1521-0103/357/1/10–16$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

http://dx.doi.org/10.1124/jpet.115.230417
J Pharmacol Exp Ther 357:10–16, April 2016

Ethanol Regulation of Synaptic $GABA_A$ $\alpha4$ Receptors Is Prevented by Protein Kinase A Activation

Stephen L. Carlson, John Peyton Bohnsack, and A. Leslie Morrow

Departments of Psychiatry and Pharmacology, Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, Chapel Hill, North Carolina

Received November 3, 2015; accepted January 28, 2016

ABSTRACT

Ethanol alters $GABA_A$ receptor trafficking and function through activation of protein kinases, and these changes may underlie ethanol dependence and withdrawal. In this study, we used subsynaptic fraction techniques and patch-clamp electrophysiology to investigate the biochemical and functional effects of protein kinase A (PKA) and protein kinase C (PKC) activation by ethanol on synaptic $GABA_A$ $\alpha4$ receptors, a key target of ethanol-induced changes. Rat cerebral cortical neurons were grown for 18 days in vitro and exposed to ethanol and/or kinase modulators for 4 hours, a paradigm that recapitulates GABAergic changes found after chronic ethanol exposure in vivo. PKA activation by forskolin or rolipram during ethanol exposure prevented increases in P2 fraction $\alpha4$ subunit abundance, whereas inhibiting PKA had no effect. Similarly, in the synaptic fraction, activation of PKA by rolipram in the presence of ethanol prevented the increase in synaptic $\alpha4$ subunit abundance, whereas inhibiting PKA in the presence of ethanol was ineffective. Conversely, PKC inhibition in the presence of ethanol prevented the ethanol-induced increases in synaptic $\alpha4$ subunit abundance. Finally, we found that either activating PKA or inhibiting PKC in the presence of ethanol prevented the ethanol-induced decrease in GABA miniature inhibitory postsynaptic current decay τ_1, whereas inhibiting PKA had no effect. We conclude that PKA and PKC have opposing effects in the regulation of synaptic $\alpha4$ receptors, with PKA activation negatively modulating, and PKC activation positively modulating, synaptic $\alpha4$ subunit abundance and function. These results suggest potential targets for restoring normal GABAergic functioning in the treatment of alcohol use disorders.

Introduction

Ethanol causes adaptations in $GABA_A$ receptors that are associated with alcohol dependence and withdrawal (Kumar et al., 2009). $GABA_A$ receptors are ligand-gated chloride channels mediating the majority of inhibitory neurotransmission in the brain through both phasic and tonic currents (Farrant and Nusser, 2005). These channels consist of five subunits typically composed of two α subunits (1–6), two β subunits (1–3), and a γ (1–2) or δ subunit (Tretter and Moss, 2008). Two important targets of ethanol regulation in particular are the synaptic $\alpha4\beta\gamma2$ and extrasynaptic $\alpha4\beta\delta$ $GABA_A$ receptors mediating a portion of phasic and tonic inhibition, respectively (Olsen and Sieghart, 2009). The extrasynaptic $\alpha4$ receptors have been the subject of much study, owing to their responses to relatively low doses of ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004); however, surprisingly little is known about the regulation of synaptic $\alpha4$ receptors.

Although early studies showed that ethanol regulates the abundance of $\alpha4$ subunit mRNA and protein expression (Devaud et al., 1996, 1997; Papadeas et al., 2001; Cagetti et al., 2003), the question of whether this regulation reflected synaptic or extrasynaptic receptor adaptations depended on subsequent functional studies of synaptic $\alpha4$ receptor kinetics. Synaptic $\alpha4$ receptors are upregulated after both acute (Liang et al., 2007) and chronic (Liang et al., 2006; Werner et al., 2011) ethanol exposure in the rat hippocampus and cortex. More recently, we demonstrated ethanol regulation of synaptic $\alpha4$ subunits in C57BL/6J mice after an acute injection of ethanol, using subcellular fractionation to isolate synaptic versus subsynaptic receptors (Carlson et al., 2014). Isolating the precise physiologic and behavioral ramifications of these changes in synaptic $\alpha4$ receptors is difficult because there are no selective pharmacological agents targeting these receptors. The $\alpha4\beta\gamma2$ receptors are benzodiazepine insensitive, although they show relatively high affinity for the GABA inverse agonist Ro15-4513 (ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-1,4-benzodiazepine-3-carboxylate) (Knoflach et al., 1996). However, Ro15-4513 also antagonizes effects of ethanol on $\alpha4\beta\delta$ receptors (Hanchar et al., 2006) that likely mediate effects of ethanol on tonic inhibition.

Recent studies from our laboratory have uncovered that kinase activation by ethanol plays a major role in $GABA_A$ receptor regulation, and that protein kinase A (PKA) and protein kinase C (PKC) may have opposing effects on GABAergic trafficking and function. PKA activity positively

This research was supported by the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grants P60-AA11605 and T32-AA007573] and the University of North Carolina Bowles Center for Alcohol Studies.
dx.doi.org/10.1124/jpet.115.230417.

ABBREVIATIONS: ANOVA, analysis of variance; CalC, calphostin C; mIPSC, miniature inhibitory postsynaptic current; PdBu, phorbol-12,13-dibutyrate; PKA, protein kinase A; PKC, protein kinase C; Ro15-4513, ethyl-8-azido-5,6-dihydro-5-methyl-6-oxo-4*H*-imidazo-1,4-benzodiazepine-3-carboxylate; Rp-cAMP, Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine.

