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Authors
Lindemeyer, A Kerstin
Liang, Jing
Marty, Vincent N
et al.

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ORIGINAL PAPER

Ethanol-Induced Plasticity of $GABA_A$ Receptors in the Basolateral Amygdala

A. Kerstin Lindemeyer · Jing Liang · Vincent N. Marty · Edward M. Meyer · Asha Suryanarayanan · Richard W. Olsen · Igor Spigelman

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Abstract Acute and chronic ethanol (EtOH) administration is known to affect function, surface expression, and subunit composition of γ-aminobutyric acid (A) receptors ($GABA_A$Rs) in different parts of the brain, which is believed to play a major role in alcohol dependence and withdrawal symptoms. The basolateral amygdala (BLA) participates in anxiety-like behaviors including those induced by alcohol withdrawal. In the present study we assessed the changes in cell surface levels of select $GABA_A$R subunits in the BLA of a rat model of alcohol dependence induced by chronic intermittent EtOH (CIE) treatment and long-term (>40 days) withdrawal and investigated the time-course of such changes after a single dose of EtOH (5 g/kg, gavage). We found an early decrease in surface expression of α4 and δ subunits at 1 h following single dose EtOH treatment. At 48 h post-EtOH and after CIE treatment there was an increase in α4 and γ2, while α1, α2, and δ surface expression were decreased. To relate functional changes in $GABA_A$Rs to changes in their subunit composition we analyzed miniature inhibitory postsynaptic currents (mIPSCs) and the picrotoxin-sensitive tonic current (I_{tonic}) 48 h after EtOH intoxication. The I_{tonic} magnitude and most of the mIPSC kinetic parameters (except faster mIPSC decay) were unchanged at 48 h post-EtOH. At the same time, I_{tonic} potentiation by acute EtOH was greatly reduced, whereas mIPSCs became significantly more sensitive to potentiation by acute EtOH. These results suggest that EtOH intoxication-induced $GABA_A$R plasticity in the BLA might contribute to the diminished sedative/hypnotic and maintained anxiolytic effectiveness of EtOH.

Keywords Alcohol · Amygdala · Dependence · Synaptic · Tonic · Subunits · GABA · Plasticity · CIE

Introduction

Elevated anxiety and low tolerance to stress are major factors in the development of alcohol dependence. The basolateral nucleus of the amygdala (BLA) mediates emotions such as anxiety and is involved in drug craving and drug-related relapse [1, 2]. γ-Aminobutyric acid type A receptors ($GABA_A$Rs) have been shown to be important contributors to the regulation of BLA-related anxiety [3–5]. $GABA_A$Rs are ligand-gated heteropentameric chloride-channels composed of several classes of subunits (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ1-3) with usually two α- and β-subunits and one γ or δ subunit associated in one pentamer [6]. The channel kinetics, physiological function and pharmacological sensitivity of $GABA_A$Rs are largely dictated by their subunit composition [6] and subject to some fine tuning by phosphorylation/dephosphorylation states, e.g., [7]. The most abundant subunits in the BLA are α1, α2, β1-3 and γ2. $GABA_A$R subunits α3, α4, α5, γ3 and δ are also expressed [8]. The major synaptic subunits mediating phasic inhibition in the BLA are α1 and α2 [9], while α3 and α4/δ are extrasynaptic and mediate the tonic GABA current [10, 11].

Acutely, alcohol (ethanol, EtOH) at pharmacologically relevant concentrations (≤60 mM) is known to potentiate

A. K. Lindemeyer · J. Liang · A. Suryanarayanan · R. W. Olsen
Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

J. Liang · V. N. Marty · E. M. Meyer · A. Suryanarayanan · I. Spigelman (✉)
Division of Oral Biology and Medicine, School of Dentistry, University of California, 10833 Le Conte Avenue, 63-078 CHS, Los Angeles, CA 90095-1668, USA
e-mail: igor@ucla.edu

$GABA_A$Rs, particularly those composed of the extrasynaptic subunit combinations containing α4 and δ [12, 13], whereas chronic EtOH administration results in decreased $GABA_A$R activity with concomitant changes in their subunit composition in various brain regions such as the cerebral cortex [14–16], hippocampus [16–18], and amygdala [19–21], reviewed in [22].

In the rat BLA, withdrawal from chronic intermittent ethanol treatment (CIE) has previously been reported to affect $GABA_A$R subunit peptide levels including decreases in the α1 and increases in α4 subunit surface proteins and γ2 total protein [19]. These changes are similar to those found in rat hippocampus after CIE treatment [17, 18] and at 2 days after a single intoxicating dose of EtOH (5 g/kg, gavage) [18]. Examining the time course after one-dose ethanol intoxication in rat hippocampal slices and neuronal cultures, there was an early decrease in the surface levels of the α4 and the δ subunit after 1 h. After 2 days the δ subunit was still decreased and also α1 was decreased, while α4 and γ2 membrane expression was increased compared to vehicle-treated controls. All these changes went back to normal after 2 weeks [18, 23].

