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Ethanol activation of PKA regulates $GABA_A$ α1 receptor function and trafficking in cultured cerebral cortical neurons

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Running Title: PKA mitigates GABAergic adaptations to ethanol

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Nonstandard Abbreviations:

Rp-cAMP – Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine

Sp-cAMP – Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine

CalC – Calphostin-C

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Abstract

Ethanol exposure produces alterations in GABAergic signaling that are associated with dependence and withdrawal. Previously, we demonstrated that ethanol-induced protein kinase Cγ (PKCγ) signaling selectively contributes to changes in $GABA_A$ α1 synaptic receptor activity and surface expression. Here, we demonstrate that protein kinase A (PKA) exerts opposing effects on $GABA_A$ receptor adaptations during brief ethanol exposure. Cerebral cortical neurons from day 0-1 rat pups were tested after 18 days in culture. Receptor trafficking was assessed by western blot analysis and functional changes were measured using whole cell patch clamp recordings of evoked and mIPSC responses. One-hour ethanol exposure increased membrane-associated PKC and PKA, but steady-state $GABA_A$ α1 subunit levels were maintained. Activation of PKA by Sp-cAMP alone increased $GABA_A$ α1 subunit surface expression and zolpidem potentiation of GABA responses, while co-exposure of ethanol with the PKA inhibitor Rp-cAMP decreased α1 subunit expression and zolpidem responses. Exposure to the PKC inhibitor calphostin-C with ethanol mimicked the effect of direct PKA activation. The effects of PKA modulation on mIPSC decay τ were consistent with its effects on GABA currents evoked in the presence of zolpidem. Overall, the results suggest that PKA acts in opposition to PKC on α1-containing $GABA_A$ receptors, mediating the GABAergic effects of ethanol exposure, and may provide an important target for the treatment of alcohol dependence/withdrawal.

Introduction

Ethanol elicits a number of adaptations in GABAergic signaling following both acute and chronic exposure. Evidence suggests acute ethanol intoxication increases overall GABAergic inhibition, while chronic ethanol leads to deficits in GABA functioning (reviewed in (Kumar et al., 2004; Kumar et al., 2009). Numerous studies suggest the major changes associated with GABAergic deficits following chronic ethanol exposure include a decrease in surface expression of $GABA_A$ receptor α1 subunits both in the cerebral cortex (Devaud et al., 1997; Kumar et al., 2003) and hippocampus (Cagetti et al., 2003; Liang et al., 2004; Liang et al., 2006; Liang et al., 2007). Chronic ethanol exposure produces a withdrawal syndrome upon discontinuation of ethanol that is mimicked, in part, by genetic deletion of α1 subunits in mice that were never exposed to ethanol. This suggests that the loss of $GABA_A$ α1 receptor surface expression is involved in tremor and enhanced seizure susceptibility associated with ethanol dependence (Kralic et al., 2002; Kralic et al., 2005). Additionally, similar alterations in $GABA_A$ α1 subunit levels are found in other disorders, including models of epilepsy and benzodiazepine tolerance (Chen et al., 1999; Zeng and Tietz, 1999; Gonzalez and Brooks-Kayal, 2011). Therefore, a better understanding of $GABA_A$ receptor regulation following ethanol exposure may provide both valuable insight into the general mechanism of $GABA_A$ receptor regulation, as well as potential therapeutic targets for pathologies linked to these disorders.

Recently, we and others have shown that $GABA_A$ receptor adaptations to ethanol are recapitulated *in vitro* following a brief ethanol exposure in cultured cortical and hippocampal neurons (Kumar et al., 2010; Shen et al., 2011; Werner et al., 2011). The effect of ethanol is dependent upon the selective activation of PKCγ to induce the internalization of $GABA_A$ α1 subunits (Kumar et al., 2010), consistent with the ability of ethanol to activate PKCγ and reduce $GABA_A$ α1 subunit surface expression *in vivo*. However, there is a mismatch between the time dependent effects of ethanol on PKCγ and $GABA_A$ α1 receptors, suggesting the involvement of other mechanisms (Kumar et al., 2010).

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Ethanol has long been known to directly activate PKA (Dohrman et al., 1996). *In vivo* studies implicate PKA as playing a role in the behavioral effects of ethanol (Thiele et al., 2000; Pandey et al., 2003; Lai et al., 2007). Additionally, PKA has been shown to directly phosphorylate and modulate $GABA_A$ receptor activity (Ives et al., 2002; Brandon et al., 2003). Recently, we found that ethanol dose- and time-dependently increased PKA membrane levels in rat cerebral cortex, which corresponded to increased $GABA_A$ α1 subunit expression (Kumar et al., 2012). The precise physiological consequences of ethanol regulation of GABAergic inhibition via PKA, however, are unclear. As recent studies from our lab suggest both PKC and PKA activation may influence $GABA_A$ receptor activity during ethanol exposure, we investigated the dualistic role of PKA and PKC in modulating the actions of ethanol on $GABA_A$ receptor α1 subunit expression and function in cortical cultured neurons.

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. Cultured cerebral cortical neurons were isolated as described elsewhere (Kumar et al., 2010). Briefly, 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 CO_2-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% CO_2 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 $GABA_A$ receptors. Cultures were maintained for at least 17 days before conducting experiments, as 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 chamber within the incubator. 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 vapor chamber with a beaker containing water. Cells were exposed to ethanol for various times (0 – 60 min). To examine PKA involvement, the PKA activator Sp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Sp-cAMP, 70µM) and PKA inhibitor Rp-adenosine 3',5'-cyclic monophosphothioate triethylamine (Rp-cAMP, 50µM) were added to the cell

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media. These doses were chosen based on previous studies (Zhang and Pandey, 2003). To examine PKC involvement, the PKC inhibitor calphostin C (CalC, 0.3μM in 0.1% dimethyl sulfoxide, final concentrations) was used as previously described (Kumar et al., 2010).

P2 Fractionation and Biotinylation

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. The final pellet was resuspended in PBS. Protein concentrations for the isolated P2 fraction were made using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA). Biotinylation experiments were performed as previously described (Kumar et al., 2010). Briefly, cells were washed twice with ice-cold PBS, followed by addition of sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate diluted in PBS, and incubated for 30 min at 4°C. Unbound biotin was inactivated using a quenching solution. Cells were scraped and spun three times at 500g in Tris-buffered saline. Biotin-labeled (surface) and flow-through (cytosolic) proteins were separated using NeutrAvidin slurry. Biotinylated proteins were eluted from the beads by incubation for 60 min at 22^0C in Laemmli SDS-PAGE sample buffer. Samples were then subjected to gel electrophoresis and western blotting.

