

# Regulation of Extrasynaptic GABA<sub>A</sub> $\alpha$ 4 Receptors by Ethanol-Induced Protein Kinase A, but Not Protein Kinase C Activation in Cultured Rat Cerebral Cortical Neurons

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## ABSTRACT

Ethanol produces changes in GABA<sub>A</sub> receptor trafficking and function that contribute to ethanol dependence symptomatology. Extrasynaptic  $\gamma$ -aminobutyric acid A receptors (GABA<sub>A</sub>-R) mediate inhibitory tonic current and are of particular interest because they are potentiated by physiologically relevant doses of ethanol. Here, we isolate GABA<sub>A</sub>  $\alpha$ 4 $\delta$  receptors by western blotting in subsynaptic fractions to investigate protein kinase A (PKA) and protein kinase C (PKC) modulation of ethanol-induced receptor trafficking, while extrasynaptic receptor function is determined by measurement of tonic inhibition and responses evoked by 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol (THIP). Rat cerebral cortical neurons were grown for 18 days in vitro and exposed to ethanol and/or PKA/PKC modulators. Ethanol exposure (1 hour) did not alter GABA<sub>A</sub>  $\alpha$ 4 receptor abundance,

but it increased tonic current amplitude, an effect that was prevented by inhibiting PKA, but not PKC. Direct activation of PKA, but not PKC, increased the abundance and tonic current of extrasynaptic  $\alpha$ 4 $\delta$  receptors. In contrast, prolonged ethanol exposure (4 hours) reduced  $\alpha$ 4 $\delta$  receptor abundance as well as tonic current, and this effect was also PKA dependent. Finally, PKC activation by ethanol or phorbol-12,13-dibutyrate (PdBu) had no effect on extrasynaptic  $\alpha$ 4 $\delta$  subunit abundance or activity. We conclude that ethanol alters extrasynaptic  $\alpha$ 4 $\delta$  receptor function and expression in cortical neurons in a PKA-dependent manner, but ethanol activation of PKC does not influence these receptors. These results could have clinical relevance for therapeutic strategies to restore normal GABAergic functioning for the treatment of alcohol use disorders.

## Introduction

Ethanol exposure has been shown to produce adaptations in GABA<sub>A</sub> receptor trafficking that are associated with alcohol dependence and withdrawal (Kumar et al., 2009). GABA<sub>A</sub> receptors are heteropentameric ion channels, with the majority of receptors consisting of two  $\alpha$  (1–6), two  $\beta$  (1–3), and a  $\gamma$  (1–2) or  $\delta$  subunit (Tretter and Moss, 2008). As the major inhibitory receptors in the brain, GABA<sub>A</sub> transmission involves both phasic and tonic inhibition, which are conducted by synaptic and extrasynaptic receptors, respectively (Farrant and Nusser, 2005). Previous studies have elucidated that synaptic and extrasynaptic populations of receptors are differentially regulated by ethanol. Extrasynaptic  $\alpha$ 4 $\beta$  $\delta$  GABA<sub>A</sub> in particular appear to be important mediators of the acute physiological effects of ethanol (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004), though this effect is controversial (Borghese et al. 2006). In both hippocampus and cerebral cortex, extrasynaptic  $\alpha$ 4-containing receptors are down-regulated

by ethanol, producing a decrease in the overall inhibitory “tone” (Liang et al., 2004; Liang et al., 2007; Carlson et al., 2014). Dysregulation of GABAergic receptors in the cerebral cortex are likely to contribute to the molecular underpinnings of decreased cognitive abilities, reduced seizure threshold, and increased anxiety observed in alcohol dependence (Koob, 2003). However, the precise mechanism for down-regulation of extrasynaptic  $\alpha$ 4-containing receptors is still unknown.

Recent studies by our laboratory have elucidated that protein kinases play a major role in facilitating the regulation of GABA<sub>A</sub> receptors after ethanol exposure (Kumar et al., 2010; Werner et al., 2011; Kumar et al., 2012). Ethanol has long been known to activate both protein kinase A (PKA) (Dohrman et al., 1996) and protein kinase C (PKC) (Messing et al., 1991), and kinases are important regulators of the behavioral effects of ethanol (Harris et al., 1995; Hodge et al., 1999; Thiele et al., 2000; Choi et al., 2008). PKC $\gamma$  activation by ethanol is requisite for the increase in GABA<sub>A</sub>  $\alpha$ 4 surface expression after 4 hours of ethanol exposure in cultured cerebral cortical neurons (Werner et al., 2011). This change in surface expression was postulated to be due to an increase in synaptic  $\alpha$ 4 $\beta$  $\gamma$ 2 receptors because GABA<sub>A</sub>  $\delta$  subunit surface expression remained unchanged. Interestingly, the trafficking changes mediated by PKC are

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**ABBREVIATIONS:** ANOVA, analysis of variance; CalC, calphostin-C; GABA<sub>A</sub>-R,  $\gamma$ -aminobutyric acid A receptor; PdBu, phorbol-12,13-dibutyrate; Pen-Strep, penicillin and streptomycin; PKA, protein kinase A; PKC, protein kinase C; RII $\alpha$ , protein kinase A regulatory subunit II $\alpha$ ; RII $\beta$ , protein kinase A regulatory subunit II $\beta$ ; Rp-cAMP, Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine; Sp-cAMP, Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol.

mitigated by concurrent activation of PKA by ethanol both in vivo and in vitro (Kumar et al., 2012; Carlson et al., 2013). Furthermore, we found that mice lacking the PKA regulatory subunit RIIβ gene have enhanced vulnerability to reductions in extrasynaptic GABA<sub>A</sub> α4 subunit trafficking after acute ethanol challenge (Carlson et al., 2014). Taken together, this evidence suggests that PKA may play a major role in regulating extrasynaptic α4-containing GABA<sub>A</sub> receptor subtypes in the cortex.

The present study sought to clarify the role of PKA and PKC in ethanol regulation of extrasynaptic α4-containing GABA<sub>A</sub> receptor populations in cerebral cortical cultured neurons. These cells exhibit the same ethanol adaptations that have been described in the cerebral cortex of ethanol-dependent rats (Devaud et al., 1997; Kumar et al., 2003) but allow for investigation of mechanistic questions that could not be addressed in vivo. Receptor adaptations were determined using subcellular fractionation of subsynaptic membrane fractions coupled with western blot analysis (Carlson et al., 2014), while functional changes were measured using whole-cell patch clamp analysis of tonic currents. We examined the time-dependent effects of ethanol on extrasynaptic GABA<sub>A</sub> α4 receptor function and trafficking because time-dependent effects of ethanol were observed on synaptic GABA<sub>A</sub> receptors as well as ethanol activation of PKC and PKA (Kumar et al., 2012; Carlson et al., 2013).

