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Plasticity of $GABA_A$ Receptors after Ethanol Pre-Exposure in Cultured Hippocampal Neurons

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ABSTRACT

Alcohol use causes many physiological changes in brain with behavioral sequelae. We previously observed (*J Neurosci* **27:** 12367–12377, 2007) plastic changes in hippocampal slice 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 to 15 days in vitro demonstrated $GABA_A$R δ subunit expression and sensitivity to enhancement by short-term exposure to 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 short-term 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 short-term EtOH; in addition, $GABA_A$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 and reduced α5-containing $GABA_A$Rs at 1 h, followed by a larger reduction of normally synaptic α1 subunit at 4 h, and then 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.

Introduction

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

Rapid inhibitory synaptic transmission is mediated by γ2 subunit-containing $GABA_A$R subtypes, whereas tonic inhibition primarily depends on extrasynaptic δ subunit-containing $GABA_A$Rs (Farrant and Nusser, 2005). The δ-containing $GABA_A$Rs have unusual properties, such as high affinity but low efficacy for GABA, slow desensitization kinetics, benzodiazepine insensitivity, and high sensitivity for EtOH in both recombinant expression cells (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003) and brain slices (Wei et al., 2004; Hanchar et al., 2005; Liang et al., 2006). Several groups have observed enhancement of inhibitory synaptic transmission in

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ABBREVIATIONS: $GABA_A$R, GABA type A receptor; EtOH, ethanol; CA, cornu ammonis; DIV, days in vitro; I_{tonic}, tonic current; mIPSCs, miniature inhibitory postsynaptic currents; PBS, phosphate-buffered saline; ANOVA, analysis of variance.

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.

Previous reports have shown that $GABA_AR$ function and expression are altered after long-term administration of EtOH in vivo and in vitro, including hippocampus (Mhatre and Ticku, 1992; Kang et al., 1998; Kumar et al., 2009). 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 $\alpha4$ 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). Long-term EtOH exposure decreases $GABA_AR$ $\alpha1$ and δ subunit expression and increases the $\alpha4$, $\gamma1$, and $\gamma2$ subunits (Cagetti et al., 2003; Marutha 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 $GABA_ARs$ and slower decrease of surface synaptic $GABA_ARs$, followed by increased protein expression of hippocampal $\alpha4$ and $\gamma2$ $GABA_AR$ 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, has 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 we grew the embryonic cells for enough time in culture [embryonic day 18 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 in vitro exposure of primary cultured neurons to a drug of abuse 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 versus extrasynaptic positioning for $GABA_ARs$, after one brief exposure to an intoxicating dose of EtOH. Furthermore, 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 $\alpha4\beta\delta$-type $GABA_AR$ 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, Carlsbad, CA) and B27 supplement as reported previously (Stowell and Craig, 1999). In brief, 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 Hanks' buffered salt solution, pH 7.45. Tissues were dissociated by papain digestion followed by trituration through a Pasteur pipette and papain 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, St. Louis, MO).

Dissociated cells were then plated at a density of 0.3×10^5 cells/cm^2 onto 12-mm round coverslips in 24-well plates (for patch-clamp recording) and/or at a density of 0.5×10^5 cells/cm^2 in six-well plates (for Western blot and biotinylation assays) coated with poly-D-lysine (50 μg/ml; Sigma). Cultures were kept at 37°C in a 5% CO_2 humidified incubator. Thereafter, one third to half of the medium was replaced twice per 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 (DIV 13–14) was replaced with Neurobasal culture medium containing 120 mM EtOH (final EtOH concentration, 60 mM) for 30 min, and then the entire medium was 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 to 3 h and induced significant plasticity in $GABA_ARs$ and drug tolerance (Liang et al., 2007). This dose models levels observed in human binge drinking and also that used in our long-term studies on a rat model of alcoholism, chronic intermittent ethanol, in which rats receive a daily intermittent regimen of intoxication and 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% (w/v) (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 three times with PBS, the coverslips were mounted on slides with ProLong Gold antifade reagent with 4′,6-diamidino-2-phenylindole (Invitrogen) to estimate total cell number. Propidium iodide- and 4′,6-diamidino-2-phenylindole-positive cells were counted and their ratio in percentage 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 40× objective (with numerical aperture 0.75) of a fluorescent microscope (BX60; Olympus, Tokyo, Japan).