Western Blot Analysis

$GABA_A$ receptor α1 subunits, PKA subunits and PKC isoforms were analyzed by western blotting as described elsewhere (Kumar et al., 2010). 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 $GABA_A$ receptor α1 subunit (Millipore, Billerica, MA), PKA RIIα, RIIβ, or PKCγ (BD Biosciences, Franklin Lakes, NJ) antibodies. Blots were then exposed to an antibody for β-actin for normalization. Proteins were detected with enhanced chemilumnesence (GE Healthcare, Amersham, UK). Membranes were exposed to film under non-saturating conditions. Densitometric analysis was conducted using NIH Image 1.57.

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Comparisons were made within blots and expressed as % averaged control values. Statistics were conducted using ANOVA or Student's t-test.

Electrophysiology

Whole-cell voltage clamp recordings were used to assess evoked currents and 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 $MgCl_2$, 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 $CaCl_2$, 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 (CNQX; 10 μM, Sigma), D-2-amino-5-phosphonopentanoic 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). Drugs were diluted in the extracellular solution and applied using a U-tube apparatus. Zolpidem (100 nM) was co-applied with GABA (1 or 10 μM) to observe potentiation. Potentiation by zolpidem is expressed as the current in response to (GABA + zolpidem/GABA alone · 100). Dose-response curves were generated from GABA-evoked responses at 1 μM, 3 μM, 10 μM, 30 μM, 100 μM, and 1 mM GABA. Current amplitudes were normalized to the averaged control current response to 1 mM GABA and fit using GraphPad Prism software (San Diego, CA) with the sigmoidal dose-response equation:

$$Y = min + (max – min)/(1+10^(LogEC50 – X))$$

Miniature inhibitory post-synaptic currents (mIPSCs) were analyzed using the mini-Analysis 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 and amplitude were 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 in order to negate artifacts due to event summation. Only recordings with a minimum of 25 events fitting these criteria were analyzed. Decay time constants were

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obtained by using a double exponential fit for the average of the mIPSCs in a single recording. Numerical data are presented as mean ± SEM. Statistics were conducted using ANOVA or Student's t test.

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Results

PKA activation by ethanol modulates $GABA_A$ receptor trafficking and activity

Cultured cerebral cortical neurons were exposed to ethanol (50 mM) for 1 hr to test for its effects on membrane expression of $GABA_A$ α1 subunit and protein kinase levels (Fig. 1). Whereas 4 hr ethanol exposure produces a decrease in $GABA_A$ α1 levels (Kumar et al., 2010), no effects on this subunit are observed at the 1 hr time point (Fig. 1A). However, 1 hr ethanol exposure produced a significant increase in the abundance of both PKCγ (Fig. 1B, 78.1 ± 21.0, n=7, $p<0.05$, Student's t test) and the PKA regulatory subunits RIIα (Fig. 1C, 35.5 ± 12.7, n=6, $p<0.05$, Student's t test) and RIIβ (Fig. 1D, 36.4 ± 11.1, n=6, $p<0.05$, Student's t test) in the P2 fraction. Table 1 provides a comparison of the $GABA_A$ α1 and protein kinase trafficking changes induced by 1 hr and 4 hr ethanol exposure in cerebral cortical neurons. While $GABA_A$ α1 membrane levels are unchanged at 1 hr and decreased following 4 hr ethanol, PKCγ levels are elevated at both time points. Conversely, while PKA RIIα and RIIβ are elevated at the 1 hr time point, PKA levels return to baseline after 4 hr ethanol (Table 1).

To determine the effects of ethanol that are mediated by PKA, $GABA_A$ receptor subunit levels were assessed after either direct activation of PKA or ethanol exposure in the presence of a PKA inhibitor. Additionally, whole cell patch clamp recordings were used to measure functional changes in $GABA_A$ receptor electrophysiological responses. GABA (1-1000 μM) responses were first determined and then zolpidem (100 nM) enhancement of GABA (10 μM) responses was used as a pharmacological indicator for altered synaptic $GABA_A$ α1 containing receptor activity (Liang et al., 2004; Kumar et al., 2010). Currents evoked by GABA alone were compared to currents evoked by co-application of GABA + zolpidem to measure percent potentiation.

Exposure to the PKA activator Sp-cAMP (50 μM) for 1 hr produced a significant increase in $GABA_A$ α1 subunits relative to control values in the P2 fraction (Fig. 2A, 58.4 ± 13.7, n=7, $p<0.05$, Student's t test), with a corresponding increase in surface biotinylated protein (Fig. 2B, 50.48 ± 18.45, n=5, $p<0.05$, Student's t test) and decrease in the cytosolic fraction (Fig. 2C, 54.03 ± 10.74, n=5, $p<0.05$, Student's t test). Sp-cAMP exposure had no effect on the EC50 or amplitude of GABA-evoked responses (Fig. 2D) or whole cell GABA-

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evoked current amplitude at the dose used to test zolpidem enhancement of GABA responses (Fig. 2E). Sp-cAMP exposure did, however, increase zolpidem potentiation of GABA responses by 78.1 ± 9.4% (Fig. 2F-G; n=6 per group, Student's t test, $p<0.05$) compared to control cells. Currents evoked over the course of 1 hr during Sp-cAMP exposure revealed that direct PKA activation resulted in a rapid increase in potentiation by zolpidem that was sustained over the course of the hour (n=4, repeated measures ANOVA, $f=22.03$, $p<0.05$, significantly increased at t=12-60 min, Bonferroni post test, $p<0.05$), while current amplitude was stable for currents evoked under control conditions (Fig. 3).

While ethanol alone did not significantly alter $GABA_A$ α1 levels after 1 hr, co-exposure of ethanol and the PKA inhibitor Rp-cAMP decreased $GABA_A$ α1 membrane levels 33% (Fig. 4A, n=4, one-way ANOVA, $f=12.42$, $p<0.05$, Newman-Keuls post test, $p<0.05$). Ethanol, ethanol+Rp-cAMP, or Rp-cAMP exposure had no effect on the GABA dose-response (Fig. 4B) or GABA-evoked current amplitude (Fig. 4C). Reduced membrane levels of α1 subunits corresponded to reduced zolpidem potentiation of GABA responses in ethanol + Rp-cAMP exposed cells (Fig. 4D-E, n=6-11, one-way ANOVA, $f=4.031$, $p<0.05$, Newman-Keuls post test, $p<0.05$). Rp-cAMP exposure alone had no effect on $GABA_A$ α1 subunit levels or zolpidem potentiation.

