Ethanol regulation of synaptic γ-aminobutyric acid (GABA)\(_A\) α4 receptors is prevented by PKA activation

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Running Title Page

Running Title: Ethanol regulation of synaptic GABA_4 receptors

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Non-Standard Abbreviations:
Rp-cAMP: Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine
CalC: Calphostin-C
PdBu: phorbol-12,13-dibutyrate
Abstract

Ethanol alters GABA_A receptor trafficking and function through activation of protein kinases, and these changes may underlie ethanol dependence and withdrawal. In the present study, we utilized subsynaptic fraction techniques and patch clamp electrophysiology to investigate the biochemical and functional effects of protein kinase A (PKA) and PKC activation by ethanol on synaptic GABA_A α4 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 h, 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 α4 subunit abundance, while 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 α4 subunit abundance while inhibiting PKA in the presence of ethanol was ineffective. Conversely, PKC inhibition in the presence of ethanol prevented the ethanol-induced increases in synaptic α4 subunit abundance. Finally, we found that either activating PKA or inhibiting PKC in the presence of ethanol prevented the ethanol-induced decrease in GABA mIPSC decay τ_1, while inhibiting PKA had no effect. We conclude that PKA and PKC have opposing effects in the regulation of synaptic α4 receptors, with PKA activation negatively modulating, and PKC activation positively modulating, synaptic α4 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<sub>A</sub> receptors that are associated with alcohol dependence and withdrawal (Kumar et al., 2009). GABA<sub>A</sub> 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 (1-6), two β (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 α<sub>4</sub>βγ<sub>2</sub> and extrasynaptic α<sub>4</sub>βδ GABA<sub>A</sub> receptors mediating a portion of phasic and tonic inhibition, respectively (Olsen and Sieghart, 2009). The extrasynaptic α<sub>4</sub> 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), surprisingly little is known about the regulation of synaptic α<sub>4</sub> receptors.

While early studies showed that ethanol regulates the abundance of α<sub>4</sub> subunit mRNA and protein expression (Devaud et al., 1996; Devaud et al., 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 α<sub>4</sub> receptor kinetics. Synaptic α<sub>4</sub> receptors are upregulated following both acute (Liang et al., 2007) and chronic ethanol exposure (Liang et al., 2006; Werner et al., 2011) in rat hippocampus and cortex. More recently, we have demonstrated ethanol regulation of synaptic α<sub>4</sub> subunits in C57BL/6 mice following an acute injection of ethanol, using subcellular fractionation to isolate synaptic vs. subsynaptic receptors (Carlson et al., 2014). Isolating the precise physiological and behavioral ramifications of these changes in synaptic α<sub>4</sub> receptors is difficult as there are no selective pharmacological agents targeting these receptors. The α<sub>4</sub>βγ<sub>2</sub> receptors are benzodiazepine insensitive, though they show relatively high affinity for the GABA inverse agonist Ro 15-4513 (Knoflach et al., 1996). However, Ro 15-4513 also antagonizes effects of ethanol on α<sub>4</sub>βδ receptors (Hanchar et al., 2006) that likely mediate effects of ethanol on tonic inhibition.

Recent studies from our lab have uncovered that kinase activation by ethanol plays a major role in GABA<sub>A</sub> receptor regulation, and that PKA and PKC may have opposing effects on GABAergic trafficking and function. PKA activity positively regulates synaptic GABA<sub>A</sub> α1 subunit abundance and function in cortical
neurons, while PKCγ activity negatively regulates these receptors (Kumar et al., 2010; Carlson et al., 2013).

PKA activation also positively regulates extrasynaptic GABA_4α and δ subunits in cortical neurons, while 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_4α subunits, though it is unclear if this effect was 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.

