Chronic Ethanol and Withdrawal Differentially Modulate Basolateral Amygdala Paracapsular and Local GABAergic Synapses

Marvin R. Diaz, Daniel T. Christian, Nancy J. Anderson, and Brian A. McCool

Department of Physiology and Pharmacology

Wake Forest University School of Medicine, Winston-Salem, NC
(MRD, DTC, NJA, BAM)
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Author for Correspondence:
Brian A. McCool, Ph.D.
Associate Professor
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157
Phone: 336-716-8608
Fax: 336-716-8501
E-mail: bmmcool@wfubmc.edu

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Non-Standard Abbreviations: BLA – lateral/basolateral amygdala; BS3 – bis(sulfosuccinimidyl)suberate; CIE – chronic intermittent ethanol; EC – external capsule; GABA – γ-aminobutyric acid; GABA-RAP – GABA receptor associated protein; LPC – lateral paracapsular cells; mIPSC – miniature inhibitory postsynaptic current; WD – withdrawal

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Abstract

Withdrawal-related anxiety is cited as a major contributor to relapse in recovering alcoholics. Changes in basolateral amygdala (BLA) neurotransmission could directly influence anxiety-like behaviors following chronic ethanol exposure and withdrawal. We have recently shown that these treatments enhance BLA glutamatergic function and neurotransmission. However, the BLA GABAergic system tightly controls the expression of anxiety-like behavior; and, additional neuro-adaptations in this system are potentially important as well. The intrinsic BLA GABAergic system consists of at least two populations of interneurons – local feed-back interneurons scattered throughout the region and feed-forward interneurons concentrated within groups found in the lateral/paracapsular region of the BLA. In the present study, we found that withdrawal from chronic ethanol robustly decreased presynaptic function at feed-forward GABA synapses but did not alter neurotransmitter release from local interneurons. Differential presynaptic changes at these synapses were complemented by decreased zolpidem sensitivity at feed-forward synapses and decreased midazolam sensitivity at local synapses. Consistent with this, chronic ethanol/withdrawal decreased expression of GABA $\alpha_1$ subunit total protein and increased surface expression of $\alpha_4$ subunit protein. We also found transient increases in GABA-RAP protein levels and persistent increases in $\gamma_2$ subunit and gephyrin proteins that would suggest alterations in GABA$_A$ receptor trafficking that might help regulate changes in $\alpha_4$ subunit localization. These data together suggest that chronic ethanol and withdrawal differentially modulate local and LPC GABAergic synapses via distinct pre- and post-synaptic mechanisms. These findings extend our understanding of the neurobiological mechanisms governing changes in anxiety-like behavior following chronic ethanol exposure and withdrawal.
Introduction

Dependence-associated anxiety is a significant risk factor for relapse in human alcoholics. This withdrawal-related anxiety has been recapitulated in many different rodent models of ethanol dependence. Although many brain regions are likely to regulate withdrawal-associated anxiety, the amygdala plays an important role in both fear-learning and innate anxiety-like behaviors across many species (Davis et al., 2010) and appears to regulate the expression of withdrawal anxiety as well (Lack et al., 2007). The lateral and basolateral subdivisions of the amygdala (BLA) serve as a primary input into the fear/anxiety circuit and are critically important to drug-related behaviors like relapse (See, 2005). The BLA neurotransmitter systems that are altered by ethanol exposure and that help regulate withdrawal-associated behaviors like anxiety have only recently been explored.

We have previously shown that chronic ethanol/withdrawal produces robust increases in glutamatergic synaptic and receptor function measured from BLA principal neurons (Lack et al., 2007; Lack et al., 2009), and this may contribute to the expression of withdrawal anxiety. However, intoxicated animals express decreased, not increased, anxiety-like behavior immediately following a chronic exposure despite exposure-induced increases in glutamatergic synaptic function. Since BLA glutamatergic synaptic responses are relatively insensitive to acute ethanol (Lack et al., 2008), the maintenance of an anxiolytic phenotype suggests contributions by neurotransmitter systems other than glutamate. In this regard, the expression of anxiety-like behavior (Sanders and Shekhar, 1995) and the activity of BLA principal neurons (Woodruff et al., 2006) are tightly regulated by GABAergic neurotransmission. Given that GABAergic neurotransmission in this brain region is robustly enhanced by acute ethanol (Silberman et al., 2008), we hypothesized that exposure-related changes in the BLA GABAergic
system might ultimately influence the expression of anxiety-like behavior in intoxicated animals and during withdrawal.

There is an extensive literature demonstrating that chronic ethanol exposure results in robust changes to GABA\(_A\) receptor pharmacology, expression, and function. Several excellent reviews on this topic are available (Siggins et al., 2005; Kumar et al., 2009). In general, chronic ethanol exposure appears to alter GABA\(_A\) receptor subunit composition in a manner that changes the pharmacology and biophysical properties of the channel. Recent studies demonstrating altered lateral amygdala GABA\(_A\) receptor pharmacology and mRNA expression (Floyd et al., 2004) in non-human primates following long-term ethanol self-administration are consistent with this. Similarly, chronic exposure to a liquid ethanol diet increased the functional expression of GABA\(_A\) receptors measured in acutely isolated rat BLA neurons (McCool et al., 2003). While these data suggest that chronic ethanol may modulate the BLA GABA system, GABAergic synaptic neurotransmission has not been specifically examined.

The GABAergic system in the BLA is comprised of at least two anatomically and functionally distinct populations of interneurons. Lateral paracapsular cells (LPC) are GABAergic interneurons concentrated in ‘islands’ along the external capsule and provide feed-forward inhibitory synapses onto the distal dendrites of BLA principal neurons (Marowsky et al., 2005). In contrast, local interneurons are scattered throughout the subdivision and provide feedback inhibitory synapses onto peri-somatic areas of BLA principal neurons (Woodruff and Sah, 2007). A similar dichotomy of GABAergic neurocircuitry has also been found in the hippocampus (Weiner et al., 1997; Poelchen et al., 2000) and the cerebellum (Mameli et al., 2008); and the acute effects of ethanol are distinct at these different GABAergic inputs. Nevertheless, these anatomically distinct BLA GABAergic synapses arising from different
interneuron populations can be independently activated during in vitro electrophysiological recordings using specific placement of the stimulating electrodes (Silberman et al., 2008; Diaz et al., 2010). This provides the opportunity to study the effects of chronic ethanol and withdrawal on distinct populations of BLA GABAergic synapses.