Inhibition of PKC in the presence of ethanol mimics direct PKA activation

As ethanol is known to activate PKC and inhibition of PKC prevents the GABAergic changes seen after 4 hr ethanol exposure (Kumar et al., 2010; Werner et al., 2011), P2 fractions levels of $GABA_A$ subunits were measured following 1 hr ethanol exposure in the presence of the PKC inhibitor calphostin-C (0.3 μM). Co-exposure of ethanol and calphostin C uncovered effects not normally observed at 1 hr. $GABA_A$ α1 membrane levels were increased 56% (Fig. 5A, n=6, one-way ANOVA, $f=11.91$, $p<0.05$, Newman-Keuls post-hoc test, $p<0.05$).

The regulation of $GABA_A$ receptor α1 subunit expression in the P2 fraction was corroborated by the effects of ethanol plus kinase inhibitors on GABA-evoked currents (compare Figs. 3 and 5B-C). Ethanol (50 mM) was applied via the extracellular solution at the start of the time course, while CalC or Rp-cAMP were in the patch pipette. Currents evoked by GABA and zolpidem over the course of 1 hr were compared to currents

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averaged for 10 min prior to drug exposure. Current amplitudes were stable prior to application of ethanol and kinase inhibitors (Fig. 5). Current amplitude was not significantly altered in the presence of ethanol alone (Fig. 5B-C). Co-application of ethanol + CalC, however, rapidly increased the current amplitude produced by GABA+ zolpidem, mimicking the results of direct PKA activation shown above (n=3, repeated measures ANOVA, f=3.863, significantly increased at t=14-60 min, Bonferroni post test, $p<0.05$). Conversely, co-application of ethanol + Rp-cAMP resulted in a rapid decrease in current amplitude that was also sustained for the duration of 1 hr (n=3, repeated measures ANOVA, f=11.10, significantly decreased at t=8-60 min, Bonferroni post test, $p<0.05$).

PKA activation by ethanol regulates zolpidem modulation of mIPSC decay τ_2

As ethanol exposure has been shown to alter mIPSC decay kinetics, which is related to changes in $GABA_A$ receptor subunit expression (Liang et al., 2006; Fleming et al., 2009; Werner et al., 2011), recordings of mIPSCs were made to further investigate the functional consequences of ethanol activation of PKA on synaptic $GABA_A$ receptor signaling. While there appeared to be a slight shift in the mIPSC decay kinetics suggestive of an altered synaptic $GABA_A$ receptor expression profile, there was not a significant direct effect of 1 hr ethanol and/or PKA modulation on GABA mIPSC kinetics (Table 2 and Figure 6A). To further investigate the potential for receptor subtype-specific effects on mIPSCs, recordings were also made in the presence of 100 nM zolpidem. Under control conditions zolpidem increased decay τ_2 as expected (comparison of control with and without zolpidem, $p<0.05$, Student's t test). Further, decay τ_2 in the presence of zolpidem (Table 2 and Fig. 6B) was decreased after ethanol + Rp-cAMP exposure (24.7 ± 3.2, n=6) and increased after Sp-cAMP exposure (68.8 ± 9.0, n=6) relative to controls (46.1 ± 3.7, n=8, one-way ANOVA, f=9.673, $p<0.05$, Newman-Keuls post test, $p<0.05$ for both comparisons). No other mIPSC characteristics were altered in the presence of zolpidem following modulation of PKA activity (Table 2).

Discussion

The present results demonstrate that ethanol activation of PKA plays an active role in $GABA_A$ receptor trafficking and function. PKA activation results in an increase in $GABA_A$ α1 subunit expression in P2 and biotinylated fractions of surface receptors in cultured cortical neurons. The finding that these alterations corresponded to functional changes in zolpidem enhancement of GABA responses corroborates this conclusion. Additionally, our results highly implicate oppositional roles of PKA and PKC in $GABA_A$ receptor regulation. Ethanol is known to activate both PKA and PKC pathways, and our past results coupled with the present studies indicate that such changes in activity are accompanied by increases in PKA and PKC abundance in the P2 fraction. We demonstrate that inhibiting the activity of either kinase pathway in the presence of ethanol results in $GABA_A$ α1 subunit alterations that are dependent on the sum of the activity of these protein kinase pathways. Specifically, inhibition of PKC in the presence of ethanol results in increased $GABA_A$ α1 surface expression, similar to that observed following exposure to PKA activators alone. Furthermore, a decrease in $GABA_A$ receptor α1 subunit levels similar to that found in models of ethanol dependence *in vivo* (Devaud et al., 1997; Cagetti et al., 2003; Liang et al., 2004) or 4hr ethanol exposure *in vitro* (Kumar et al., 2010; Werner et al., 2011) could be observed after 1 hr ethanol exposure in the presence of a PKA antagonist.

The results suggest that ethanol activates at least two opposing pathways in the cerebral cortex: PKA and PKC. While PKCγ activation results in decreased $GABA_A$ receptor α1 subunit (Kumar et al., 2010), PKA appears to counteract these changes. Such opposition is consistent with previous studies that have found an apparent antagonistic role of PKA and PKC working in other CNS systems (Vaello et al., 1994; Brandon et al., 2000; Grey and Chang, 2011). The data further suggest that these kinases are under time-dependent regulation by ethanol. While PKA and PKC are both active at 1 hr of ethanol exposure, no overall change in $GABA_A$ α1 subunit expression is observed. By four hours, however, PKA activity has returned to baseline while PKC remains active, and trafficking of $GABA_A$ α1 receptors is observed (Kumar et al., 2010; Werner et al., 2011). An increase in PKA translocation to the P2 synaptosomal region is consistent with ethanol-induced

activation of PKA observed in other cellular systems (Diamond and Gordon, 1997). While the exact role of increased PKA subunits in the synaptosomal region is unclear, the effects of direct activation by Sp-cAMP, or inactivation by Rp-cAMP in the presence of ethanol, would suggest that ethanol is increasing overall catalytic activity by PKA. Future studies might investigate the specific phosphorylation states regulating trafficking of α1 subunits by the oppositional PKA and PKC activation by ethanol.

The present results extend several recent studies characterizing altered $GABA_A$ regulation following ethanol exposure. These alterations include a shift in the normal synaptic and extrasynaptic $GABA_A$ receptor trafficking. Studies in the hippocampus have found a decrease in synaptic α1 and extrasynaptic α4δ receptors, and a corresponding increase in synaptic α4 $GABA_A$ receptors following both acute (Liang et al., 2007) and chronic (Cagetti et al., 2003; Liang et al., 2004) ethanol exposure thereby producing an overall decrease in GABAergic inhibition and increasing CNS hyperexcitability. The present results suggest that these processes are conserved in the cerebral cortex as well (Kumar et al., 2010; Werner et al., 2011). The lack of an effect of brief (1hr) ethanol exposure on $GABA_A$ α1 receptors in the present study is consistent with prior reports in cerebral cortex and in cultured cortical neurons (Kumar et al., 2003; Kumar et al., 2010); however, more rapid effects of ethanol have been observed in rat hippocampus (Liang et al., 2007), suggesting that the time course of ethanol regulation of $GABA_A$ receptors may differ across brain regions. We have previously shown that ethanol activation of PKC differs in rat cortex and hippocampus (Kumar et al., 2006). Further studies are needed to determine if regional differences in the time course of ethanol regulation of $GABA_A$ receptors are secondary to regional differences in ethanol activation of protein kinases.

