$\alpha 2$ Subunit-Containing GABA_A Receptor Subtypes Are Upregulated and Contribute to Alcohol-Induced Functional Plasticity in the Rat Hippocampus

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ABSTRACT

Alcohol (EtOH) intoxication causes changes in the rodent brain γ -aminobutyric acid receptor (GABA_AR) subunit composition and function, playing a crucial role in EtOH withdrawal symptoms and dependence. Building evidence indicates that withdrawal from acute EtOH and chronic intermittent EtOH (CIE) results in decreased EtOH-enhanced GABA_AR δ subunit-containing extrasynaptic and EtOH-insensitive $\alpha 1\beta\gamma 2$ subtype synaptic GABA_ARs but increased synaptic $\alpha 4\beta\gamma 2$ subtype, and increased EtOH sensitivity of GABA_AR miniature postsynaptic currents (mIPSCs) correlated with EtOH dependence. Here we demonstrate that after acute EtOH intoxication and CIE, upregulation of hippocampal $\alpha 4\beta\gamma 2$ subtypes, as well as increased cell-surface levels of GABA_AR $\alpha 2$ and $\gamma 1$ subunits, along with increased $\alpha 2\beta 1\gamma 1$ GABA_AR pentamers in hippocampal slices using cell-surface cross-linking, followed by Western blot and coimmunoprecipitation. One-dose and two-dose acute EtOH treatments

produced temporal plastic changes in EtOH-induced anxiolysis or withdrawal anxiety, and the presence or absence of EtOH-sensitive synaptic currents correlated with cell surface peptide levels of both $\alpha 4$ and $\gamma 1$ (new $\alpha 2$) subunits. CIE increased the abundance of novel mIPSC patterns differing in activation/deactivation kinetics, charge transfer, and sensitivity to EtOH. The different mIPSC patterns in CIE could be correlated with upregulated highly EtOH-sensitive $\alpha 2\beta\gamma$ subtypes and EtOH-sensitive $\alpha 4\beta\gamma 2$ subtypes. Naïve $\alpha 4$ subunit knockout mice express EtOH-sensitive mIPSCs in hippocampal slices, correlating with upregulated GABA_AR $\alpha 2$ (and not $\alpha 4$) subunits. Consistent with $\alpha 2$, $\beta 1$, and $\gamma 1$ subunits genetically linked to alcoholism in humans, our findings indicate that these new $\alpha 2$ -containing synaptic GABA_ARs could mediate the maintained anxiolytic response to EtOH in dependent individuals, rat or human, contributing to elevated EtOH consumption.

Introduction

γ -Aminobutyric acid type A receptors (GABA_ARs) are major targets of ethanol (EtOH) action in the brain. GABA_ARs are heteromeric chloride channels of the Cys-loop pentameric ligand-gated ion channel family that mediate most of the inhibitory neurotransmission in the mammalian central nervous system. More than 20 GABA_AR subunit combinations have been identified, derived from a family of 19 subunit genes divided into eight classes according to sequence homology: $\alpha 1$ -6, $\beta 1$ -3, $\gamma 1$ -3, δ , ϵ , θ , π , and $\rho 1$ -3 (Olsen and Sieghart, 2008), many found organized in clusters on human chromosomes 4 ($\alpha 4$, $\beta 1$, $\alpha 2$, $\gamma 1$), 5 ($\alpha 6$, $\beta 2$, $\alpha 1$, $\gamma 2$), 15 ($\beta 3$, $\alpha 5$, $\gamma 3$), and X (θ , $\alpha 3$, ϵ) (McLean et al., 1995; Simon et al., 2004). In the forebrain,

typically, 2α and 2β are present with either one γ or one δ subunit in each pentamer.

According to the subunits present, distinct GABA_ARs have different pharmacology, channel kinetics, and topography, with resulting functional and behavioral consequences (Puia et al., 1991; Whiting et al., 1999; Olsen and Sieghart, 2008). Primarily in combination with $\alpha 2$ or $\alpha 1$, $\gamma 2$ -containing subtypes cluster to a greater extent at postsynaptic sites, interacting with the inhibitory postsynaptic scaffold protein gephyrin (Essrich et al., 1998). The presence of the δ subunit confers extrasynaptic localization (Nusser et al., 1998), producing EtOH sensitivity of the tonic inhibitory current (I_{tonic}). We and others have shown that low millimolar EtOH enhances δ -GABA_AR tonic inhibition in neurons (Hancher et al., 2005; Liang et al., 2006; Mody et al., 2007) and in recombinant systems (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). This EtOH enhancement in slices is interpreted by some as indirect action on GABA release (Borghese et al., 2006; Weiner and Valenzuela, 2006; Lovinger

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ABBREVIATIONS: ACSF, artificial cerebrospinal fluid; CIE, chronic intermittent ethanol; CIV, chronic intermittent vehicle; co-IP, coimmunoprecipitation; DG, dentate gyrus; DGC, dentate gyrus granule cell; EPM, elevated plus maze; EtOH, ethanol; GABA, γ -aminobutyric acid; GABA_AR, γ -aminobutyric acid type A receptor (ionotropic); MANOVA, multivariate analysis of variance; mIPSC, miniature inhibitory postsynaptic current; WT, wild-type.

and Homanics, 2007). GABA_ARs and their EtOH-induced plastic changes have long been implicated in EtOH's acute effects and dependence (Follesa et al., 2004; Kumar et al., 2009; Olsen and Spigelman, 2012). Moreover, chronic intermittent ethanol (CIE) administration to rodents produces long-lasting alterations in hippocampal GABA_AR subunit expression at cell surfaces and corresponding changes in GABA_AR-mediated inhibitory synaptic and extrasynaptic tonic currents, pharmacology, and behavior (Cagetti et al., 2003; Liang et al., 2004, 2006). CIE administration results in a persistent decrease of $\alpha 1$ and δ and increased $\alpha 4$ and $\gamma 2$ subunits. The observed net subunit switch ($\alpha 1$ to $\alpha 4$ and δ to $\gamma 2$) suggests a decrease of mostly $\alpha 1\beta\gamma 2$ (synaptic) and $\alpha 4\beta\delta$ (extrasynaptic) GABA_ARs and an increase of $\alpha 4\beta\gamma 2$ GABA_ARs, including $\alpha 4$ appearing at central locations of symmetric synapses. I_{tonic} and its enhancement by EtOH are reduced, but the miniature inhibitory postsynaptic currents (mIPSCs) become sensitive to enhancement by EtOH (Liang et al., 2006). Accompanying behavioral alterations are consistent with alcohol dependence, including increased anxiety and seizure susceptibility and tolerance to the sedative-hypnotic, but not anxiolytic, effects of EtOH and other GABAergic modulatory sleep aids (Liang et al., 2009; Olsen and Spigelman, 2012). Rats administered intermittent regimens of EtOH have demonstrated increased EtOH preference in voluntary consumption assays (Rimondini et al., 2003; O'Dell et al., 2004; Simms et al., 2008). Similar, but transient, hippocampal changes in GABA_AR subunits and inhibitory currents can be observed immediately ($\alpha 4\beta\delta$ -receptor downregulation after 1 hour), continuing to 48 hours (delayed downregulation of $\alpha 1\beta\gamma 2$ -receptors and upregulation of $\alpha 4\beta\gamma 2$ -receptors) after one large dose of EtOH in vivo (Liang et al., 2007; Gonzalez et al., 2012). The GABA_AR $\alpha 4$ subunit is upregulated in numerous hyperexcitability animal models involving drug withdrawal and/or epilepsy (Schwarzer et al., 1997; Smith and Gong, 2005; Lagrange et al., 2007; Olsen and Spigelman, 2012).

After acute EtOH intoxication and CIE, we investigated: 1) possible increased partnerships between GABA_AR $\alpha 4$ and $\gamma 2$ subunits and whether other subtypes were altered and 2) which GABA_ARs mediate EtOH-sensitive mIPSCs in hippocampal slices from EtOH-treated rats. Previously, recombinant GABA_ARs with differing α subunits and native GABA_ARs in cells with dominant expression of specific α subunits have been distinguished on the basis of activation and inactivation rates, τ , in the order $\alpha 4 < \alpha 1 < \alpha 2$, and $\gamma 2 < \gamma 1$ (Lavoie et al., 1997; McClellan and Twyman, 1999; Smith and Gong, 2005; Liang et al., 2006; Dixon et al., 2014). We performed coimmunoprecipitation experiments to determine which GABA_AR subtypes were altered after EtOH intoxication. We then investigated the time course of plastic changes in EtOH-sensitive mIPSCs relative to subunit and behavioral changes by examining mIPSC recordings using new software for spontaneous synaptic current pattern recognition analysis. We interpret the EtOH-induced, upregulated, EtOH-sensitive GABA_ARs as likely candidates for a role in the anxiolytic response to EtOH, increased EtOH drinking in the dependent individual, and development of dependence.