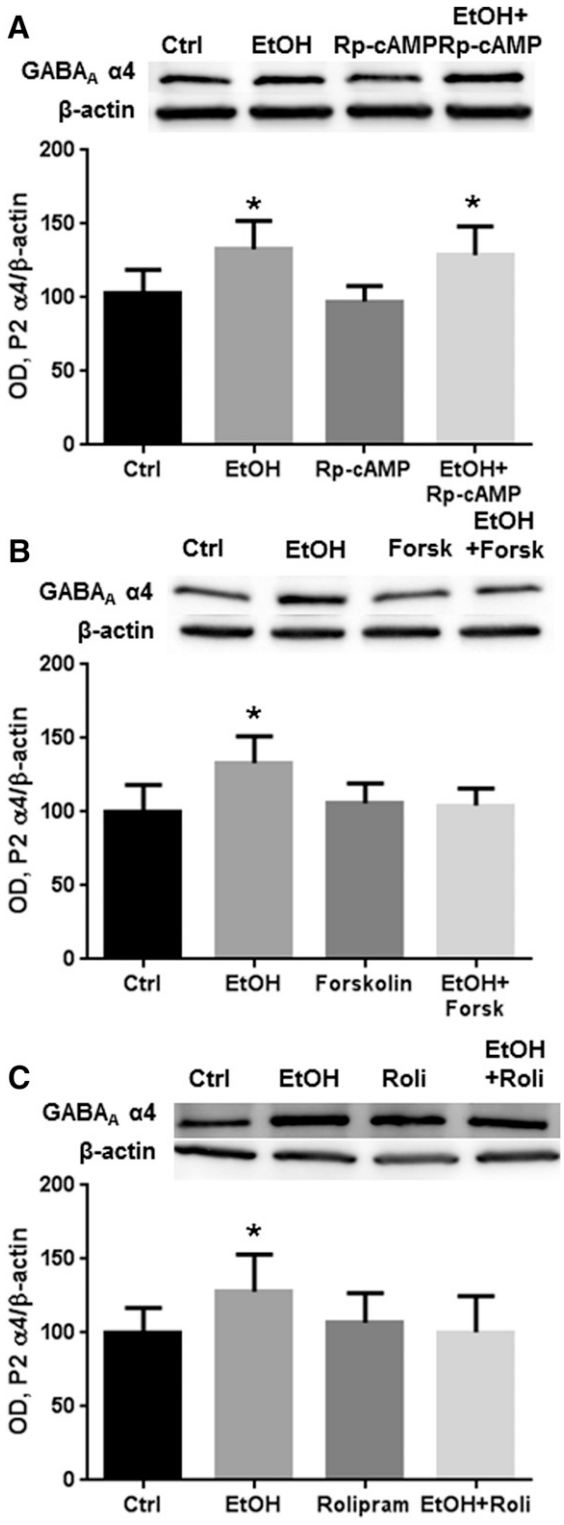

Fig. 1. PKA activation prevents ethanol-induced increases in P2 fraction GABA$_A$ α4 subunits. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 μM), forskolin (10 μM), and/or rolipram (10 μM) for 4 hours, followed by P2 fractionation and western blot analysis. (A) Exposure to ethanol for 4 hours increased P2 fraction levels of the GABA$_A$ α4 subunit, which was not affected by coexposure with Rp-cAMP. (B and C) Exposure to the adenylyl cyclase activator forskolin (B) or the phosphodiesterase inhibitor rolipram (C) with ethanol prevented the increase in P2 fraction levels of α4 subunits induced by ethanol. *$P < 0.05$ (one-way ANOVA, Bonferroni post-test, n = 6–8 per group). Ctrl, control; EtOH, ethanol; Forsk, forskolin; OD, optical density; Roli, rolipram.

regulates synaptic GABA$_A$ α1 subunit abundance and function in cortical neurons, whereas PKCγ activity negatively regulates these receptors (Kumar et al., 2010; Carlson et al., 2013). PKA activation also positively regulates extrasynaptic GABA$_A$ α4 and δ subunits in cortical neurons, whereas PKC is not involved in regulating these receptors in this brain region (Carlson et al., 2016). Finally, PKCγ activation by ethanol causes an increase in GABA$_A$ α4 subunits, although it is unclear whether this effect is specific to the synaptic population of receptors (Werner et al., 2011). It is also unclear what role PKA activity may play in regulating this subset of α4 receptors.

This study elucidates the role of PKA and PKC in ethanol regulation of synaptic α4-containing GABA$_A$ receptors in cerebral cortical cultured neurons. We used a 4-hour ethanol exposure paradigm that recapitulates many of the GABAergic adaptations observed after chronic ethanol exposure in vivo (Devaud et al., 1997; Kumar et al., 2003). We measured changes in subunit abundance using a subcellular fractionation technique that enriches synaptic proteins combined with western blot analysis, whereas functional changes were measured using whole-cell patch-clamp analysis of GABA miniature inhibitory postsynaptic currents (mIPSCs).

Materials and Methods

Cultured Cerebral Cortical Neurons. All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee at the University of North Carolina. Mixed-sex rat pups from Sprague-Dawley breeding pairs (Harlan, Indianapolis, IN) were decapitated on postnatal day 0–1. Brains were rapidly dissected and the cerebral cortices were isolated. Cortical halves were minced into fine pieces and tissue was incubated in CO_2-independent media containing papain (50 U/ml; Worthington, Lakewood, NJ) and L-cysteine and DNase (both from Sigma-Aldrich, St. Louis, MO) for 30 minutes at 37°C. Tissue pieces were gently washed, followed by gentle trituration in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY) containing 10% horse serum, penicillin-streptomycin, and DNase. Cells used for biochemistry were plated onto poly(D-lysine)–coated flasks, whereas cells used for electrophysiology were plated onto poly (D-lysine)–coated coverslips in 12-well plates. Cells were maintained in a 5% CO_2 humidified incubator. After day 3, cells were fed with serum-free medium containing B27 and penicillin-streptomycin (10,000 U/ml; final concentration 50 U per flask). Media were changed twice per week with no more than one-third of the media being removed during exchanges. For all experiments, penicillin-streptomycin was removed from cultures on day 14 to prevent interactions with GABA$_A$ receptors. Cultures were maintained for 18 days before conducting experiments, because prior studies determined that GABA$_A$ receptor expression was stable between 15 and 19 days in vitro.