Previous studies have shown that PKA and PKC activation have direct effects on GABA responses that were not observed in the present study (Kittler and Moss, 2003). This is likely due to the use of different model systems and the fact that we focused on the $GABA_A$ α1 receptors in cerebral cortex that are sensitive to 100nM zolpidem. To isolate effects on these receptors in cultured neurons, we used an IC10 concentration of GABA. It is quite possible that effects of PKA or PKC inhibition may have been observed at higher concentrations of GABA, however, such an experiment would involve actions at additional receptor subtypes.

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Although effects of PKA on $GABA_A$ receptor function have been previously observed (Brandon et al., 2003; Lilly et al., 2003), this is the first evidence that PKA specifically regulates trafficking and function of zolpidem-sensitive $GABA_A$ α1 receptors. The observation that inhibition of PKA or PKC alone had no effect on α1 receptor abundance or function, however, suggests that these kinases do not constitutively regulate $GABA_A$ α1 receptors. Rather, these pathways may only become active regulators of GABAergic inhibition following physiological insults such as ethanol exposure or processes associated with epileptogenesis (Gonzalez and Brooks-Kayal, 2011).

Although our present results demonstrate modulation of $GABA_A$ receptor α1 subunit abundance by PKA *in vitro*, our previous results suggest the same processes occur *in vivo* (Kumar et al., 2012). Recently, we found $GABA_A$ α1 levels in rat cerebral cortex were increased following an acute ethanol injection, concurrent with an increase in PKA, whereas PKC was unchanged. Further, behavioral studies have well established that PKA is a key mediator of ethanol sensitivity. Infusion of Sp-cAMP into the lateral ventricles prior to systemic ethanol administration increased LORR (loss of righting reflex) duration (Kumar et al., 2012). Conversely, inhibition of PKA prior to ethanol administration decreased LORR duration (Lai et al., 2007) and withdrawal-induced anxiety (Pandey et al., 2003). Studies utilizing mutant mouse lines with reduced PKA activity mimic results found with pharmacological inhibition of PKA (Thiele et al., 2000; Naassila et al., 2002; Kim et al., 2011). The precise role of PKA in mediating these behaviors is complex, however, as some studies have found increased ethanol sensitivity in animals with diminished PKA activity (Wand et al., 2001; Yang et al., 2003; Maas et al., 2005). While the precise role remains equivocal, collectively these studies clearly establish the importance of PKA activation in mediating the behavioral effects of ethanol, potentially through $GABA_A$ receptor regulation. The present results underscore the clinical relevance of understanding the role of PKA in modulating the effects of ethanol for the development of drugs for the treatment of alcoholism.

The relatively rapid time course over which changes in zolpidem potentiation were observed suggests a post-translational mechanism of $GABA_A$ receptor regulation (Brandon et al., 2002). Previous studies in cultured cerebral cortical neurons have found changes in $GABA_A$ receptor activity via phosphorylation and

dephosphorylation to be due entirely to changes in channel gating and conductance rather than channel number (Brandon et al., 2000). The fact that the functional changes were observed prior to alterations in surface receptor expression suggest that multiple mechanisms of GABA regulation may be involved, including both direct effects on channel conductance as well as receptor trafficking. Further studies investigating single channel conductance states and utilizing phosphorylation state dependent antibodies will help to elucidate these mechanisms. Additionally, the observation that whole cell GABA-evoked current amplitude was unchanged would suggest bidirectional regulation of GABA receptor subtypes by PKA, as well as ethanol. Thus, while PKA activation leads to increased surface expression of $GABA_A$ α1 receptors, there is likely a concurrent decrease in other subtypes. Previous studies have observed bidirectional regulation of synaptic $GABA_A$ α1 and α4 containing receptors by PKCγ (Kumar et al., 2010; Werner et al., 2011), and studies are currently underway to investigate bidirectional regulation of $GABA_A$ α4 receptors by PKA.

Overall, the present study elucidates a novel role for PKA in the subcellular pathways mediating the effects of ethanol. While PKC and PKA play important roles in altered $GABA_A$ receptor trafficking following ethanol exposure, it is possible that other kinases or phosphatases are also active. In particular, ethanol-induced adaptations in other brain regions may be governed by different subcellular mechanisms, which could, in part, account for some of the brain region-specific effects of ethanol. This area requires further study to fully elucidate the physiology of ethanol intoxication and withdrawal. Nonetheless, the current results suggest that PKA activation may delay some ethanol-induced adaptations in GABAergic signaling. Additionally, PKA may provide a promising target for the development of drugs aimed at restoring normal $GABA_A$ receptor functioning for the treatment of multiple pathologies involving similar $GABA_A$ receptor adaptations including alcohol dependence, epilepsies and benzodiazepine abuse.

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Authorship Contributions

Participated in research design: Carlson, Kumar, Morrow

Conducted experiments: Carlson, Kumar, Comerford

Contributed new reagents or analytic tools: N/A

Performed data analysis: Carlson, Kumar, Comerford

Wrote or contributed to the writing of the manuscript: Carlson, Werner, Comerford, Morrow

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Footnotes

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Figure Legends

Figure 1. One hour ethanol (50 mM) alters expression of protein kinase subunits in the P2 fraction of cultured cortical neurons. Cortical neurons were exposed to ethanol (50 mM for 60 min) followed by preparation of P2 fractions. Western blot analysis of P2 fraction of cortical neurons revealed P2 fraction levels of (A) $GABA_A$ α1 were unchanged, while (B) PKCγ subunit levels were increased by 78.1 ± 25.2%, (C) PKA RIIα were increased by 35.5 ± 12.7%, and (D) PKA RIIβ subunit levels were increased by 36.4 ± 11.1% following ethanol exposure. Graphs show the mean ± SEM of percent control optical density values normalized to β-actin levels (n=4-7 per group). ***** $p < 0.05$, compared to vehicle (Student's *t* test).