Immunocytochemistry. Cultured hippocampal neurons (DIV 14) 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 subunits, unspecific binding was blocked under nonpermeabilizing conditions with PBS containing 4% normal goat serum (Vector Laboratories, Burlingame, CA) 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) (Thermo Fisher Scientific, Waltham, MA) 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 (Invitrogen) were used as secondary antibodies. Olympus fluorescent microscope BX60 with a digital camera (Hamamatsu Corporation, Bridgewater, NJ) was used for image, and Wasabi software (version 1.5; Hamamatsu) 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, Hamden, CT) and visualized with an inverted microscope (TE200; Nikon, Tokyo, Japan). 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 137 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$,1 mM $MgCl_2$, 20 mM glucose, and 10 mM HEPES (310–320 mOsM, pH adjusted to 7.40 with NaOH). Glass pipettes were filled with internal solution containing 137 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM EGTA, 10 mM HEPES, and 3 mM ATP (290–300 mOsM, pH adjusted to 7.30 with CsOH), with an input resistance of 4 to 7 MΩ. All reagents were from Sigma-Aldrich unless specified otherwise. $GABA_AR$-mediated miniature inhibitory postsynaptic currents (mIPSCs) were pharmacologically isolated by adding tetrodotoxin (0.5 μM), D(−)-2-amino-5-phosphonopentanoate (40 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (10 μM), and [*S*-(*R**,*R**)]-3-[[1-(3,4-dichlorophenyl)ethyl]amino]-2-hydroxypropyl](cyclohexylmethyl) phosphinic acid [CGP54626; 1 μM ($GABA_B$ receptor antagonist)] to the extracellular solution from stock solutions. Stock solutions of CGP54626 were made with pure dimethyl sulfoxide. Final concentrations of dimethyl sulfoxide 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, Inc., Berkeley, CA). The flow rate of the perfusion system was approximately 0.3 to 0.5 ml/min, and the total volume of the recording chamber was 3 ml. Electrical signals were amplified using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). After establishing the whole-cell configuration, at least 5 to 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), digitized at 20 kHz (Digidata 1440A; Molecular Devices), and analyzed using the Clampfit software (version 10.2; Molecular Devices) and the Mini Analysis Program (version 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). In brief, the recordings were low-pass-filtered off-line (Clampfit software) at 2 kHz. The mIPSCs were detected off-line using the Mini Analysis Program. 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 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 $GABA_ARs$, 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 (DIV 14) and hippocampi from $GABA_AR$ δ subunit knockout mice (UCLA colony, following Mihalek et al., 1999) were lysed in lysis buffer containing 1% Triton X-100, 0.1% 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, and Complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). The lysate was centrifuged for 15 min (14,000*g*, 4°C), and the supernatant was collected for Western blot analysis. Protein concentrations were determined with BCA Protein Assay Kit (Pierce Protein Research Products, Rockford, IL) according to the manufacturer's instructions.

Biotinylation Assay for Cell-Surface Receptors. Cultured hippocampal neurons (DIV 14) 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 [sulfosuccinimidyl-6-(biotinamido) hexanoate; ProteoChem, Denver, CO]. After rinsing with Tris-buffered saline to quench the biotin reaction, neurons were lysed in 150 μl of modified lysis buffer (see *Western Blot*). The homogenates were centrifuged for 15 min (14,000*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 Protein Research Products) 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, Inc., Littleton, CO), 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, Medical University Vienna, Vienna, Austria) and mouse anti-β-actin (Sigma) followed by horseradish peroxidase-conjugated secondary antibodies. Bands were detected using Enhanced Chemiluminescence detection kit (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) 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 [sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate; Pierce Protein Research Products] 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 after 20 min withdrawal at 37°C (the same treatment protocol as mentioned under *EtOH or Vehicle Exposure*). 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, and 10% fetal calf serum, pH 8.5–9.0). After washing with PBS, cells were lysed with PBS containing Complete protease inhibitor (Roche Applied Science), 0.5 mM phenylmethylsulfonyl fluoride, 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 Protein Research Products) as 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 horseradish peroxidase-conjugated secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA).