Materials and Methods

Animals. All protocols followed National Institutes of Health guidelines and were approved by the University of California Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Harlan/Envigo, www.envigo.com), 200–250 g, were housed in the vivarium under a 12-hour light/dark cycle and had free access to food

and water. The $\alpha 4$ knockout and wild-type (WT) littermate control mice were produced from heterozygous breeding pairs on a C57BL/6J N7 genetic background at the University of California Los Angeles in an Association for Assessment and Accreditation of Laboratory Animal Care–approved facility. Mouse production and genotyping have been previously reported (Chandra et al., 2006). Only adult male (3 to 4 months old) mice were used in the experiments.

EtOH Treatment. To study acute EtOH effects, rats were administered a single dose of EtOH by gavage (5 g/kg of body weight), as a 25% (w/v) solution in water (Pharmco Products, Brookfield, CT). Control rats were underwent gavage with drinking water (20 ml/kg of body weight). For CIE treatment, rats underwent gavage with 5 g/kg, 25% (w/v) EtOH for the first five doses once every other day, followed by 55 doses of 6 g/kg EtOH 30%, once daily. This CIE paradigm led rats to experience multiple cycles of intoxication and withdrawal. Chronic intermittent vehicle (CIV) rats, as controls, underwent gavage with drinking water (20 ml/kg of body weight) in parallel (Liang et al., 2006). Rats were euthanized (1–48 hours after acute treatment or 40–60 days after CIV or CIE treatment, if not stated otherwise).

Measurement of Surface Receptor Subunit Expression by Cross-Linking. To measure cell surface protein levels of slices from in vivo treated rats, cross-linking experiments followed by Western blot analysis were performed, as described previously (Grosshans et al., 2002). Briefly, 400- μ m-thick coronal hippocampal slices were prepared using a vibrating blade microtome (VT1200S; Leica Microsystems, Bannockburn, IL), and the dentate gyrus (DG) and CA1 regions were microdissected. Slices were incubated either in ice-cold artificial cerebrospinal fluid (ACSF) composed of 124 mM NaCl, 3 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1 mM KH₂PO₄, 10 mM D-glucose, 26 mM NaHCO₃, and oxygenated with 95% O₂/5% CO₂, pH 7.4) or ACSF containing 1 mg/ml bis(sulfosuccinimidyl)suberate (BS³) (Pierce, Rockford, IL) at 4°C for 45 minutes. The cell-impermeable BS³ bifunctionally cross-links all surface proteins, leading to large-molecular-weight aggregates that do not reliably migrate in the gel. Therefore, only internal proteins show up in the gel of the BS³ sample. The cross-link reaction was quenched with 20 mM Tris wash buffer, pH 7.6, and the slices were homogenized in homogenizing buffer containing 10 mM Tris, pH 8.0, 1 mM EDTA, and 1% SDS. Protein concentrations were determined using the BCA Protein Assay Kit (Pierce).

Coimmunoprecipitation. Crude membrane proteins were obtained from freshly isolated DG and CA1 hippocampal regions. The tissue was homogenized at 4°C in homogenizing buffer containing 10 mM HEPES, pH 7.5, 1 mM EDTA, 300 mM sucrose, complete protease inhibitor (Roche, Basel, Switzerland), 0.5 mM PMSF, 5 mM sodium fluoride, and 5 mM sodium orthovanadate. The homogenate was centrifuged at 1000g for 10 minutes at 4°C to remove pelleted nuclear fraction. To obtain crude membrane fraction, the supernatant was centrifuged at 100,000g at 4°C for 30 minutes, and the pellet was resuspended in solubilization buffer containing 50 mM Tris, pH 8.0, 50 mM KCl, 1 mM EDTA, complete protease inhibitor (Roche), 0.5 mM PMSF, 5 mM sodium fluoride, 5 mM sodium orthovanadate, 1.0% Triton X-100, and 0.1% SDS for 30 minutes and was then cleared by ultracentrifugation (100,000g at 4°C for 30 minutes). Solubilized proteins were incubated with 10 μ g of the respective antibody (see following discussion) for 1 hour at 4°C. Then 50 μ l of protein G agarose (Sigma) was added overnight at 4°C. Precipitate was washed with washing buffer containing 20 mM Tris, pH 8.0, and 15% solubilization buffer. Precipitated proteins were eluted with Laemmli sample buffer (Bio-Rad). β -Mercaptoethanol was added after elution. A negative control with 10 μ g of normal rabbit IgG (Cell Signaling Technology, Danvers, MA) was performed in parallel for each experiment. For detection of low-affinity or transient bound interaction partners, slices were incubated in ACSF containing 2.5 mM 3,3'-dithiobis(sulfosuccinimidylpropionate) (Pierce), and 3 mM dithiobis(succinimidylpropionate) (Pierce) for 2 hours at 4°C to reversibly cross-link extracellular and intracellular proteins, respectively, before to cell lysis and coimmunoprecipitation (co-IP). The reaction was quenched with 50 mM Tris, pH 7.6, for 30 minutes at 4°C.

To reverse cross-linking, samples were boiled in 5% β -mercaptoethanol in SDS-PAGE sample buffer (Bio Rad) for 5 minutes at 95°C.

SDS-PAGE and Western Blot Analysis. Proteins were separated on SDS-PAGE gels (Bio-Rad, Hercules, CA) using the Bio-Rad Mini-Protein 3 cell system. Proteins were transferred on a polyvinylidene difluoride membrane (Bio-Rad) and blocked with 4% nonfat dry milk in TTBS. Blots were incubated overnight at 4°C with primary polyclonal rabbit antibodies against the following: $\alpha 1$ (NB 300-207; 1:1000; Novus Biologicals, Littleton, CO), $\alpha 3$ (1:500; Abcam, 72446), $\alpha 2$ (aa 416–424) (Zezula et al., 1991) [$\alpha 2$ (aa322–357) (Pörtl et al., 2003) was used for co-IP], $\alpha 4$ (aa379–421) (Ebert et al., 1996), $\alpha 5$ (aa 337–380) (Pörtl et al., 2003), $\beta 1$ (aa 350–404), $\beta 2$ (aa 351–405) (Jechlinger et al., 1998), $\beta 3$ (aa345–408) (Slany et al., 1995), $\gamma 1$ (aa 1–39) (Pörtl et al., 2003), $\gamma 2$ (aa 319–366) (Tretter et al., 1997), δ (aa 1–44) (Jones et al., 1997) (all at 1 μ g/ml, gift from W. Sieghart); gephyrin (TXsc-14003, 1:500; Santa Cruz, Dallas, TX), or mouse monoclonal β -actin (A2228; 1:1000; Sigma), followed by horseradish peroxidase-conjugated secondary antibodies (1:5000, Rockland Antibodies & Assays, Limerick, PA) or Clean-Blot IP Detection Reagent (Thermo Scientific, Somerset, NJ) for 2 hours at room temperature. Bands were detected using ECL detection kit (Amersham Pharmacia UK, Littlefont, UK) and exposed to X-ray films (MidSci, St. Louis, MO).

Electrophysiological Recordings. Whole-cell patch-clamp recordings were obtained from neurons located in the dentate granule cell layer in transverse slices (400 μ m thick) of dorsal hippocampus from rats or mice. The slices were perfused with ACSF ($34 \pm 0.5^\circ\text{C}$) equilibrated with carbogen of 95% O_2 /5% CO_2 to ensure adequate oxygenation of slices and a pH of 7.4. Patch pipettes were filled with solution containing (in mM) 135 cesium gluconate (or 135 CsCl), 2 MgCl_2 , 1 CaCl_2 , 11 EGTA, 10 HEPES, 2 ATP- Na_2 , and 0.2 GTP- Na_2 , pH adjusted to 7.25 with CsOH. GABA_A-mediated 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 CGP 54626 (1 μM) to the ACSF. Whole-cell currents were recorded (voltage clamp at 0 mV or –70 mV) with an amplifier (Axoclamp 2B; Molecular Devices, Union City, CA). Whole-cell access resistances were in the range of 10–25 M Ω and electrically compensated by $\sim 70\%$. During voltage-clamp recordings, access resistance was monitored by measuring the size of the capacitive transient in response to a 5-mV step command. Intracellular signals were low-pass-filtered at 3 kHz (Axoclamp 2B) and digitized at 20 kHz with Digidata 1200 and software Clampex (Molecular Devices).