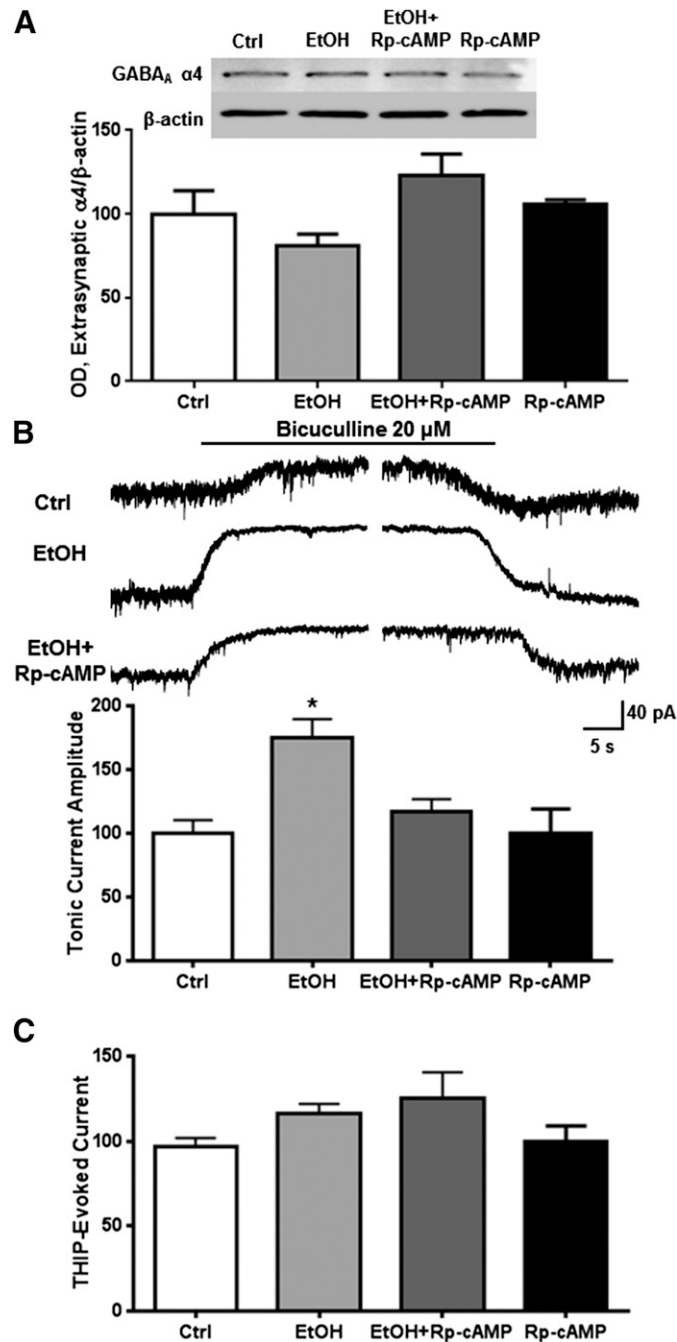
## Materials and Methods

**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. The brains were rapidly dissected, and the cerebral cortices were isolated. The cortical halves were minced into fine pieces, and the tissue was incubated in CO<sub>2</sub>-independent medium containing papain (50 U/ml; Worthington, Lakewood, NJ), 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 and streptomycin (Pen-Strep), and DNase. Cells used for biochemistry were plated onto poly-D-lysine-coated flasks, and cells used for electrophysiology were plated onto poly-D-lysine-coated cover slips in 12-well plates. Cells were maintained in a 5% CO<sub>2</sub> humidified incubator.

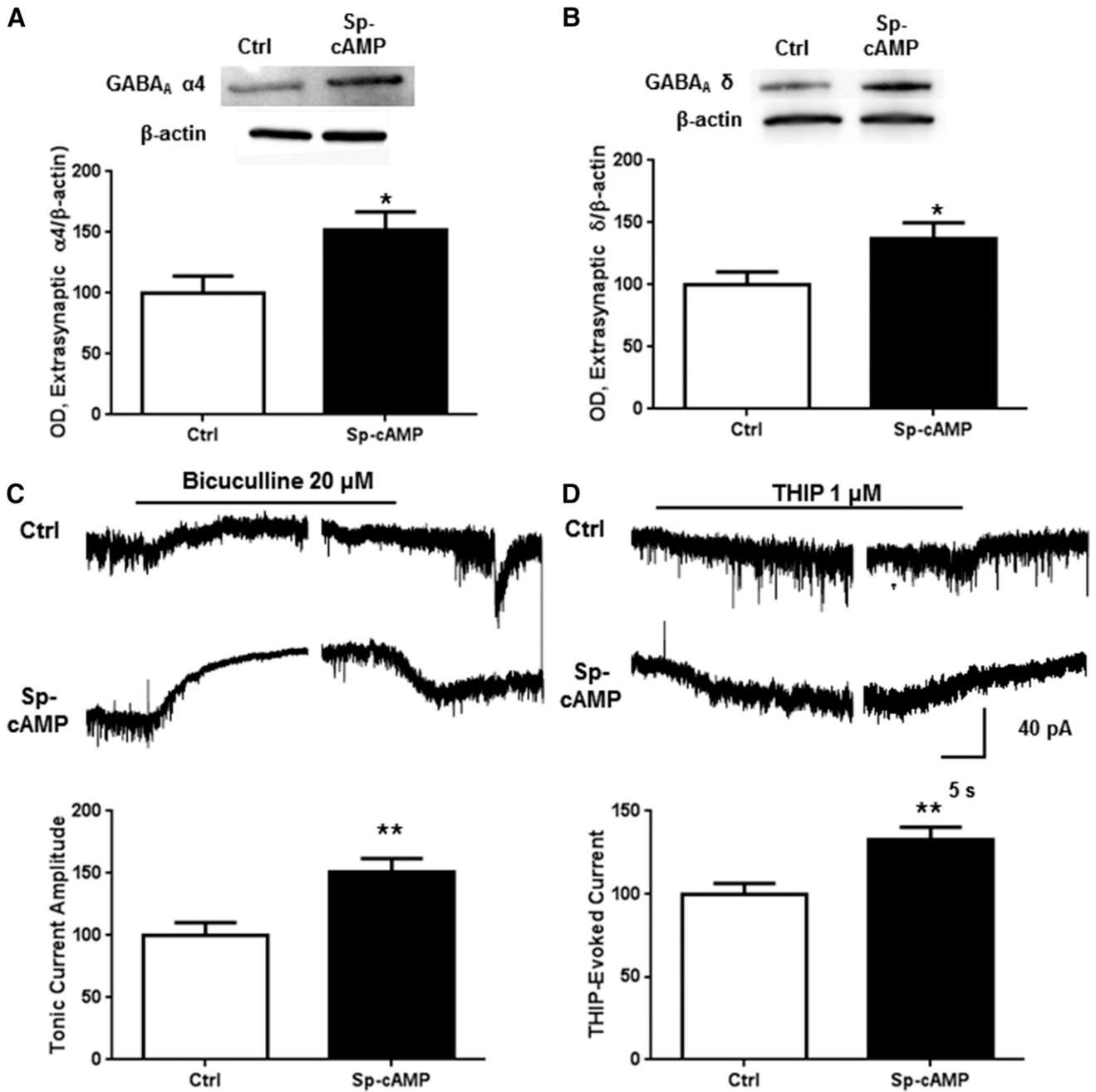
After day 3, the cells were fed with serum-free medium containing B27 and Pen-Strep (10,000 U/ml; final concentration 50 U per flask). The medium was changed twice per week, with no more than one-third of the medium being removed during exchanges. For all experiments, Pen-Strep was removed from cultures on day 14 to prevent interactions with GABA<sub>A</sub> receptors. The cultures were maintained for 18 days before conducting experiments, as prior studies had determined that neurons at this time point show stable GABA<sub>A</sub> receptor expression, with GABA<sub>A</sub> receptor populations mirroring the electrophysiologic and pharmacologic properties and responses to ethanol of adult neurons.