Statistical Analysis. Data were from three to five independent preparations of neuron cultures. Sigmaplot (Windows version 10.1; Systat Software, Inc., San Jose, CA), SigmaStat (Windows version 3.5; Systat Software, Inc.), and SAS (version 9.2; SAS Institute, Cary, NC) were used for data display and statistical analysis. Data were expressed as mean ± S.E.M., and all values included in the statistics represent data from individual 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 whether EtOH exposure and withdrawal induces cell death, we compared, with the use of propidium iodide fluorescence, the proportion of dead neurons found in control cultures with those exposed to 60 mM EtOH for 30 min and withdrawn for 24 h. 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$_A$R δ Subunit Expression in Cultured Hippocampal Neurons. We previously reported that the δ subunit is a critical component of GABA$_A$R-mediated extrasynaptic inhibition, which is sensitive to EtOH (Hanchar et al., 2005; Liang et al., 2006). 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 ≥13) cultured hippocampal neurons. Results from both immunostaining and Western blot showed GABA$_A$R δ subunit expression in cultured hippocampal neurons at DIV 14 (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 that the cells in these cultures expressed the GABA$_A$R 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).

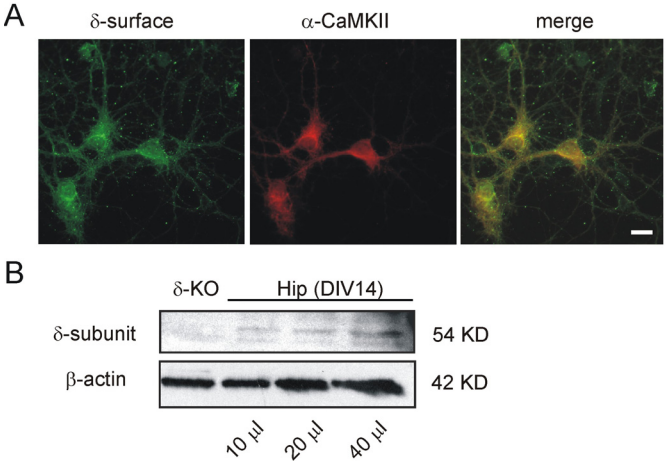

Fig. 1. GABA$_A$R δ subunit is expressed in cultured hippocampal neurons at DIV 14. A, immunocytochemistry on nonpermeabilized neurons shows δ expression on cell surface (green) of α-CaMKII-positive neurons (red). The merged pictures are shown on the right. Scale bar, 10 μm. B, Western blot of cell lysate from cultured hippocampal neurons (hip) shows δ expression [40 μg of protein/lane (10 μl), 80 μg/lane (20 μl), and 160 μg/lane (40 μl)]. Hippocampus of δ knockout (KO) mouse (40 μg of protein/lane) is used as a negative control. β-Actin is used as a loading control.

EtOH Sensitivity of GABA$_A$R-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 DIV 11 to 15 in the presence of tetrodotoxin (0.5 μM), D(−)-2-amino-5-phosphonopentanoate (40 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (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$_A$R-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 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 DIV 11, neurons showed very small total charge transfer of mIPSCs, I_{tonic} magnitudes, and no significant enhancement by short-term EtOH (60 mM) of I_{tonic} (potentiated by 10.8 ± 10.7%; there were hardly any cell responses to short-term EtOH; for example, only one of nine total neurons showed potentiation by EtOH). At DIV 15, mature neurons showed much larger total charge transfer of mIPSCs and I_{tonic} magnitudes, and significant enhancement by short-term EtOH on I_{tonic} (potentiated 116.5 ± 25.5%, more than twice the control current, with seven of eight neurons sensitive to EtOH). Between DIV 11 and 15, I_{tonic} from these neurons showed sensitivity to EtOH gradually increasing with age, whereas total charge transfer of mIPSCs in those neurons was not significantly potentiated by short-term EtOH. The δ subunit total expression measured by Western blot over time was also increased during neuronal development (Fig. 2A, left). In addition, biotinylation results showed clearly that δ subunit expression occurs at the cell surface in mature neurons (DIV 14) (Fig. 2A, right). 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 short-term EtOH are increased in parallel with the development of cultured hippocampal neurons, which show higher δ subunit expression and higher short-term EtOH sensitivity as mature neurons at DIV 14 to 15.

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 a short-

term 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 short-term EtOH application to untreated neurons (Fig. 3, A and C), and virtually no enhancement (30–60 mM) of mIPSCs (Fig. 3, A and B) compared with no EtOH. Approximately 75% of the neurons recorded (n = 25) showed this EtOH enhancement, consistent with variable gene expression typical of cultured neurons at DIV 14 to 15. However, when short-term 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. 3, A and 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. 3, A and 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). Short-term 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).