Detection and General Analysis of mIPSCs and GABA_A-Mediated I_{tonic} . mIPSCs were detected with amplitude and area threshold criteria using MiniAnalysis Program (version 6.0.7; Synaptosoft Inc. Fort Lee, NJ). The frequency of mIPSCs was determined from all automatically detected events in a given 100-second recording period. For kinetic analysis, only single-event mIPSCs with a stable baseline, sharp rising phase, and exponential decay were used. mIPSCs were scaled averaged and aligned with half rise time. Decay time constants were obtained by fitting exponentials to the falling phase of the averaged mIPSCs. The GABA_A-mediated I_{tonic} magnitudes were obtained as the difference between the averaged baseline current of a given recording period minus the averaged baseline current in the presence of picrotoxin (100 μM , a GABA_A antagonist).

The mIPSC Pattern Recognition Analysis Method. An optimally scaled template algorithm (Clements and Bekkers, 1997) is implemented in DataView software (V9.3, WJ Heitler, University of St Andrews, St Andrews, Scotland). This method finds synaptic events of particular waveforms (shapes) within data trace records. We define a search template by taking an example of the desired mIPSC pattern from a current trace record or taking the average of similar mIPSCs. An acceptable error level is set, which is the degree of similarity an event must have to the templates to be included in the search results. The program searches through the record for similar waveform patterns. During the search, the software scales and offsets the template to find the best match in shape, but not necessarily in size or threshold, and then accepts the match if it falls within the specified

error tolerance. We performed pattern recognition (classification) on several data files with a given template.

Anxiety Assay. Anxiety associated with EtOH treatment was measured on an elevated plus maze (EPM) with four arms of 51-cm-long, 11.5-cm-wide arranged at right angles (Liang et al., 2004). Briefly, each animal was placed in the center of the maze facing an open arm and allowed to explore for 5 minutes. During this 5-minute test session, the animal's behavior was recorded on a camcorder. Numbers of arm entries and time spent in each arm per entry were analyzed. An increase in time (%) spent in the closed arms (time in open arms/total time in open and closed arms) in EPM indicates an enhanced anxiety level.

Data Analysis. Biochemistry protein signals were analyzed by densitometry using ImageQuant5.2 (Molecular Dynamics). For cross-linking experiments, the signal intensity of bands was normalized to the corresponding β -actin signal after background subtraction. Surface protein levels were calculated by subtraction of the internal protein signal from the respective total protein signal. Signals for co-IPs were calculated as percentage protein co-IP/protein IP. Sets of control and treatment experiments were always performed in parallel and run on the same gel. Subunit data were analyzed with multivariate analysis of variance (MANOVA) using Proc GLM of SAS program (V9.4; SAS Institute, Cary, NC). Wilks' λ value and its P value for every MANOVA family are reported. P values for comparison of treatment versus control groups were also calculated within MANOVA model. GABAergic currents recorded by whole-cell patch-clamp, behavioral data and subunit measurements for multiple time points were analyzed with one-way or two-way ANOVA, followed by post hoc multiple comparison based on Dunnett's method to determine significant levels between treatments and single controls or Holm-Sidak's method to determine significant levels among groups (pairwise) using SigmaStat (Systat Software Inc. San Jose, CA) or SAS (details in figure legends). $P < 0.05$ was considered statistically significant. All values are shown as mean $\pm$ S.E.M., with n representing the number of experiments using different sets of animals.

Results

EtOH Treatment of Rats Induces a Net Switch of GABA_AR Subtypes in the Hippocampal Formation.

First, changes in cell-surface expression of $\alpha 1$, $\alpha 4$, δ , and $\gamma 2$ GABA_AR subunits were measured 24 hours after rats were administered a single intoxicating dose of EtOH (EtOH 24 hours: 5 g/kg via gavage) using cross-linking experiments on microdissected slices of the DG (Fig. 1, A and B). Compared with vehicle-treated controls, surface $\alpha 1$ levels were decreased to $54.8\% \pm 8.7\%$ ($n = 5$) in DG. In contrast, the surface levels of the GABA_A $\alpha 4$ subunit in DG were increased to $214.6\% \pm 16.8\%$ ($n = 5$) compared with vehicle-treated controls (Fig. 1, A and B). Surface δ subunit expression in DG was reduced to $61.4\% \pm 6.4\%$ ($n = 5$), whereas surface $\gamma 2$ was increased to $246.1\% \pm 47.4\%$ ($n = 5$) of control (Fig. 1, A and B). Next, we investigated $\alpha 1$ and $\alpha 4$ surface expression in the CA1 hippocampal region 24 hours after EtOH administration (Fig. 1C). Compared with vehicle-treated rats, we found a decrease in the $\alpha 1$ subunit to $46.6\% \pm 7.6\%$ ($n = 5$) and an increase in $\alpha 4$ to $188.4\% \pm 28.6\%$ ($n = 5$) in CA1 (Fig. 1C). Surface expression of the δ subunit was decreased to $70.7\% \pm 3.3\%$ ($n = 5$), and the $\gamma 2$ subunit on the cell surface showed a $238.2\% \pm 25.5\%$ ($n = 5$) increase in the CA1 (Fig. 1C). These observations are consistent with what has been observed 48 hours after EtOH intoxication and ≥ 40 days after CIE treatment in CA1 and DG slices (Liang et al., 2007). They also agree with previous studies on cultured hippocampal neurons, where similar plastic changes in GABA_AR subunit surface levels were reported 24 hours after EtOH exposure (Shen et al., 2011).

Fig. 1. Acute and chronic EtOH treatments induce a long-term increase in the amount of $\alpha 4\gamma 2$ -containing GABA_ARs and a decrease in $\alpha 1\gamma 2$ - and $\alpha 4\delta$ -containing GABA_ARs in rat CA1 + DG. (A) Representative Western blots after cell-surface cross-linking show changes in GABA_AR $\alpha 1$ and $\alpha 4$ (same blot), left, and in δ and $\gamma 2$ subunits (same blot), right. (B) Quantification of the surface levels of $\alpha 1$, $\alpha 4$, δ , and $\gamma 2$ in the DG 24 hours after EtOH intoxication compared with their controls (ctrl). MANOVA analysis, Wilks' $\lambda = 0.0295$, $P = 0.0005$, $n = 5$. (C) Quantification of the surface levels of $\alpha 1$, $\alpha 4$, δ , and $\gamma 2$ in the CA1 24 hours after EtOH intoxication compared with their controls. MANOVA, Wilks' $\lambda = 0.0635$, $P = 0.0034$, $n = 5$. tot, amount of total protein; int, amount of internal protein; molecular weight is indicated in kDa. Note: Surface levels are calculated by subtracting amount of internal protein from amount of total protein; for details, see *Materials and Methods*. (D) Representative Western blots (WB) for GABA_AR $\alpha 4$, $\alpha 1$, and $\gamma 2$ subunits after co-IP with a $\gamma 2$ -specific antibody (left) and quantification (right) 24 hours after acute EtOH (E24h)/vehicle. MANOVA, Wilks' $\lambda = 0.217$, $P = 0.022$, $n = 4$. (E) Quantification of the increased association of $\alpha 4$ and the decreased association of $\alpha 1$ with the $\gamma 2$ subunit after CIE in DG and CA1. MANOVA, Wilks' $\lambda = 0.0837$, $P = 0.002$, $n = 4$. (F) Representative Western blots for GABA_AR $\gamma 2$, δ , and $\alpha 4$ subunits after co-IP with a $\alpha 4$ -specific antibody (left) and quantification (right) after CIV/CIE. MANOVA, Wilks' $\lambda = 0.392$, $P = 0.038$, $n = 5$. (G) Quantification of the increased association of $\gamma 2$ and the decreased association of δ with the $\alpha 4$ subunit 24 hours after one dose of EtOH treatment. Wilks' $\lambda = 0.130$, $P = 0.0062$, $n = 4$. Vehicle-treated controls are set as 1.0. Data are mean $\pm$ S.E.M. *Significant difference ($P < 0.05$, calculated within MANOVA model) between the treatment and the control.