Figure 2. PKA activator Sp-cAMP increases $GABA_A$ α1 subunit expression and zolpidem potentiation in cultured cortical neurons. Cortical neurons were exposed to vehicle or SP-cAMP for 60 min followed by preparation of cellular fractions for Western blot analysis or patch clamp recording. Activation of PKA increased $GABA_A$ receptor α1 subunit expression in the P2 fraction (A; 58.4 ± 13.7%) and in the biotinylated surface protein fraction (B; 50.5 ± 18.5) compared to vehicle controls. There was a corresponding decrease in α1 subunit levels in the cytosolic fraction following Sp-cAMP (C; 54.0 ± 10.7%). (D) There was no effect of Sp-cAMP exposure on GABA dose-response (control EC50=15.2 μM; Sp-cAMP EC50=17.5 μM) or (E) whole cell current amplitude in response to 10 μM GABA. Sp-cAMP exposure significantly increased zolpidem potentiation relative to controls (F; 78.1 ± 9.4%). (G) Representative GABA-evoked (10 μM) current trace for Control and Sp-cAMP exposure with and without 100 nM zolpidem are shown. Data are presented as mean ± SEM. Representative blots are shown. * $p < 0.05$, Student's t-test. n = 6-18 per group.

Figure 3. PKA activation produces rapid increases in zolpidem potentiation of GABA responses. Whole cell currents were evoked by application of GABA (1 μM) and zolpidem (100 nM) at two minute intervals over the course of one hour with vehicle or Sp-cAMP (50 μM) exposure. (A) Currents evoked in Sp-cAMP-exposed cells were increased from 12-60 min. (repeated measures ANOVA, $f=3.648$, $p<0.05$, Bonferroni post test),

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while control cells were unaffected. Data are presented as mean ± SEM. n = 3-4. (B) Representative whole cell current traces elicited by GABA and zolpidem before (t=0) and after (t=60 min) exposure.

Figure 4. PKA inhibitor RP-cAMP decreases $GABA_A$ α1 expression and zolpidem potentiation in the presence of ethanol. Neurons were exposed to vehicle, ethanol (50 mM), ethanol with Rp-cAMP (50 μM), or Rp-cAMP alone for 60 min followed by preparation of P2 fractions for Western blot analysis or patch clamp recording. Ethanol or Rp-cAMP exposure alone did not alter $GABA_A$ receptor α1 subunit expression; however, ethanol exposure in the presence of the PKA inhibitor Rp-cAMP decreased $GABA_A$ receptor α1 subunit expression by 33% (A; one-way ANOVA, f=12.42, p<0.05, Newman-Keuls post test). There was no effect of drug exposure on (B) GABA dose-response (not all dose-response curves shown for visual simplicity; EtOH+Rp-cAMP EC50=16.4 μM) or (C) whole cell current amplitude in response to 10 μM GABA. (D) EtOH+Rp-cAMP decreased zolpidem potentiation (one-way ANOVA, f=4.031, p<0.05, Newman-Keuls post test) of GABA-evoked currents compared to vehicle control, while EtOH or Rp-cAMP had no effect. (E) Representative GABA-evoked (10 μM) current trace for Control and EtOH+Rp-cAMP exposure with and without 100 nM zolpidem. Data are presented as mean ± SEM. Optical density values normalized to β-actin. Representative blots are shown. * p < 0.05, one-way ANOVA, Newman-Keuls post-hoc test. n = 3-21 per group.

Figure 5. PKA and PKC activation by ethanol produce opposing effects on $GABA_A$ α1 expression and zolpidem potentiation of GABA responses. (A) Neurons were exposed to vehicle, ethanol (50 mM) or ethanol with calphostin C (CalC, 0.3 μM) for 60 min followed by preparation of P2 fractions and Western blot analysis. Ethanol or CalC exposure alone did not alter $GABA_A$ receptor α1 subunit expression; however, ethanol exposure in the presence of the PKC inhibitor CalC increased $GABA_A$ receptor α1 subunit expression by 56% (one-way ANOVA, f=11.91, p<0.05, Newman-Keuls post-hoc test) (B-C) Whole cell currents were evoked by application of GABA (1 μM) and zolpidem (100 nM) at two minute intervals over the course of one

hour during ethanol exposure (50 mM), ethanol and Rp-cAMP (50 μM), or ethanol and Calphostin-C (CalC; 3 μM). Currents were stable for the 10 min period prior to bath application of ethanol and during diffusion of CalC or Rp-cAMP via the recording pipette. Currents evoked by EtOH+CalC were significantly increased from 14 - 60 min (repeated measures ANOVA, $f=3.863$, $p<0.05$, Bonferroni post test). Currents evoked by EtOH+Rp-cAMP were significantly decreased at 8 - 60 min (repeated measures ANOVA, $f=11.10$, $p<0.01$, Bonferroni post test). (C) Representative whole cell current traces elicited by GABA and zolpidem before (t=0) and after (t=60 min) ethanol and drug exposure. Data are presented as mean ± SEM. n = 3-6 per group.

Figure 6. Ethanol and PKA modulation of $GABA_A$ mIPSC decay kinetics in the presence of zolpidem. Recordings were made in the presence of TTX, CNQX, and AP-5 to pharmacologically isolate GABA mIPSCs. Representative averaged mIPSC traces, following exposure to vehicle (control) or drug and standardized for peak amplitude, are shown. (A) Representative control (no zolpidem) traces following control, ethanol, Sp-cAMP, or ethanol+Rp-cAMP exposure. (B) Representative traces in the presence of zolpidem (100 nM) following control, Sp-cAMP, or ethanol+Rp-cAMP exposure. Summarized data are shown in Table 2.

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Table 1. Comparison of effects of ethanol at 1 and 4 hr on P2 fraction protein levels.

Protein	1 Hr EtOH	4 Hr EtOH
$GABA_A$ α1	- 109.4 ± 9.6	↓ 59.8 ± 15.0 *
PKCγ	↑ 178.1 ± 21.0 *	↑ 152.3 ± 19.0 *
PKA RIIα	↑ 135.5 ± 12.7 *	- 97.1 ± 5.3
PKA RIIβ	↑ 136.4 ± 11.1 *	- 100.7 ± 4.2

*$p < 0.05$, compared to Controls, Student's t test. Data for 4 hr time point levels of $GABA_A$ α1 and PKCγ are from Kumar et al. (2010).

