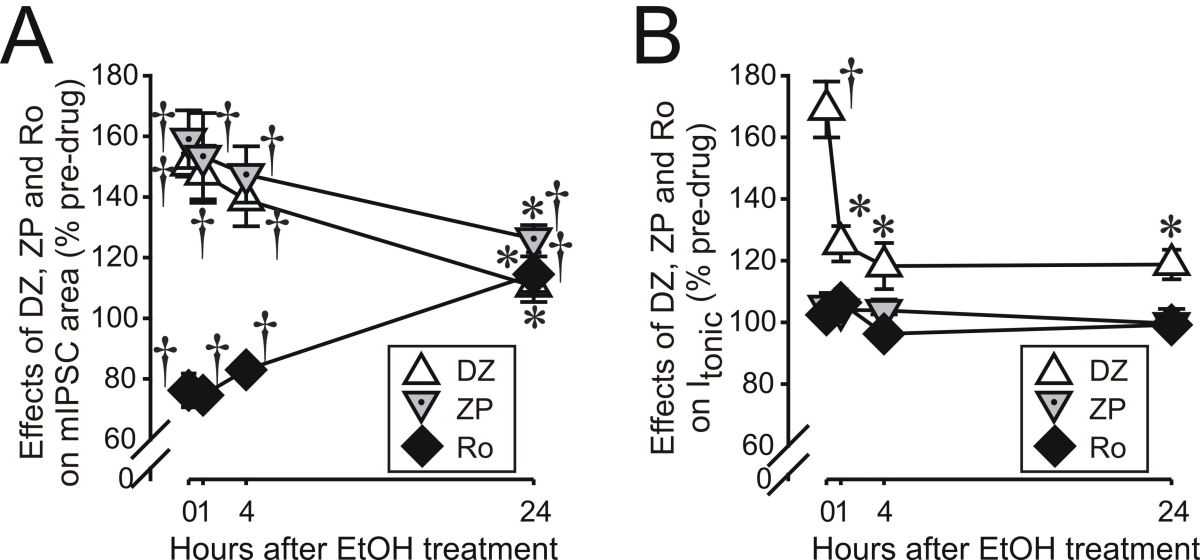

A
B
Effects of DZ, ZP and Ro on mIPSC area (% pre-drug)
Effects of DZ, ZP and Ro on I_{tonic} (% pre-drug)
180
160
140
120
100
80
60
0
01
4
24
Hours after EtOH treatment
DZ
ZP
Ro

Figure 6

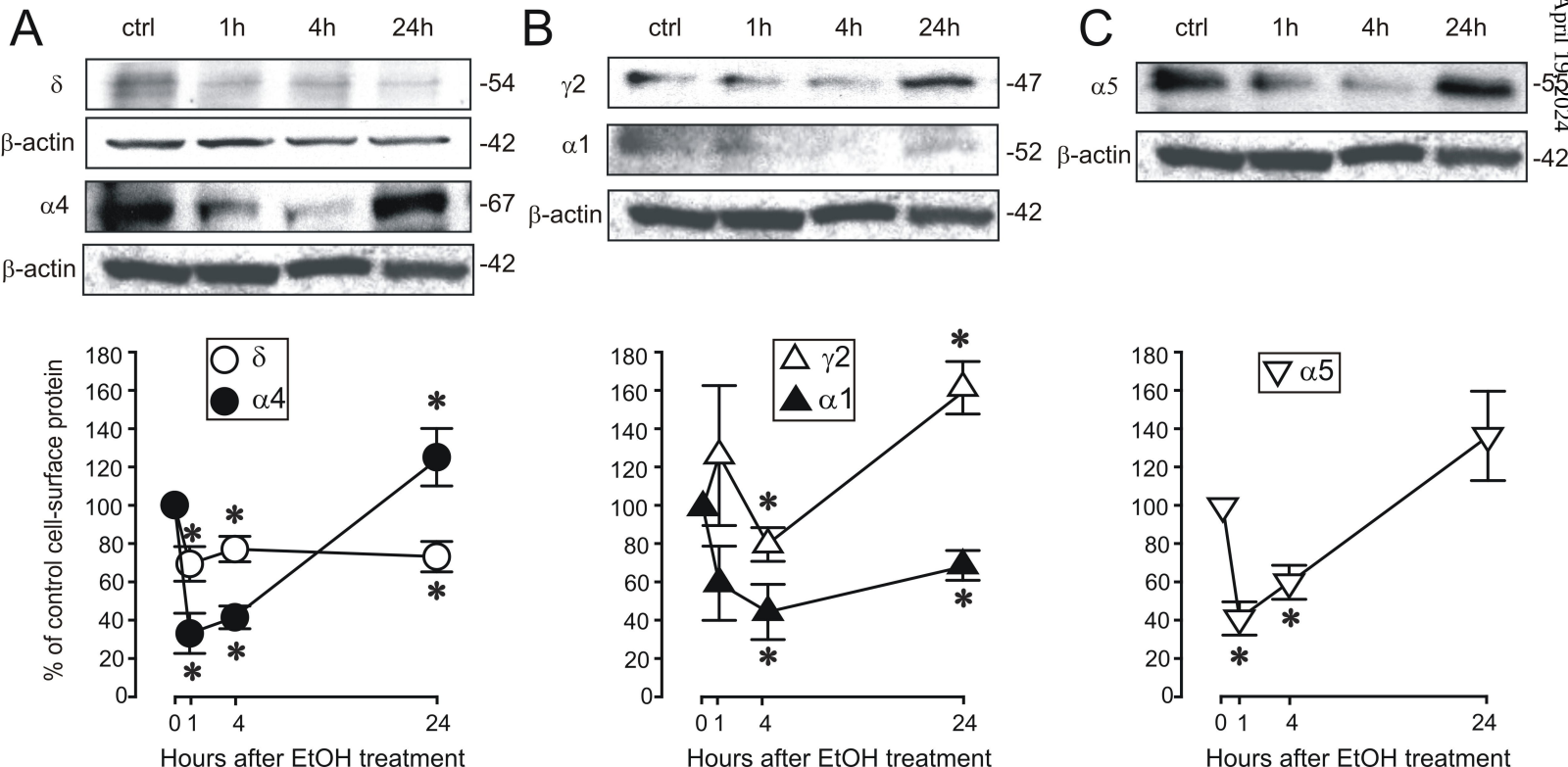


Figure 7