To verify suggestions from earlier studies that EtOH induced a net switch from GABA_ARs composed of $\alpha 4\beta\delta$ and $\alpha 1\beta\gamma 2$ to $\alpha 4\beta\gamma 2$ -containing receptors, coimmunoprecipitation (co-IP) experiments were carried out on solubilized hippocampal membrane proteins from rats. Co-IP experiments using a $\gamma 2$ -specific antibody after one-dose EtOH showed $\alpha 4$ partnering with $\gamma 2$ was increased to $217.0\% \pm 32.4\%$ ($n = 4$), and $\alpha 1$ partnering with $\gamma 2$ was decreased to $50.6\% \pm 11.1\%$ ($n = 4$) (Fig. 1D). After CIE, $\alpha 4$ showed an increase in $\gamma 2$ co-IP to $173.7\% \pm 16.3\%$ ($n = 4$), whereas $\alpha 1$ and $\gamma 2$ co-IP was decreased to $52.6\% \pm 7.0\%$ ($n = 5$) (Fig. 1E). Next, we performed co-IPs with an antibody against the GABA_AR $\alpha 4$ subunit after CIE, revealing an increase in $\gamma 2$ partnering to $164.6\% \pm 21.5\%$ ($n = 5$); at the same time, association with the δ subunit was reduced to $54.1\% \pm 8.7\%$ ($n = 5$) compared with CIV-treated controls (Fig. 1F). Likewise, 24 hours after one-dose EtOH treatment, the $\alpha 4$ co-IP with the $\gamma 2$ subunit was elevated to $180.9\% \pm 32.0\%$ ($n = 4$) of vehicle-treated controls; partnering with δ was decreased to $57.9\% \pm 6.6\%$ ($n = 4$) (Fig. 1G).

Acute and Chronic EtOH Intoxication Increases Surface Levels of GABA_AR $\alpha 2$ and $\gamma 1$ Subunit Protein. After reports of GABA_AR subunit changes in the post-mortem brain of human alcoholics and animals treated with EtOH (Devaud et al., 1997; Cagetti et al., 2003; Follesa et al.,

2004; Jin et al., 2012), we asked whether we could also find changes in total and cell-surface expression of important GABA_AR subunits (Tretter et al., 2008) $\alpha 2$ and $\gamma 1$ in the rat hippocampus after CIE or a single dose of EtOH. Cross-linking experiments on slices from the DG 24 hours after a single dose of EtOH (5 g/kg) revealed increases in cell surface expression of $\alpha 2$ and $\gamma 1$ compared with controls (Fig. 2A). The amount of the $\alpha 2$ subunit on the cell surface was increased to $176.7\% \pm 22.9\%$ ($n = 5$), and the amount of the $\gamma 1$ subunit was increased to $213.4\% \pm 28.0\%$ ($n = 5$) in the plasma membrane of the DG (Fig. 2A). In the DG, the total protein level of the $\alpha 2$ subunit was unaltered 24 hours after EtOH ($94.0 \pm 14.1\%$, $n = 5$). The total amount of $\gamma 1$ protein was increased to $199.0 \pm 37.4\%$ ($n = 5$) in the DG (Fig. 2B). We next looked at the CA1 and found an increase in cell surface $\alpha 2$ to $240.4\% \pm 50.7\%$ ($n = 6$) and an increase in cell surface $\gamma 1$ to $242.9\% \pm 49.9\%$ ($n = 6$) (Fig. 2C). Total protein levels of $\alpha 2$ were not altered ($119.8\% \pm 8.8\%$, $n = 5$), and total $\gamma 1$ in CA1 was increased to 140.0 ± 11.3 ($n = 5$) (Fig. 2B). For CIE experiments, we did not separate the brain regions and used slices from the DG combined with the adjacent CA1. CIE treatment increased cell-surface $\alpha 2$ to $228.8\% \pm 40.4\%$ ($n = 5$) and $\gamma 1$ to $173.8\% \pm 19.2\%$ ($n = 5$) (Fig. 2E). Total $\alpha 2$ was not changed after CIE ($89.5\% \pm 17.6\%$, $n = 4$, $P = 0.665$), whereas total $\gamma 1$ was increased to $228.4\% \pm 48.0\%$ ($n = 4$) (Fig. 2F).

Fig. 2. Administration of acute and chronic EtOH induces an increase in GABA_A $\alpha 2$ and $\gamma 1$ subunit surface levels. (A) Representative Western blots for $\alpha 2$, $\gamma 1$, and β -actin after cell-surface cross-linking (left) and quantification (right) 24 hours after a single intoxicating dose of EtOH in the DG. MANOVA, Wilks' $\lambda = 0.226$, $P = 0.0055$, $n = 5$. tot, amount of total protein; int, amount of internal protein. (B) Quantification of the total amount of $\alpha 2$ and $\gamma 1$ protein measured in DG 24 hours after EtOH intoxication. Wilks' $\lambda = 0.554$, $P = 0.126$, $n = 5$. ctrl, control. (C) Representative Western blots for $\alpha 2$, $\gamma 1$, and β -actin after cell-surface cross-linking (left) and quantification (right) 24 hours after a single intoxicating dose of EtOH in the CA1. Wilks' $\lambda = 0.443$, $P = 0.0255$, $n = 6$. (D) Quantification of the total amount of $\alpha 2$ and $\gamma 1$ protein measured in CA1 24 hours after EtOH intoxication. Wilks' $\lambda = 0.478$, $P = 0.0754$, $n = 5$. (E) Representative Western blots for $\alpha 2$, $\gamma 1$, and β -actin after cell-surface cross-linking (left) and quantification (right) after CIE in the DG + CA1. Wilks' $\lambda = 0.246$, $P = 0.0073$, $n = 5$. (F) Quantification of the total amount of $\alpha 2$ and $\gamma 1$ protein measured after CIE in DG + CA1; tot, total amount of protein; int, intracellular protein content. Wilks' $\lambda = 0.457$, $P = 0.141$, $n = 4$. Controls are set as 1.0%. Data are mean $\pm$ S.E.M. *Significant difference ($P < 0.05$, calculated within the MANOVA model) between the treatment versus the control.

CIE and One-Dose EtOH Administration Upregulate GABA_ARs Composed of $\alpha 2\beta 1\gamma 1$ Subunits that Bind to Gephyrin. Co-IP experiments were performed to determine which subunits partner with $\gamma 1$. Therefore, $\gamma 1$ co-IPs were carried out in parallel with $\gamma 2$ co-IPs as a positive control. Western blots were probed for $\alpha 1$, $\alpha 2$, $\alpha 4$, and $\alpha 5$. In contrast to $\gamma 2$, which was found to associate with different α subunits, $\gamma 1$ primarily coassembled with the $\alpha 2$ subunit (Fig. 3A). The figure also shows that the $\gamma 1$ antibody did not co-IP $\gamma 2$ and vice versa. We next attempted to identify the preferred β subunit partner for the $\alpha 2\gamma 1$ -containing GABA_ARs. Membrane proteins were coimmunoprecipitated with $\beta 1$ -, $\beta 2$ -, or $\beta 3$ -specific antibodies and probed for $\gamma 1$ and $\gamma 2$. We found that $\gamma 1$ preferred to form a receptor complex with the $\beta 1$ subunit, although it was also found to a small extent with $\beta 3$. Moreover, as is illustrated in Fig. 3B, we found that the $\gamma 2$ equally partnered with $\beta 1$ and $\beta 3$ and somewhat less with $\beta 2$. These data identify GABA_ARs composed of $\alpha 2$, $\beta 1$, and $\gamma 1$ subunits in hippocampal CA1 and the DG regions, which are upregulated after CIE and single-dose EtOH intoxication. The selective partnering of $\gamma 1$ with $\alpha 2$ allows use of $\gamma 1$ as a marker for the upregulated pool of cell-surface $\alpha 2$ subunits.

The 93-kDa tubulin-binding protein gephyrin is a marker for GABAergic postsynaptic sites and is assumed to predominantly cluster GABA_A $\gamma 2$ subunit-containing receptors (Essrich et al., 1998); however, direct interaction is mediated by α subunits (i.e., $\alpha 1$, $\alpha 2$, $\alpha 3$) (Tretter et al., 2008; Saiepour

et al., 2010). Since $\gamma 1$ subunits co-IP with the gephyrin-binding $\alpha 2$ subunit (Fig. 3A), we tested the possibility that $\gamma 1$ -containing GABA_ARs might also bind to gephyrin and found that the hydrophobic interaction between gephyrin and $\alpha 2$ is sensitive to detergents (Tretter et al., 2008). Therefore, we performed co-IP experiments after reversibly cross-linking proteins that are in close vicinity in acute slices to retain unstable, weak interactions (see Materials and Methods). Western blotting with a gephyrin antibody gave the expected 93-kDa signal after using $\gamma 2$ antibody for co-IP, as well as for the $\gamma 1$ antibody, whereas the IgG control showed no band (Fig. 3C), suggesting at least some postsynaptic localization of $\gamma 1$ -containing receptors at inhibitory synapses.