Ethanol and Drug Exposure. Cultured cells were exposed to 50 mM ethanol and placed in a plastic vapor chamber within the incubator. This concentration was chosen because it produces changes in GABAergic inhibition consistent with in vivo models (Devaud et al., 1997; Kumar et al., 2003). A beaker of water with 50 mM ethanol was used to maintain stable ethanol concentrations in the chamber. Control cells were exposed to an equivalent amount of water and placed in a plastic vapor chamber with a beaker containing water. Cells were exposed to ethanol for 4 hours. To examine PKA involvement, the PKA inhibitor Rp-adenosine 3′,5′-cyclic monophosphothioate triethylamine (Rp-cAMP; 50 μM), the adenylyl cyclase activator forskolin (10 μM; Tocris Bioscience, Minneapolis, MN), or the phosphodiesterase inhibitor rolipram (10 μM; Sigma-Aldrich) was added to the cell media. To examine PKC involvement, the PKC inhibitor calphostin C (CalC; 0.3 μM in 0.1% dimethylsulfoxide, final

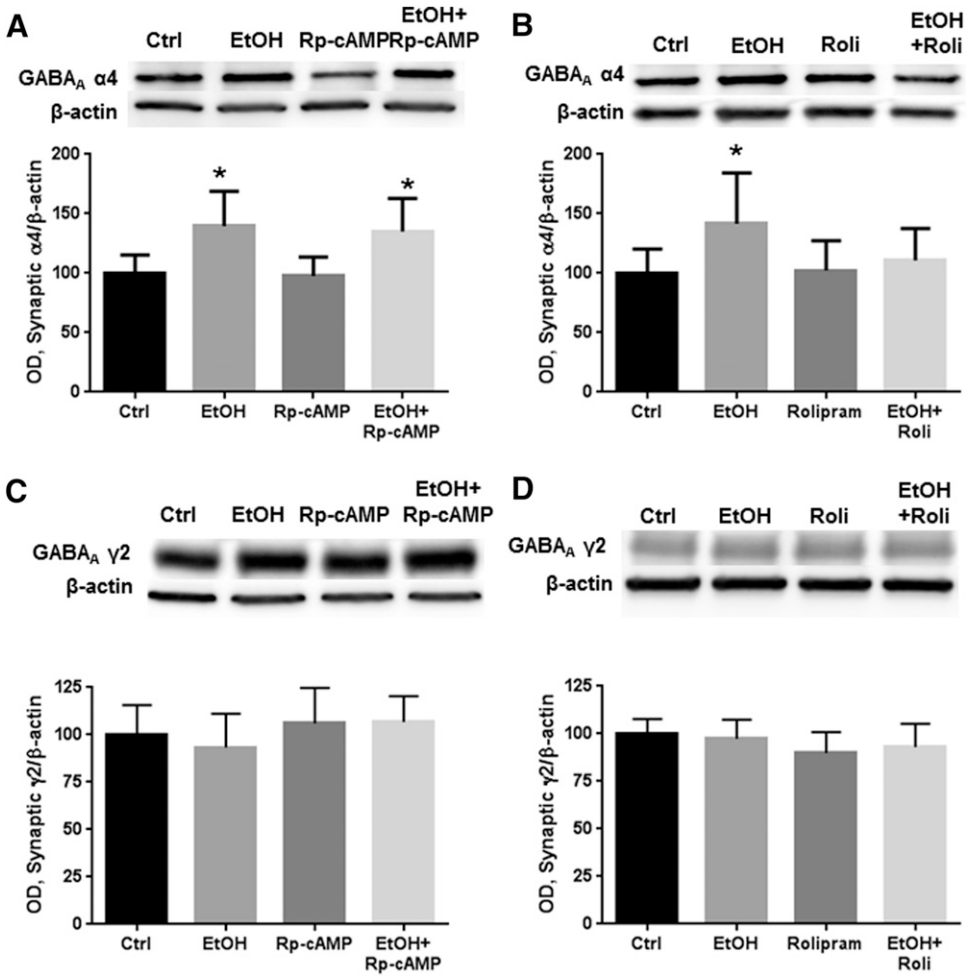

Fig. 2. PKA activation prevents the increased abundance of synaptic α4 receptors induced by ethanol. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 μM), and/or rolipram (10 μM) for 4 hours, followed by synaptic fractionation and western blot analysis. (A) Ethanol decreased synaptic $GABA_A$ α4 abundance, an effect that was not altered by coexposure with Rp-cAMP. (B) Coexposure with rolipram prevented ethanol-induced increases in synaptic α4 abundance. (C and D) $GABA_A$ γ2 abundance was not altered by exposure to ethanol or either Rp-cAMP (C) or rolipram (D). $^*P < 0.05$ (one-way ANOVA, Bonferroni post-test, n = 6–10). Ctrl, control; EtOH, ethanol; OD, optical density; Roli, rolipram.

concentrations; Sigma-Aldrich) or the activator phorbol-12,13-dibutyrate (0.1 μM; Sigma-Aldrich) was added to the cell media. The concentrations of PKA and PKC modulators were chosen based on previous studies (Zhang and Pandey, 2003; Carlson et al., 2013).

Subcellular Fractionation. After experiments, the reactions were stopped by placing the flasks on ice. Cells were washed with cold phosphate-buffered saline, scraped, centrifuged at 1000*g* for 18 minutes, and stored at −80°C until fractionation. Cell pellets were homogenized in 0.32 M sucrose and centrifuged at 1000*g* for 10 minutes. The supernatant was then centrifuged twice for 30 minutes at 12,000*g* to yield the P2 fraction pellet. For experiments examining the synaptic fraction, the P2 fraction was further purified into the synaptic fraction according to the methods of Goebel-Goody et al. (2009) as previously described (Carlson et al., 2014, 2016). The fractions were separated by 30-minute incubation in 0.5% Triton-X, followed by two centrifugations at 32,000*g* for 30 minutes. The resulting pellet was resuspended to yield the synaptic fraction. Protein concentrations for isolated P2 fraction or synaptic fractions were calculated using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Samples were then subjected to gel electrophoresis and western blot analysis.