Methods

Animals. All animal procedures were performed in accordance with protocols approved by Wake Forest University School of Medicine Animal Care and Use Committee and were consistent with the National Institutes of Health animal care and use policy. 120-150g male Sprague–Dawley peri-adolescent rats (~5 weeks of age; (McCutcheon and Marinelli, 2009)) were obtained from Harlan (Indianapolis, IN, U.S.A.) and were housed in an animal care facility at 23°C with a 12-h light/dark cycle and given food and water without restriction. Rats were weighed daily to ensure that ≥80% of their free-feeding weight was maintained during vapor chamber ethanol exposure.

Chronic Ethanol Exposure. Ethanol exposure was accomplished via an ethanol vapor chamber as previously described (Lack et al., 2007; Lack et al., 2009). Briefly, rats were housed in groups of four in large, standard polycarbonate cages. To achieve the ethanol exposure, these home cages were placed in large, custom-built Plexiglas chambers (Triad Plastics, Winston-Salem, NC, U.S.A.) similar to those previously described (Lack et al., 2009). At the beginning of the light cycle (lights on at 9 pm EST), animals were exposed to either ethanol vapor or only room air (control or CON group) for 12 hours during the light cycle for 10 days. Using calibrated pressure gauges, we mixed ethanol vapor with room air to achieve the desired vapor concentration (~45mg EtOH/L air) in the ethanol chamber. Vapor levels were tested daily. Animals receiving the chronic intermittent ethanol vapor were further divided into two
experimental groups: some animals were sacrificed immediately after the last ethanol exposure while they were still intoxicated (chronic intermittent ethanol or CIE group); the remaining animals remained in the chamber but were withdrawn from ethanol for 24hr prior to sacrifice (withdrawal or WD group). Blood was collected at sacrifice from the CIE group; and blood ethanol levels were 194±11mg/dl (n=45) as determined by a commercially available alcohol dehydrogenase assay (Genzyme, Middleton WI, U.S.A.).

Slice Preparation. Animals were anesthetized with halothane and decapitated according to a protocol approved by an Institutional Animal Care and Use Committee. The brains were quickly removed and incubated in ice-cold sucrose/artificial CSF (aCSF) equilibrated with 95% O₂ and 5% CO₂ containing (in mM): 180 sucrose, 30 NaCl, 4.5 KCl, 1 MgCl₂·6H₂O, 26 NaHCO₃, 1.2 NaH₂PO₄, 10 glucose, and 0.1 ketamine. Coronal brain slices (400µm) were prepared using a Vibratome Series 3000 (Vibratome, St. Louis, MO, U.S.A.) and submerged in room-temperature (~25°C), oxygenated standard aCSF containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 MgSO₄, 26 NaHCO₃, 10 glucose, and 2 CaCl₂·2H₂O. Slices were maintained in aCSF for ~1 hour before recording. All experiments were performed 1-4 hours after preparation of the BLA slices. All chemicals for slice preparation and electrophysiology were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise noted.

Whole-cell patch-clamp electrophysiology. Methods used for whole-cell patch-clamp electrophysiology were similar to those previously described (Lack et al., 2007). Slices were placed in a recording chamber and perfused with room temperature aCSF at a rate of 2mL/min. Patch electrodes were filled with an internal solution containing (in mM): 122 Cs-gluconate, 10 CsCl, 10 HEPES, 1 EGTA, 5 NaCl, 0.1 CaCl₂, 4 Mg-ATP, 0.3 Na-GTP, and 2 QX314-(Cl) and had an open-tip of 8-12 MΩ. All recordings were made from principal BLA neurons as
suggested by their initial membrane resistance of <50 MΩ (Lack et al., 2007). Inclusion criteria for analysis was that access resistance and baseline holding currents did not change more than 20% throughout the duration of any experiment. Analysis of holding currents revealed a significant treatment-dependent decrease in holding current in neurons recorded from CIE and WD slices relative to the CON neurons (one-way ANOVA, F=4.16 dF=2, P<0.05; data not shown). Data were acquired at 10kHz with an Axopatch 200B amplifier (Molecular Devices, Foster City, CA, U.S.A.) and analyzed using Clampex software (Molecular Devices). Electrically evoked GABA-IPSCs were pharmacologically isolated with the glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3(1H,4H)-dione (DNQX, 20µM) and D/L-2-amino-5-phosphono-pentanoic acid (APV, 50µM).

For some experiments, GABAergic IPSCs were electrically evoked using platinum/iridium concentric bipolar stimulating electrodes (FHC, Bowdoinham, ME, U.S.A.) with an inner pole diameter of 25µm. Stimulating electrodes were placed in the external capsule to stimulate paracapsular GABAergic synapses and within the BLA, medial to the recording site, to stimulate local GABAergic synapses (Silberman et al., 2008; Diaz et al., 2010). Stimulation intensities used at both stimulation sites achieved a GABAergic response of ~100pA; this level of stimulation represents less than 20% of electrically-evoked maximum responses (data not shown) and preferentially activates these distinct interneuron populations (Silberman et al., 2008). For electrically-evoked experiments, the LPC and local IPSCs were both measured in a given BLA principal neuron. The consecutive order of stimulation sites was alternated between neurons; and, in some cases, these sites were consecutively stimulated during the same recording epoch. BLA neurons were maintained at a holding potential of -10mV.
Paired-Pulse Ratios. Paired-electrical stimuli were given to each stimulation site at interpulse intervals of 50 and 250 msec. These intervals were chosen to examine treatment-related changes in GABA release probability (50 msec) and presynaptic autoreceptor function (250 msec). A normalized paired-pulse ratio for each stimulation site was calculated as \( \frac{\text{Amplitude}_{\text{IPSC}_2} - \text{Amplitude}_{\text{IPSC}_1}}{\text{Amplitude}_{\text{IPSC}_1}} \). These ratios were expressed as means ± SEM, and compared across treatment groups using a one-way ANOVA and a Newman-Keul’s post-hoc test, with P<0.05 considered statistically significant.