Time-Dependent Changes of $\alpha 4$ - and $\alpha 2\gamma 1$ -Containing GABA_AR Subtypes Are Tightly Correlated with Upregulation and Downregulation of EtOH-Sensitive mIPSCs and Withdrawal Anxiety after One or Two Doses of EtOH. As presented already herein, two relatively rare GABA_AR subtypes, $\alpha 4\beta 2$ and $\alpha 2\beta 1\gamma 1$, were found to be upregulated in the hippocampus postsynaptic membrane after CIE and after a single dose of EtOH in rats. At the same time, mIPSCs became sensitive to enhancement by acute EtOH (50 mM) perfused into the recording chamber, exhibiting a prolonged decay time constant τ and/or an increase in area (charge transfer) of mIPSCs. EtOH-sensitive GABA_ARs are subject to rapid internalization after exposure to high EtOH concentrations (Liang et al., 2007; Shen et al., 2011;

Fig. 3. $\gamma 1$ -Receptors contain $\alpha 2$ and $\beta 1$ subunits and bind to gephyrin. Solubilized membrane proteins from DG+CA1 slices after CIE were co-immunoprecipitated with antibodies against A. $\gamma 1$, $\gamma 2$, or IgG and immunoblotted with antibodies against $\alpha 1$, $\alpha 2$, $\alpha 4$, $\alpha 5$, $\gamma 1$, and $\gamma 2$ or B. $\beta 1$, $\beta 2$, $\beta 3$, or IgG and immunoblotted with antibodies against $\gamma 1$, $\gamma 2$, $\beta 1$, $\beta 2$ and $\beta 3$ or C. $\gamma 1$, $\gamma 2$, and IgG and immunoblotted with antibodies against gephyrin, $\gamma 1$, and $\gamma 2$ on the same blot. For C, co-IP experiments were performed after membrane proteins were reversibly cross-linked in acute slices (see Material and Methods). Note, that the $\beta 1$ antibody gives a signal with $\beta 2$ and $\beta 3$; and $\beta 3$ with $\beta 1$.

Suryanarayanan et al., 2011; Gonzalez et al., 2012). To identify EtOH-sensitive synaptic GABA_AR dynamic changes after EtOH exposure, we gave rats a “two-pulse” EtOH treatment 48 hours apart (Fig. 4A). At 48 hours (designated 1E48h) after rats received the first EtOH dose (1E), rats received a second EtOH dose (2E) via gavage. GABAergic tonic currents (I_{tonic}) and mIPSCs were patch-clamp-recorded from dentate gyrus granule cells (DGCs) in hippocampal slices obtained from control rats and in rats 48 hours after the first EtOH dose (1E48h), as well as 1 hour (2E1h) and 48 hours (2E48h) after the second dose of EtOH. EtOH sensitivity of GABA_ARs in these four conditions was measured as the responses of I_{tonic} and mIPSCs to acute EtOH (50 mM, E50) perfusion in the recording chamber (Fig. 4A). In controls, the I_{tonic} in response to perfused EtOH was potentiated from 18.9 ± 0.5 to 33.4 ± 1.2 pA ($174.9\% \pm 6.2\%$ of pre-EtOH) but was not changed at 1E48h (E0: 4.0 ± 0.7 pA, E50: 4.3 ± 0.7 pA; $109.9\% \pm 11.7\%$ of E0), 2E1h (E0: 6.0 ± 0.6 pA, E50: 6.3 ± 0.6 pA; $105.7\% \pm 3.0\%$ of E0), and at 2E48h (E0: 7.1 ± 1.2 pA, E50: 7.9 ± 1.9 pA; $103.9\% \pm 9.5\%$ of pre-EtOH) (Fig. 4, B and C). By contrast, in controls, the mIPSC area (charge transfer) was unchanged by EtOH perfusion ($103.5\% \pm 1.3\%$ of E0) but was increased at 1E48h ($159.8\% \pm 12.0\%$ of E0), and at 2E48h ($172.5\% \pm 9.7\%$ of E0). Compared with the 1E48h and 2E48h groups, bath perfused EtOH at 2E1h did not change mIPSC area ($109.8\% \pm 1.7\%$ of pre-EtOH) (Fig. 4, B and C).

To relate the electrophysiology data to GABA_AR subunit composition, we next measured the cell-surface content of $\alpha 4$ (as a marker for $\alpha 4\beta\gamma 2$ receptors) and $\gamma 1$ (for $\alpha 2\beta 1\gamma 1$) subtypes at 1E48h, 2E1h, and 2E48h (Fig. 4D). At 2E1h, surface $\alpha 4$ -containing GABA_ARs were significantly decreased compared with 1E48h and 2E48h (Fig. 4E). At 2E1h, surface $\gamma 1$ -containing GABA_ARs also robustly decreased compared with 1E48h and 2E48h (Fig. 4E). This finding suggests that both receptor subtypes are affected and become internalized within 1 hour after a second intoxicating EtOH dose; however, this change is transient in that the subtypes reappear 48 hours later. Notably, their cell surface expression is both upregulated and downregulated in concert with the increased EtOH sensitivity of mIPSCs, suggesting that these GABA_AR subtypes might mediate EtOH sensitivity of mIPSCs.

Changes in anxiety-like behaviors have been previously linked to alcohol-induced changes in GABA_AR function (Liang et al., 2006). We measured basal anxiety with the elevated plus maze assay. Control rats (drinking water gavage) spent

$49.6\% \pm 2.3\%$ of total time in open arms versus $50.4\% \pm 2.3\%$ time in closed arms. The first EtOH dose increased the time spent in open arms to $88.9\% \pm 3.1\%$ of total time ($11.1 \pm 3.1\%$ time in closed arms) in the 1E1h group, but 1E48h induced a rebound increase in anxiety as the rat stayed in open arms $8.8\% \pm 3.8\%$ of total time ($91.2\% \pm 3.8\%$ in closed arms). This rebound increase in anxiety was alleviated by the second EtOH dose. The 2E1h group spent $63.3\% \pm 5.2\%$ of total time in open arms ($36.8\% \pm 5.3\%$ in closed arms), which was not statistically significant compared with $49.6\% \pm 2.3\%$ in open arms in control rats; however, 48 hours after the second EtOH dose, basal anxiety was again increased as the rat stayed $23.9\% \pm 6.2\%$ of total time in open arms ($76.6\% \pm 6.2\%$ in closed arms). Summary statistics of the EPM assays are shown in Fig. 4F. These results suggest that the anxiety levels at different time points after EtOH treatment change concurrently with the postsynaptic GABA_AR dynamic changes.

CIE Induces Upregulation of One or More GABA_AR Subtypes with Slow mIPSC Decay Kinetics. To better understand how changes in subunit combinations alter GABA_AR function and responsiveness to acute EtOH, we measured mIPSCs in DGCs from hippocampal slices of CIV (control) and CIE-treated rats. Recombinant GABA_ARs with differing α subunits, expressed as $\alpha\beta$ without γ (Mortensen and Smart, 2006) or with $\gamma 2$ (Lavoie et al., 1997; McClellan and Twyman, 1999; Vicini, 1999), could be distinguished from each other on the basis of activation and inactivation rates, $\alpha 1$ faster than $\alpha 2$, and can be detected in neurons by the peak shapes of their mIPSCs, which differ between and provide a “fingerprint” for individual α subunits, including $\alpha 2$. Recombinant $\alpha 4\beta 2\gamma 2$ have accelerated deactivation compared with their $\alpha 1$ or $\alpha 5$ counterparts, correlating with upregulated $\alpha 4$ subunit in a hyperexcitable model examining hippocampal slices in a neurosteroid-withdrawn rat (Smith and Gong, 2005). Also, the $\gamma 1$ subunit-containing receptors (especially with $\alpha 2$) exhibit slower activation and deactivation rates than the respective $\gamma 2$ -containing GABA_ARs expressed in engineered synapses, suggested to be due to a more diffuse clustering (Dixon et al., 2014). mIPSC rise time is sensitive to multiple spatial, physical variables related to synaptic transmission not due to receptor subunit composition (Capogna and Pearce, 2011); however, the decay time is less sensitive to these variables as they are rather random, and yet they are more sensitive to the nature of the postsynaptic receptor channels, such as rates of channel closing and agonist