**Ethanol and Drug Exposure.** Cultured cells were exposed to 50 mM ethanol, a concentration previously shown to produce changes in GABAergic inhibition highly consistent with in vivo models (Devaud et al., 1997; Kumar et al., 2003), and were placed in a plastic vapor chamber within the incubator. A beaker of 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

drugs for either 1 or 4 hours. To examine PKA involvement, we added the PKA activator Sp-adenosine 3',5'-cyclic monophosphate triethylamine (Sp-cAMP, 50 μM), or the PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphate triethylamine (Rp-cAMP, 50 μM) or phosphodiesterase inhibitor rolipram (10 μM) to the cell medium. These doses were chosen based on previous studies (Zhang and Pandey, 2003; Carlson et al., 2013). To examine PKC involvement, we used



**Fig. 1.** PKA mediates ethanol-induced increases in tonic current activity. Cortical neurons were exposed to vehicle, ethanol (50 mM), and/or Rp-cAMP (50 μM) for 1 hour followed by either subsynaptic fractionation and western blot analysis or whole-cell patch clamp recording. (A) There was no effect of ethanol and/or PKA inhibition on extrasynaptic α4 subunit abundance. (B) Ethanol exposure significantly increased bicuculline-blocked current, an effect that was prevented by inhibiting PKA. (C) There was no effect of ethanol or PKA inhibition on THIP-evoked current. \*  $P < 0.05$ , one-way ANOVA Bonferroni post-test,  $n = 7-12$ .



**Fig. 2.** PKA activation increases extrasynaptic  $\alpha 4$  subunit abundance and function. Cortical neurons were exposed to vehicle or Sp-cAMP ( $50 \mu\text{M}$ ) for 1 hour followed by either subsynaptic fractionation and western blot analysis or whole-cell patch clamp recording. Exposure to Sp-cAMP for 1 hour caused an increase in (A) extrasynaptic  $\alpha 4$  and (B)  $\delta$  subunit abundance as well as (C) tonic current blocked by bicuculline ( $20 \mu\text{M}$ ) or (D) THIP-evoked ( $1 \mu\text{M}$ ) current.  $*P < 0.05$ ,  $**P < 0.01$ , Student's  $t$  test,  $n = 7-13$ .

the PKC inhibitor calphostin C (CalC,  $0.3 \mu\text{M}$ ) and the activator phorbol-12,13-dibutyrate (PdBu,  $100 \text{ nM}$ ), as previously described elsewhere (Kumar et al., 2010).

**Fractionation.** After the experiments, the reactions were stopped by placing the flasks on ice. The cells were washed with cold phosphate-buffered saline, scraped, centrifuged at  $1000g$  for 18 minutes, and stored at  $-80^\circ\text{C}$  until fractionation. The cell pellets were homogenized in  $0.32\text{M}$  D-sucrose and centrifuged at  $1000g$  for 10 minutes. The supernatant was then centrifuged twice for 30 minutes at  $12,000g$ . The final pellet was resuspended in phosphate-buffered saline.

For some experiments, the P2 fraction was further purified into extrasynaptic fractions according to the methods of Goebel-Goody et al. (2009). We have previously shown that this procedure produces

enrichment of synaptic and extrasynaptic markers in their respective fractions (Carlson et al., 2014). The fractions were separated by 30 minutes of incubation in  $0.5\%$  Triton-X, followed by two centrifugations at  $32,000g$  for 30 minutes. The supernatant was incubated overnight at  $-20^\circ\text{C}$  in acetone (1:8 ratio supernatant/acetone). The resulting solution was spun twice for 30 minutes at  $12,000g$  to produce the extrasynaptic fraction. Protein concentrations for isolated P2 or extrasynaptic 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.** PKA regulatory subunit  $\text{II}\alpha$  ( $\text{RII}\alpha$ ), PKA regulatory subunit  $\text{II}\beta$  ( $\text{RII}\beta$ ), PKC $\gamma$ , and GABA $_A$  receptor  $\alpha 4$  and  $\delta$  subunits were analyzed by western blot as described elsewhere

TABLE 1

Kinase abundance in the subsynaptic fraction after 1 and 4 hours of ethanol exposure.

Values expressed as percentage relative to control.

Protein	1-h Ethanol	4-h Ethanol
PKA RII $\alpha$	122.0 $\pm$ 3.5 <sup>a</sup>	97.3 $\pm$ 5.9
PKA RII $\beta$	126.2 $\pm$ 4.1 <sup>a</sup>	102.8 $\pm$ 6.3
PKC $\gamma$	104.5 $\pm$ 6.7	96.4 $\pm$ 5.1

<sup>a</sup>*P* < 0.01, compared with control, Student's *t* test, *n* = 4.