Different brain regions can exhibit different EtOH-induced plastic changes [16, 24]. Therefore, we studied the surface expression of $GABA_A$R subunits α1, α2 α4, γ2 and δ in rat BLA slices 40 days after CIE treatment. Furthermore, we looked at the time-course of these plastic changes 1, 48 h and 2 weeks after one single intoxicating dose of EtOH (5 g/kg, gavage). Since the CIE-induced plasticity was similar to that observed at 48 h after single dose EtOH we obtained whole-cell patch clamp recordings in BLA neurons in slices from rats at 48 h after single dose EtOH or vehicle. We analyzed the picrotoxin-sensitive miniature inhibitory postsynaptic currents (mIPSCs) and tonic current (I_{tonic}) to relate functional changes in $GABA_A$Rs to $GABA_A$R subunit composition.

Materials and Methods

Animals

All protocols approved by the University of California Institutional Animal Care and Use Committee following NIH guidelines. Sprague–Dawley rats (male, Harlan), 200–350 g, were housed in the vivarium under a light/dark cycle (12 h) and had free access to food and water.

EtOH Administration

Chronic intermittent EtOH (CIE) rats were produced as follows: rats received 5 g/kg, 25 % (w/v) EtOH solution in drinking water (Pharmco Products, Brookfield, CT) for the first five times once every other day and then, for the following 55 doses, 6 g/kg EtOH 30 % (w/v) once every day. This EtOH regimen led rats to experience multiple cycles of intoxication and withdrawal. The control group, chronic intermittent vehicle (CIV) rats, was treated with water (20 ml/kg) in parallel. To study acute EtOH effects rats were administered a single dose of EtOH (5 g/kg) by gavage. Control rats received drinking water (20 ml/kg of body weight; gavage). Rats were euthanized and brain tissue collected at ≥40 days after CIE/CIV treatment or at 1 h, 48 h, or 2 weeks after acute EtOH.

Tissue Collection

Transverse brain slices (400 μm thick) containing the BLA were obtained using standard techniques. Briefly, rats were decapitated under isoflurane anesthesia, brains were quickly removed, trimmed with a razor blade and glued to the base of a cutting chamber (VT1200S, Leica Microsystems Inc., Buffalo Grove, IL) filled with cold (~4 °C) artificial cerebrospinal fluid (ACSF) composed of 125 mM NaCl, 2.5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 26 mM $NaHCO_3$, and 10 mM D-glucose (Sigma, USA). The ACSF was continuously bubbled with a 95/5 % mixture of O_2/CO_2 to ensure adequate oxygenation of slices and a pH of 7.4. Slices containing the BLA were then used for the biochemical determination of surface proteins or for electrophysiological recordings.

Measurement of Surface Expressed Receptor Subunits by Cross-Linking

To measure surface protein expression in slices from in vivo treated rats, cell surface cross-linking experiments followed by Western blot analysis were performed. The BLA was microdissected in ice-cold ACSF from individual slices with the aid of a surgical microscope (Carl Zeiss Surgical GmbH, Oberkochen, Germany) guided by anatomical landmarks as illustrated in the rat brain atlas [25]. The microdissected slices were incubated at 4 °C either in ACSF alone, or ACSF containing 1 mg/ml bis(sulfosuccinimidyl)suberate (BS^3) (Pierce) for 45 min. The cell-impermeable BS^3 cross-links all surface proteins leading to high molecular weight aggregates which do not enter the gel. Therefore, only internal proteins are detectable in the BS^3 sample. The cross-link reaction was quenched washing with 20 mM Tris wash buffer, pH 7.6, and the slices were homogenized in a 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).

SDS-PAGE and Western Blot Analysis

Proteins were separated on SDS–polyacrylamide gels using the Mini-Protean 3 cell system (Bio-Rad), transferred on a

polyvinylidene difluoride (PDVF) membrane (Bio-Rad) and blocked with 4 % non-fat dry milk in TTBS. Blots were incubated overnight at 4 °C with the following primary antibodies: anti-α1 (aa328-382), anti-α2 (aa322-357), anti-α4 (aa379-421), anti-γ2 (aa319-366), anti-δ (aa1-44) (all at 1 mg/ml, gift of W. Sieghart), anti-β-actin (Sigma, 1:1,000), or βIII-tubulin (Sigma, 1:1,000), followed by HRP-conjugated secondary antibodies (1:5,000, Rockland). The $GABA_AR$ subunit antibodies have been previously extensively verified by various methods including immunoprecipitation, Western blotting and immunocytochemistry [26–28]. Bands from different samples corresponding to the appropriate subunit were analyzed and absorbance values compared by densitometry using ImageQuant 5.2 (Molecular Dynamics) analysis systems. The surface amount of the protein of interest is calculated by the difference between the signal of internal and total protein and normalized to β-actin or βIII-tubulin.