Fig. 4. A second pulse of EtOH reveals further changes in GABA_AR physiology, pharmacology, and plasticity, correlated with anxiety-like behavior. (A) “Two-pulse” experimental protocol. Treatment regimen is indicated by black arrows. 1E: First EtOH oral gavage (5 g/kg); 2E: second EtOH gavage. Gray arrows show time points when patch-clamp recordings from DGCs in slices were performed. 1E1h: 1 hour after first EtOH gavage; 1E48h: 48 hours after first EtOH; 2E1h: 1 hour after second EtOH gavage; 2E48h: 48 hours after second EtOH. (B) Effects of acute EtOH (50 mM) applied in the recording chamber on I_{tonic} and mIPSCs. High chloride (135 mM CsCl, refer to *Materials and Methods* section) patch solution was used. The cells were whole-cell voltage-clamped at -70 mV. Gray dashed line is the basal I_{tonic} level after picrotoxin (PTX, 100 μ M) application. Representative recordings are shown (left) with the averaged mIPSCs (right) before (black trace) and during (gray trace) EtOH perfusion. ctrl, control (drinking water). (C) Summary of acute EtOH-induced changes in I_{tonic} and mIPSCs. Gray dashed line represents E0 (set as 100%). * $P < 0.05$, significant difference between EtOH treatment versus control in I_{tonic} ; #, $P < 0.05$, significant difference between EtOH treatment versus control in mIPSC area ($n = 5$ or 6/group), one-way ANOVA followed by Dunnett’s multiple comparison method comparing the four treatment groups with a single control. (D) Representative Western blots for GABA_AR $\alpha 4$ and $\gamma 1$ subunits with their β -actin signals (all from the same blot) after cell-surface cross-linking from control (ctrl), 1E48h, 2E1h, and 2E48h. tot, amount of total protein; int, amount of internal protein. (E) Quantification of surface levels measured by cross-linking experiments. Data are mean $\pm$ S.E.M., $n = 4$ –10 rats. * $P < 0.05$ significant decrease versus 1E48h and 2E48h, one-way ANOVA, followed by Holm-Sidak’s (pairwise) multiple comparisons test. (F) Anxiety assayed by elevated plus maze (EPM, $n = 6$ /group). Open bars are the time rats spent in open arms (% of total 5-minute monitor time); solid bars are the time spent in closed arms in respective treatment groups. * $P < 0.05$; time spent in open arm of EtOH treatment groups versus control group (drinking water), one-way ANOVA followed by Dunnett’s multiple comparison method comparing the four treatment groups with a single control. Note that % time spent in the closed arms is complementary to that in the open arms. Significant % time increase in open arms is equivalent to significant % time decrease in closed arms. Therefore, we omitted statistical tests on time spent in closed arms.

dissociation (Otis and Mody, 1992). Nevertheless, mIPSC shape is highly sensitive to both synaptically released peak GABA concentrations and durations (Lagrange et al., 2007); but according to Kerti-Szigeti and Nusser (2016), “[The] differential expression of GABA_AR α subtypes with either a variable or constant ratio from synapse-to-synapse and cell-to-cell, allows them to fulfil individual cellular requirements in network dynamics.”

We observed that mIPSCs exhibited a few relatively consistent waveform patterns from the recordings of hippocampal DGCs after CIV and CIE treatments (Fig. 5A). We used the optimally scaled template method (Clements and Bekkers, 1997), implemented in DataView software, to identify kinetic patterns of mIPSCs (e.g., fast rise and fast decay, slow rise and slow decay). Then we used these identified patterns as templates to detect differently shaped mIPSCs in the recording traces. An acceptable error level was set, which is the degree of similarity an event must have to the templates to be included in the search results. The detected mIPSC peak patterns were averaged, mIPSC patterns were classified (Fig.

5B, a–d) and their kinetic decay constants τ determined, and the percentage of abundance was counted (Table 1).

In CIV animals, three distinct mIPSC waveform patterns were detected (Fig. 5B): one standard pattern (a, abundance, $\sim 48\%$) and the other two displaying a slower decay pattern (c, $\sim 37\%$ and d, $\sim 16\%$). In CIE animals, we also found three distinct mIPSC shape patterns: a “fast” decay pattern (peak pattern b, $\sim 42\%$) and two apparently similar to CIV patterns with a slow decay (peak pattern c, $\sim 22\%$), and a very slow decay pattern (peak pattern d, $\sim 36\%$) (see Table 1). The standard peak pattern a seen in CIV had disappeared in CIE, whereas the ratio of c to d had reversed, from $\sim 2:1$ (CIV) to $\sim 3:1$ (CIE). Pattern d had clearly increased in abundance, and pattern c had decreased (see statistics in Table 1).

To better understand the different patterns of peaks possibly carried by particular GABA_AR subtypes, we extended this analysis to genetically engineered $\alpha 4$ KO mice (Fig. 5). The patterns of mIPSCs in WT mice, untreated pattern a, abundance 46%; pattern c, abundance 36%; pattern d, 18% (Table 1) are similar to CIV rats, whereas $\alpha 4$ KO mice showed

Fig. 5. Pattern analysis reveals different kinetic patterns of mIPSCs after CIE versus CIV and $\alpha 4$ KO versus WT. (A) Sample recordings of mIPSCs from DGCs. (B) Classifications of patterns of mIPSCs. a, b, c, and d: Averaged mIPSC patterns during recordings before (solid line) and after (dotted line) EtOH application on the hippocampal slice. All averaged mIPSC event patterns were detected by templates from recordings from CIV and CIE rats or $\alpha 4$ KO and WT mice using the optimally scaled template algorithm in software DataView (V9.3). The event no. ratios (abundance) of classified mIPSC patterns are shown in Table 1. The scale is for all panels. (C) Responses of mIPSCs charge transfer (area) to acute EtOH application in DGCs. Averaged mIPSC area (mean $\pm$ S.E.M.) of different patterns before (empty columns) and after (solid columns) EtOH application. CIV (control); $\alpha 4$ KO: $\alpha 4$ subunit knockout mice. E0: without EtOH; E50, in the presence of 50 mM EtOH. $*P < 0.05$, the area of a pattern in the presence of 50 mM EtOH is statistically different from that of 0 EtOH (two-way repeated measures ANOVA followed by Sidak's method for pairwise multiple comparisons). $n = 5$ neurons from three rats/group. $n = 4$ neurons from two mice/group.

mIPSC waveform pattern a (abundance $\sim 36\%$, Table 1), pattern c ($\sim 35\%$), with increased abundance of pattern d ($\sim 29\%$), and no pattern b (Fig. 5). The absence of pattern b is consistent with the absence of the $\alpha 4$ subtypes.

EtOH (50 mM) perfused into the recording chamber potentiated mIPSCs by prolonging decay time and/or increasing charge transfer (area under the curve), as previously observed (Liang et al., 2006) for CIE rats. We therefore examined whether EtOH (50 mM) application enhanced the current of the various types of mIPSCs detected (Fig. 5B, C). Table 2 shows the decay τ values for the various mIPSCs patterns in the presence and absence of EtOH (50 mM) and the statistical comparisons. We found that acute EtOH potentiated some specific GABA_AR mIPSCs. In CIE rats, waveform pattern b, decay τ_1 (in ms) increased from 4.0 ± 0.3 to 8.0 ± 0.3 ; decay τ_2 increased from 12.9 ± 2.2 to 26.9 ± 4.3 ; and pattern c, decay τ_1 increased from 14.6 ± 0.7 to 17.2 ± 0.3 ; pattern d, only one

decay time constant τ increased from 17.4 ± 0.7 to 19.5 ± 0.4 . EtOH had no effect on decay times in control CIV rats except pattern c, decay τ_1 increased slightly. Similarly, EtOH had no effect on decay times in control WT mice. In $\alpha 4$ KO mice, waveform pattern a, decay τ_1 increased from 7.1 ± 0.4 to 8.2 ± 0.5 ; pattern c, decay τ_1 increased from 13.8 ± 0.9 to 16.2 ± 1.1 ; pattern d, τ increased from 16.6 ± 1.1 to 18.4 ± 0.8 . The area of the mIPSCs increased greatly in CIE pattern d (Fig. 5), as did its abundance (Table 1). This in vitro sensitivity to EtOH modulation correlated in time with the upregulation and downregulation of the $\alpha 4$ - and especially the $\alpha 2$ -containing GABA_AR subtype species (Fig. 4). Figure 6 shows a reasonable hypothesis of GABA_AR plasticity induced by EtOH in rat hippocampus (updated from Liang et al., 2007), which shows how synaptic and extrasynaptic GABA_AR subtypes change rapidly in surface expression after in vivo exposure to EtOH and that the plastic changes become persistent after CIE

TABLE 1

Characterization of mIPSC waveforms and decay times in DG granule cells from hippocampal slices

Numbers are mean $\pm$ S.E.M. $\tau 1$ and $\tau 2$ are time constants when the decay of mIPSCs was fitted with two exponentials. Decay of mIPSCs in pattern d was fitted well with only one exponential. Abd (abundance): percentage of mIPSC numbers of a specific pattern (a, b, c, or d) versus total number of mIPSCs (patterns a + b + c + d), analyzed from a neuron.