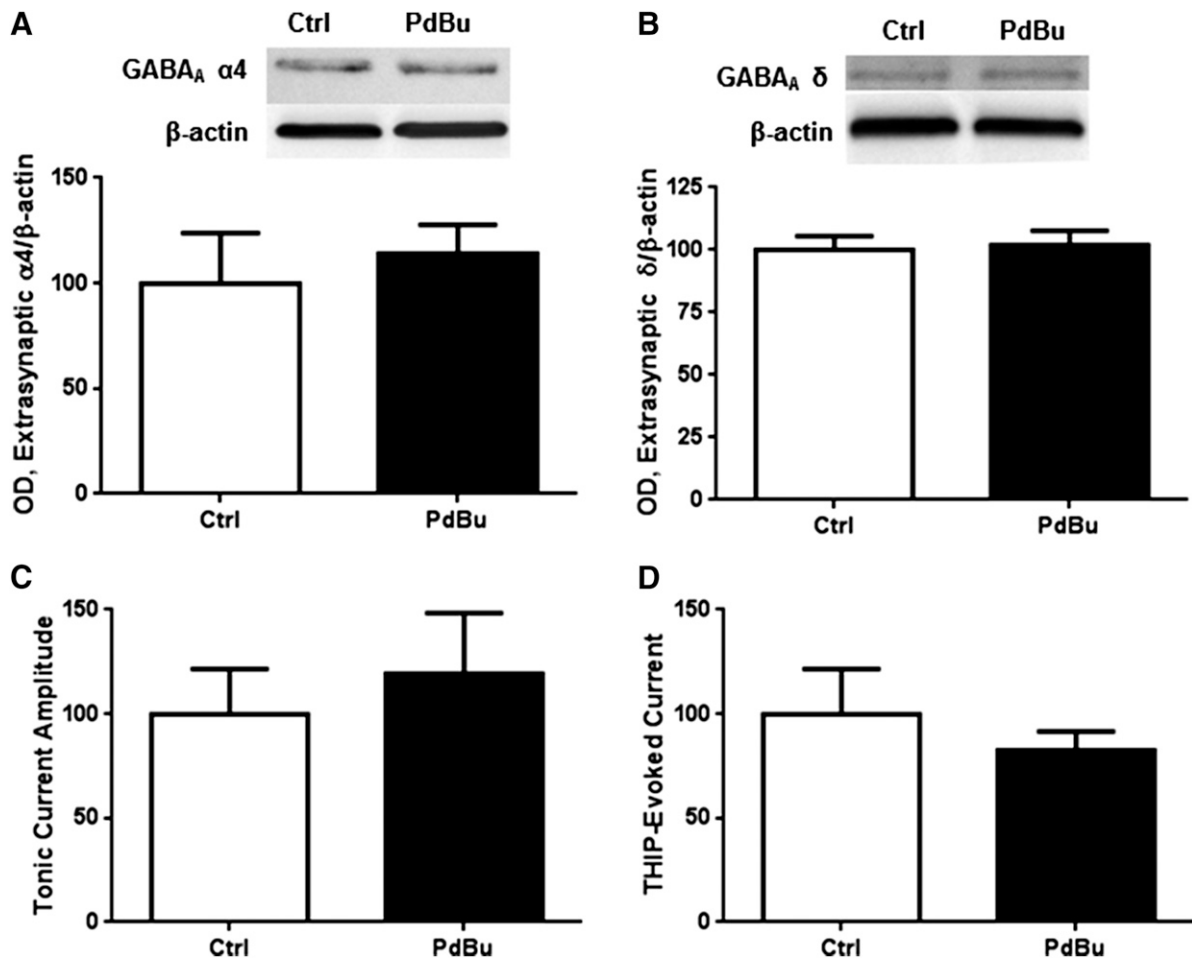
(Kumar et al., 2010). Protein samples were subjected to SDS-PAGE using Novex Tris-Glycine (8%–16%) gels and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). Membranes were probed with GABA<sub>A</sub> receptor  $\alpha$ 4 (1:500; Abcam, Cambridge, MA), or  $\delta$  (1:500; Millipore, Billerica, MA), PKA RII $\alpha$  (1:1000; Novus, Littleton, CO), PKA RII $\beta$  (Novus; 1:2000), and PKC $\gamma$  (Novus; 1:1000) antibodies. Blots were then exposed to a  $\beta$ -actin antibody (1:3000; Millipore) as a loading control. Proteins were detected with enhanced chemiluminescence (GE Healthcare, Amersham, United Kingdom). 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 percentage of averaged control values.

**Electrophysiology.** Standard whole-cell voltage-clamp recordings were made with glass electrodes, fire-polished to a resistance of

2–3 M $\Omega$  and filled with internal solution (150 mM KCl, 3.1 mM MgCl<sub>2</sub>, 15 mM HEPES, 5 mM K-ATP, 5 mM EGTA, and 15 mM phosphocreatine, adjusted to pH 7.4 with KOH). The recording chamber was perfused with external solution (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM sucrose, and 10 mM glucose, adjusted to pH 7.4 with NaOH). Drugs were diluted in external solution and applied using a U-tube apparatus. Recordings were performed at room temperature (22–23°C). The membrane potential was held at -60 mV using a patch-clamp amplifier (Axopatch 1D; Molecular Devices, Sunnyvale, CA), and data were collected with Clampex 10.2 software (Molecular Devices). Bicuculline (20  $\mu$ M) was used as an indicator of tonic current activity, while THIP (1  $\mu$ M) was used as an indicator of extrasynaptic  $\delta$ -receptor agonism. Drugs were applied for 1.5 min after a 10 s baseline activity was established, followed by 30 s washout. Tonic and THIP-evoked currents were measured as the change in current amplitude in the presence of drug to an average of current baseline amplitude and post-washout.

**Kinase Activity Assay.** Kinase activity was measured using the PepTag assay from Promega (Madison, WI). Assays were performed on cultured cells that were exposed to 50 mM ethanol for 1 or 4 hours and subjected to P2 fractionation procedures described earlier. Assays were conducted according to the manufacturer's instructions.

**Statistical Analysis.** All numerical data are presented as a percentage of control values and as mean  $\pm$  S.E.M. Significance was determined using one-way analysis of variance (ANOVA) followed by Bonferroni correction for multiple comparisons post hoc test if there were more than two groups or Student's *t* test for only two groups.



**Fig. 3.** PKC activation does not alter extrasynaptic  $\alpha$ 4 subunit abundance or function. Cortical neurons were exposed to vehicle or PdBu (100 nM) for 1 hour followed by subsynaptic fractionation and western blot analysis. (A) PdBu exposure did not alter extrasynaptic  $\alpha$ 4 or (B) extrasynaptic  $\delta$  subunit abundance, or (C) tonic or (D) THIP-evoked current.