To compare the EtOH effects on total charge transfer of mIPSCs and tonic current, we further calculated the changes

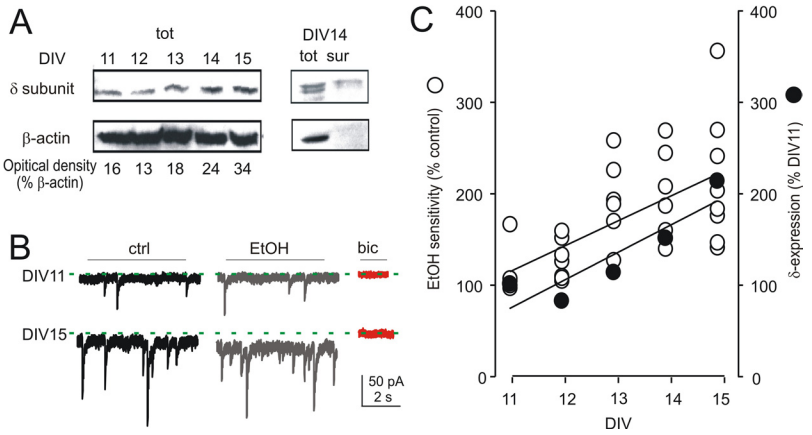

Fig. 2. Parallel increases in short-term EtOH sensitivity and $GABA_AR$ δ subunit expression during development in primary cultured hippocampal neurons (hip DIV 11–15). A, left, representative Western blot of δ total expression (tot) in hip neurons during development. Total δ amount is calculated by the optical density of δ subunit signal divided by corresponding optical density of β-actin (% β-actin). Right, biotinylation assay and Western blot of total (tot) and surface (sur) δ expression from neurons at DIV 14. B, sample traces of individual cultured hippocampal neuron recordings at DIV 11 (top, from the same neuron) and DIV 15 (bottom, from the same neuron). Data are obtained under basal conditions and after short-term 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). DIV 15 cells show larger I_{tonic} and greater EtOH enhancement. C, increased short-term EtOH sensitivity of I_{tonic} and δ subunit expression in neurons during development. Total δ expression is normalized as percentage of DIV 11 δ values. The black and gray solid lines are the linear regression fits of all data points in each experiment, respectively. The slopes of fitting curves of I_{tonic} and δ expression are 26.65 and 30.79, respectively ($p > 0.05$ for nonidentity, linear regression).

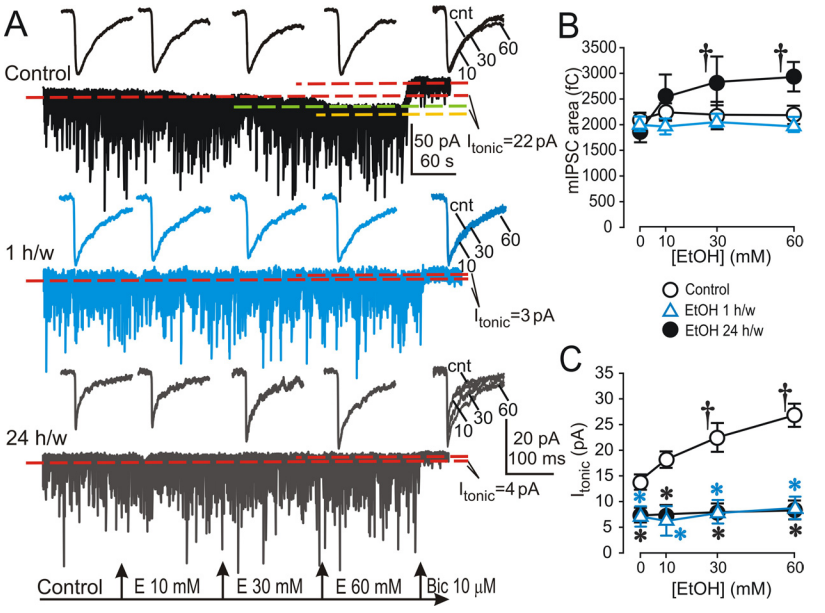

Fig. 3. Plasticity of $GABA_AR$-mIPSCs and I_{tonic} in primary cultured hippocampal neurons (DIV 14–15) after pre-exposure to EtOH. A, sample traces of individual recordings from a control neuron (top), a neuron obtained at 1 h after 30 min incubation with 60 mM EtOH (EtOH 1 h/w, middle trace), and 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 short-term EtOH 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). ○, control; △, 1 h/w; and ●, 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 ± S.E.M. value. ∗, $p < 0.05$ versus control neurons; †, $p < 0.05$ versus pre-EtOH value (two-way repeated measures ANOVA).