Electrophysiological Recordings

For recordings, individual slices containing the BLA were transferred to a custom-built Plexiglass recording chamber. Patch electrode filling solutions contained: 135 mM cesium gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 11 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM HEPES, 2 mM ATP-K_2, 0.2 mM GTP-Na_2; pH adjusted to 7.25 with CsOH. Patch electrode and probe assembly targeting the region of interest were advanced using a 4-axis motorized manipulator (MX7600, Siskiyou Corporation, Grants Pass, OR) and controller (MC1000e, Siskiyou Corp.) with the aid of a dissecting microscope (7–45×, SZ61, Olympus). Whole-cell patch clamp recordings were obtained from cells of the BLA region at 34 ± 0.5 °C during perfusion with oxygenated ACSF. Cells were voltage-clamped at 0 mV. Pharmacological separation of $GABA_AR$-mediated currents was done by application of TTX (0.5 μM), CNQX (10 μM), APV (40 μM) and CGP54626 (1 μM) in the ACSF. EtOH (10–60 mM) was applied after appropriate dilution in the ACSF.

Detection and Analysis of Miniature Inhibitory Postsynaptic Currents (mIPSCs) and Tonic Currents

The recordings were low-pass filtered off-line (Clampfit software, Molecular Devices, Sunnyvale, CA) at 2 kHz. The mIPSCs were detected (Mini Analysis Program) with threshold criteria of: 5 pA, amplitude and 20 fC, charge transfer. Frequency of mIPSCs was determined from all automatically detected events in a given 100 s recording period. For kinetic analysis, only single event mIPSCs with a stable baseline, sharp rising phase and exponential decay were chosen during visual inspection of the recording trace. Double and multiple peak mIPSCs were excluded. The mIPSC kinetics were obtained from analysis of the averaged chosen single events (>120 events/100 s recording period) aligned with half rise-time in each cell. Decay time constants were obtained by fitting a double exponential equation to the falling phase of the averaged mIPSCs of the form $I(t) = I_f * \exp(-t/\tau_f) + I_s * \exp(-t/\tau_s)$, where I_f and I_s are the amplitudes of the fast and slow decay components, and τ_f and τ_s are their respective decay time constants used to fit the data. To compare decay times, we used a weighted mean decay time constant: $\tau_w = [I_f/(I_f + I_s)] * t_f + [I_s/(I_f + I_s)] * t_s$. The tonic current magnitudes were obtained from the mean baseline current of a given recording period.

Statistical Analysis

The investigators who performed and analyzed the patch clamp recordings and Western blots were blind to the treatment (vehicle, CIE, or single dose EtOH) that the rats received. All values are presented as mean ± SEM. Group differences were evaluated by unpaired Student's t test or ANOVA with post hoc comparisons where appropriate. $P < 0.05$ was considered statistically significant.

Results

CIE-Induced Changes in $GABA_AR$ Subunit Surface Expression in the BLA

To measure the long-lasting changes in the levels of $GABA_AR$ subunits in the plasma membrane we compared Western blots of microdissected BLA slices from CIE and CIV rats incubated with or without the membrane-impermeable cross-linking reagent BS^3 (see "Materials and Methods" section). We found that the α1 $GABA_AR$ subunit surface expression was significantly decreased after CIE treatment and ≥40 days of withdrawal (concomitantly with total levels, CIV: 100.0 ± 10.4, CIE: 60.3 ± 12.5, n = 5, $p = 0.035$ unpaired t test). The surface expression of the α4 (without significant changes in total levels, CIV: 100.0 ± 19.3, CIE: 128.8 ± 27.1, n = 7, $p = 0.572$ unpaired t test) and γ2 (without significant changes in total protein levels; CIV: 100.0 ± 37.9, CIE: 118.4 ± 40.7.1, n = 5, $p = 0.749$ unpaired t test) subunits was significantly increased (Fig. 1, $p < 0.05$, unpaired t test). $GABA_AR$ δ and α2 subunits were also significantly decreased compared to CIV controls (Fig. 1, $p < 0.05$ unpaired t test). Their total protein expression was not significantly changed (δ CIV: 100.0 ± 9.9, CIE: 84.9 ± 11.9, n = 4, $p = 0.368$ unpaired t test; α2 CIV: 100.0 ± 16.0, CIE: 83.0 ± 20.1, n = 5, $p = 0.526$ unpaired t test).