Western Blot Analysis. $GABA_A$ receptor α4 subunit abundance was analyzed by western blot. Protein samples were subjected to SDS-PAGE using Novex Tris-Glycine (8%–16%) gels and were transferred to polyvinylidene fluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Membranes were probed with $GABA_A$ receptor α4 antibody (1:500 dilution; Abcam, Cambridge, MA), anti-$GABA_A$ δ (1:750 dilution; Novus, St. Louis, MO), or anti-$GABA_A$ γ2 (1:1000 dilution; Novus), followed by β-actin antibody (1:3000 dilution; Millipore, Billerica, MA) for normalization. Proteins were detected with enhanced chemiluminescence (GE Healthcare, Amersham, UK). Membranes were imaged using a LAS-4000 (GE Healthcare), and densitometric analysis was conducted using GE ImageQuant software. Comparisons were made within blots and expressed as a percentage of averaged control values.

Electrophysiology. Whole-cell voltage clamp recordings were used to assess mIPSCs. Electrodes were pulled using a PP-830 device (Narishige, Tokyo, Japan) and were fire polished to a resistance of 2 to 3 MΩ. Intracellular solution contained 150 mM KCl, 3.1 mM $MgCl_2$, 15 mM HEPES, 5 mM KATP, 5 mM EGTA, and 15 mM phosphocreatine, adjusted to pH 7.4 with KOH. Extracellular solution contained 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sucrose, and 10 mM glucose, adjusted to pH 7.4 with NaOH. For mIPSC recordings, the external solution also contained 6-cyno-7-nitroquinoxaline-2,3-dione (10 μM; Sigma-Aldrich), D-2-amino-5-phosphonopentanoic acid (40 μM; Tocris Bioscience), and tetrodotoxin (1 μM; Sigma-Aldrich). Membrane potential was held at −60 mV and currents were recorded with an Axopatch ID amplifier (Axon Instruments, Union City, CA). Data were collected using Clampex software (Axon Instruments). mIPSCs were analyzed using miniAnalysis software (version 5.6.4; Synaptosoft, Decatur, GA). mIPSCs were recorded for a minimum of 3 minutes. Minimum threshold detection was set to 5 pA. Frequency was determined using automatic detection of each recording. To assess mIPSC kinetics, the recording trace was visually inspected and only events with a stable baseline, sharp rising phase, and single peak were used. Only recordings with a minimum of 25 events fitting these criteria were analyzed. Decay time constants were obtained by using a double exponential fit for the average of the mIPSCs in a single recording.

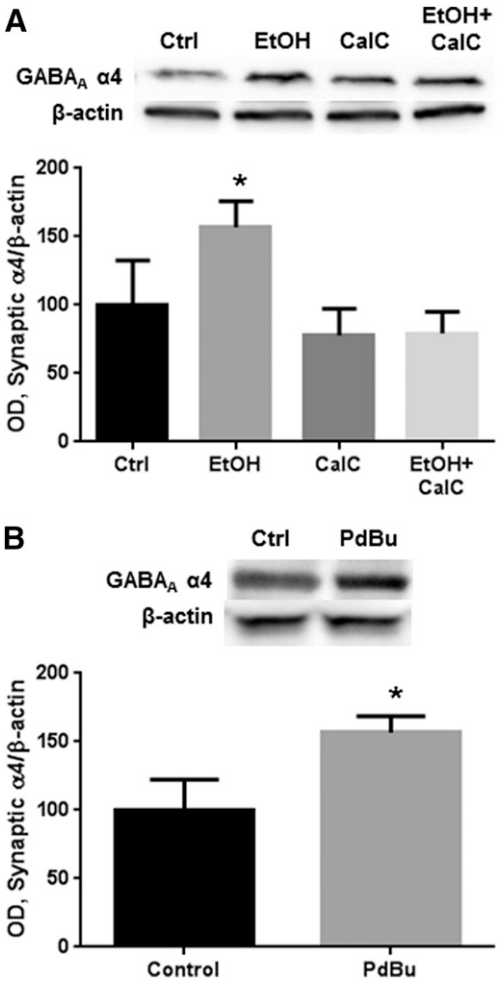

Fig. 3. PKC activation by ethanol increases GABA$_A$ α4 subunit levels in the synaptic fraction. Cortical neurons were exposed to vehicle, ethanol (50 mM), CalC (0.3 μM), and/or PdBu (0.1 μM) for 4 hours followed by synaptic fractionation and western blot analysis. (A) Exposure to ethanol for 4 hours increased synaptic fraction levels of the GABA$_A$ α4 subunit, which was prevented by coexposure with CalC. (B) Exposure to PdBu mimicked the effect of ethanol on synaptic GABA$_A$ α4 levels. *$P < 0.05$ (t test or one-way ANOVA, Bonferroni post-test, n = 5 to 6 per group). Ctrl, control; EtOH, ethanol; OD, optical density; PdBu, phorbol-12,13-dibutyrate; CalC, Calphostin C.

Statistical Analysis. Numerical data are presented as means ± S.E.M. Analyses were conducted using analysis of variance (ANOVA) and the Bonferroni post-test or t test.

Results

Direct PKA Activation Prevents the Effects of 4-Hour Ethanol Exposure on Total GABA$_A$ α4 Abundance in the P2 Fraction. We first examined the effect of PKA modulation on GABA$_A$ α4 subunits during ethanol exposure. Exposure to ethanol for 4 hours increased GABA$_A$ α4 subunit levels in the P2 fraction of cerebral cortical neurons as expected based on previous studies (Fig. 1). Inhibition of PKA by Rp-cAMP did not affect ethanol-induced increases in α4 abundance (Fig. 1A). Conversely, activation of PKA during ethanol exposure by either the adenylyl cyclase activator forskolin (10 μM; Fig. 1B, $P < 0.01$, one-way ANOVA, $F = 6.330$, $P < 0.01$, Bonferroni post-test, n = 6 to 7 per group) or the phosphodiesterase inhibitor rolipram (10 μM; Fig. 1C, $P < 0.05$, one-way ANOVA, $F = 3.107$, $P < 0.05$, Bonferroni post-test, n = 7 to 8 per group) prevented the increase of α4 subunit abundance. None of the PKA modulators alone had any effect on GABA$_A$ α4 subunit abundance.