of total charge transfer of mIPSCs and I_{tonic} in 100-s 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), whereas 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 short-term 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 short-term 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 short-term 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. 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 short-term 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$_A$R-Mediated Currents Induced by Withdrawal from EtOH Exposure. It is known that GABA$_A$Rs with different subunit compositions have distinct pharmacological properties; for example, diazepam has no agonist activity at α4-containing GABA$_A$Rs but potentiates other $\alpha x\beta\gamma 2$, x = 1,2,3,5 GABA$_A$Rs (Rudolph et al., 2001), whereas zolpidem is an allosteric positive modulator of GABA$_A$R function with selectivity for the α1-containing GABA$_A$Rs, with intermediate potency on α2/3-containing GABA$_A$Rs and no effect on α5-containing GABA$_A$Rs (Olsen and Sieghart, 2008). To provide additional evidence in support of the hypothesis that GABA$_A$R subunit alterations mediate the switch in responsiveness of synaptic

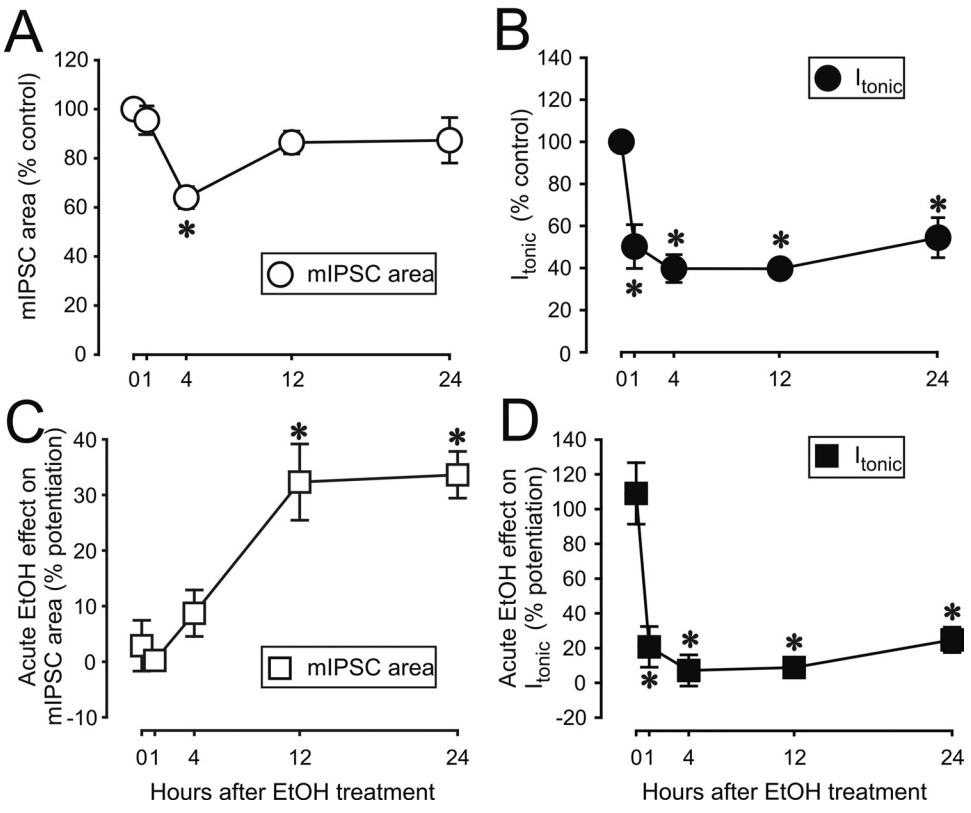

Fig. 4. Time course of changes in total charge transfer of mIPSCs and I_{tonic} magnitude as well as short-term EtOH sensitivity in primary cultured hippocampal neurons (DIV 14–15) at various times after 30-min incubation with 60 mM EtOH. A, changes in total charge transfer of mIPSCs recorded in control 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, short-term 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, short-term EtOH sensitivity (60 mM) in I_{tonic} magnitude after EtOH withdrawal from the same recordings as in C (n = 9–24). Data are mean ± S.E.M. of values. *, $p < 0.05$ versus control neurons (one-way ANOVA).

and extrasynaptic $GABA_A$Rs to short-term EtOH after withdrawal from 30 min of 60 mM 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). Short-term 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). Likewise, 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). It is noteworthy that 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 change transfer of mIPSCs, causing 23.8 ± 5.3, 25.4 ± 3.7, and 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). In addition, 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 after 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 with 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 coassembles 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., 2002). 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; 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 with control (Fig. 6B). These data are consistent with previous studies (Liang et al., 2004, 2006, 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, because α1βxγ2 $GABA_A$R 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 $GABA_A$Rs 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,

TABLE 1

Time course of changes in mIPSC kinetics and I_{tonic} magnitudes after EtOH exposure/withdrawal

Data (mean ± S.E.M.) 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.