	Pattern a Abd			Pattern b			Pattern c			Pattern d	
	$\tau 1$ (ms)	$\tau 2$ (ms)	Abd (%)	$\tau 1$ (ms)	$\tau 2$ (ms)	Abd (%)	$\tau 1$ (ms)	$\tau 2$ (ms)	Abd (%)	τ (ms)	Abd (%)
CIV	7.8 $\pm$ 0.4	32.0 $\pm$ 11.1	47.8 $\pm$ 2.6				14.1 $\pm$ 1.2	54.9 $\pm$ 11.3	36.6 $\pm$ 1.9	16.9 $\pm$ 0.6	15.6 $\pm$ 1.9
CIE				4.0 $\pm$ 0.3	12.9 $\pm$ 2.2	42.6 $\pm$ 0.9	14.6 $\pm$ 0.7	70.4 $\pm$ 9.2	21.8 $\pm$ 1.8*	17.4 $\pm$ 0.7	35.6 $\pm$ 2.4*
WT	7.6 $\pm$ 0.5	37.1 $\pm$ 10.5	46.2 $\pm$ 2.2				13.7 $\pm$ 0.6	35.9 $\pm$ 8.9	36.3 $\pm$ 1.9	17.0 $\pm$ 0.5	17.5 $\pm$ 0.7
$\alpha 4$ KO	7.1 $\pm$ 0.4	28.0 $\pm$ 8.6	35.5 $\pm$ 1.9†				13.8 $\pm$ 0.9	35.4 $\pm$ 8.6	35.3 $\pm$ 2.9	16.6 $\pm$ 1.1	29.2 $\pm$ 1.1†

† $P < 0.05$, Abd of a pattern in $\alpha 4$ KO group statistically different from that of WT group (one-way ANOVA followed by Sidak method for pairwise multiple comparisons).

*Abd of every pattern is not statistically significant between CIV and WT group. $n = 6$ neurons from three rats/group, $n = 5$ neurons from two mice/group.

treatment. Note that in this simplified cartoon we have grouped all the game players in a single synapse, which is not likely to be the actual situation.

Discussion

We have shown that two novel GABA_AR subtypes are upregulated after acute EtOH treatment and CIE. Cell-surface levels of both subtypes are tightly synchronized over one- or two-dose EtOH administration, with changes in anxiety behavior and the abundance of EtOH-enhanced mIPSCs. We directly related changes in surface expression of GABA_AR subunits (downregulation of $\alpha 1$ and δ , upregulation of $\alpha 4$, $\alpha 2$, $\gamma 1$, and $\gamma 2$) with a decrease in heteropentameric $\alpha 4\beta\delta$ - and $\alpha 1\beta\gamma 2$ -containing GABA_ARs and an increase in postsynaptic $\alpha 4\beta\gamma 2$ - and $\alpha 2\beta 1\gamma 1$ -containing GABA_ARs in hippocampal neurons (Fig. 6). EtOH-induced decreases in $\alpha 1$ and δ subunits and increased $\alpha 4$ and $\gamma 2$ are also found in other brain regions, including the basolateral amygdala (Diaz et al., 2011; Lindemeyer et al., 2014) and nucleus accumbens (Liang et al., 2014), whereas $\alpha 2$ was downregulated in the amygdala and $\alpha 5$ was upregulated in the nucleus accumbens. In this study, decreased extrasynaptic $\alpha 4\beta\delta$ GABA_ARs correlated with decreased GABA_AR-mediated I_{tonic} and loss of its EtOH sensitivity. Upregulated $\alpha 2$ subtypes correlated with the appearance of synaptic currents enhanced by EtOH (>10 mM). EtOH-enhanced mIPSCs have also been observed in untreated $\alpha 4$ KO mice (Liang et al., 2008; Suryanarayanan et al., 2011), in which the EtOH-sensitive subtype cannot contain $\alpha 4$. The $\alpha 2$ subunit is colocalized with gephyrin and presynaptic glutamic

acid decarboxylase at both DGC cell bodies and axon initial segments (Kerti-Szigeti and Nusser, 2016) and is upregulated in the hippocampus of $\alpha 4$ KO mice (Liang et al., 2008; Suryanarayanan et al., 2011).

The decrease in $\alpha 1\beta\gamma 2$ - and gain of $\alpha 4\beta\gamma 2$ - and $\alpha 2\beta 1\gamma 1$ -containing GABA_ARs change the kinetics and pharmacologic properties of mIPSCs. We previously found decreased diazepam or zolpidem enhancement of mIPSC decay constants and a markedly increased area by the imidazobenzodiazepine partial inverse agonist Ro15-4513 in hippocampal slices after CIE (Cagetti et al., 2003; Liang et al., 2004, 2007, 2009) and single-dose treatment in vivo (Liang et al., 2007). These pharmacologic and subunit changes were reproduced in primary cultured embryonic hippocampal neurons after 15 days in vitro, 24 hours after exposure for 30 minutes to EtOH (50 mM) (Shen et al., 2011). In recombinant systems, GABA_ARs containing $\alpha 4/\alpha 6$ when expressed with the $\gamma 2$ subunit are insensitive to traditional benzodiazepine drugs and zolpidem, whereas Ro15-4513 acts as an agonist (Knoflach et al., 1996; Wafford et al., 1996). Recombinant GABA_ARs containing the $\gamma 1$ subunit show slightly different BZ pharmacology from $\gamma 2$, including agonism by Ro15-4513 (Puia et al., 1991; Wafford et al., 1993). Ro15-4513 at 1 μ M slightly potentiates $\alpha 2\beta 1\gamma 1$ receptors expressed in *Xenopus* oocytes (Wafford et al., 1993), which indicates that $\alpha 2\beta 1\gamma 1$ and $\alpha 4\beta\gamma 2$ receptor subtypes have a similar, not easily distinguishable, pharmacologic profile.

We tried to identify the EtOH-sensitive GABA_AR (presumably either $\alpha 4\beta\gamma 2$ or $\alpha 2\beta 1\gamma 1$) by two-pulse EtOH experiments using the assumption, that subtypes responding to EtOH will become internalized within 1 hour, as demonstrated for δ -containing

TABLE 2

Responses of mIPSCs to acute drug (EtOH) application in DGCs

$\tau 1$ and $\tau 2$ are time constants (mean $\pm$ S.E.M.) when the decay of mIPSCs was fitted with two exponentials. Decay of mIPSCs in pattern d was fitted well with only one exponential (MiniAnalysis program V6.07). $\alpha 4$ KO, $\alpha 4$ subunit knockout mice; E0, without EtOH; E50, in the presence of 50 mM EtOH.

	Pattern a		Pattern b		Pattern c		Pattern d
	$\tau 1$ (ms)	$\tau 2$ (ms)	$\tau 1$ (ms)	$\tau 2$ (ms)	$\tau 1$ (ms)	$\tau 2$ (ms)	τ (ms)
CIV E0	7.8 $\pm$ 0.4	32.0 $\pm$ 11.1			14.1 $\pm$ 1.2	54.9 $\pm$ 11.3	16.9 $\pm$ 0.6
E50	8.0 $\pm$ 0.4	60.3 $\pm$ 11.6			16.3 $\pm$ 0.7*	58.4 $\pm$ 14.6	18.2 $\pm$ 0.5
CIE E0			4.0 $\pm$ 0.3	12.9 $\pm$ 2.2	14.6 $\pm$ 0.7	70.4 $\pm$ 9.2	17.4 $\pm$ 0.7
E50			8.0 $\pm$ 0.3*	26.9 $\pm$ 4.3*	17.2 $\pm$ 0.3*	73.1 $\pm$ 12.5	19.5 $\pm$ 0.4*
WT E0	7.6 $\pm$ 0.5	37.1 $\pm$ 10.5			13.7 $\pm$ 0.6	35.9 $\pm$ 8.9	17.0 $\pm$ 0.5
E50	7.7 $\pm$ 0.4	53.9 $\pm$ 3.6			14.8 $\pm$ 0.5	50.7 $\pm$ 13.5	18.0 $\pm$ 0.5
$\alpha 4$ KO E0	7.1 $\pm$ 0.4	28.0 $\pm$ 8.6			13.8 $\pm$ 0.9	35.4 $\pm$ 8.6	16.6 $\pm$ 1.1
E50	8.2 $\pm$ 0.5*	48.0 $\pm$ 12.4			16.2 $\pm$ 1.1*	63.7 $\pm$ 19.9	18.4 $\pm$ 0.8*

* $P < 0.05$, the decay time constant of a pattern in the presence of 50 mM EtOH is statistically different from that of 0 EtOH (two-way repeated measures ANOVA, followed by Sidak method for pairwise multiple comparison). $n = 6$ neurons from three rats/group, $n = 5$ neurons from two mice/group.