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Table 2. $GABA_A$ mIPSC kinetics following exposure to PKA modulators and ethanol

Measure	Control	EtOH	EtOH + Rp-cAMP	Sp-cAMP
Rise Time (ms)	3.6 ± 1.5	1.2 ± 0.8	4.0 ± 1.5	1.3 ± 0.9
Decay 90-37 (ms)	14.4 ± 2.6	21.7 ± 1.6	14.9 ± 2.2	16.7 ± 2.2
Half-width (ms)	12.6 ± 2.5	20.1 ± 0.7	14.5 ± 3.2	16.4 ± 2.0
Amplitude (pA)	25.3 ± 0.8	23.4 ± 1	23.2 ± 1.1	21.8 ± 1.8
Frequenzy (Hz)	2.6 ± 0.9	1.6 ± 0.2	1.6 ± 0.5	1.5 ± 0.3
Decay τ_1	13.2 ± 2.4	18.3 ± 2.0	15.0 ± 3.2	16.8 ± 1.7
Decay τ_2	31.0 ± 6.4	36.6 ± 5.8	26.3 ± 2.5	45.6 ± 10
Decay τ_2 with zolpidem	$^{\#}$ 46.1 ± 3.7	46.2 ± 6.0	*** 24.7 ± 3.2**	*** 68.8 ± 9.0**
n	8	6	6	6

*$p < 0.05$, compared to Controls with zolpidem, one-way ANOVA, Newman-Keuls post test.

$^{\#}p < 0.05$, compared to Control Decay τ_2 (without zolpidem), Student's t test.