The present study elucidates the role of PKA and PKC in ethanol regulation of synaptic α4-containing GABA_4 receptors in cerebral cortical cultured neurons. We used a 4 h ethanol exposure paradigm that recapitulates many of the GABAergic adaptations observed following 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, while functional changes were measured using whole cell patch clamp analysis of GABA mIPSCs.
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, USA) 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 CO₂-independent media containing papain (50 U/mL, Worthington, Lakewood, NJ, USA), L-cysteine and DNase (both from Sigma, St. Louis, MO, USA) for 30 min at 37°C. Tissue pieces were gently washed followed by gentle trituration in Dulbecco’s modified eagle’s medium (DMEM, Gibco, Grand Island, NY) containing 10% horse serum, penicillin-streptomycin (Pen-Strep) and DNase. Cells used for biochemistry were plated onto poly-D-lysine–coated flasks, while cells used for electrophysiology were plated onto poly-D-lysine–coated cover slips in 12-well plates. Cells were maintained in a 5% CO₂ humidified incubator. After day 3, cells were fed with serum-free medium containing B27 and Pen-Strep (10 000 U/mL; final concentration 50 U per flask). Media was changed twice per week with no more than one-third of the media being removed during exchanges. For all experiments, Pen-Strep was removed from cultures on day 14 to prevent interactions with GABAₐ receptors. Cultures were maintained for 18 days before conducting experiments, as prior studies determined that GABAₐ 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 as 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 h. To examine PKA involvement, the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Rp-cAMP, 50 µM), the adenyl cyclase activator forskolin (10 µM; Tocris), or the
phosphodiesterase inhibitor rolipram (10 µM; Sigma) were added to the cell media. To examine PKC involvement, the PKC inhibitor calphostin C (Cal-C, 0.3 µM in 0.1% dimethyl sulfoxide, final concentrations; Sigma) or the activator phorbol-12,13-dibutyrate (PdBu, 0.1 µM; Sigma) were 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 (PBS), scraped, centrifuged at 1000 g for 18 min, and stored at -80°C until fractionation. Cell pellets were homogenized in 0.32M sucrose and centrifuged at 1000 g for 10 min. The supernatant was then centrifuged twice for 30 min 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 (Goebel-Goody et al., 2009) as previously described (Carlson et al., 2014; Carlson et al., 2016). The fractions were separated by 30 min incubation in 0.5% Triton-X, followed by two centrifugations at 32,000 g for 30 min. 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<sub>A</sub> receptor α4 subunit abundance was analyzed by western blot. Protein samples were subjected to SDS-PAGE using Novex Tris-Glycine (8-16%) gels and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were probed with GABA<sub>A</sub> receptor α4 (1:500 dilution, Abcam, Cambridge, MA) antibody), anti-GABA<sub>A</sub> δ (1:750, Novus, St. Louis, MO), or anti-GABA<sub>A</sub> γ2 (1:1000, Novus, St. Louis, MO), followed by β-actin antibody (1:3,000 dilution, Millipore) for normalization. Proteins were detected with enhanced chemiluminescence (GE Healthcare, Amersham, UK). Membranes were imaged using LAS-4000 (GE Healthcare), and densitometric analysis was conducted using GE ImageQuant software. Comparisons were made within blots and expressed as a percent of averaged control values.
Electrophysiology

Whole-cell voltage clamp recordings were used to assess mIPSCs. Electrodes were pulled using a PP-830 (Narishige, Japan) and fire polished to a resistance of 2-3 MΩ. Intracellular solution contained 150 mM KCl, 3.1 mM MgCl₂, 15 mM HEPES, 5 mM KATP, 5 mM EGTA, and 15 mM phosphocreatine, adjusted to pH 7.4 with KOH, while extracellular solution contained 145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl₂, 1 mM MgCl₂, 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 (CNQX; 10 μM, Sigma), D-2-amino-5-phophonopentanoic acid (AP-5; 40 μM ,Tocris), and tetrodotoxin (TTX; 1 μM, Sigma). Membrane potential was held at -60 mV and currents were recorded with an Axopatch ID (Axon Instruments, Union City, CA, USA) amplifier. Data were collected using Clampex software (Axon Instruments). Miniature inhibitory post-synaptic currents (mIPSCs) were analyzed using the miniAnalysis software (v5.6.4; Synaptosoft, Decatur, GA, USA). mIPSCs were recorded for a minimum of 3 min. 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.