Fig. 6. Hypothetical model of GABA_AR subunit plasticity seen within two days following a single intoxicating dose of EtOH to rats leading to changes of physiopharmacologic properties in GABAergic ionotropic receptor-mediated inhibitory synaptic transmission in hippocampus.

GABA_ARs after one-pulse EtOH (Shen et al., 2011); the reversible loss of EtOH sensitivity of mIPSCs at 1 hour after the second EtOH dose paralleled reversible decreases in both $\alpha 4$ - and $\alpha 2\gamma 1$ -containing surface-expressed GABA_ARs. In addition, the increased anxiety 48 hours after the first dose of EtOH could be partially alleviated by the second EtOH dose, measured after 1 hour. Therefore, the two-pulse EtOH experiments failed to distinguish which receptor subtypes mediate EtOH sensitivity; however, mIPSC pattern recognition analysis suggested that both $\alpha 2$ and $\alpha 4$ subtypes are involved.

CIE-Induced mIPSCs with Slow Decay Kinetics, Enhanced by EtOH in the Recording Chamber, Correlate with Upregulated $\alpha 2$ Subunits. We previously reported (Liang et al., 2006) a difference between the decay rates of mIPSCs in DGCs in CIE compared with CIV controls. The control mIPSCs, which are sensitive to BZ drugs and insensitive to EtOH, were downregulated by CIE treatment in parallel with the $\alpha 1$ subunit. The CIE-induced averaged mIPSCs were sharper, faster decaying, insensitive to BZ, but enhanced by Ro15-4513 and enhanced EtOH, and correlated with upregulated $\alpha 4$ subunit.

In the present study, we analyzed patch-clamp recordings from hippocampal DGCs in CIE-treated rats and controls using an optimally scaled template algorithm (Clements and Bekkers, 1997) implemented in DataView software. We mentioned above that although mIPSC kinetics are sensitive to multiple anatomic

variables affecting transmitter concentrations and duration during synaptic transmission, the decay time is very sensitive to the nature of the postsynaptic receptor channels and can give clues to the nature of GABA_AR α subtypes (Otis and Mody, 1992; McClellan and Twyman, 1999; Vicini, 1999; Lagrange et al., 2007; Kerti-Szigei and Nusser, 2016).

We were able to detect mIPSCs of distinct waveforms consistent with the heterogeneity of GABA_ARs formed from different α subunits. These were found consistently over multiple recordings in different DGCs and thus are unlikely to result from anatomic variables. We confirmed that control, CIV, and WT animals were enriched in typically shaped mIPSCs (peak a in Fig. 5 and Table 1), which showed no enhancement by EtOH in the recording chamber and downregulated by CIE, consistent with the $\alpha 1\beta \gamma 2$ GABA_AR subtype. CIE treatment induced a new rapidly decaying peak, now described as pattern b in Fig. 5, which correlated with the appearance of upregulated $\alpha 4\beta \gamma 2$ GABA_AR subtype, showing some enhancement by EtOH. Consistent with this, pattern b is missing in the $\alpha 4$ KO mouse (Fig. 5).

The averaged shapes must contain some contribution from $\alpha 2$. Numerous mIPSC waveform events with slow decay consistent with the presence of $\alpha 2$ GABA_ARs were detectable in CIV controls, and these were increased in CIE rats. Since $\alpha 2$ is upregulated in CIE rats, as are EtOH enhanced, slow-decay mIPSCs, and likewise in untreated $\alpha 4$ KO mice as shown by Western blot analysis (Suryanarayanan et al., 2011) and by

immunohistochemistry (Houser CR, Liang J, and Olsen RW, unpublished), we posit that $\alpha 2$ contributes to these mIPSCs. Peak pattern c- and d-like slow-decay mIPSCs were found in CIV and CIE rats, as well as WT and $\alpha 4$ KO mice; both were enhanced by mM EtOH in the recording chamber. Comparing the abundance of kinetic patterns in the four conditions (Table 1), we conclude that the sum of pattern c and d correlate with upregulated $\alpha 2$ subunit-containing GABA_ARs. Further, the abundance ratio of peaks d to c is elevated in both CIE rats and $\alpha 4$ KO mice (Fig. 5; Table 1), and this upregulation of peak 'd' correlates with the averaged mIPSCs exhibiting greater EtOH enhancement (Fig. 6). It is likely that the EtOH-sensitive mIPSCs with unusually slow decay (peak pattern d) are due to $\alpha 2$, perhaps demonstrating an unusual subunit combination (e.g., $\alpha 2\beta 1\gamma 1$), or some other 'abnormal' postsynaptic membrane structure. Interestingly, in DGCs of both untreated δ - and $\alpha 4$ KO mice, mIPSC charge transfer is increased by acute EtOH in the recording chamber, which is not observed in WT animals (Liang et al., 2006; Liang et al., 2008; Suryanarayanan et al., 2011). We find an increased amount of the GABA_A $\gamma 1$ subunit in δ KO and $\alpha 4$ KO mice compared with WT by Western blot analysis (Lindemeyer AK, Liang J, and Olsen RW, unpublished). It is tempting to speculate that $\alpha 2\beta 1\gamma 1$ receptors may mediate EtOH sensitivity in these animals. Future work on genetically engineered rodents should allow proof of which GABA_AR subtypes are critical for the relevant plasticity.

Relationship between Alcohol Use Disorder and the Gene Cluster Encoding GABA_AR Subunits $\alpha 2$, $\beta 1$, $\gamma 1$, and $\alpha 4$ on Human Chromosome 4. There is growing evidence that alcoholism as a complicated behavioral disorder has complex genetic involvement. In particular, genes encoding the chromosome 4 cluster of GABA_AR subunits *GABRA4*, *GABRA2*, *GABRB1*, and *GABRG1* are genetically linked to certain aspects of alcoholism in humans. Because coregulation of GABA_AR subunits within gene clusters has been reported during development (Fillman et al., 2010), there may be some combination of these gene products acting together functionally in some way to affect alcohol behavior. Single nucleotide polymorphisms in the chromosome 4 GABA_AR subunit genes are highly associated with alcohol abuse and dependence (Porjesz et al., 2002; Edenberg et al., 2004; Enoch et al., 2009; Borghese and Harris, 2012). Postmortem brain of human alcoholics exhibits elevated $\alpha 1$, $\alpha 4$, $\alpha 5$, and especially $\beta 1$ and $\gamma 1$ mRNA in the hippocampus (Jin et al., 2012).

The $\alpha 2$ subunit has been shown by mouse genetic engineering to be required for the anxiolytic efficacy and dependence liability of benzodiazepines (Rudolph and Knoflach, 2011; Engin et al., 2016). Gephyrin-binding subunits ($\alpha 2$) of GABA_ARs are important for receptor dynamics critical for plasticity phenomena such as inhibitory LTP (Petrini et al., 2014; Tyagarajan and Fritschy, 2014). The $\alpha 2$ candidate partner (but rare) $\gamma 1$ subunit is abundant and functional in the central amygdala (Esmaeili et al., 2009); knockdown of $\alpha 2$ expression in this area resulted in reduction of binge drinking (Liu et al., 2011). The hippocampus likewise participates in neurocircuitry of drug craving and relapse (Koob and Volkow, 2010). Our studies reveal GABA_ARs as novel targets of neuronal adaptations in response to EtOH, probably important for understanding alcohol-induced memory loss and drug-seeking behavior during abstinence and facilitating the development of therapeutic alcohol use disorder treatment.

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Authorship Contributions

Participated in research design: Lindemeyer, Shen, Shao, Spigelman, Olsen, Liang.

Conducted experiments: Lindemeyer (biochemistry), Shen (electrophysiology), Yazdani (behavior), Spigelman (electrophysiology), Liang (electrophysiology and behavior).

Performed data analysis: Lindemeyer, Shen, Shao, Spigelman, Olsen, Liang.

Wrote or contributed to the writing of the manuscript: Lindemeyer, Shao, Spigelman, Davies, Olsen, Liang.

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