PKA Activation Prevents the Effects of 4-Hour Ethanol Exposure on Synaptic Fraction GABA$_A$ α4 Abundance. To resolve whether the effects of ethanol and PKA on GABA$_A$ α4 subunits in the P2 fraction represent synaptic receptor regulation, we expanded the study shown in Fig. 1 to determine whether these effects occur within the synaptic fraction purified by subcellular fractionation (Carlson et al., 2014, 2016; Bohnsack et al., 2016). Coexposure of Rp-cAMP with ethanol did not prevent ethanol-induced increases in synaptic GABA$_A$ α4 abundance (Fig. 2A, $P < 0.01$, one-way ANOVA, $F = 5.776$, $P < 0.05$, Bonferroni post-test, n = 6–8 per group). Conversely, coexposure of rolipram with ethanol prevented increases in synaptic GABA$_A$ α4 abundance (Fig. 2B, $P < 0.05$, one-way ANOVA, $F = 3.107$, $P < 0.05$, Bonferroni post-test, $n = 7$

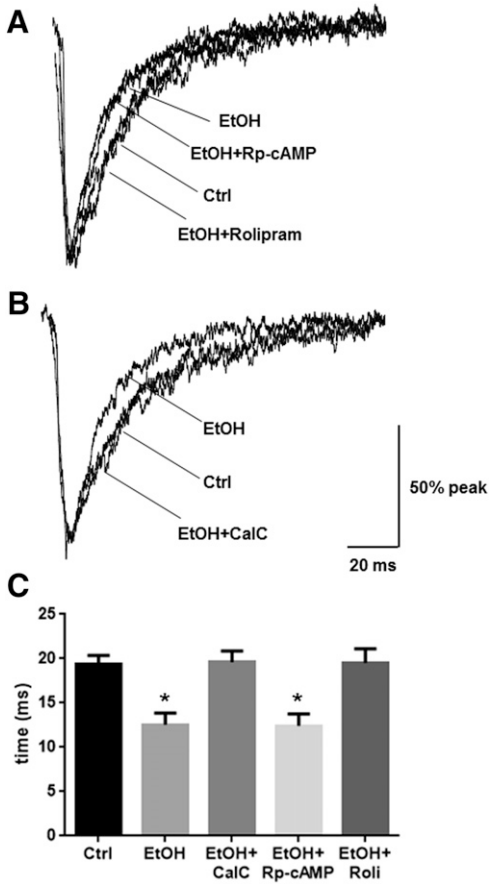

Fig. 4. PKA activation or PKC inhibition mitigate ethanol-induced alterations in mIPSC responses. Whole-cell patch-clamp recordings of cortical neurons were made in the presence of TTX (1 μM), CNQX (10 μM), and AP-5 (40 μM) to isolate GABA mIPSCs after 4-hour exposure to ethanol and/or kinase modulatory drugs. (A) Decay time (decay τ_1) was significantly decreased by ethanol exposure, an affect that was not affected by coexposure of ethanol with Rp-cAMP; however, coexposure of ethanol with rolipram prevented this decrease. (B) Coexposure of ethanol with CalC also prevented the decrease in decay rate induced by ethanol alone. (C) Quantification of changes in decay τ_1 is shown. (*$P < 0.05$ compared with control, EtOH plus CalC, or EtOH plus rolipram groups; one-way ANOVA, Bonferroni post-test). Summarized mIPSC metrics are presented in Table 1. AP-5, D-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyno-7-nitroquinoxaline-2,3-dione; Ctrl, control; EtOH, ethanol; Roli, rolipram; TTX, tetrodotoxin.

TABLE 1
GABA mIPSC decay kinetics after exposure to PKA modulators and ethanol
Data are presented as means ± S.E.M.

Measure	Control	Ethanol	Ethanol Plus CalC	Ethanol Plus Rp-cAMP	Ethanol Plus Rolipram
Rise time (ms)	2.4 ± 1.0	3.1 ± 1.4	2.8 ± 0.9	1.9 ± 1.0	2.6 ± 0.6
Amplitude (pA)	22.1 ± 1.2	23.5 ± 1.0	22.8 ± 1.9	21.6 ± 0.9	22.5 ± 1.1
Frequency (Hz)	1.9 ± 0.4	1.5 ± 0.8	2.1 ± 0.6	1.8 ± 0.4	2.0 ± 0.5
Decay τ_1	19.4 ± 1.0	12.5 ± 1.3*	19.6 ± 1.3	12.4 ± 1.4*	19.5 ± 1.6
Decay τ_2	30.1 ± 2.3	32.1 ± 2.6	31.2 ± 2.5	32.0 ± 1.9	31.6 ± 1.4
Number	14	11	11	11	11

*$P < 0.05$ (compared with control, ethanol plus CalC, or ethanol plus rolipram groups, one-way ANOVA, Bonferroni post-test).

to 8 per group). No changes in $GABA_A$ $\gamma2$ subunit abundance were observed in response to ethanol or PKA modulators (Fig. 2, C and D). No δ subunits were detected in the synaptic fraction (data not shown), although δ subunit abundance is known to decrease after 4-hour ethanol exposure in the extrasynaptic fraction (Carlson et al., 2016).

PKC Activity Mediates Ethanol-Induced Increases in Synaptic $GABA_A$ $\alpha4$ Subunits. Although we previously found increases in P2 fraction levels of $GABA_A$ $\alpha4$ to be caused by PKC activity (Werner et al., 2011), we next used subcellular fractionation to confirm that these changes occur in the synaptic fraction. Ethanol increased synaptic $GABA_A$ $\alpha4$ levels, an effect that was prevented by inhibiting PKC with CalC (0.3 μM; Fig. 3A, $P < 0.01$, one-way ANOVA, $F = 12.53$, $P < 0.01$, Bonferroni post-test, $n = 6$ per group). CalC alone had no effect on $GABA_A$ $\alpha4$ subunit abundance. Direct activation of PKC with the phorbol ester phorbol-12,13-dibutyrate mimicked the effect of ethanol in increasing synaptic $GABA_A$ $\alpha4$ abundance (Fig. 3B, $P < 0.01$, t test, $n = 5$ to 6 per group).