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

* $p < 0.05$ vs. control neurons (one-way ANOVA).

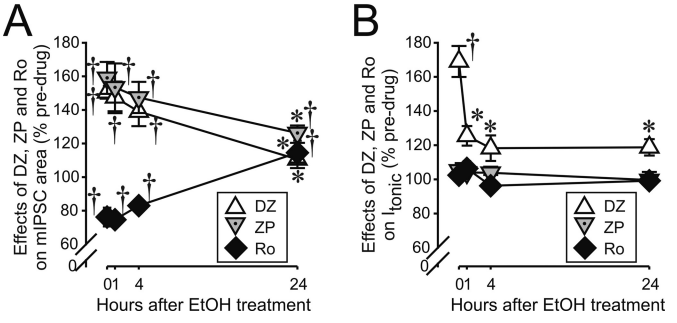

Fig. 5. Time course of changes in effects of diazepam (DZ, 0.3 μM), zolpidem (ZP, 0.3 μM), and Ro15-4513 (Ro, 0.3 μM) on total charge transfer of mIPSCs and I_{tonic} magnitude in primary cultured hippocampal neurons (DIV 14–15) at various times after withdrawal from EtOH exposure. A, DZ, ZP, and Ro effects on total charge transfer of mIPSCs (percentage of 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). *, $p < 0.05$ versus control neurons; †, $p < 0.05$ versus pre-EtOH value (two-way repeated measures ANOVA).

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$_A$R α4 and δ Subunits after EtOH Exposure. We hypothesized that the decreased magnitude of I_{tonic} and tolerance to short-term EtOH enhancement at 1 h after EtOH exposure are most likely due to α4βδ GABA$_A$R overactivation followed by internalization. However, a decrease in cell-surface expression of α4βδ GABA$_A$Rs 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 tested whether the alterations in surface GABA$_A$Rs 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 with 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 and 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$_A$Rs.

Discussion

A Single Pre-Exposure to EtOH Alters GABA$_A$R 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$_A$R 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$_A$R-mediated mIPSCs and extrasynaptic GABA$_A$R-mediated I_{tonic} currents, consistent with previous literature (Craig et al., 1994; Mangan et al., 2005). Such studies showed that there are sufficient glutamate decarboxylase-positive interneurons in the cultures to not only produce inhibitory synapses on the principal cells but produce sufficient GABA in the extracellular space to allow detection of these GABA$_A$R-mediated currents. Second, our tonic inhibitory currents at DIV 14 were significantly enhanced by short-term application of modest 30 to 60 mM EtOH. Furthermore, the results show that within 1 h after withdrawal from EtOH exposure (60 mM, 30 min), the extrasynaptic GABA$_A$R-mediated I_{tonic} and its potentiation by short-term EtOH is significantly reduced. At 4 h, there is a reduction of synaptic GABA$_A$R-mediated mIPSCs, followed at 24 h after EtOH exposure by restored mIPSCs, but with altered pharmacological responsiveness to modulators including EtOH, diaze-