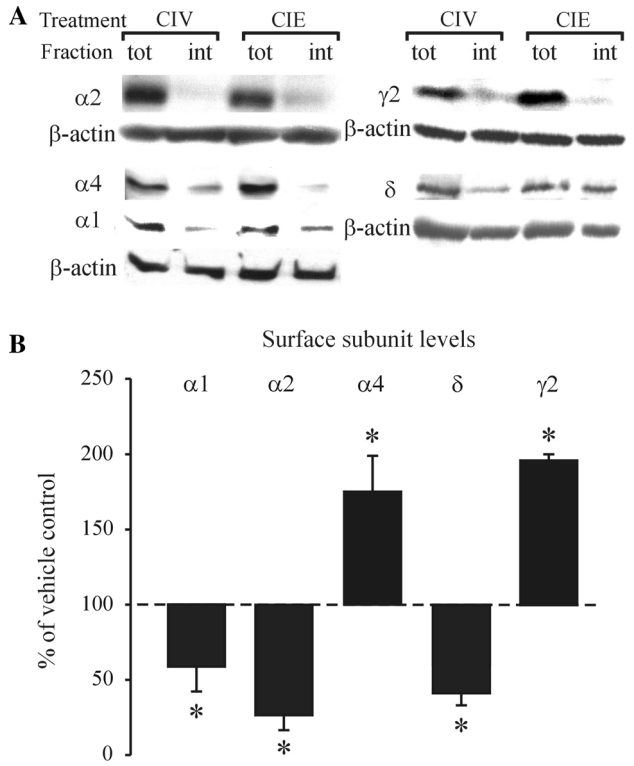

Fig. 1 Long-lasting changes in the surface expression of the α1, α2, α4, δ and γ2 $GABA_AR$ subunits in the BLA after CIE treatment. **a** Representative Western blots for α1, α2, α4, δ, γ2 $GABA_AR$ subunits and β-actin after cell surface cross-linking; *tot* total amount of protein/sample, *int* intracellular protein/sample. **b** *Bar graph* summarizing the subunit alterations after CIE treatment and > 40 -days of withdrawal compared to CIV-treated controls, set as 100 % (*dashed line*). $*p < 0.05$ (unpaired t test) compared to CIV-treated controls, n = 5–6 rats/group

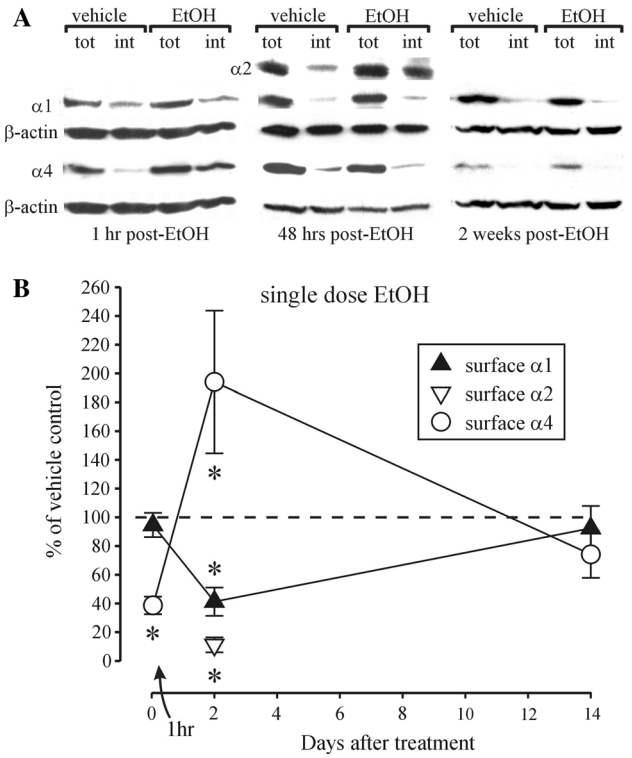

Fig. 2 Reversible changes in α1 and α4 $GABA_AR$ subunits in the BLA after single-dose EtOH intoxication. **a** Representative Western blots for α1, α4 and β-actin after cell surface cross-linking at 1 h, 48 h and 2 weeks after single-dose EtOH intoxication. Cell-surface content was also measured for the α2 subunit 48 h after EtOH treatment; *tot* total amount of protein/sample, *int* intracellular protein/sample. **b** Summary graph showing the alterations in cell surface expression of the subunits compared to vehicle-treated controls, set as 100 % (*dashed line*). $*p < 0.05$ (unpaired t test) compared to vehicle-treated controls, n = 4–7 rats/group

Time-Dependent Changes in BLA $GABA_AR$ Subunit Surface Expression After a Single Intoxicating Dose of EtOH