Spontaneous GABAAergic Synaptic Events. Miniature inhibitory postsynaptic currents (mIPSCs), were acquired at 20 kHz, and were filtered at 2 kHz. For these experiments, we used a holding membrane potential of -60mV and an internal solution consisting of (in mM): 135 KCl, 10 HEPES, 2MgCl2, 0.5 EGTA, 5 Mg-ATP, 1 Na-GTP, and 1 QX314-(Cl), pH 7.25, osmolarity 280-290 mOsm. After the onset of the recordings, 1µM tetrodotoxin (TTX) was applied for >5 minutes before recording spontaneous activity. mIPSCs were recorded for 1 minute following a baseline period (~5min). Event amplitude, frequency, charge transfer, and decay time (including \( \tau \) measures) were measured using MiniAnalysis (SynaptSoft, Inc.). Median values of these measures from individual cells were averaged within treatment groups (Lack et al., 2007), reported as mean ± SEM, and analyzed using one-way ANOVA with Newman-Keul’s post-hoc test. P<0.05 was considered statistically significant.

Zolpidem, Midazolam, and Ethanol Pharmacology. After collecting a baseline of evoked GABA-IPSCs, 100nM zolpidem (a GABA\(_A\) \( \alpha1 \) subunit-selective modulator), or 1µM midazolam (a benzodiazepine allosteric modulator), or 80mM ethanol was perfused onto slices until the drug effect reached steady state (typically within 10min). Drug effects were calculated as percent
change from baseline±SEM and were subjected to a one-way ANOVA with Newman-Keul’s post-hoc test. P < 0.05 was considered statistically significant.

**Western Blots.** Lysis buffer (50mM Tris pH 7.4, 0.5% sodium dodecyl sulfate, 1mM EDTA pH 8, and protease inhibitors for mammalian tissue (Sigma, St. Louis, MO)) was added to BLA dissected from CON, CIE, and WD coronal slices at 5μl/mg tissue; and tissue was disrupted by brief sonication and incubated at 4°C on a rotisserie mixer for 2 hours. Protein yield was quantified using the BCA assay (Thermal Scientific, Rockford, IL, U.S.A.). Fifteen micrograms of total protein was loaded on to 4-20% sodium dodecyl sulfate precast polyacrylamide gels (Thermal Scientific), separated, and transferred to a nitrocellulose membrane (Hybond N; Amersham, Piscataway, NJ, U.S.A.). The membrane was blocked with 10% nonfat dry milk (NFM) in Tris-buffered saline (TBS-T; 150mM NaCl, 5.2 mM Na2HPO4, 1.7mM KH2PO4, 0.05% Tween-20). Blots were incubated overnight at 4°C in TBS-T/0.5% NFM containing a rabbit polyclonal primary antibody: GABAA α1 subunit, 1:3000 dilution (Millipore, Temecula, CA, U.S.A.); GABAA α4 subunit-N terminus, 1:650 dilution (PhosphoSolutions, Aurora, CO, U.S.A.); GABAA γ2 subunit, 1:500 dilution (Sigma); Gephyrin, 0.5ug/mL (Millipore); GABA RAP, 1:300 (Novus Biologicals, LLC, Littleton, CO, U.S.A.). Following extensive washing with TBS-T, the blots were exposed to a goat anti-rabbit, HRP-labeled secondary antibody (1:3000 dilution; Sigma) for one hour at room temperature with agitation. Detection of bound secondary antibody was performed using SuperSignal West Dura Extended Duration Substrate (Thermal Scientific). To normalize expression between experiments, the blots were probed with mouse monoclonal antibody directed against β-actin, 1:50,000 to 1:100,000 dilution (Millipore) followed by peroxidase-labeled goat anti-mouse secondary antibody, 1:10,000 dilution (Sigma). Band intensity was quantified from digital
images captured on a CCD camera using a Bio Rad ChemiDoc XRS Imaging System with Quantity One Analysis Software (Hercules, CA, U.S.A.).

*Surface Cross-linking with BS³.* To examine surface-expression of specific GABA_A receptor subunits, we utilized the membrane-impermeable cross-linker bis(sulfosuccinimidyl)suberate (BS³, Thermal Scientific) as previously described (Grosshans et al., 2002) with slight modifications. Slices from individual CON, CIE, and WD animals were allowed to stabilize for 1 hour after preparation and subsequently transferred to aCSF ± 1mg/ml BS³ and allowed to incubate for 1 hour at 4°C. We then rinsed slices three times with aCSF containing 20mM Tris (pH = 7.4). The BLA was dissected and frozen until tissue was used for Western Blot analysis. Western Blot methods were the same as described above except that 20μg total protein was loaded onto precast 8-16% SDS-polyacrylamide gels. BS³-insensitive intracellular protein was compared to total protein from the untreated samples collected in parallel to calculate the percent protein found on the surface for each animal. Percent ‘cell surface’ receptors from individual animals were averaged within the treatment groups and compared using a one-way ANOVA with Newman-Keuls multiple comparisons post-test.