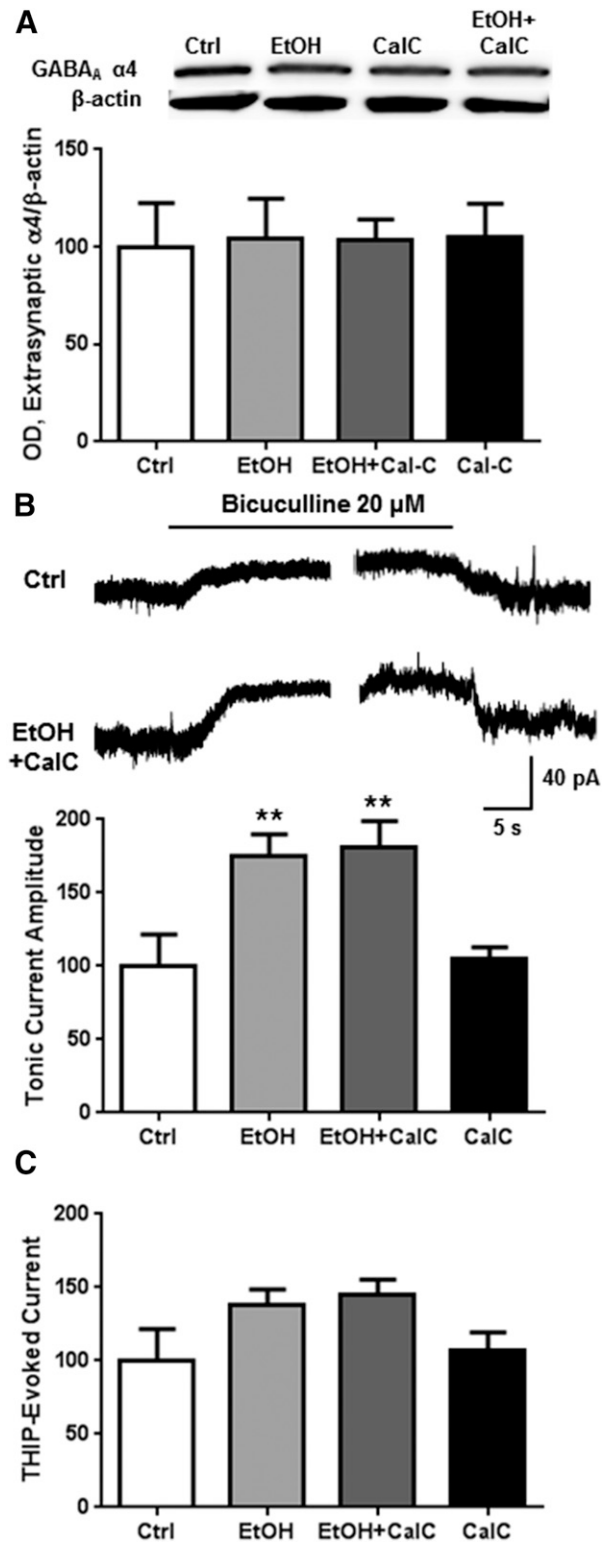
## Results

**Acute Ethanol Exposure Increases Tonic Current Activity in a PKA-Dependent Manner with No Effect on Extrasynaptic  $\alpha 4$  Subunit Abundance.** We used a subsynaptic preparation we have previously shown to enrich indicators of extrasynaptic proteins (Carlson et al., 2014) to examine the effects of ethanol exposure (50 mM) for 1 hour to determine its effect on receptor function and expression. Neither exposure to ethanol alone nor coexposure of ethanol and Rp-cAMP altered extrasynaptic abundance of the GABA<sub>A</sub>  $\alpha 4$  subunits (Fig. 1A). However, ethanol increased the baseline tonic activity, blocked by bicuculline in neurons by  $74.8\% \pm 15.1\%$  (Fig. 1B; one-way ANOVA,  $f = 7.624$ ,  $P < 0.01$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 5$ –12 per group), with no effect on current evoked by THIP (1  $\mu$ M). Bicuculline effectively blocks the constitutively active baseline GABA conductance, whereas THIP shows high preference for  $\alpha 4\beta 3\delta$  receptors at 1  $\mu$ M (Mortensen et al., 2010). The ethanol effect on tonic current was prevented by coexposure with Rp-cAMP. No effect was observed from ethanol or PKA inhibition on THIP-evoked current amplitude (Fig. 1C).

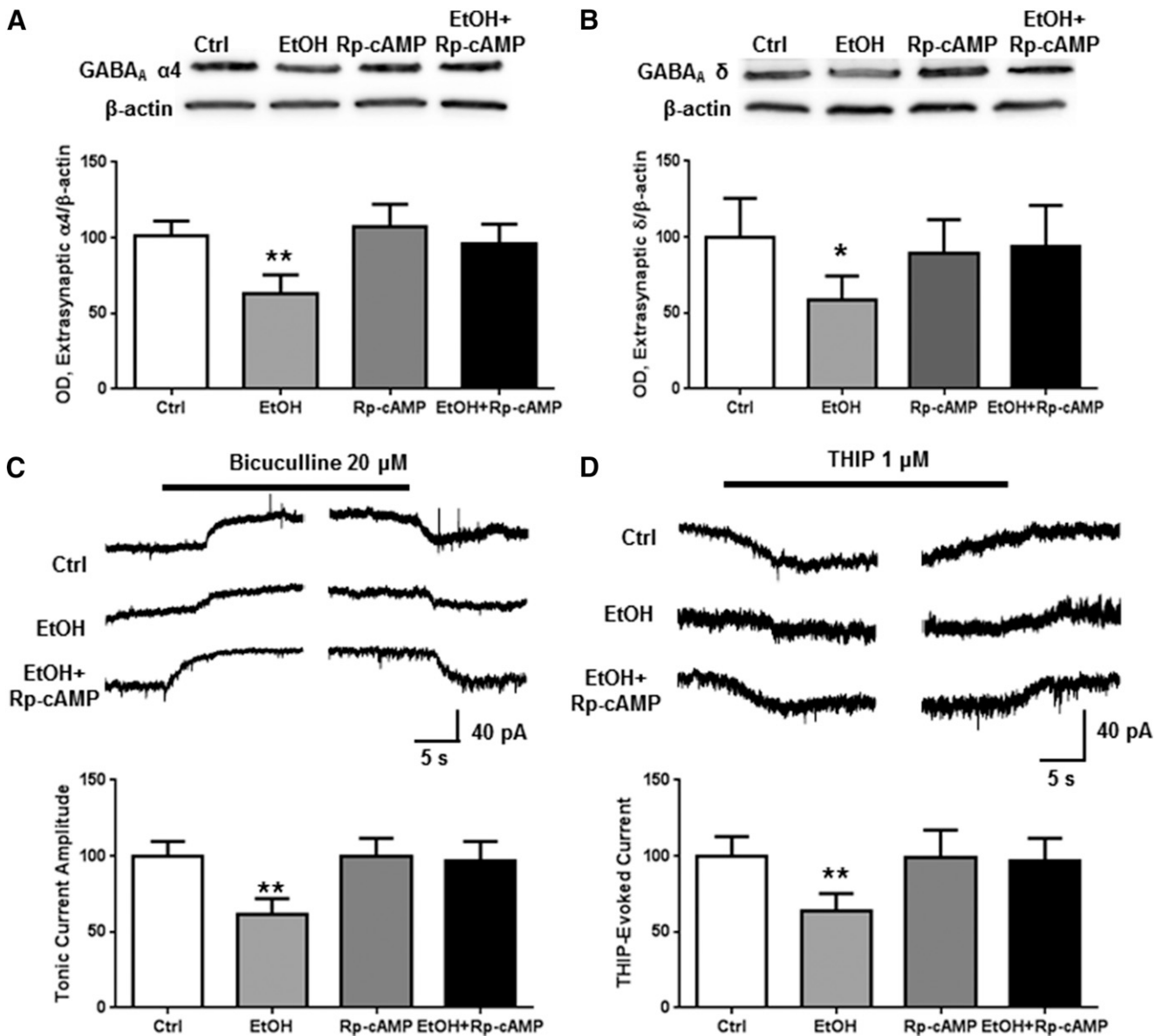
**Direct PKA Activation Increases Membrane Expression and Function of Extrasynaptic  $\alpha 4\delta$  Receptors.** We next tested the effects of direct PKA activation on extrasynaptic GABA<sub>A</sub>  $\alpha 4$  abundance and function. Activation of PKA by exposure to Sp-cAMP (50  $\mu$ M) for 1 hour increased  $\alpha 4$  subunit abundance in the extrasynaptic fraction by  $52.4\% \pm 14.3\%$  (Fig. 2A,  $P < 0.05$ , Student's  $t$  test,  $n = 10$ ). Similarly, Sp-cAMP increased  $\delta$  subunit abundance by  $36.6\% \pm 13.2\%$  (Fig. 2B,  $P < 0.05$ , Student's  $t$  test,  $n = 8$ ). The comprehensive GABA antagonist bicuculline (20  $\mu$ M) and the  $\delta$  subunit-preferring agonist THIP (1  $\mu$ M) were used as physiological indicators of altered tonic current activity. Tonic and THIP-evoked currents were both significantly increased after 1 hour Sp-cAMP exposure (Fig. 2, C and D;  $P < 0.01$ , Student's  $t$  test,  $n = 9$ –13).