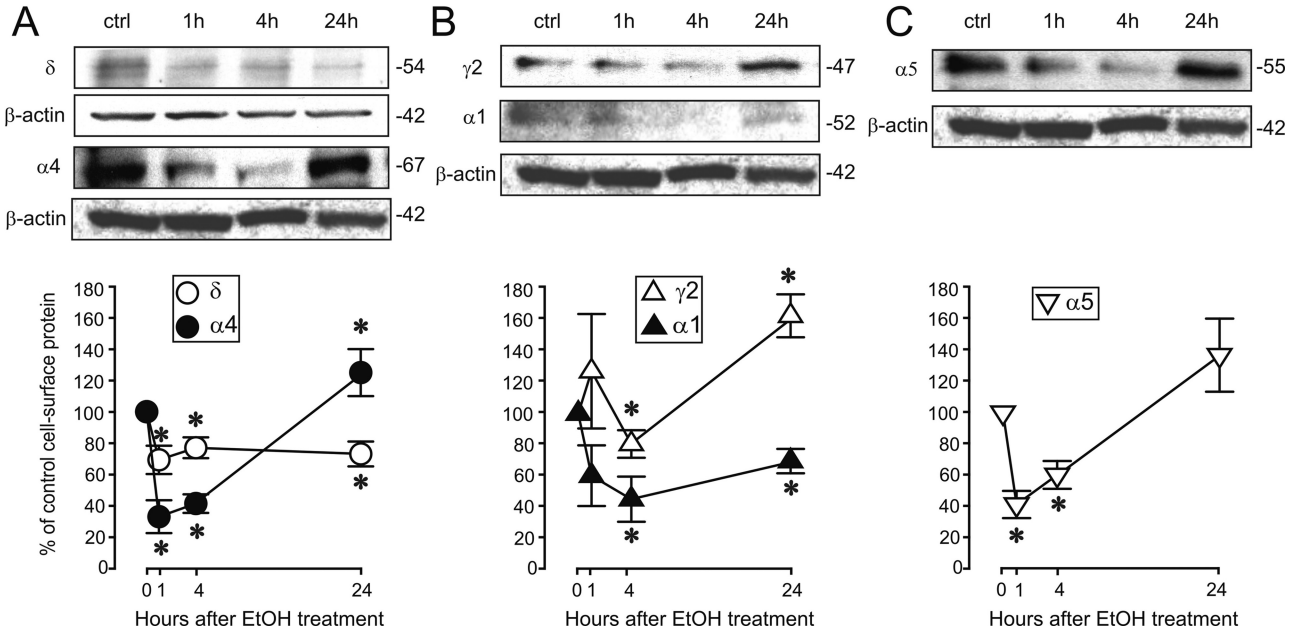

Fig. 6. Time course of changes in cell-surface expression of GABA$_A$R subunits after withdrawal from EtOH exposure measured by biotinylation assay in cultured hippocampal neurons. A, top, representative Western blot of the biotinylation assay for δ (54 kDa) and α4 (67 kDa) subunit cell-surface expression obtained at 1, 4, and 24 h after respective treatments compared with vehicle-treated controls (ctrl). β-Actin (42 kDa) of total cell lysate is used as a loading control. Bottom, 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 signal divided by optical density of the corresponding β-actin signal of the total cell lysate (percentage of β-actin) and compared with control, which is set at 100%. B, top, cell-surface expressed γ2 (47 kDa) and α1 (52 kDa) subunits. Bottom, quantification of changes in cell-surface expressed γ2 and α1 subunits. C, top, cell-surface expressed α5 (55 kDa) subunit. Bottom, quantification of changes in cell-surface expressed α5 subunit. Data are mean ± S.E.M. (n = 3–5). Note that the representative bands of α1, α4, α5, and γ2 are from the same Western blot experiment. *, $p < 0.05$ versus control neurons (one-way ANOVA).

pam, zolpidem, and Ro15-4513. It is noteworthy that as found in hippocampal slices from EtOH-exposed rats, the rapid reduction in I_{tonic} magnitude and development of tolerance to short-term 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 $GABA_ARs$. In addition, at 24 h, significant up-regulation of total and cell-surface $\alpha4$ and $\gamma2$ subunits is observed, which seems 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

Tonic versus Phasic Inhibitory Currents, and Role of the δ Subunit. Tonic inhibitory currents are generated by extrasynaptic $GABA_ARs$, activated by ~0.2 to 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 $\alpha5\beta\gamma2$ $GABA_ARs$ in hippocampal CA1 pyramidal neurons (Caraiscos et al., 2004; Mangan et al., 2005) and by $\alpha4\beta\delta$ $GABA_ARs$ 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 $GABA_ARs$ 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 (DIV 14–15) as well as the other $GABA_AR$ subunits normally expressed in the hippocampus in situ (Sperk et al., 1997). Electrophysiology revealed I_{tonic} sensitivity to short-term EtOH increased in a parallel manner with increased δ subunit expression in embryonic cultured neurons during neuronal development (DIV 11–15). Biotinylation experiments indicate that this $GABA_AR$ 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 $GABA_ARs$. 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 $\alpha4\beta\delta$ $GABA_ARs$. In our recordings from neuronal cultures, I_{tonic} is consistently observed without manipulation of [GABA], without any drugs, or with application of short-term EtOH and other modulators, as in brain slices (Liang et al., 2006, 2007). Using biotinylation assay, we show significant reduction in cell-surface expression of δ, $\alpha4$, and $\alpha5$ 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 $\alpha4$ and δ, but not $\alpha5$ or $\alpha1$ 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 $\alpha4$ and δ subunits provides strong evidence that extrasynaptic $\alpha4\beta\delta$ receptors are involved in EtOH-induced $GABA_AR$ plasticity. The $\alpha4\beta\delta$ $GABA_ARs$ are particularly sensitive to EtOH (low millimolar) 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 $\alpha4$ subunit after EtOH can be masked by a fairly rapid compensatory increase in $\alpha4$ readily observed at later times (Mhatre and Ticku, 1992; Kumar et al., 2009), as also seen after long-term benzodiazepine (Follesa et al., 2001) and neurosteroid (Gulinello et al., 2001) administration/withdrawal. This striking plasticity of the $\alpha4$ subunit gene expression has been explained by demonstration that exposure to EtOH (10–60 mM) in cultured cortical neurons for 1 to 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 $\alpha1$ expression has been observed after EtOH (Kumar et al.,