Statistical Analysis

Numerical data are presented as mean ± SEM. Analyses were conducted using ANOVA and Bonferroni posttest or Student’s t test.
Results

Direct PKA activation prevents the effects of 4 hr ethanol exposure on total GABA\textsubscript{A} $\alpha$4 abundance in the P2 fraction

We first examined the effect of PKA modulation on GABA\textsubscript{A} $\alpha$4 subunits during ethanol exposure. Exposure to ethanol for 4 h increased GABA\textsubscript{A} $\alpha$4 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 $\alpha$4 abundance (Fig. 1A). Conversely, activation of PKA during ethanol exposure by either the adenylyl cyclase activator forskolin (10 $\mu$M; Fig. 1B, p<0.01, one-way ANOVA, $f$=6.330, p<0.01, Bonferroni posttest, n=6-7 per group) or the phosphodiesterase inhibitor rolipram (10 $\mu$M; Fig. 1C, p<0.05, one-way ANOVA, $f$=3.107, p<0.05, Bonferroni posttest, n=7-8 per group) prevented the increase of $\alpha$4 subunit abundance. None of the PKA modulators alone had any effect on GABA\textsubscript{A} $\alpha$4 subunit abundance.

PKA activation prevents the effects of 4 hr ethanol exposure on synaptic fraction GABA\textsubscript{A} $\alpha$4 abundance

To resolve whether the effects of ethanol and PKA on GABA\textsubscript{A} $\alpha$4 subunits in the P2 fraction represent synaptic receptor regulation, we expanded the study shown in figure 1 to determine if these effects occur within the synaptic fraction purified by subcellular fractionation (Carlson et al., 2014; Bohnsack et al., 2016; Carlson et al., 2016). Co-exposure of Rp-cAMP with ethanol did not prevent ethanol-induced increases in synaptic GABA\textsubscript{A} $\alpha$4 abundance (Fig. 2A, p<0.01, one-way ANOVA, $f$=5.776, p<0.05, Bonferroni posttest, n=6-8 per group). Conversely, co-exposure of rolipram with ethanol prevented increases in synaptic GABA\textsubscript{A} $\alpha$4 abundance (Fig. 2B, p<0.05, one-way ANOVA, $f$=3.107, p<0.05, Bonferroni posttest, n=7-8 per group). No changes in GABA\textsubscript{A} $\gamma$2 subunit abundance were observed in response to ethanol or PKA modulators (Fig. 2C and 2D). No $\delta$ subunits were detected in the synaptic fraction (data not shown), while $\delta$ subunit abundance is known to decrease after 4 h ethanol exposure in the extrasynaptic fraction (Carlson et al., 2016).

PKC activity mediates ethanol-induced increases in synaptic GABA\textsubscript{A} $\alpha$4 subunits

While we previously found increases in P2 fraction levels of GABA\textsubscript{A} $\alpha$4 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\textsubscript{A} $\alpha$4 levels, an effect which 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 posttest, n=6 per group). CalC alone had no effect on GABA<sub>α4</sub> subunit abundance. Direct activation of PKC with the phorbol ester PdBu mimicked the effect of ethanol in increasing synaptic GABA<sub>α4</sub> abundance (Fig. 3B, p<0.01, Student's t test, n=5-6 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 hr ethanol exposure. We previously demonstrated that GABA mIPSC decay τ₁ is decreased following 4 hr ethanol exposure (Werner et al., 2011). Co-exposure of ethanol with Rp-cAMP had no effect on the ethanol-induced decrease in GABA mIPSC decay τ₁, while co-exposure with rolipram prevented the decrease (Fig. 4A &C, p<0.001, one-way ANOVA, f=9.955, p<0.001, Bonferroni posttest, n=11-14 per group). Co-exposure of ethanol with CalC prevented the decrease in GABA mIPSC decay τ₁ (Fig. 4B &C, p<0.001, one-way ANOVA, f=11.78, p<0.001, Bonferroni posttest, n=11-14 per group). There were no other effects on mIPSC kinetics (Table 1).
Discussion

In the present study we demonstrate that PKA and PKC have opposing effects on synaptic GABA$_\alpha$4 subunit abundance and function. We elucidate that PKC activation by ethanol upregulates synaptic GABA$_\alpha$4 abundance to reduce mIPSC decay rate, while maintaining PKA activation through phosphodiesterase inhibition prevents these effects. These data further characterize the regulation of a poorly understood GABA$_\alpha$ receptor that may be an important mediator of the chronic effects of ethanol, as well as enhance our understanding of kinase regulation of GABA$_\alpha$ receptors by ethanol (Table 2). Thus, the findings implicate potential therapeutic methods for restoring normal GABAergic functioning after chronic alcohol misuse.