**Results**

*Differential Presynaptic Adaptations at Paracapsular and Local GABAergic Synapses*

To measure presynaptic changes from both paracapsular and local synapses, we employed two stimulating electrodes, one placed along the border of the BLA within the external capsule and on placed within the BLA medial to the recording site. This arrangement functionally separates GABAergic synapses from paracapsular and local interneurons (Lack et al., 2007; Silberman et al., 2008; Silberman et al., 2009). To measure presynaptic function from each synapse, we used paired electrical stimuli and calculated the ratio between the first and
second synaptic response (see Methods; Fig. 1A). At the paracapsular GABAergic synapses, we found ethanol exposure/withdrawal significantly increased the paired-pulse ratio at the 50msec inter-stimulus interval (Fig. 1B; One-way ANOVA, F=4.53, dF=2, P<0.05) and significantly decreased the ratio at the 250msec inter-stimulus interval (One-way ANOVA, F=3.90, dF=2, P<0.05). Neuman-Kuels multiple comparison posttest indicated that ratios obtained from BLA neurons in the withdrawal group (WD) was significantly different from the control (CON) at both stimulus intervals (P<0.05). With the 250msec interval, WD paracapsular response ratios were also significantly different from the chronic intermittent ethanol group (CIE; P<0.05). In contrast to the effects of withdrawal at the paracapsular GABAergic inputs, there were no treatment effects on the paired pulse ratio recorded from local GABAergic synapses (Fig. 1C; one-way ANOVA, P>0.05 at both inter-stimulus intervals). These data suggest that withdrawal from chronic ethanol exposure differentially modulates the presynaptic function of paracapsular and local GABAergic inputs onto BLA pyramidal neurons.

Recent evidence suggests that tonic GABAB receptor activity regulates LPC GABAergic synapses in the BLA (Silberman et al., 2008; Silberman et al., 2009). Although this modulation did not involve apparent presynaptic mechanisms in naïve animals, it is possible that chronic ethanol exposure and withdrawal altered the presynaptic contributions by GABAB receptors at LPC synapses. To test this directly, we measured the effects of the GABAB antagonist CGP55845 (10μM; Tocris Bioscience) on paired-pulse responses from LPC synapses using the 250msec interstimulus interval. In these experiments, the paired-pulse ratio at 250msec in WD neurons was not significantly different ±CGP55845 (baseline = -0.27±0.15, +CGP55845 = -0.32±0.11, P>0.05, paired t-test, n=9 cells). Likewise, CGP55845 did not significantly alter the 250msec ratio in either CON (n=4) or CIE (n=7) neurons (not shown, P>0.05, paired t-test).
Together, these data are very similar to that reported previously in a study using the GABA_B antagonist SCH50911 (Silberman et al., 2009) and suggest that treatment-related changes in LPC paired-pulse ratios at the 250msec stimulus interval are not related to changes in GABA_B receptor function.

To confirm that presynaptic transmitter release from local synapses was not altered by chronic ethanol or withdrawal, we quantified the frequency and amplitude of spontaneous IPSCs in the presence of tetrodotoxin (1μM TTX). In BLA principal neurons, miniature IPSCs (mIPSCs) recorded arise solely from the GABAergic terminals of local feed-back interneurons (Silberman et al., 2009). Consistent with the paired-stimulus data, we found no treatment-related changes in mIPSC frequency (Fig. 2A; one-way ANOVA, P>0.05, F=1.33, Df=2). There was a trend of increased mIPSC amplitude in the WD group (21.85±1.74pA, n=7) relative to CON (17.75±1.61pA, n=5) and CIE (18.49±1.17pA, n=5); but this did not reach statistical significance (one-way ANOVA, P>0.05). However, we found decay times from WD mIPSCs were significantly longer than both CON and CIE mIPSCs (Fig. 2C&D; one-way ANOVA, F=6.21, dF= 2, P<0.05; *--P<0.05 compared to CON, #--P<0.05 compared to CIE, using Newman-Keuls multiple comparison test). This was paralleled by a significant increase in the mIPSC charge transfer (area) in the WD group (Fig. 2E, one-way ANOVA; F=5.14, dF= 2, P<0.05; *--P<0.05 compared to CON, #--P<0.05 compared to CIE from Newman-Keuls multiple comparisons test). These data indicate that withdrawal from chronic ethanol may change postsynaptic GABA_A receptor function.

Withdrawal decreases benzodiazepine sensitivity at local synapses and alters α4 subunit expression and localization.
The WD-related changes in mIPSC kinetics suggest that chronic ethanol exposure or withdrawal might alter the properties of postsynaptic GABA$_A$ receptors. To examine this in more detail, we characterized the pharmacological properties of LPC and local GABAergic synapses in the CON, CIE, and WD treatment groups using the benzodiazepine, midazolam (1μM). There was no apparent treatment-related alteration in the percent effect of midazolam at LPC synapses (Fig. 3A$_2$, one-way ANOVA, P>0.05, F=1.05, dF=2). In contrast, we did find that midazolam modulation of the local GABAergic IPSC was significantly reduced in WD slices relative to CON and CIE (Fig. 3A$_1$ & A$_2$; one-way ANOVA, F=4.76, dF=2, P<0.05; *--P<0.05 relative to CON from Newman-Keul’s multiple comparison post-hoc analysis). These data suggest that withdrawal decreases the contribution by benzodiazepine-sensitive GABA$_A$ receptors at local but not LPC GABAergic inputs.