Previous studies in the rat hippocampus demonstrated that the CIE-induced $GABA_AR$ plasticity could be reproduced, albeit transiently, by a single high dose of EtOH [18]. Since function and pharmacological sensitivity of $GABA_ARs$ are largely dictated by their subunit composition [6], this allowed some insight into the mechanisms of EtOH-induced $GABA_AR$ alterations. Applying similar principles to the BLA, we studied the time-course of $GABA_AR$ subunit changes at 1 h, 48 h and 2 weeks after administering a single dose of EtOH (5 g/kg; gavage). At 1 h after EtOH dosing the intracellular levels of the α4 subunit significantly increased (Fig. 2a, $p < 0.05$, unpaired t test), without significant changes in total levels (vehicle: 100.0 ± 30.1, EtOH: 106.6 ± 39.0, n = 5, $p = 0.871$ unpaired t test), suggesting rapid internalization of $GABA_ARs$ containing this subunit. However, by 48 h after EtOH α4 subunit surface levels (but not total levels, vehicle: 100.0 ± 22.5, EtOH: 122.6 ± 24.3, n = 5, $p = 0.508$ unpaired t test) were increased compared to vehicle-treated controls, with return to control levels at 2 weeks following EtOH treatment (Fig. 2). By contrast, there was no detectable change in the α1 subunit surface levels at 1 h after EtOH, but they were significantly decreased after 48 h (without significant decreases in total protein level; vehicle: 100.0 ± 47.1, EtOH: 80.5 ± 48.5, n = 5, $p = 0.782$ unpaired t test) with return to control levels at 2 weeks following EtOH treatment (Fig. 2). Although we did not examine all three time points, at 48 h post-EtOH the α2 subunit surface levels were also significantly decreased compared to vehicle-treated controls (Fig. 2). No changes in total protein amounts of α2 were detectable (vehicle: 100.0 ± 14.9, EtOH: 113.9 ± 12.8, n = 5, $p = 0.498$ unpaired t test).

Estimates of the δ subunit surface levels revealed their significant decreases at 1 h post-EtOH (Fig. 3). Similar to the α4 subunit changes at this time point, the decreases in δ

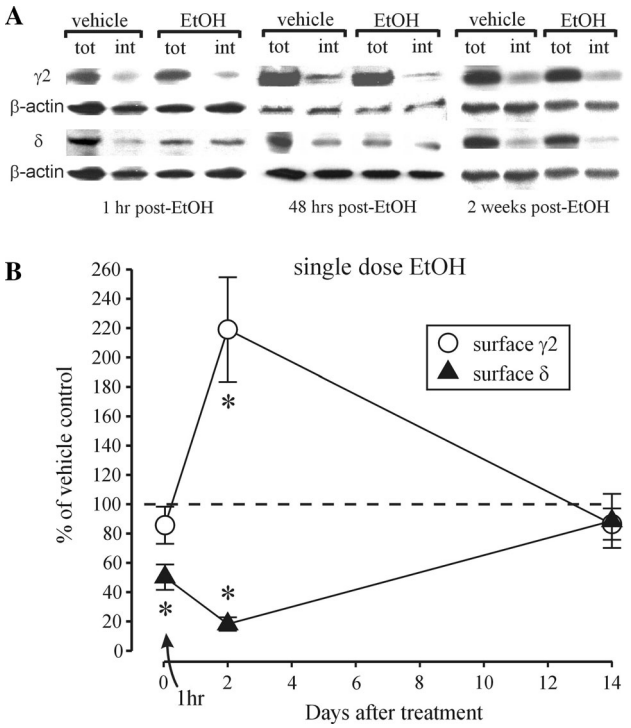

Fig. 3 Reversible changes in δ and γ2 in the BLA following single-dose EtOH treatment. **a** Representative Western blots for δ, γ2 and β-actin $GABA_AR$ subunits after cell surface cross-linking at 1 h, 48 h and 2 weeks after single-dose EtOH intoxication; *tot* total amount of protein/sample, *int* intracellular protein/sample. **b** Summary graph showing the alterations in cell surface expression of δ and γ2 subunits at 1 h, 48 h and 2 weeks after single-dose intoxication with EtOH compared to vehicle-treated controls, set as 100 % (*dashed line*). *$p < 0.05$ (unpaired t test) compared to vehicle-treated controls, n = 6–7 rats/group

subunit surface levels were associated with an increase in their intracellular fraction, but not in the total levels (vehicle: 100.0 ± 20.0; EtOH: 112.8 ± 36.1, n = 5, $p = 0.761$ unpaired t test). The δ subunit surface levels remained decreased at 48 h after EtOH, but recovered to control levels at 2 weeks after EtOH. The γ2 subunit surface levels were unchanged 1 h after EtOH, but there was a significant increase at the 48 h time point (without significant changes in the total levels, vehicle: 100.0 ± 16.4, EtOH: 117.9 ± 22.3, n = 6, $p = 0.525$ unpaired t test), with return to control levels at two weeks following EtOH treatment (Fig. 3).