We utilized a 4 h 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; Carlson et al., 2016). It was not surprising that PKA inhibition during 4 h ethanol exposure had no effect, as we have 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 one 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$_\alpha$R$_\alpha$4 expression, it appears that constitutive PKA activity does not regulate synaptic GABA$_\alpha$R$_\alpha$4 receptors. The data suggest that PKA activation downregulates synaptic GABA$_\alpha$4 receptors, as either maintaining PKA activity through phosphodiesterase inhibition or activating PKA through adenylyl cyclase activation prevented ethanol-induced increases in GABA$_\alpha$4 subunits. As GABA$_\alpha$4$\beta_3\gamma_2$ receptors are up-regulated in other pathological conditions, including seizure disorders (Gonzalez and Brooks-Kayal, 2011) and progesterone withdrawal (Gulinello et al., 2002), PKA regulation of these receptors likely has broad implications deserving further exploration.

Previously, our lab demonstrated PKC activation by ethanol leads to an upregulation of abundance of GABA$_\alpha$4 subunits in vitro after 4 hours, purportedly due to an increase in the synaptic population of GABA$_\alpha$4 receptors (Werner et al., 2011). However, these studies did not isolate synaptic $\alpha$4 receptors since $\delta$ subunits were detected in the P2 fraction. The present results, combined with recent findings demonstrating a lack of effect of PKC on extrasynaptic GABA$_\alpha$4$\delta$ receptors (Bohnsack et al., 2016; Carlson et al., 2016),
support the hypothesis that PKC effects are specific for the synaptic GABA\textsubscript{\alpha}4 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 suggest that the P2 fraction largely consists of synaptic components and that the subsynaptic fraction, while functionally relevant (Carlson et al., 2016), may not be present in sufficient quantities to confound these results.

The present results suggest that PKA and PKC play oppositional roles in synaptic GABA\textsubscript{\alpha}4 receptor regulation, similar to our previous findings on GABA\textsubscript{\alpha}1 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 h of PKA activation, despite an increase in \alpha1 receptor abundance (Carlson et al., 2013). Similarly, the lack of change in GABA\textsubscript{\gamma}2 subunit abundance observed in the current study is likely due to opposing changes in \alpha4\beta\gamma2 \alpha1\beta\gamma2 subunits (Kumar et al., 2010). The observation that kinase inhibition in the absence of ethanol had no effect on synaptic \alpha4 subunits would suggest these receptors do not undergo constitutive regulation by these pathways, but are only altered after a physiological insult such as high concentrations of ethanol. These findings are consistent with previous studies in which the PKA RII\beta subunit did not constitutively regulate \alpha4 subunits in vivo (Carlson et al., 2014).

The more rapid mIPSC decay constants after 4 h ethanol were consistent with previous findings from our lab (Werner et al., 2011). The synaptic GABA\textsubscript{\alpha}4 receptors display more rapid decay times than GABA\textsubscript{\alpha}1 receptors in recombinant systems (Whittemore et al., 1996; Brown et al., 2002) and \alpha4 knockout mice have longer decay times compared to wildtype mice (Chandra et al., 2006). Thus, the faster decay time after chronic ethanol exposure is consistent with a higher proportion of GABA\textsubscript{\alpha}4 receptors in the synaptic GABA 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 following chronic ethanol exposure in the hippocampus (Cagetti et al., 2003; Liang et al., 2006) and during
withdrawal from 3α,5α-THP (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<sub>A</sub> mIPSC decay time suggests two possible methods of preventing pathological changes associated with ethanol dependence. Finally, the correspondence of the functional changes in mIPSCs with the change in α4 subunit expression detected by synaptic fractionation technique further validates the viability of this method of isolating synaptic proteins.