GABA$_A$ receptors containing the α4 subunit are insensitive to benzodiazepines (Wisden et al., 1991); so we examined α4 subunit expression and localization using western analysis of BLA tissue. There was no treatment-related change in total α4-subunit protein expression (Fig. 3B; one-way ANOVA, P>0.05). GABA$_A$ subunit immunoreactivity was normalized to β-actin; and, there was no significant differences in β-actin expression between CON (100.0±3.8%), CIE (90.0±4.0%), and WD samples (90.0±10.7%; n=4 animals per treatment, P>0.05 one-way ANOVA). Since the midazolam pharmacology suggested increased contributions by α4-containing receptors, we measured the expression of α4 subunit protein that was accessible to the membrane impermeant cross-linking agent, bis-(sulfosuccinimidyl) suberate (BS$_3$; see Methods). WD caused a significant increase in the BS$_3$-accessible α4-subunit protein (Fig. 3D) compared to both CON and CIE (one-way ANOVA, F=8.42, dF=2, P<0.01; **-- P<0.01 compared to CON, #--P<0.05 compared to CIE, from Newman-Keuls multiple comparisons post-
To ensure that BS$_3$-exposure did not interact with intracellular proteins, we compared β-actin levels from aCSF-exposed and BS$_3$-exposed samples and found no significant effect of the BS$_3$ treatment on β-actin immunoreactivity (BS$_3$=90±9% compared to 100±11% aCSF, n=4 each; P>0.05 paired t-test). These data suggests that WD does not alter total α4 subunit protein levels in the BLA but instead increases the amount of BS$_3$-accessible α4 subunit protein found on the cell surface. The functional decrease of local IPSC midazolam sensitivity is consistent with this interpretation.

Withdrawal-induced changes in α1-subunit function and expression at LPC synapses

Changes in GABA$_A$ α4 subunit expression are frequently associated with alterations in α1 subunit as well. To examine this directly, we used the non-benzodiazepine allosteric modulator zolpidem which is selective for GABA$_A$ α1-containing receptors. Zolpidem (100nM) robustly increased electrically-evoked GABAergic responses from both lateral paracapsular (Fig. 4A1) and local interneurons. Surprisingly, CIE and WD significantly diminished the percent-effect of zolpidem at the paracapsular synapses (Fig. 4A2; one-way-ANOVA, F=12.55, dF=2; *** -- P<0.001 compared to CON, Newman-Keul’s multiple comparison post-test) but not at local GABAergic synapses (one-way ANOVA, P>0.05, F=2.57). These data illustrate that chronic ethanol exposure and withdrawal diminish the functional contributions of zolpidem-sensitive GABA$_A$ receptors specifically at BLA LPC GABAergic synapses.

To examine the mechanism responsible for decreased zolpidem sensitivity at LPC synapses, we measured total α1-subunit protein expression and localization with western analysis. In contrast to the α4 data, α1-like immunoreactivity was significantly decreased in both CIE and WD groups compared to CON (Fig. 4B; one-way-ANOVA, F=10.35, dF=2, P<0.01; *--P<0.05, **--P<0.01 compared to CON from Newman-Keuls multiple comparison
post-test). BS^3-sensitive, surface-accessible α1-like immunoreactivity was also significantly decreased but only in the WD group (Fig. 4C; one-way ANOVA, F=5.39, dF=2, P<0.05; *-- P<0.05 compared to CON and #--P<0.05 compared to CIE, from Newman-Keul’s multiple comparison post-hoc analysis). Together, these biochemical data suggest that chronic ethanol exposure and withdrawal cause a robust decrease in the expression of total α1 GABA_α receptor subunit protein that is followed during WD by decreased levels of α1-containing receptors at the cell surface. The changes in zolpidem pharmacology suggest these alterations are more robustly expressed at LPC synapses relative to local GABAergic inputs.

**Molecular mechanisms regulating postsynaptic subunit changes during CIE and WD**

The reciprocal regulation of GABA_α receptor subunit pharmacology and expression suggest complex molecular mechanisms might be involved. To test this, we examined GABA_α-associated proteins involved with receptor localization and trafficking. CIE and WD significantly increased total γ2-subunit protein expression (Fig. 5A; one-way ANOVA, F=4.742, dF=2, P<0.05; * -- P<0.05 relative to CON, from Newman-Keul’s multiple comparison test). Importantly, neither α2 (CON=100±3%, CIE = 79±4%, and WD = 88±16%; n=4 animals each, P>0.05 one-way ANOVA) nor α3 (CON=100±7%, CIE = 91±5% and WD = 93±11% of, n=4, P>0.05) total protein levels were altered by CIE or WD (not shown). We likewise found that the expression levels of total gephyrin protein was significantly increased during CIE and WD (Fig. 5B; one-way ANOVA, F=9.94, dF=2, P<0.01; **--P<0.01, *--P<0.05, Newman-Keuls multiple comparison post-test). Finally, GABA_α-receptor associated protein (GABA-RAP) protein levels were significantly increased during CIE (Fig. 4C; one-way ANOVA, P<0.05; *--P<0.05 compared to CON from Newman-Keuls multiple comparisons post-hoc analysis) but returned to CON levels when measured after 24 hours of withdrawal.
Lack of Tolerance to Acute Ethanol during CIE and WD

At local synapses we found that neither CIE nor WD altered the efficacy of 80mM ethanol relative to CON BLA neurons (Fig. 6A; one-way ANOVA, P>0.05, F=0.11, dF=2, P>0.05). Likewise at LPC GABAergic synapses, the effect of 80mM ethanol was not altered by CIE or WD at LPC synapses (Fig. 6B; one-way ANOVA, P>0.05, F=0.06, dF=2). These data suggest that there is no tolerance to acute effects of ethanol on BLA GABAergic transmission in CIE and WD animals.

Discussion

In the lateral/basolateral amygdala, the GABAergic system is robustly modulated by chronic alcohol and withdrawal. This modulation is characterized by increased paired-pulse ratios at feed-forward GABAergic synapses and differential alterations of postsynaptic contributions by \( \alpha_1 \)- and \( \alpha_4 \)-containing receptors at feed-forward LPC and feed-back local inputs, respectively. These functional measures are complemented by changes in total subunit protein expression and/or surface localization of these subunits. While receptor benzodiazepine pharmacology is altered by chronic ethanol and withdrawal, the facilitating effects of acute ethanol on GABAergic transmission at both synapses remain intact.