Changes in BLA $GABA_AR$ Function After a Single Intoxicating Dose of EtOH

$GABA_AR$ currents were recorded during pharmacological blockade of ionotropic glutamate receptors, $GABA_BRs$ and voltage-gated sodium channels. Under these recording conditions $GABA_AR$ currents could be separated into 2 components: a non-desensitizing tonic current (I_{tonic}) and phasic mIPSCs which are known to be mediated by extrasynaptic and synaptic $GABA_AR$ activation, respectively [29]. The bulk of mIPSCs detected in recordings from BLA principal neurons were previously shown to arise from local circuit GABAergic interneurons [30]. Since the surface $GABA_AR$ subunit changes after CIE treatment were remarkably similar to those observed at 48 h after a single EtOH dose, we concentrated our recordings on this latter time point. At 48 h after single dose EtOH, the picrotoxin-sensitive I_{tonic} magnitude was not significantly altered compared to vehicle-treated rats (Table 1). However there was a trend to decreased mIPSC charge transfer, primarily via speeding up of the mIPSC rise time and significantly faster weighted decay time constant (τw) of mIPSCs (Table 1). Acute application of EtOH (10–60 mM) in BLA recordings from vehicle-treated rats preferentially potentiated the picrotoxin-sensitive I_{tonic} (Fig. 4a). The phasic mIPSCs were not significantly affected by acute EtOH (10–60 mM) in these recordings (Fig. 4a). Recordings at 48 h after EtOH treatment from EtOH-treated rats showed that the mIPSCs were now significantly potentiated by 60 mM EtOH (Fig. 4c), whereas the I_{tonic} was no longer potentiated by EtOH (10-60 mM) (Fig. 4b). The acute EtOH-induced increases in mIPSC charge transfer resulted primarily from the increases in τw and the rise time without changes in mIPSC amplitude (Fig. 4d–g). The frequency of mIPSCs was unaffected by acute EtOH application in either vehicle or EtOH-treated rats (Fig. 4h).

Table 1 Kinetic properties of mIPSCs in BLA neurons from water- and EtOH (5 g/kg, gavage)-exposed rats. All data are expressed as mean ± SEM

	Post-vehicle (48 h)	Post-EtOH (48 h)
I_{tonic} (pA)	15.8 ± 2.2	19.7 ± 5.5
mIPSC area (fC)	293.2 ± 38.4	224 ± 37.1
mIPSC amplitude (pA)	13.4 ± 2.0	14.4 ± 2.1
mIPSC weighted decay τ (ms)	22.8 ± 4.8	13.7 ± 1.6*
mIPSC rise time (ms)	2.3 ± 0.4	1.6 ± 0.1
mIPSC frequency (Hz)	3.4 ± 0.6	3.2 ± 0.9
n (cells/rats)	13/10	7/4

* $p < 0.05$ from post-vehicle (unpaired t test)

Discussion

Subunit Changes and Comparison to Other Brain Regions

With the cross-linking assay and Western blot analysis in microdissected BLA slices, we observed decreases in the surface expression of the $GABA_AR$ subunits α1 and δ at