PKC Inhibition and PKA Activation Prevent Ethanol-Induced Changes in GABA mIPSCs. Finally, we investigated the ramifications of PKA and PKC activity on mIPSC kinetics during 4-hour ethanol exposure. We previously demonstrated that GABA mIPSC decay τ_1 is decreased after 4-hour ethanol exposure (Werner et al., 2011). Coexposure of ethanol with Rp-cAMP had no effect on the ethanol-induced decrease in GABA mIPSC decay τ_1, whereas coexposure with rolipram prevented the decrease (Fig. 4, A and C, $P < 0.001$, one-way ANOVA, $F = 9.955$, $P < 0.001$, Bonferroni post-test, $n = 11$–14 per group). Coexposure of ethanol with CalC prevented the decrease in GABA mIPSC decay τ_1 (Fig. 4, B and C, $P < 0.001$, one-way ANOVA, $F = 11.78$, $P < 0.001$, Bonferroni post-test, $n = 11$–14 per group). There were no other effects on mIPSC kinetics (Table 1).

Discussion

In this study, we demonstrate that PKA and PKC have opposing effects on synaptic $GABA_A$ $\alpha4$ subunit abundance and function. We elucidate that PKC activation by ethanol upregulates synaptic $GABA_A$ $\alpha4$ abundance to reduce the mIPSC decay rate, whereas maintaining PKA activation through phosphodiesterase inhibition prevents these effects. These data further characterize the regulation of a poorly understood $GABA_A$ receptor that may be an important mediator of the chronic effects of ethanol, and these results enhance our understanding of kinase regulation of $GABA_A$ receptors by ethanol (Table 2). Thus, these findings implicate potential therapeutic methods for restoring normal GABAergic functioning after chronic alcohol misuse.

We used a 4-hour in vitro ethanol exposure paradigm that recapitulates changes observed after chronic ethanol consumption and withdrawal (Devaud et al., 1997; Kumar et al., 2003; Carlson et al., 2013, 2016). It was not surprising that PKA inhibition during 4-hour ethanol exposure had no effect, because we previously found that PKA abundance (Carlson et al., 2013) and activity (Carlson et al., 2016) are not altered by ethanol exposure for 4 hours, despite the effects of ethanol at 1 hour. Nonetheless, it was possible that earlier PKA activation might produce downstream effects and this possibility was ruled out by testing the effect of PKA inhibition. Since PKA inhibition had no effect on synaptic $GABA_A$ $\alpha4$ receptor expression, it appears that constitutive PKA activity does not regulate synaptic $GABA_A$ $\alpha4$ receptors. The data suggest that PKA activation downregulates synaptic $GABA_A$ $\alpha4$ receptors, because either maintaining PKA activity through phosphodiesterase inhibition or activating PKA through adenylyl cyclase activation prevented ethanol-induced increases in $GABA_A$ $\alpha4$ subunits. Because $GABA_A$ $\alpha4\beta\gamma2$ receptors are upregulated in other pathologic conditions, including seizure disorders (González and Brooks-Kayal, 2011) and progesterone withdrawal (Gulinello et al., 2002), PKA regulation of these receptors likely has broad implications deserving further exploration.

Our laboratory previously demonstrated that PKC activation by ethanol leads to an upregulation of the abundance of $GABA_A$ $\alpha4$ subunits in vitro after 4 hours, purportedly owing to an increase in the synaptic population of $GABA_A$ $\alpha4$ receptors (Werner et al., 2011). However, these studies did not isolate synaptic $\alpha4$ receptors since δ subunits were

TABLE 2
Summary of findings on modulation of $GABA_A$ $\alpha1$ and $\alpha4$ subunits by ethanol and kinases
Data are presented as means ± S.E.M. Downward arrows indicate negative modulation, whereas upward arrows indicate positive modulation. The dash indicates no change.

Subunit	Chronic Ethanol	PKC	PKA
$GABA_A$ $\alpha1$	↓ (Kumar et al., 2003)	↓ (Kumar et al., 2010)	↑ (Carlson et al., 2013)
Extrasynaptic $GABA_A$ $\alpha4$	↓ (Liang et al., 2006)[a]	— (Carlson et al., 2016)	↑ (Carlson et al., 2016)
Synaptic $GABA_A$ $\alpha4$	↑ (Liang et al., 2006)[a]	↑ (This study)	↓ (This study)

[a]Note that these studies are in the hippocampus.

detected in the P2 fraction. Our current results, combined with recent findings demonstrating a lack of effect of PKC on extrasynaptic $GABA_A$ $\alpha4\delta$ receptors (Bohnsack et al., 2016; Carlson et al., 2016), support the hypothesis that PKC effects are specific for the synaptic $GABA_A$ $\alpha4$ receptors in cortical neurons and that synaptic and extrasynaptic populations of receptors are subject to different methods of regulation. The similarity of ethanol effects in the synaptic fraction and P2 fraction suggests that the P2 fraction largely consists of synaptic components and that the subsynaptic fraction, although functionally relevant (Carlson et al., 2016), may not be present in sufficient quantities to confound these results.