Our paired-pulse findings suggests that withdrawal from chronic ethanol suppresses presynaptic function at BLA paracapsular GABAergic. Since synaptic responses to closely paired electrical stimuli are modulated presynaptic environments (reviewed in (Zucker and Regehr, 2002)), the WD-dependent increase in the paired-pulse ratio at LPC inputs may reflect decreased GABA release from these synapses. Importantly, these presynaptic effects were specific to paracapsular interneurons since the paired-pulse ratio did not change at local GABAergic synapses. Current evidence suggests that LPC interneurons are the predominant
source of feed-forward inhibition in the BLA (Marowsky et al., 2005) while local interneurons appear to provide the dominant source of feed-back inhibition in this brain region (Lang and Pare, 1998). Anatomical data suggests that lateral paracapsular synapses are segregated to the distal dendritic processes of BLA principal neurons (Marowsky et al., 2005); and recent pharmacological evidence is consistent with this hypothesis (Silberman et al., 2008; Diaz et al., 2010; Silberman et al., 2010). This suggests that dominant contributions by the perisomatic GABAergic synapses arising from local interneurons would likely mask any spontaneous activity arising from LPC interneuron synapses (Muller et al., 2006). The precise mechanisms governing down-regulation of GABA release from feed-forward LPC synapses are therefore not currently clear.

Our presynaptic findings contrast to some degree with previous work in the central amygdala and hippocampus. For example, chronic ethanol exposure increases GABAergic neurotransmission in the central amygdala (Roberto et al., 2003). However, the neuroanatomy of the BLA and CeA GABAergic synapses differ considerably. There are also numerous GABAergic neuronal populations within the CeA (Pitkanen and Amaral, 1994), and chronic ethanol modulation of these distinct neuronal phenotypes remains to be specifically examined. In contrast, chronic ethanol exposure decreases mIPSC frequency and amplitude in hippocampal neurons (Cagetti et al., 2003). Methodological differences between these studies and the current work might play some role in these apparent regional differences.

It is worth noting that the results from the paired-pulse 250 experiments at LPC synapses could suggest changes in autoreceptor function. In fact, tonic GABA$_B$ receptor activity regulates electrically-evoked IPSCs from LPC synapses (Silberman et al., 2008) but this does not appear to involve presynaptic mechanisms (Silberman et al., 2009). Similar to this, we found no
treatment-dependent changes in the sensitivity of paired-pulse responses to a GABA<sub>B</sub> antagonist. This suggests that the treatment-related changes in paired-pulse ratios at LPC synapses do not involve GABA<sub>B</sub> receptors. Similarly, the acute effects of ethanol on LPC GABAergic responses do not appear to be presynaptic (Silberman et al., 2009). The specific mechanisms underlying changes in paired-pulse responses at longer inter-stimulus intervals remain to be precisely identified.

While presynaptic neuroadaptations in the GABA system appear to occur exclusively at lateral paracapsular synapses, postsynaptic alterations occur at both GABAergic inputs. At LPC synapses, we found that CIE and WD decreased zolpidem-sensitivity. This was associated with a decrease in both total and surface (BS<sup>3</sup>)-accessible α<sub>1</sub> subunit immunoreactivity. While western analysis does not specifically measure changes in GABA<sub>A</sub> receptor subunits at synaptic sites, the coincidental depression of both zolpidem modulation and α<sub>1</sub>-subunit expression/localization measured are remarkably consistent. There are several potential mechanisms that could alter surface-expression of GABA<sub>A</sub> receptor subunit proteins. The BS<sup>3</sup>-resistant populations might consist of mature subunit proteins sequestered to intracellular compartments or may be related to immature forms of the subunit protein that have yet to reach the cell surface. Chronic ethanol modulation of either process would alter the quantity of BS<sup>3</sup>-accessible protein. In addition, BLA α<sub>1</sub>-mRNA decreases following long-term exposure/withdrawal in non-human primates (Floyd et al., 2004) and in rats (Falco et al., 2008).

Although these findings are similar to other brain regions (Cagetti et al., 2003), their apparent functional segregation to one subtype of BLA GABAergic input suggests that generalized decreases in GABA<sub>A</sub> subunit protein or mRNA expression might have a more localized impact at specific synapses. Since there was no significant exposure-related change in the midazolam
modulation at LPC GABAergic synapses, contributions by additional benzodiazepine-sensitive alpha subunits might compensate for the loss of $\alpha_1$-containing receptors. The lack of any treatment-effect on $\alpha_2$- or $\alpha_3$-total protein expression is consistent with this.

In contrast to the LPC inputs, we found a decrease in midazolam modulation by chronic ethanol exposure/withdrawal at local BLA GABAergic synapses. This suggests increased functional contributions by benzodiazepine-insensitive receptors at these inputs. The GABA$_A$ $\alpha_4$ subunit confers a benzodiazepine-insensitive phenotype to the channel, and $\alpha_4$ expression is robustly modulated by chronic ethanol. In the hippocampus, chronic intermittent ethanol exposure causes a ‘switch’ from benzodiazepine-sensitive GABA$_A$ receptors to more-insensitive receptors (Liang et al., 2006); and, this functional alteration is associated with down-regulation of $\alpha_1$- and up-regulation of $\alpha_4$-subunit protein (Cagetti et al., 2003). The decreased midazolam sensitivity of local BLA GABA synapses is likewise consistent with increased contributions by $\alpha_4$-containing receptors. Our biochemical data support this by showing increased levels of plasma membrane-associated $\alpha_4$ subunit protein (as measured by BS$_3$-accessible protein). Whether this represents an increased accumulation of mature, $\alpha_4$-containing protein specifically at synaptic sites or increased maturation rates for newly synthesized $\alpha_4$ subunits is not yet clear. It is also worth noting that there was a trend toward a decrease in zolpidem-sensitivity at local GABAergic inputs during CIE that did not reach statistical significance. However, it remains to be established whether longer exposures/withdrawals might spread contributions by benzodiazepine-insensitive subunit contributions to LPC GABAergic synapses or decrease zolpidem-sensitive subunit contributions at local GABAergic inputs.