**PKC Is Not Involved in Ethanol Regulation of Extrasynaptic GABA<sub>A</sub>  $\alpha 4$  Receptors.** Given that our prior work indicates that PKA and PKC have antagonistic actions on GABA<sub>A</sub> receptor  $\alpha 1$  subunit abundance (Werner et al., 2011; Carlson et al., 2013), we investigated the effect of PKC activity on extrasynaptic GABA<sub>A</sub>  $\alpha 4$  abundance and function. Although PKC $\gamma$  is not directly increased in the subsynaptic fraction (Table 1), we cannot discount downstream or non-site-specific effects of PKC. Activation of PKC for 1 hour using PdBu (100 nM) had no effect on extrasynaptic  $\alpha 4$  (Fig. 3A) or  $\delta$  subunit abundance (Fig. 3B). PKC has previously been shown to regulate synaptic  $\alpha 4$  current in cortical neurons (Werner et al., 2011), so we investigated whether PKC activation altered tonic current. PdBu exposure did not alter tonic current (Fig. 3C) or THIP-evoked current amplitude (Fig. 3D).

We investigated the inhibition of PKC in the presence of ethanol to determine whether PKC might be involved in the effects of ethanol on extrasynaptic GABA<sub>A</sub>  $\alpha 4$  receptors. Inhibition of PKC by CalC (300 nM) in the presence of ethanol had no effect on extrasynaptic GABA<sub>A</sub>  $\alpha 4$  subunit abundance (Fig. 4A). Similar to our previous data (Fig. 1B), ethanol exposure increased tonic current, an effect that was not prevented by coexposure with the PKC inhibitor CalC (Fig. 4B; one-way ANOVA,  $f = 6.6$ ,  $P < 0.01$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 6$ –9). Ethanol alone or coexposure of ethanol with CalC did not alter THIP-evoked current amplitude (Fig. 4C).



**Fig. 4.** PKC is not involved in 1-hour ethanol regulation of tonic current activity. Cortical neurons were exposed to vehicle, ethanol (50 mM), and/or CalC (0.3  $\mu$ M) for 1 hour followed by subsynaptic fractionation and western blot analysis or whole-cell patch clamp recording. (A) There was no change in extrasynaptic GABA<sub>A</sub>  $\alpha 4$  abundance at 1 hour for any of the exposures. (B) Ethanol and ethanol + CalC treatment increased bicuculline-blocked tonic current activity, but (C) had no effect on THIP-evoked current. \*\* $P < 0.01$ , one-way ANOVA Bonferroni post-test,  $n = 5$ –9.