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Figure 1

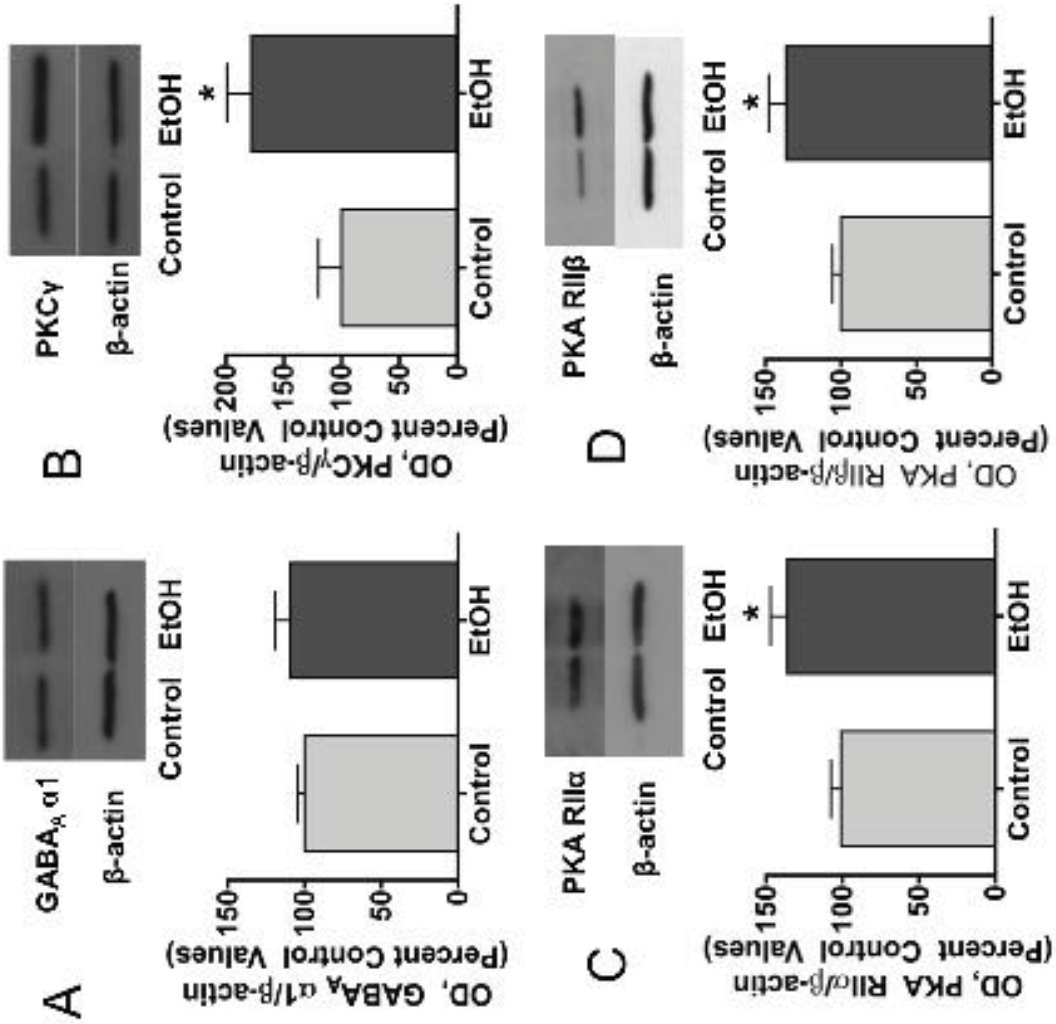

Figure 2

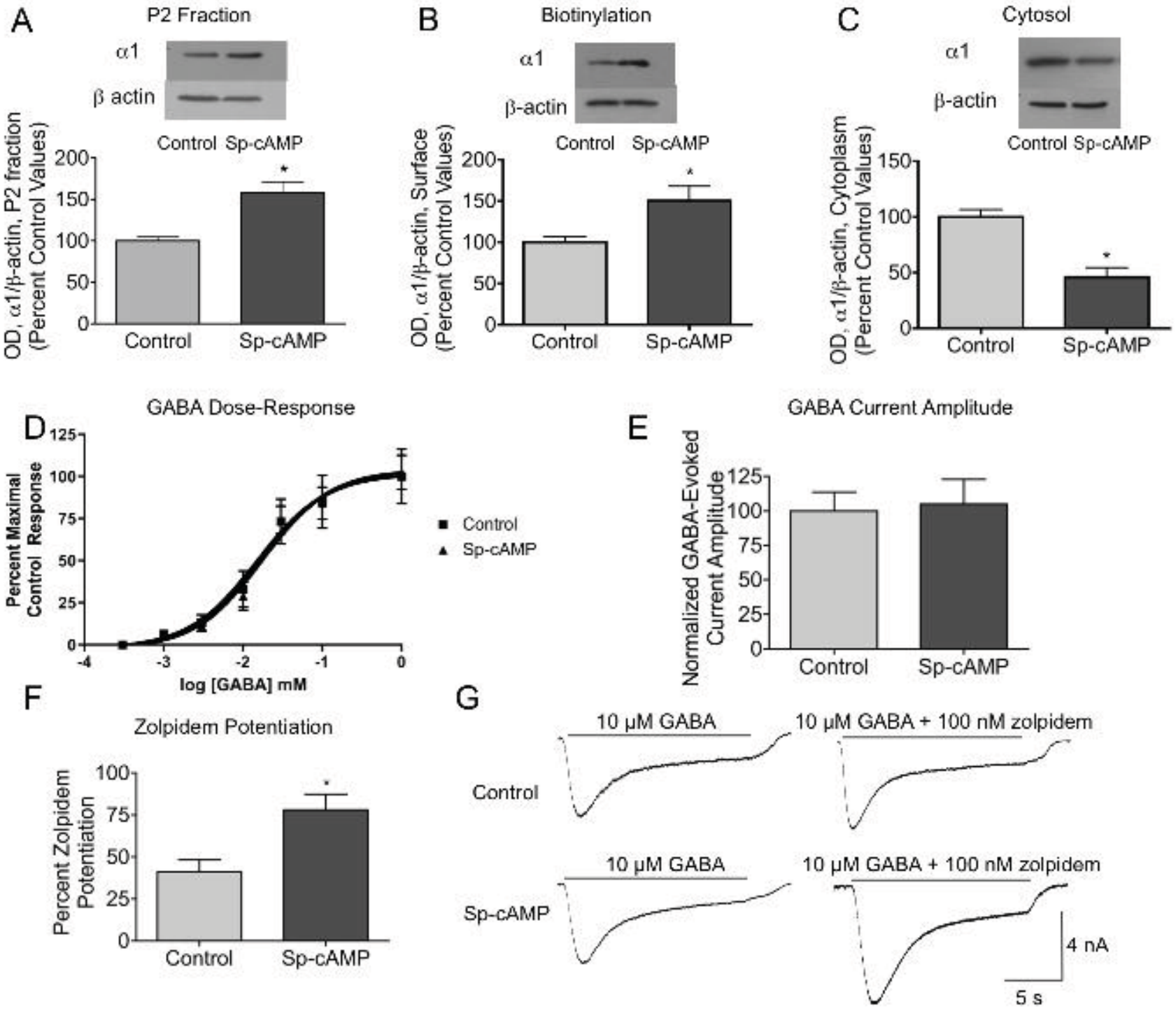

Figure 3