Our results suggest that PKA and PKC play oppositional roles in synaptic $GABA_A$ $\alpha4$ receptor regulation, similar to our previous findings on $GABA_A$ $\alpha1$ receptor regulation (Carlson et al., 2013). These results are consistent with other studies demonstrating oppositional roles of PKC and PKA activity on GABA receptor functioning (Poisbeau et al., 1999; Brandon et al., 2000; Bohnsack et al., 2016). The observation that PKA activation decreases synaptic $\alpha4$ abundance could explain why we previously found no difference in whole-cell GABA-evoked current amplitude or GABA dose response after 1 hour of PKA activation, despite an increase in $\alpha1$ receptor abundance (Carlson et al., 2013). Similarly, the lack of change in $GABA_A$ $\gamma2$ subunit abundance observed in this study is likely attributable to opposing changes in $\alpha4\beta\gamma2$ vs. $\alpha1\beta\gamma2$ receptors (Kumar et al., 2010). The observation that kinase inhibition in the absence of ethanol had no effect on synaptic $\alpha4$ subunits suggests that these receptors do not undergo constitutive regulation by these pathways but are only altered after a physiologic insult such as high concentrations of ethanol. These findings are consistent with previous studies in which the PKA RIIβ subunit did not constitutively regulate $\alpha4$ subunits in vivo (Carlson et al., 2014).

The more rapid mIPSC decay constants after 4-hour ethanol exposure were consistent with previous findings from our laboratory (Werner et al., 2011). The synaptic $GABA_A$ $\alpha4$ receptors display more rapid decay times than $GABA_A$ $\alpha1$ receptors in recombinant systems (Whittemore et al., 1996; Brown et al., 2002) and $\alpha4$ knockout mice have longer decay times compared with wild-type mice (Chandra et al., 2006). Thus, the faster decay time after chronic ethanol exposure is consistent with a higher proportion of $GABA_A$ $\alpha4$ receptors in the synaptic GABA receptor population; however, it will be important to confirm this conclusion in recombinant systems that can isolate synaptic $\alpha4$ receptors physiologically. These results mirror similar studies uncovering reduced decay times after chronic ethanol exposure in the hippocampus (Cagetti et al., 2003; Liang et al., 2006) and during withdrawal from the neuroactive steroid allopregnanolone (Hsu et al., 2003). These changes are correlated with a reduction in the anxiolytic and sleep-inducing effects of ethanol associated with withdrawal and dependence. Thus, the finding that PKC inhibition or PKA activation in the presence of ethanol prevented the faster $GABA_A$ mIPSC decay time suggests two possible methods of preventing pathologic changes associated with ethanol dependence. Finally, the correspondence of the functional changes in mIPSCs with the change in $\alpha4$ subunit expression detected by synaptic fractionation technique further validates the viability of this method of isolating synaptic proteins.

Our results underscore the potential therapeutic relevance for phosphodiesterase inhibition using drugs such as rolipram. Recent studies in rodent models have demonstrated decreased drinking behavior in animals given phosphodiesterase inhibitors (Hu et al., 2011; Wen et al., 2012; Blednov et al., 2014; Franklin et al., 2015). Together, these studies suggest that phosphodiesterase inhibition provides a promising target for the treatment of alcohol use disorders. Future in vivo studies examining effects of coadministration of ethanol and rolipram (or administration of rolipram after chronic ethanol) on GABAergic trafficking and GABA-related behavior would be a logical extension of these data.

This study expands our understanding of kinase signaling in modulating the GABAergic effects of ethanol. The data suggest that PKA activity may prevent alterations of GABAergic inhibition associated with chronic alcohol misuse, whereas PKC activity may facilitate them. These second messenger pathways could provide important targets for treatments to prevent or restore normal $GABA_A$ receptor functioning associated with alcohol tolerance, dependence, and withdrawal.

Acknowledgments

The authors thank Todd K. O'Buckley, Raechel McKinley, and Danielle Morrow for technical assistance.

Authorship Contributions

Participated in research design: Carlson, Bohnsack, Morrow.
Conducted experiments: Carlson, Bohnsack.
Performed data analysis: Carlson, Bohnsack.
Wrote or contributed to the writing of the manuscript: Carlson, Bohnsack, Morrow.

References

Blednov YA, Benavidez JM, Black M, and Harris RA (2014) Inhibition of phosphodiesterase 4 reduces ethanol intake and preference in C57BL/6J mice. *Front Neurosci* **8**:129.

Bohnsack JP, Carlson SL, and Morrow AL (2016) Differential regulation of synaptic and extrasynaptic α4 GABA(A) receptor populations by protein kinase A and protein kinase C in cultured cortical neurons. *Neuropharmacology* **105**:124–132.

Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG, and Moss SJ (2000) $GABA_A$ receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. *J Biol Chem* **275**: 38856–38862.

Brown N, Kerby J, Bonnert TP, Whiting PJ, and Wafford KA (2002) Pharmacological characterization of a novel cell line expressing human α(4)β(3)δ $GABA_(A)$ receptors. *Br J Pharmacol* **136**:965–974.

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

Carlson SL, Bohnsack JP, Patel V, and Morrow AL (2016) Regulation of extrasynaptic GABAA α4 receptors by ethanol-induced protein kinase A, but not protein kinase C activation in cultured rat cerebral cortical neurons. *J Pharmacol Exp Ther* **356**:148–156.

Carlson SL, Kumar S, Werner DF, Comerford CE, and Morrow AL (2013) Ethanol activation of protein kinase A regulates GABAA α1 receptor function and trafficking in cultured cerebral cortical neurons. *J Pharmacol Exp Ther* **345**:317–325.

Carlson SL, O'Buckley TK, Thomas R, Thiele TE, and Morrow AL (2014) Altered GABAA receptor expression and seizure threshold following acute ethanol challenge in mice lacking the RIIβ subunit of PKA. *Neurochem Res* **39**:1079–1087.

Chandra D, Jia F, Liang J, Peng Z, Suryanarayanan A, Werner DF, Spigelman I, Houser CR, Olsen RW, and Harrison NL, et al. (2006) $GABA_A$ receptor α 4 subunits mediate extrasynaptic inhibition in thalamus and dentate gyrus and the action of gaboxadol. *Proc Natl Acad Sci USA* **103**:15230–15235.