In addition to these shifts in pharmacological sensitivity, it is worth noting that we also found that the decay times of TTX-insensitive spontaneous IPSCs were modestly but
significantly increased following chronic ethanol exposure/withdrawal. This finding contrasts with previous results in hippocampus showing significantly shorter decay times following chronic intermittent ethanol exposure (Cagetti et al., 2003). Given the coincidence between altered midazolam sensitivity at local synapses and the changes in mIPSC decay kinetics, α4-containing receptors may contribute to longer decay times measured in the current work. In support of this hypothesis, deactivation times of recombinant receptors following very brief (~1ms) applications of saturating GABA concentrations are longer in α4-containing receptors relative to α1-containing GABA<sub>A</sub> receptors (Lagrange et al., 2007). Unlike the conditions needed to produce receptor desensitization (prolonged exposure to high agonist concentrations), the constraints associated with this brief GABA application are similar to central GABAergic synapses where GABA concentrations are 1.2-5mM and clearance rates are estimated to be <1msec (Overstreet and Westbrook, 2003; Barberis et al., 2004). We believe the slower mIPSC decay times following ethanol exposure/withdrawal are consistent with slower deactivation due to increased α4-subunit contributions at the local GABAergic synapses. Given the paucity of pharmacological tools, neither the current study nor previous studies can rule out the possibility that altered mIPSC kinetics may be related to GABA<sub>A</sub> subunits other than α4.

The precise molecular mechanism for altered functional contributions by zolpidem- and midazolam-sensitive GABA<sub>A</sub> receptors is not totally clear. However, the increased levels of BLA γ2-subunit and gephyrin protein during CIE and WD may suggest a potential mechanism. γ2 subunits are important for GABA<sub>A</sub> receptor clustering at synaptic specializations (Schweizer et al., 2003), apparently via their close association with the postsynaptic density protein gephyrin (Essrich et al., 1998). We also found a significant increase in GABA-RAP expression; but this was transient and found only in the BLA from the CIE slices. GABA-RAP interacts with
gephyrin (Kneussel and Betz, 2000) within intracellular compartments and dissociates from GABA_A receptors once they are found at the synapse (Kneussel and Betz, 2000). In our system, GABA-RAP might help regulate the exposure-related trafficking of GABA_A receptors to-and-from intracellular pools to the plasma membrane (Kittler et al., 2001). Withdrawal could then consolidate these receptors at synaptic sites in a GABA-RAP-independent fashion.

In the BLA, the GABA and glutamate systems work intimately together to regulate BLA-mediated behaviors. Although glutamatergic function is elevated during CIE (Lack et al., 2007; Lack et al., 2009), both the chronic ethanol-resistant local GABAergic synapses and the continued sensitivity of GABAergic neurotransmission to acute ethanol appear sufficient to offset this elevated glutamatergic function while the animal is intoxicated. During withdrawal however, the BLA GABA system is markedly suppressed as a result of decreased GABAergic inhibition from LPC synapses and the notable absence of any ethanol in the system. Under these conditions, enhanced glutamatergic function would tend to drive the observed increases in anxiety-like behaviors (Lack et al., 2007). Our study therefore emphasizes the importance of the BLA GABA system and its control over the BLA principal neurons during chronic alcohol and withdrawal.

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Authorship Contributions

Marvin R. Diaz – participated in research design; conducted experiments; wrote or contributed to the writing of the manuscript

Daniel T. Christian – conducted experiments; wrote or contributed to the writing of the manuscript
Nancy J. Anderson – conducted experiments; wrote or contributed to the writing of the manuscript

Brian A. McCool – participated in research design; wrote or contributed to the writing of the manuscript


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Please direct reprint requests to:
Brian A. McCool, Ph.D.
Associate Professor
Wake Forest University School of Medicine
Medical Center Boulevard
Winston-Salem, NC 27157
Phone: 336-716-8608
Fax: 336-716-8501
E-mail: bmmcool@wfubmc.edu
Legends to the Figures

Figure 1: Chronic ethanol and withdrawal differentially modulate LPC and local interneuron synaptic responses to paired electrical stimuli. (A) Sample paired-pulse (PP) traces from local and LPC neurons across treatment groups. P1 and P2 denote the first and second response, respectively. Stimulation artifacts have been removed for clarity. Dotted lines used to compare peak of the first response to the second. These continuous traces sampled both local and LPC GABAergic synapses in the same BLA principal neuron using two stimulating electrodes (see Methods). (B) Normalized paired-pulse ratios (Methods) from LPC interneurons across the treatment groups at 50 and 250msec interpulse intervals (IPI) show a significant changes in WD treated neurons (n=11) relative to CON (n=21; one-way ANOVA P<0.05, *--P<0.05 with Newman-Keuls multiple comparison post-test). There was no significant difference between CON and CIE neurons (n=17). (C) Paired-pulse ratios measured in the same neurons as in (B) were evoked from local interneurons and were not different between the treatment groups at either stimulus interval.

Figure 2: Chronic ethanol and withdrawal differentially modulate pre- and post-synaptic properties of mIPSCs. (A) Exemplar traces of mIPSCs collected from individual principal cells at each exposure group (A1; CON, CIE, and WD). mIPSC amplitudes (A2) and inter-event intervals (A3) were not significantly different between CON (n=7), CIE (n=5), or WD (n=7) BLA neurons. (B) mIPSCs from each neuron were averaged to yield a normalized mIPSC current. Exemplar traces from representative CON and WD neurons are shown (B1). The normalized mIPSC decays were fit to a single exponential; amplitudes from these representative traces have been normalized to emphasize the decay phase of the response. Time constants ("Tau", B2) were significantly larger in both CIE and WD mIPSCs relative to CON (one-way...
ANOVA, P<0.01, **--P<0.01 relative to CON with Newman-Keul’s multiple comparison post-test).