The present results underscore the potential therapeutic relevance for phosphodiesterase inhibition using drugs like 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 phosphodiesterase inhibition provides a promising target for the treatment of alcohol use disorders. Future in vivo studies examining effects of co-administration 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.

The current 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, while PKC activity may facilitate them. These second messenger pathways could provide important targets for treatments to prevent or restore normal GABA<sub>A</sub> receptor functioning associated with alcohol tolerance, dependence, and withdrawal.
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Authorship Contributions

*Participated in research design:* Carlson, Bohnsack, Morrow

*Conducted Experiments:* Carlson, Bohnsack

*Contributed new reagents or analytic tools:* N/A

*Performed data analysis:* Carlson, Bohnsack

*Wrote or contributed to the writing of the manuscript:* Carlson, Bohnsack, Morrow
References

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Footnotes

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Figure Legends

Figure 1. PKA activation prevents ethanol-induced increases in P2 fraction GABA\textsubscript{A} \(\alpha\)4 subunits. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 \(\mu\)M), forskolin (10 \(\mu\)M), and/or rolipram (10 \(\mu\)M) for 4 h followed by P2 fractionation and western blot analysis. (A) Exposure to ethanol for 4 h increased P2 fraction levels of the GABA\textsubscript{A} \(\alpha\)4 subunit, which was not affected by co-exposure with Rp-cAMP. Exposure to the (B) adenylyl cyclase activator forskolin or the (C) phosphodiesterase inhibitor rolipram with ethanol prevented the increase in P2 fraction levels of \(\alpha\)4 subunits induced by ethanol.

\* \(p<0.05\), one-way ANOVA, Bonferroni post-test, \(n=6-8\) per group.

Figure 2. PKA activation prevents the increased abundance of synaptic \(\alpha\)4 receptors induced by ethanol. Cortical neurons were exposed to vehicle, ethanol (50 mM), Rp-cAMP (50 \(\mu\)M), and/or rolipram (10 \(\mu\)M) for 4 h followed by synaptic fractionation and western blot analysis. (A) Ethanol decreased in synaptic GABA\textsubscript{A} \(\alpha\)4 abundance, an effect which was not altered by co-exposure with Rp-cAMP; however, (B) co-exposure with rolipram prevented ethanol-induced increases in synaptic \(\alpha\)4 abundance. GABA\textsubscript{A} \(\gamma\)2 abundance was not altered by exposure to ethanol or either (C) Rp-cAMP or (D) rolipram.

\(p<0.05\), one-way ANOVA, Bonferroni post-test, \(n=6-10\).

Figure 3. PKC activation by ethanol increases GABA\textsubscript{A} \(\alpha\)4 subunit levels in the synaptic fraction. Cortical neurons were exposed to vehicle, ethanol (50 mM), CalC (0.3 \(\mu\)M), and/or PdBu (0.1 \(\mu\)M) for 4 h followed by synaptic fractionation and western blot analysis. (A) Exposure to ethanol for 4 h increased synaptic fraction levels of the GABA\textsubscript{A} \(\alpha\)4 subunit, which was prevented by co-exposure with CalC. (B) Exposure to PdBu mimicked the effect of ethanol on synaptic GABA\textsubscript{A} \(\alpha\)4 levels.

\* \(p<0.05\), Student’s t test or one-way ANOVA, Bonferroni post-test, \(n=5-6\) per group.
Figure 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 following 4 h exposure to ethanol and/or kinase modulatory drugs. (A) Decay time (decay $\tau_1$) was significantly decreased by ethanol exposure, an affect which was not affected by co-exposure of ethanol with Rp-cAMP; however, co-exposure of ethanol with rolipram prevented this decrease. (B) Co-exposure of ethanol with CalC also prevented the decrease in decay rate induced by ethanol alone. Quantification of changes in decay $\tau_1$ are shown in Fig. 4C and summarized mIPSC metrics are found in Table 1.
Table 1. GABA mIPSC decay kinetics following exposure to PKA modulators and ethanol