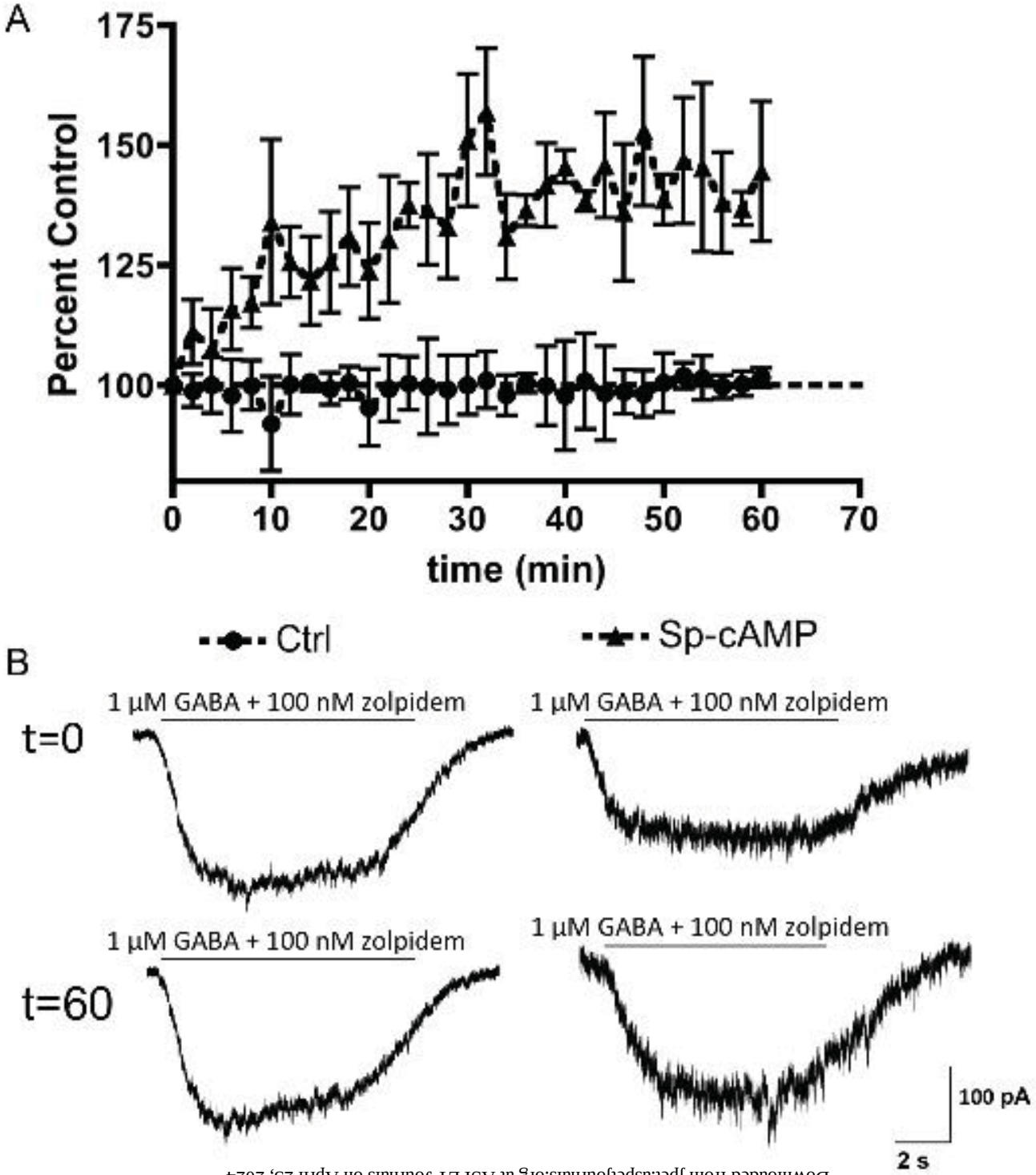

Figure 4

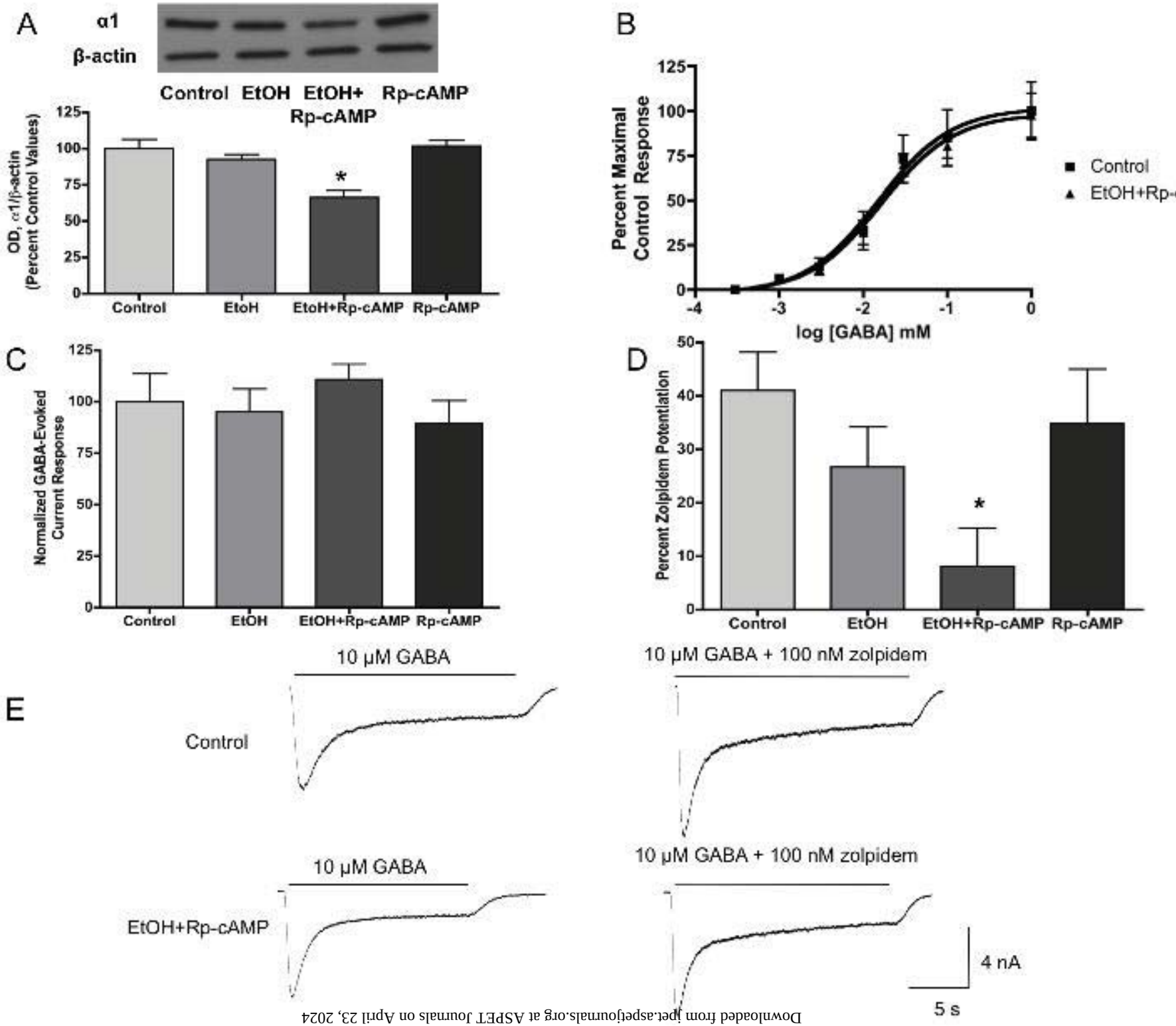

Figure 5

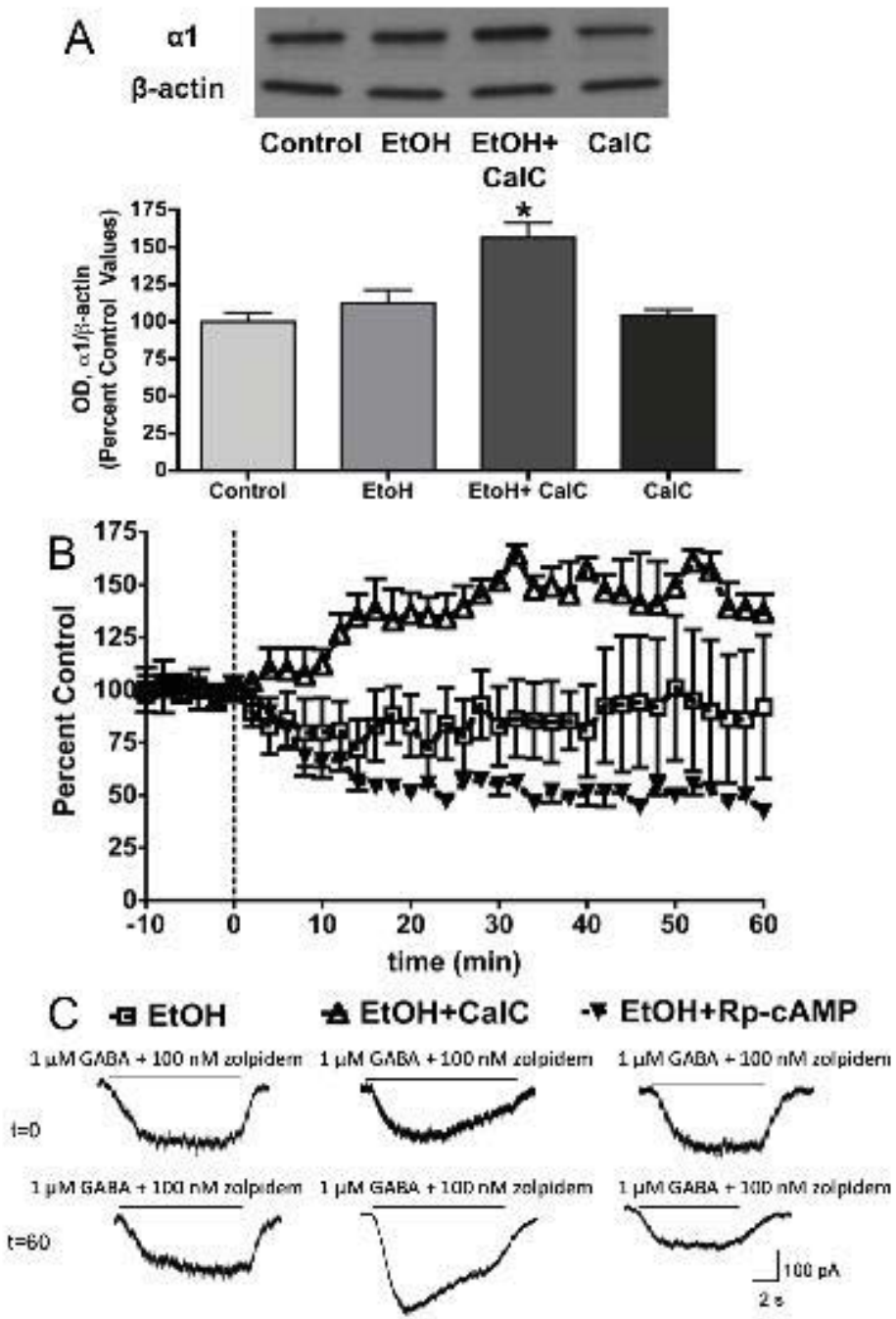

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Figure 6