**Figure 3:** Midazolam sensitivity of ‘local’ IPSCs and α4-subunit expression are modulated by chronic ethanol exposure/withdrawal. (A) Midazolam sensitivity decreases at local GABAergic synapses but not paracapsular GABAergic inputs. Exemplar traces of the effects of midazolam (1µM) on electrically-evoked GABA IPSCs from local interneurons from CON, CIE, and WD treated slices (A1). Summary of data (A2) shows that the midazolam sensitivity of local IPSCs (“Local”) was significantly decreased in principal neurons from WD slices (n=9; one-way ANOVA, *--P<0.05 vs CON, Newman-Keuls multiple comparison post-test) relative to CON (n=10) and CIE (n=9) BLA neurons. The effect of midazolam on evoked-IPSCs from LPC interneurons (“LPC”) was not altered in principal neurons from CIE or WD. (B) Total α4 protein levels were not significantly changed across treatment groups (n=4 animals each). Representative immunoreactive bands from α4 total protein and β-actin correspond to the treatment groups in the above bar graphs. (C) Surface-accessible, BS3-sensitive α4 subunit protein levels were significantly elevated in WD BLA (n=4 animals in each group), compared to CON BLA (one-way ANOVA, **--P<0.01 with Newman-Keuls multiple comparison posttest) and CIE BLA (#--P<0.05, Newman-Keuls). Representative aCSF-treated and BS3-treated samples from each treatment are show below the bar graph. The intracellular, BS3-insensitive pool of α4 subunit protein is shown (see Methods for details).

**Figure 4:** Zolpidem sensitivity and α1-subunit expression are robustly diminished by chronic ethanol and withdrawal. (A) Zopidem (100nM) modulation of LPC IPSCs is decreased in CIE and WD BLA principal neurons. Sample traces (A1) from LPC inputs illustrate the treatment
Summary of zolpidem data (A2) show that CIE (LPC: n=7, local:n=9) and WD (LPC: n=9, local: n=9) significantly diminish modulation at LPC but not local IPSCs relative to CON (LPC: n=9, local: n=9; one-way ANOVA, ***--P<0.001 vs CON, Newman-Keuls multiple comparison posttest). (B) Total α1-subunit protein levels were significantly reduced in CIE and WD BLA relative to CON (n=4 animals per group; one-way ANOVA, *--P<0.05, **--P<0.01 vs CON with Newman-Keuls posthoc). Representative immunoreactive bands from total α1 protein and β-actin correspond to the treatment groups in the above bar graphs. (C) BS3-sensitive, surface accessible α1-subunit protein levels were significantly reduced in WD BLA relative to both CON and CIE (n=4 animals/treatment; one-way ANOVA, *--P<0.05 compared to CON, #--P<0.05 compared to CIE, Newman-Keuls posthoc). Representative aCSF-treated and BS3-treated samples from each treatment are show below the bar graph. The intracellular, BS3-insensitive pool of α1 subunit protein is shown (see Methods for details).

**Figure 5:** CIE and WD significantly alter protein expression of several GABA<sub>A</sub> receptor association proteins. (A) Total γ2 subunit protein levels were robustly elevated in CIE and WD slices relative to CON (n=4 animals/treatment; one-way ANOVA, *--P<0.05 vs CON from Newman-Keuls posttest). (B) Total gephrin protein levels were also significantly increased in both CIE (**--P<0.01 relative to CON) and WD BLA slices (*--P<0.05 vs CON, Newman-Keuls posthoc). (C) Total GABA-RAP protein expression was significantly increased in CIE slices (*--P<0.05, Newman-Keuls posthoc) but returned to CON levels by 24 hours of withdrawal. Representative protein and β-actin immunoreactivity from each treatment group is shown at right.
Figure 6: CIE and WD-treatments do not alter acute ethanol modulation of BLA GABAergic synapses. Graphs demonstrate that the effect of 80mM acute ethanol on electrically evoked GABAergic transmission from LPC (A) and local (B) synapses is different between CON (n=6 at each input), CIE (n=11), and WD (n=10; P>0.05, one-way ANOVA) principal neurons. Sample traces correspond to the bar graphs.
Figure 1
Figure 2

A1

CON
CIE
WD

20pA
400msec

A2

mIPSC Amplitude

\[\begin{array}{ccc}
\text{pA} & \text{CON} & \text{CIE} & \text{WD} \\
0 & 15 & 20 & 25 \\
5 & 20 & 25 & 30 \\
10 & 25 & 30 & 35 \\
15 & 30 & 35 & 40 \\
20 & 35 & 40 & 45 \\
25 & 40 & 45 & 50 \\
\end{array}\]

A3

Inter-event Interval

\[\begin{array}{ccc}
\text{msec} & \text{CON} & \text{CIE} & \text{WD} \\
0 & 100 & 150 & 200 \\
50 & 150 & 200 & 250 \\
100 & 200 & 250 & 300 \\
150 & 250 & 300 & 350 \\
200 & 300 & 350 & 400 \\
250 & 350 & 400 & 450 \\
\end{array}\]

B1

CON
WD

20msec

B2

mIPSC Decay

\[\begin{array}{ccc}
\text{Tau (msec)} & \text{CON} & \text{CIE} & \text{WD} \\
0 & 5 & 10 & 15 \\
5 & 10 & 15 & 20 \\
10 & 15 & 20 & 25 \\
15 & 20 & 25 & 30 \\
20 & 25 & 30 & 35 \\
25 & 30 & 35 & 40 \\
\end{array}\]
Figure 3

A1

Midazolam → Baseline → CON
Midazolam → Baseline → CIE
Midazolam → Baseline → WD

A2

1μM Midazolam

% from Baseline

Local
LPC

CON
CIE
WD

B

Total Protein

Alpha 4

% from Control

CON
CIE
WD

C

Surface Expression

Alpha 4

% BS3-Accessible

CON
CIE
WD

Alpha 4 (64kD)

Actin

aCSF BS3

# **
Figure 6

A1

A2

LPC IPSCs

% from Baseline

CON CIE WD

B1

B2

Local IPSCs

% from Baseline

CON CIE WD