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control</th>
<th>EtOH</th>
<th>EtOH + CalC</th>
<th>EtOH + Rp-cAMP</th>
<th>EtOH + Rolipram</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rise time (ms)</td>
<td>2.4 ± 1.0</td>
<td>3.1 ± 1.4</td>
<td>2.8 ± 0.9</td>
<td>1.9 ± 1.0</td>
<td>2.6 ± 0.6</td>
</tr>
<tr>
<td>Amplitude (pA)</td>
<td>22.1 ± 1.2</td>
<td>23.5 ± 1.0</td>
<td>22.8 ± 1.9</td>
<td>21.6 ± 0.9</td>
<td>22.5 ± 1.1</td>
</tr>
<tr>
<td>Frequency (Hz)</td>
<td>1.9 ± 0.4</td>
<td>1.5 ± 0.8</td>
<td>2.1 ± 0.6</td>
<td>1.8 ± 0.4</td>
<td>2.0 ± 0.5</td>
</tr>
<tr>
<td>Decay $\tau_1$</td>
<td>19.4 ± 1.0</td>
<td>* 12.5 ± 1.3</td>
<td>19.6 ± 1.3</td>
<td>* 12.4 ± 1.4</td>
<td>19.5 ± 1.6</td>
</tr>
<tr>
<td>Decay $\tau_2$</td>
<td>30.1 ± 2.3</td>
<td>32.1 ± 2.6</td>
<td>31.2 ± 2.5</td>
<td>32.0 ± 1.9</td>
<td>31.6 ± 1.4</td>
</tr>
<tr>
<td>n</td>
<td>14</td>
<td>11</td>
<td>11</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

*p < 0.05, compared to Control, EtOH + CalC, or EtOH + Rolipram groups, one-way ANOVA, Bonferroni post-test.
Table 2. Summary of findings on modulation of GABA<sub>A</sub> α1 and α4 subunits by ethanol and kinases

<table>
<thead>
<tr>
<th></th>
<th>Chronic EtOH</th>
<th>PKC</th>
<th>PKA</th>
</tr>
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<tbody>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; α1</td>
<td>↓ (Kumar et al. 2003)</td>
<td>↓ (Kumar et al. 2010)</td>
<td>↑ (Carlson et al. 2013)</td>
</tr>
<tr>
<td>Extrasynaptic GABA&lt;sub&gt;A&lt;/sub&gt; α4</td>
<td>↓ (Liang et al. 2006)*</td>
<td>- (Carlson et al. 2015)</td>
<td>↑ (Carlson et al. 2015)</td>
</tr>
<tr>
<td>Synaptic GABA&lt;sub&gt;A&lt;/sub&gt; α4</td>
<td>↑ (Liang et al. 2006)*</td>
<td>↑ (present study)</td>
<td>↓ (present study)</td>
</tr>
</tbody>
</table>

* - note that these studies are in hippocampus.
Figure 2

(A) Western blot analysis of GABA<sub>A</sub> α4 subunit levels in Ctrl, EtOH, Rp-cAMP, and EtOH+ Rp-cAMP treated groups. 

(B) Western blot analysis of GABA<sub>A</sub> α4 subunit levels in Ctrl, EtOH, Roli, EtOH+ Roli treated groups. 

(C) Western blot analysis of GABA<sub>A</sub> γ2 subunit levels in Ctrl, EtOH, Rp-cAMP, and EtOH+ Rp-cAMP treated groups. 

(D) Western blot analysis of GABA<sub>A</sub> γ2 subunit levels in Ctrl, EtOH, Roli, EtOH+ Roli treated groups. 

Graphs show the OD of synaptosomal α4/β-actin and γ2/β-actin in each condition. 

* indicates statistical significance compared to Ctrl.
Figure 4

(A) Graph showing the effect of different treatments on a response. Treatments include EtOH, EtOH+Rp-cAMP, Ctrl, and EtOH+Rolipram.

(B) Graph showing the effect of different treatments on a response. Treatments include EtOH, Ctrl, and EtOH+CalC.

(C) Bar graph showing the time (ms) for different treatments. Treatments include Ctrl, EtOH, EtOH+CalC, EtOH+Rp-cAMP, and EtOH+Rolipram.

Legend: * indicates significant difference from Ctrl.