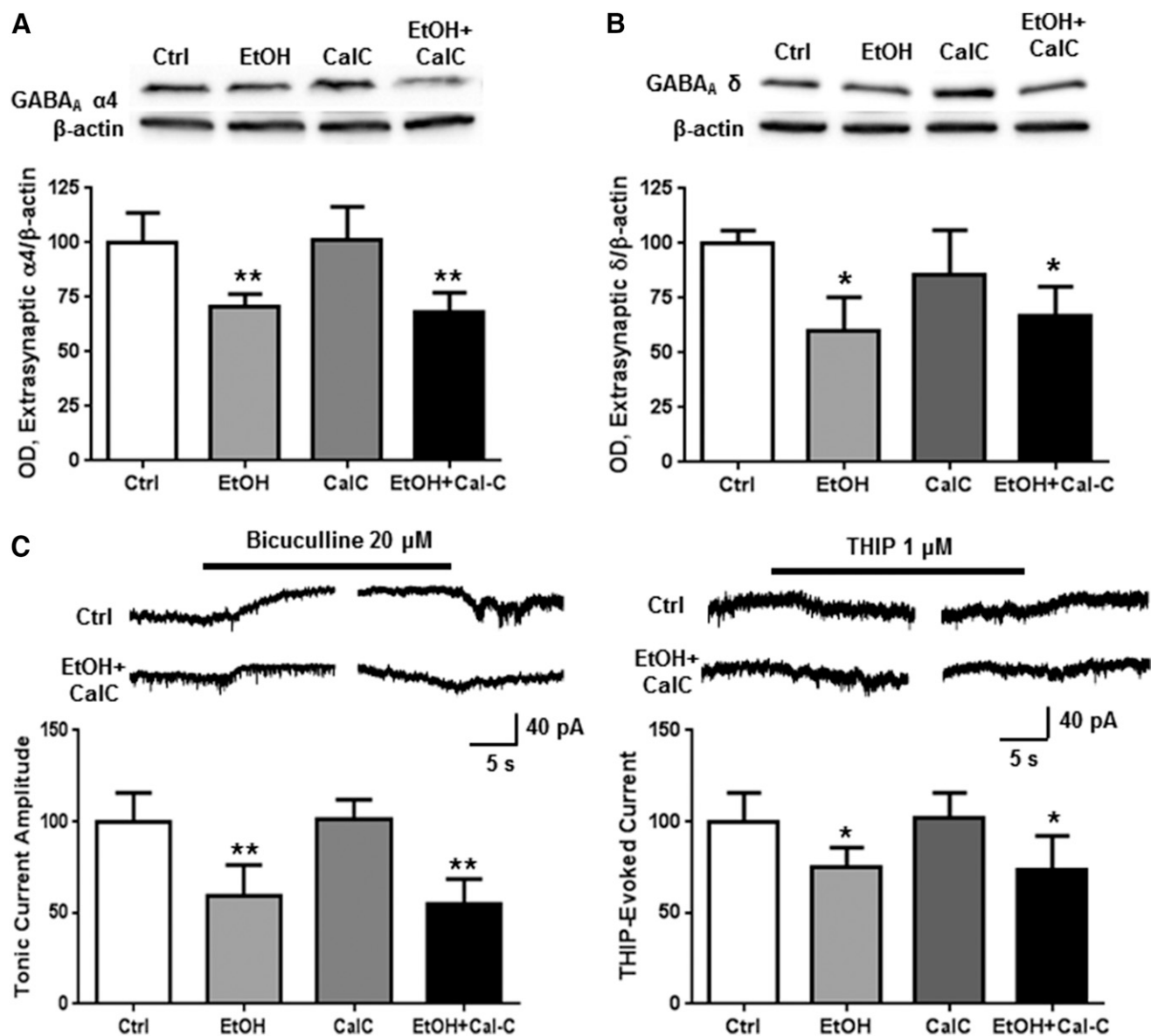


**Fig. 5.** Four-hour ethanol exposure causes a decrease in extrasynaptic α4 subunit abundance and function through a PKA-dependent mechanism. Cortical neurons were exposed to vehicle, ethanol (50 mM) and/or Rp-cAMP (50 μM) for 4 hours followed by either extrasynaptic fractionation and western blot analysis or whole-cell patch clamp recording. Ethanol caused a decrease in extrasynaptic GABA<sub>A</sub> receptor (A) α4 and (B) δ subunit abundance, (C) tonic current blocked by bicuculline, and (D) THIP-evoked current, all of which were prevented by Rp-cAMP coexposure. \* $P < 0.05$ , \*\* $P < 0.01$ , one-way ANOVA Bonferroni post-test,  $n = 7-12$ .

**PKA Is Involved in Functional and Biochemical Changes in Extrasynaptic GABA<sub>A</sub> α4 after 4-Hour Ethanol Exposure.** We next investigated the role of PKA in mediating ethanol adaptations after 4 hours of ethanol exposure, a paradigm that recapitulates in cultured cortical neurons the changes seen after chronic ethanol exposure in vivo (Kumar et al., 2010; Werner et al., 2011). Ethanol produced a  $37.2\% \pm 5.2\%$  decrease in extrasynaptic GABA<sub>A</sub> α4 subunits (Fig. 5A; one-way ANOVA,  $f = 14.51$ ,  $P < 0.0001$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 6$  per group) and  $41.3\% \pm 6.4\%$  decrease in GABA<sub>A</sub> δ subunits (Fig. 5B; one-way ANOVA,  $f = 3.818$ ,  $P < 0.05$ , Bonferroni posttest,  $P < 0.05$ ,  $n = 6-7$  per group), and both effects were prevented by coexposure with Rp-cAMP. Similarly, 4 hours of ethanol exposure decreased tonic current by  $38.7\% \pm 6.3\%$  (Fig. 5C; one-way ANOVA,  $f = 17.63$ ,  $P < 0.0001$ , Bonferroni posttest,

$P < 0.01$ ,  $n = 6-7$  per group) and THIP-induced current by  $36.3\% \pm 5.4\%$  (Fig. 5D; one-way ANOVA,  $f = 10.18$ ,  $P < 0.001$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 7$  per group). Both effects were prevented by coexposure with Rp-cAMP.

PKC is known to be active in cultured cortical neurons after 4-hour ethanol exposure (Kumar et al., 2010; Werner et al., 2011), so we investigated whether PKC might also be involved in the decreased abundance and functioning of extrasynaptic GABA<sub>A</sub> α4 subunits at this time point. Coexposure with CalC failed to prevent ethanol-induced decreases in extrasynaptic GABA<sub>A</sub> α4 (Fig. 6A; one-way ANOVA,  $f = 14.53$ ,  $P < 0.0001$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 7$  per group) or δ (Fig. 6B; one-way ANOVA,  $f = 8.802$ ,  $P < 0.01$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 5-6$  per group) subunit levels, tonic current (Fig. 6C; one-way ANOVA,  $f = 18.64$ ,  $P < 0.0001$ , Bonferroni posttest,  $P < 0.01$ ,  $n = 6-7$  per group), or THIP-evoked current



**Fig. 6.** PKC is not involved in decreased extrasynaptic  $\alpha 4$  abundance and function after 4-hour ethanol. Cortical neurons were exposed to vehicle, ethanol (50 mM), and/or CalC (0.3  $\mu$ M) for 4 hours followed by subsynaptic fractionation and western blot analysis or whole-cell patch clamp recording. There was a decrease in extrasynaptic GABA<sub>A</sub> receptor (A)  $\alpha 4$  and (B)  $\delta$  subunit abundance, (C) tonic current blocked by bicuculline, and (D) THIP-evoked current in the ethanol and ethanol + CalC-exposed groups for all experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , one-way ANOVA Bonferroni post-test,  $n = 6-7$ .

amplitude (Fig. 6D; one-way ANOVA,  $f = 6.454$ ,  $P < 0.01$ , Bonferroni posttest,  $P < 0.05$ ,  $n = 6-7$  per group).

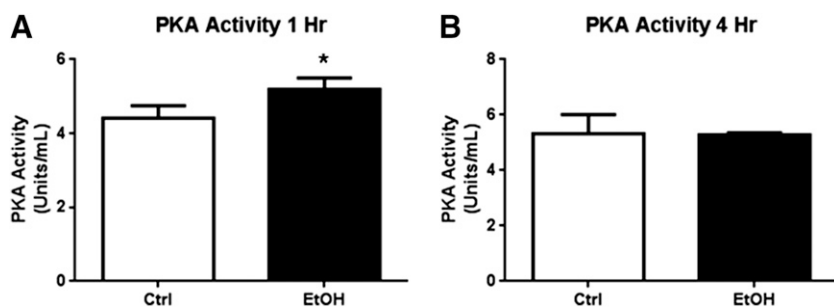
**Ethanol Increases PKA Activity after 1 Hour of Exposure but Not 4 Hours.** We previously found that PKA regulatory subunits and PKC $\gamma$  are enriched in P2 membrane fractions after 1 hour of ethanol exposure, but only PKC $\gamma$  is increased after 4 hours (Kumar et al., 2010; Carlson et al., 2013). The PKA regulatory subunits RII $\alpha$  and RII $\beta$  were both increased in the subsynaptic fraction after 1 hour of ethanol exposure ( $P < 0.01$ , Student's  $t$  test,  $n = 4$ ), but PKC $\gamma$  levels were unaffected (Table 1). Protein levels for all three kinase subunits were unaffected by 4 hours of ethanol exposure.

Because the PKA regulatory subunits show increased abundance in the extrasynaptic membrane fractions after 1 hour of ethanol exposure but not 4 hours of ethanol, we next assayed the level of PKA kinase activity at these time points

to determine the functional significance of the contrasting effect of 1-hour versus 4-hour ethanol exposure. Ethanol increased PKA kinase activity after 1 hour by  $17.6\% \pm 4.3\%$  (Fig. 7A; Student's  $t$  test,  $P < 0.05$ ,  $n = 3$ ) but not after 4 hours of ethanol exposure (Fig. 7B).

## Discussion

Our study demonstrates that ethanol modulation of extrasynaptic GABA<sub>A</sub>  $\alpha 4$  and  $\delta$  subunits is PKA dependent and that PKA has direct but independent actions on the function and expression of these receptors in cortical neurons. Conversely, PKC activation by ethanol is not involved in the regulation of extrasynaptic  $\alpha 4$  and  $\delta$  subunits in cortical neurons. The present results provide further characterization of the subcellular mechanisms through which alcohol regulates



**Fig. 7.** PKA activity is increased by 1-hour but not 4-hour ethanol exposure. Cortical neurons were exposed to ethanol (50 mM) followed by P2 fractionation and PepTag kinase activity assay. (A) PKA activity was increased after 1-hour ethanol exposure, but (B) not 4-hour ethanol exposure. \* $P < 0.05$ , Student's  $t$  test,  $n = 3$ .