Devaud LL, Fritschy JM, Sieghart W, and Morrow AL (1997) Bidirectional alterations of $GABA_(A)$ receptor subunit peptide levels in rat cortex during chronic ethanol consumption and withdrawal. *J Neurochem* **69**:126–130.

Devaud LL, Purdy RH, Finn DA, and Morrow AL (1996) Sensitization of γ-aminobutyric $acid_A$ receptors to neuroactive steroids in rats during ethanol withdrawal. *J Pharmacol Exp Ther* **278**:510–517.

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

Franklin KM, Hauser SR, Lasek AW, McClintick J, Ding ZM, McBride WJ, and Bell RL (2015) Reduction of alcohol drinking of alcohol-preferring (P) and high-alcohol drinking (HAD1) rats by targeting phosphodiesterase-4 (PDE4). *Psychopharmacology (Berl)* **232**:2251–2262.

Goebel-Goody SM, Davies KD, Alvestad Linger RM, Freund RK, and Browning MD (2009) Phospho-regulation of synaptic and extrasynaptic N-methyl-d-aspartate receptors in adult hippocampal slices. *Neuroscience* **158**:1446–1459.

González MI and Brooks-Kayal A (2011) Altered GABA(A) receptor expression during epileptogenesis. *Neurosci Lett* **497**:218–222.

Gulinello M, Gong QH, and Smith SS (2002) Progesterone withdrawal increases the alpha4 subunit of the GABA(A) receptor in male rats in association with anxiety and altered pharmacology - a comparison with female rats. *Neuropharmacology* **43**: 701–714.

Hanchar HJ, Chutsrinopkun P, Meera P, Supavilai P, Sieghart W, Wallner M, and Olsen RW (2006) Ethanol potently and competitively inhibits binding of the alcohol antagonist Ro15-4513 to alpha4/6beta3delta $GABA_A$ receptors. *Proc Natl Acad Sci USA* **103**:8546–8551.

Hsu FC, Waldeck R, Faber DS, and Smith SS (2003) Neurosteroid effects on GABAergic synaptic plasticity in hippocampus. *J Neurophysiol* **89**:1929–1940.

Hu W, Lu T, Chen A, Huang Y, Hansen R, Chandler LJ, and Zhang HT (2011) Inhibition of phosphodiesterase-4 decreases ethanol intake in mice. *Psychopharmacology (Berl)* **218**:331–339.

Knoflach F, Benke D, Wang Y, Scheurer L, Lüddens H, Hamilton BJ, Carter DB, Mohler H, and Benson JA (1996) Pharmacological modulation of the diazepam-insensitive recombinant gamma-aminobutyric acidA receptors alpha 4 beta 2 gamma 2 and alpha 6 beta 2 gamma 2. *Mol Pharmacol* **50**:1253–1261.

Kumar S, Kralic JE, O'Buckley TK, Grobin AC, and Morrow AL (2003) Chronic ethanol consumption enhances internalization of α1 subunit-containing $GABA_A$ receptors in cerebral cortex. *J Neurochem* **86**:700–708.

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

Kumar S, Suryanarayanan A, Boyd KN, Comerford CE, Lai MA, Ren Q, and Morrow AL (2010) Ethanol reduces GABAA alpha1 subunit receptor surface expression by a protein kinase Cγ-dependent mechanism in cultured cerebral cortical neurons. *Mol Pharmacol* **77**:793–803.

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

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

Olsen RW and Sieghart W (2009) $GABA_A$ receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* **56**:141–148.

Papadeas S, Grobin AC, and Morrow AL (2001) Chronic ethanol consumption differentially alters GABA(A) receptor alpha1 and alpha4 subunit peptide expression and GABA(A) receptor-mediated 36 Cl(-) uptake in mesocorticolimbic regions of rat brain. *Alcohol Clin Exp Res* **25**:1270–1275.

Poisbeau P, Cheney MC, Browning MD, and Mody I (1999) Modulation of synaptic $GABA_A$ receptor function by PKA and PKC in adult hippocampal neurons. *J Neurosci* **19**:674–683.

Sundstrom-Poromaa I, Smith DH, Gong QH, Sabado TN, Li X, Light A, Wiedmann M, Williams K, and Smith SS (2002) Hormonally regulated $\alpha_4\beta_2\delta$ $GABA_A$ receptors are a target for alcohol. *Nat Neurosci* **5**:721–722.

Tretter V and Moss SJ (2008) GABA(A) receptor dynamics and constructing GABAergic synapses. *Front Mol Neurosci* **1**:7.

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

Wei W, Faria LC, and Mody I (2004) Low ethanol concentrations selectively augment the tonic inhibition mediated by delta subunit-containing $GABA_A$ receptors in hippocampal neurons. *J Neurosci* **24**:8379–8382.

Wen RT, Zhang M, Qin WJ, Liu Q, Wang WP, Lawrence AJ, Zhang HT, and Liang JH (2012) The phosphodiesterase-4 (PDE4) inhibitor rolipram decreases ethanol seeking and consumption in alcohol-preferring Fawn-Hooded rats. *Alcohol Clin Exp Res* **36**:2157–2167.

Werner DF, Kumar S, Criswell HE, Suryanarayanan A, Fetzer JA, Comerford CE, and Morrow AL (2011) PKCγ is required for ethanol-induced increases in GABA(A) receptor α4 subunit expression in cultured cerebral cortical neurons. *J Neurochem* **116**:554–563.

Whittemore ER, Yang W, Drewe JA, and Woodward RM (1996) Pharmacology of the human gamma-aminobutyric acidA receptor alpha 4 subunit expressed in Xenopus laevis oocytes. *Mol Pharmacol* **50**:1364–1375.

Zhang H and Pandey SC (2003) Effects of PKA modulation on the expression of neuropeptide Y in rat amygdaloid structures during ethanol withdrawal. *Peptides* **24**:1397–1402.

Address correspondence to: Dr. A. Leslie Morrow, Departments of Psychiatry and Pharmacology, Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, 3027 Thurston-Bowles Building, CB 7178, Chapel Hill, NC 27599. E-mail: morrow@med.unc.edu