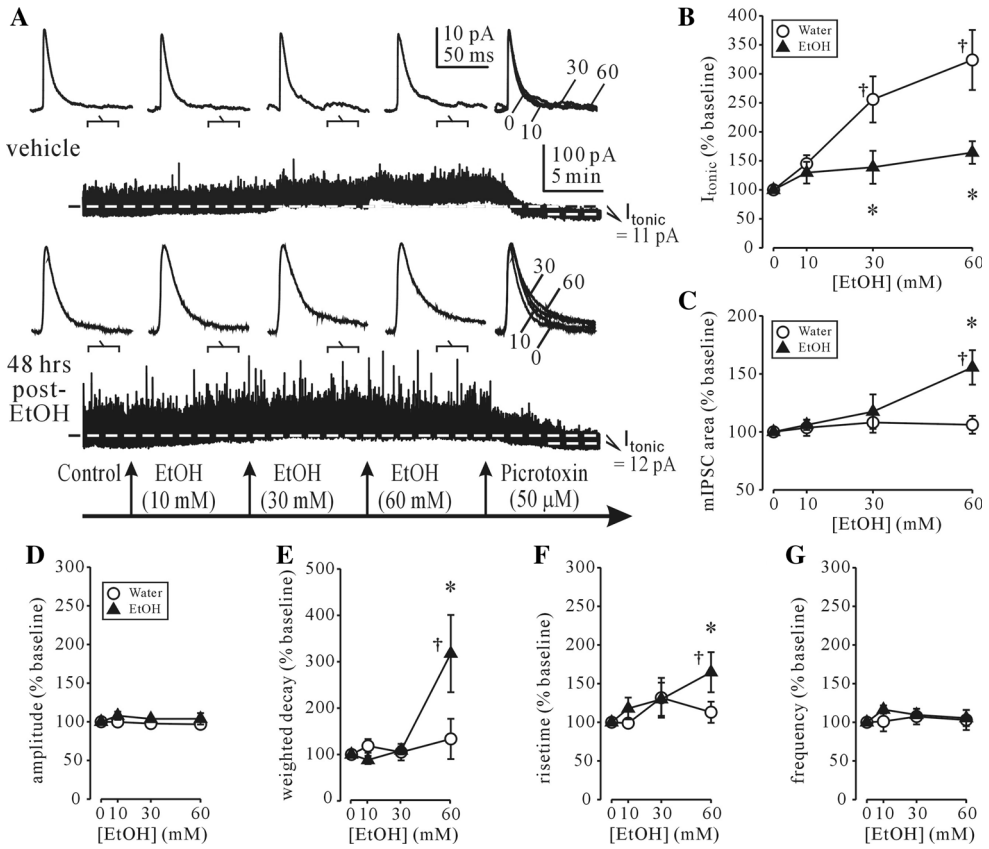

Fig. 4 Altered EtOH sensitivity of synaptic and tonic $GABA_AR$ currents after single-dose EtOH intoxication. **a** Examples of individual BLA neuron recordings from vehicle-treated (*upper traces*) and single dose EtOH-treated rats (*lower traces*). The holding current (I_{hold}) needed to clamp the voltage at 0 mV before EtOH application is indicated by a *dashed line*. In a control recording, the kinetics of mIPSCs (*top traces*) averaged over the indicated 100 s periods during continuous recordings (*lower trace*) are unaffected, while I_{hold} is visibly potentiated by EtOH (10–60 mM). Subsequent application of picrotoxin (50 μM) reveals the $GABA_AR$-mediated tonic current component (I_{tonic}). At 48 h post-EtOH, I_{tonic} (*bottom trace*) potentiation is reduced and mIPSC potentiation by acute EtOH is increased. **b** I_{tonic} is significantly potentiated by acute application of EtOH (10–60 mM) from vehicle- but not EtOH-treated rats. **c** mIPSC area in EtOH-treated but not vehicle-treated rats is significantly potentiated by EtOH (60 mM) application. **d–g** summary graphs of acute EtOH effects on other kinetic parameters of mIPSCs from vehicle- and single dose EtOH (5 g/kg, gavage)-treated rats. For all panels, each point represents a mean of % baseline ± SEM value from 4-13 neurons (4–9 rats/group). †$p < 0.05$ from pre-drug; *$p < 0.05$ from vehicle-treated group (two-way ANOVA with post hoc Bonferroni multiple comparisons)

≥40 days after CIE treatment. In contrast, α4 and γ2 subunit surface expression was increased. These changes are similar to what has been shown previously after ≥40 days withdrawal from CIE treatment for hippocampal CA1 and dentate gyrus regions [18]. The increase in surface α4- and the decrease in surface α1-containing $GABA_ARs$ we observed in the BLA at 40 days after CIE is in agreement with what has been previously reported for BLA at 24 h after CIE treatment using vapor chambers [19].

Studying the time-course after a single intoxicating dose of EtOH in the BLA there were early (1 h) decreases in cell-surface α4 and δ subunits. The α4 and δ subunits often co-assemble in one pentamer at extrasynaptic $GABA_ARs$ which mediate the tonic current and have been suggested to display high EtOH sensitivity [12, 13]. Under control conditions we found that the GABAergic I_{tonic} is enhanced by EtOH in BLA principal neurons. EtOH has been shown to promote binding of the clathrin adapter protein AP2-μ2 to δ-subunits [31], suggesting agonist-induced endocytosis after EtOH intoxication. The δ subunit is still down-regulated 48 h after EtOH. At this point we found that the I_{tonic} is no longer enhanced by EtOH. Like in the hippocampus, the α1 subunit surface expression is decreased 48 h after single-dose EtOH and CIE, and the α4 and γ2 subunits are both up-regulated suggesting less partnering of γ2 with α1, but forming γ2/α4-containing receptors, which have been shown to localize at synaptic sites [32]. In cultured cortical neurons, EtOH-induced increases in α4 and decreases in α1 surface subunits were shown to be dependent on an intricate time-dependent interplay between activities of protein kinase A and different isoforms of protein kinase C at

$GABA_A$Rs [33–36]. The BLA subunit alterations likely account for the faster rise and decay kinetics and the trend towards decreased charge transfer of mIPSCs observed in the present study (Table 1), and similar to the changes observed after CIE and single dose EtOH treatment in hippocampal neurons [18]. Another parallel to the hippocampus is the reversibility of all the plastic changes in $GABA_A$R subunits after a single dose of EtOH, returning to control conditions in the BLA as well, while still present ≥40 d after CIE.

The bulk of the GABAergic postsynaptic currents in the BLA are mediated through α1- and α2-containing $GABA_A$Rs [9]. We measured α1 and α2 surface levels ≥40 d after CIE and 48 h after single-dose EtOH and observed decreases in both subunits at both time-points. Decreases in BLA α2, α3, and a trend to decreased α1 mRNA expression have been reported in non-human primates after long-term EtOH self-administration [37], consistent with the decreases in benzodiazepine (flunitrazepam) sensitivity in BLA recordings from such animals [38]. In the rat cerebral cortex decreases in α1, α2, and α5 subunit mRNA have been reported after chronic EtOH [39]. In cultured rat hippocampal neurons [40] and cultured cerebellar granule cells [41] increases in α2 mRNA have been demonstrated. This suggests, that not all brain regions have the exact same plasticity, which is also dependent on the animal species, cell-type, neuromodulators, G-proteins, other neurotransmitter systems or co-transmitters or the subunits expressed.