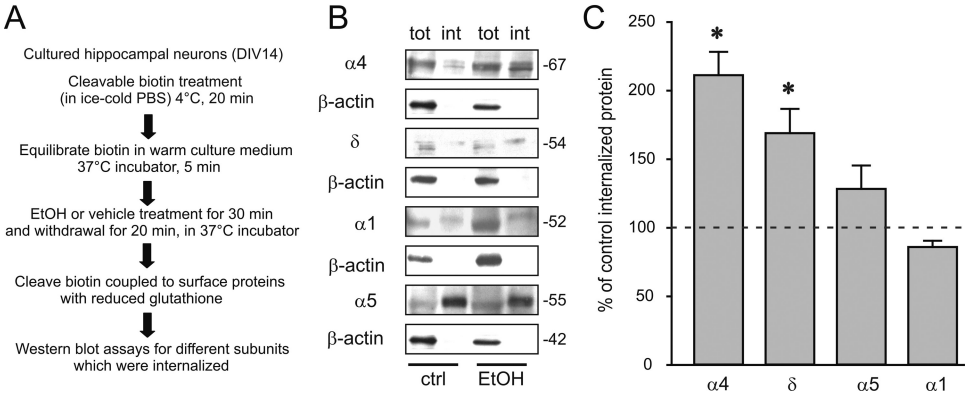

Fig. 7. Differential internalization of $GABA_AR$ subunits demonstrated by cleavable biotinylation assay in cultured hippocampal neurons at 20 min after withdrawal from EtOH exposure compared with vehicle-treated controls (ctrl). A, the timeline for experimental protocol. B, representative Western blots for total (tot) and internalized (int) $GABA_AR$ $\alpha4$, δ, $\alpha5$, and $\alpha1$ 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 $\alpha4$, δ, $\alpha5$, and $\alpha1$ subunits relative to control neurons (dashed line). The optical density of each subunit divided by the respective total amount equals the internalized protein amount. Data are mean ± S.E.M. of values (n = 3–5). *, $p < 0.05$ versus control neurons (one-way ANOVA).

2010), as has a drop in δ subunit (Marutha Ravindran et al., 2007), although these reports were not as tightly linked to electrophysiological and behavioral changes as ours (Liang et al., 2006, 2007, 2009).

Basal I_{tonic} is potentiated by diazepam but insensitive to zolpidem, suggesting a contribution from extrasynaptic α5-containing GABA$_A$Rs. In addition to tolerance to short-term EtOH, the potentiation of I_{tonic} by diazepam is also reduced at 1 h after EtOH exposure, 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. In addition, cell-surface α5 returns to control levels at 24 h after EtOH (Fig. 6C), whereas I_{tonic} recovers only slightly. Furthermore, 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 to 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 their pharmacology and probably not their subunit composition. By 12 to 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 suggest, not unexpectedly, that the mechanisms of response to EtOH are different. mISPC kinetics were unchanged after withdrawal of 1 to 4 h, but at 12 to 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, 2006).

"Subunit Switches" and Importance of Receptor Subunit Localization to Function: EtOH-Induced GABA$_A$R Plasticity Involves Trafficking and Protein Synthesis

The EtOH-induced alterations of GABA$_A$R functions in cultured neurons, as 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). Long-term ethanol (or benzodiazepine) 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).

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Authorship Contributions

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

Conducted experiments: Shen and Lindemeyer.

Contributed new reagents or analytic tools: Sieghart.

Performed data analysis: Shen, Lindemeyer, and Liang.

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