GABAergic trafficking, which should have implications for cortical circuit functioning and could provide targets for restoring normal GABA<sub>A</sub> functioning during ethanol withdrawal.

Interestingly, although activation of PKA and PKC by ethanol has directly opposing effects on synaptic  $\alpha$ 1 receptors (Carlson et al., 2013), our results suggest that PKC does not play a role in regulating the extrasynaptic  $\alpha$ 4 $\delta$  receptor population. This finding is consistent with previous studies in our laboratory that showed no effect of PKC activation on  $\delta$  subunit expression in P2 membrane fractions (Werner et al., 2011). However, this result contrasts with previous studies that have found that PKC has a role in regulating extrasynaptic  $\alpha$ 4 receptor populations in the thalamus and hippocampus (Choi et al., 2008; Abramian et al., 2010; Bright and Smart, 2013). This discrepancy could be explained by a difference in regulation by brain region, cell type, or the slice preparation. One study found PKC $\delta$  enhanced tonic GABA currents in mouse hippocampal and thalamic slices (Choi et al., 2008); however, PKC $\delta$  was not found to associate with GABA<sub>A</sub>  $\alpha$ 4 receptors after chronic ethanol in the cerebral cortex (Kumar et al., 2002). Further, PKC $\delta$  is not detectable by western blot in our cerebral cortical cultures (unpublished results), which could also explain the lack of an effect of PKC on tonic current. Another study found brief PKC activation reduced tonic current activity in the hippocampus and thalamus only at physiologic temperatures (Bright and Smart, 2013). As the electrophysiologic recordings in our study were made at room temperature, we cannot rule out a temperature-specific effect of PKC on tonic GABA currents. The Bright and Smart (2013) study was also performed at 10 minutes of PKC activation, whereas our study found no effects at either 1 or 4 hours, so it is possible that there are also time-dependent effects. Other studies in cerebellar granule cells have found that differences in PKC activity can either enhance or inhibit tonic current in the presence of ethanol with variations attributable to genotype, suggesting PKC regulation is complex (Kaplan et al., 2013).

A decrease in tonic current inhibition after ethanol exposure is believed to be linked to many of the deleterious symptoms associated with alcohol withdrawal, such as increased seizure susceptibility (Liang et al., 2004). Dysfunctions in extrasynaptic GABA<sub>A</sub> receptors have also been linked to a variety of pathologic conditions including epilepsy, traumatic brain injury, depression, and schizophrenia (Whissell et al., 2015). Thus, PKA activation may provide a method for restoring tonic current functioning to normal levels during ethanol withdrawal as well as a variety of disease states. Indeed, the loss of PKA activation at 4 hours may suggest that restoration of PKA activation could reverse the effect of ethanol at this time point. Recent studies finding a reduction in drinking of rodents coexposed to the phosphodiesterase inhibitor

rolipram further underscore the potential therapeutic relevance of manipulating PKA signaling in the treatment of alcohol use disorders (Hu et al., 2011; Wen et al., 2012).

The finding that acute ethanol exposure increased tonic current activity is consistent with previous studies in other brain regions (Jia et al., 2007; Santhakumar et al., 2007; Shen et al., 2011; Herman et al., 2013); however, to our knowledge this is the first report of increased tonic current inhibition after acute ethanol treatment in cortical neurons. If the direct involvement of PKA in mediating this increase is generalized to other brain regions, this finding could have broad implications for a potential role of PKA in ethanol effects on these receptors.

Surprisingly, whereas direct PKA activation increased abundance of both the extrasynaptic  $\alpha$ 4 and  $\delta$  subunits corresponding with an increase in both tonic current and THIP-evoked current, ethanol activation of PKA did not appear to increase THIP-evoked current, despite the increase in tonic current. One explanation for this discrepancy is that ethanol did not alter receptor abundance after 1 hour of exposure. Alternatively, some of the ethanol-induced increase in tonic current may be mediated by  $\alpha$ 1 receptors.

Recently a novel form of tonic current activity has been characterized that is mediated by  $\alpha$ 1 $\beta$  $\gamma$  receptors (Herman et al., 2013), known to be up-regulated by ethanol activation of PKA (Carlson et al., 2013). An additional explanation is that ethanol-induced PKA activation may alter the conformation of  $\alpha$ 4 $\delta$  receptors in a manner that does not support enhanced function by THIP. Further, ethanol is a known positive allosteric modulator of GABA<sub>A</sub>-Rs and may potentiate GABA<sub>A</sub>-Rs at the dose used in the current study. Finally, ethanol may be acting on other intracellular pathways whose activity is masking an effect of PKA on THIP-evoked current (Freund and Palmer, 1997; Marutha Ravindran and Ticku, 2006). It is possible that ethanol is acting via kinase or phosphatase pathways whose actions have not been clearly defined within our experimental parameters (Kittler and Moss, 2003). Future studies aimed at further characterization of both the altered tonic current as well as the activity of other kinase and phosphatase pathways could resolve this issue.

The finding that PKA mediates an increase in tonic current after 1 hour of ethanol exposure was unsurprising, as PKA has been shown to mediate increased spontaneous channel opening of GABA<sub>A</sub>  $\alpha$ 4 receptors (Tang et al., 2010); however, the observation that PKA activity mediates a decrease in extrasynaptic GABA<sub>A</sub>  $\alpha$ 4 abundance and function after 4 hours of ethanol exposure was unexpected. There are two likely explanations for the discrepancy observed between increased activity at 1-hour but decreased activity at the 4-hour time point. It is possible that activation of PKA by ethanol at the



1-hour time point leads to activation of other pathways downstream of PKA, which leads to the alterations in GABAergic functioning. It is also possible that the observed decrease in abundance and function may be caused by a compensatory decrease after sustained increases caused by PKA at earlier time points. Future studies investigating other subcellular pathways, including other kinases, phosphatases, or phosphodiesterases could clarify this issue.

Overall, the present results further underscore the relevance of PKA signaling in modulating the GABAergic effects of ethanol. The data suggest that PKA activity may enhance GABAergic inhibition that is down-regulated by chronic ethanol exposure. Thus, this pathway could provide an important target for treatments aimed at restoring normal GABA<sub>A</sub> receptor functioning after chronic alcohol misuse. Given that extrasynaptic GABA<sub>A</sub>  $\alpha 4$  receptors are altered in a variety of pathologic conditions (Whissell et al., 2015), the present results could have broad implications in treating disease states.

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#### Authorship Contributions

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

*Conducted experiments:* Carlson, Bohnsack, Patel.

*Performed data analysis:* Carlson, Bohnsack.

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

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