Interestingly, no changes in α2 total protein levels were found in rats after CIE treatment [19]. We also detected no differences in the amount of total α2 protein, but its surface level was significantly decreased. This suggests that even in the absence of changes in expression and production of total α2 protein, EtOH treatment may result in the preferential trafficking of selective subunit combinations of $GABA_A$Rs to the membrane surface.

Compared to other brain regions the α3 subunit has a notably strong expression in the BLA [42], participating to a major amount in the tonic current [10]. However, this subunit, most likely associated with γ2 is also found at synaptic sites and has been reported to interact with the postsynaptic protein gephyrin at inhibitory synapses [43]. After EtOH treatment there might be compensation not only by α4/γ2 but also by α3/γ2, explaining an increase in surface γ2 after CIE and 48 h after single dose EtOH even though surface α1 and α2 are down-regulated. Since total α3 protein levels were reported unchanged in the BLA after CIE treatment [19], this hypothesized compensation would involve selective increases in surface α3 subunits. Compensatory increases in the surface levels of α4/γ2 and α3/γ2 might also account for the lack of changes in the magnitude of I_{tonic} observed at 48 h post-EtOH in this study.

Possible Behavioral Consequences

$GABA_A$Rs play a crucial role in the BLA. Blocking $GABA_A$Rs induces anxious behaviors [4], while enhancing $GABA_A$Rs attenuates fear and anxiety-like behaviors [3, 44]. The down-regulation of certain $GABA_A$Rs subtypes we observed in the BLA after EtOH could be a possible cellular substrate of withdrawal-anxiety. Both the α1 and the α2 subunits are down-regulated. Different groups of interneurons signal through different $GABA_A$R subtypes, so that they participate in distinct neurocircuits [9]. For example, the α1 subunit is primarily located at cholecystokinin/cannabinoid 1 receptor-positive synapses, while other GABAergic synapses in the BLA exhibit greater expression of α2 subunit-containing $GABA_A$Rs [9]. Parvalbumin-expressing interneurons can coordinate and synchronize firing of the principal neurons and may underlie network oscillations in the BLA related to fear [45, 46].

Mice expressing α2 point-mutations in the benzodiazepine binding pocket are resistant to anxiolytic-like effects of benzodiazepines and therefore have been thought to be involved in anxiolysis [47]. A decrease in conditioned fear has been suggested to be correlated with an up-regulation of the α2 subunit in the amygdala [48]. Thus, decreases in the surface α2 subunit in the BLA might contribute to the increased anxiety profile of rats during withdrawal from alcohol intoxication [32] and possibly to the emergence of a heightened response to stress in rats during protracted withdrawal from CIE treatment [49]. Studies with global α1 point-mutated knock-in (diazepam-insensitive) mice have shown that the sedative-like but not anxiolytic actions of benzodiazepines are mediated by the α1 subunit [50] and studies using amygdala-specific knockdown techniques confirmed that the α1 subunit mediates sedative and anticonvulsant, but not anxiolytic properties of benzodiazepines [51]. Thus, decreases in the α1 subunit in the BLA might contribute to the hyperexcitability, decreased seizure threshold [52], and disruption of sleep patterns [53] during withdrawal from alcohol.

Despite clear changes in the relative levels of individual $GABA_A$R subunits, we did not detect any significant changes in the magnitude of I_{tonic} or the kinetics of mIPSCs at 48 h after single EtOH dosing. Others have also reported small increases in mIPSC total charge transfer after withdrawal from CIE treatment [19]. The bulk of mIPSCs detected in recordings from BLA principal neurons arise from input of local circuit GABAergic interneuron rather than the paracapsular interneurons [30]. Taken together, these observations suggest that the increases in anxiety observed during withdrawal from single dose EtOH or CIE treatment might not be mediated by a functional decrement in postsynaptic $GABA_A$R function within the BLA.

However, previous studies have also demonstrated that glutamatergic neurotransmission is enhanced in the BLA after withdrawal from CIE treatment [54, 55]. Since the GABA and glutamate systems work in concert to drive BLA-mediated behaviors it is likely that the CIE-induced increases in anxiety behaviors are mediated by the enhanced glutamatergic function [54].

Despite the lack of change in the magnitude of I_{tonic} and in mIPSC kinetics, their sensitivity to acute EtOH application was clearly altered at 48 h after single EtOH dosing. The "switch" in acute EtOH sensitivity from extrasynaptic to synaptic $GABA_A$Rs was previously demonstrated in hippocampal CA1 pyramidal and dentate granule cells after single dose EtOH and CIE treatment [18, 32]. This sensitivity switch appears to account for the development of tolerance to the sedative/anesthetic effects of EtOH and its maintained anxiolytic effectiveness after CIE treatment [32, 56]. Analogous maintenance of EtOH's anxiolytic effectiveness of may account for the high rates of stress-induced relapse to drinking in alcohol-dependent individuals.

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