

Ethanol reduces GABA<sub>A</sub>  $\alpha$ 1 subunit receptor surface expression by a PKC $\gamma$  dependent mechanism in cultured cerebral cortical neurons

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***Abbreviations used in the text:*** GABA $_A$ ,  $\gamma$ -aminobutyric acid $_A$ ; PKC, protein kinase C; PBS, phosphate buffered saline; SDS, sodium dodecyl sulfate; RACK, receptor for activated kinase C; PAGE, polyacrylamide gel electrophoresis.

## Abstract

Prolonged ethanol exposure causes central nervous system hyperexcitability that involves a loss of GABAergic inhibition. We previously demonstrated that chronic ethanol exposure enhances the internalization of synaptic GABA<sub>A</sub> receptors, comprised of  $\alpha 1\beta 2/3\gamma 2$  subunits. However, the mechanisms of ethanol-mediated internalization are unknown. This study explored the effect of ethanol on surface expression of GABA<sub>A</sub>  $\alpha 1$  subunit-containing receptors in cultured cerebral cortical neurons and the role of protein kinase C (PKC)  $\beta$ ,  $\gamma$  and  $\epsilon$  isoforms in their trafficking. Cultured neurons were prepared from rat pups on postnatal day 1 (PD1) and maintained for 18 days. Cells were exposed to ethanol and surface receptors were isolated by biotinylation and P2 fractionation while functional analysis was conducted by whole cell patch clamp recording of GABA- and zolpidem- evoked responses. Ethanol exposure for 4 hours decreased biotinylated surface expression of GABA<sub>A</sub> receptor  $\alpha 1$  subunits and reduced zolpidem (100 nM) enhancement of GABA evoked currents. The PKC activator, PDBu, mimicked the effect of ethanol, and the selective PKC inhibitor calphostin C prevented ethanol-induced internalization of these receptors. Ethanol exposure for 4 hours also increased the co-localization and co-immunoprecipitation of PKC $\gamma$  with  $\alpha 1$  subunits while PKC $\beta/\alpha 1$  association and PKC $\epsilon/\alpha 1$  co-localization were not altered by ethanol exposure. Selective PKC $\gamma$  inhibition by transfection of selective PKC $\gamma$  siRNAs blocked ethanol-induced internalization of GABA<sub>A</sub> receptor  $\alpha 1$  subunits whereas PKC $\beta$  inhibition using pseudo-PKC $\beta$  did not have any effect. These findings suggest that ethanol exposure selectively alters PKC $\gamma$  translocation to GABA<sub>A</sub> receptors and PKC $\gamma$  regulates GABA<sub>A</sub>  $\alpha 1$  receptor trafficking following ethanol exposure.

## Introduction

Chronic ethanol exposure causes CNS hyperexcitability and tremor that has been partly attributed to a loss of GABA<sub>A</sub>  $\alpha$ 1 receptor subunit expression *in vivo* (see Kumar, 2009 for review) (Kralic et al., 2005; Kralic et al., 2002; Kumar et al., 2009). This effect of ethanol is recapitulated in cultured cortical neurons *in vitro* (Sanna et al., 2003; Sheela Rani and Ticku, 2006). The reduction of GABA<sub>A</sub>  $\alpha$ 1 subunit protein has been determined to involve the internalization of GABA<sub>A</sub>  $\alpha$ 1 subunit receptors in the cerebral cortex (Kumar et al., 2003) and the hippocampus (Liang et al., 2007) where a reduction in surface expression of these receptors has been demonstrated. Furthermore, ethanol exposure produces a reduction in sensitivity to the  $\alpha$ 1 GABA<sub>A</sub> receptor-selective modulator, zolpidem in hippocampal neurons both *in vivo* (Liang et al., 2004) and *in vitro* (Sanna et al., 2003), consistent with the interpretation that ethanol promotes  $\alpha$ 1 subunit – containing GABA<sub>A</sub> receptor internalization.

GABA<sub>A</sub> receptors are heteropentameric ligand-gated ion channels that mediate inhibition in the central nervous system. The  $\alpha$ 1 subunit receptors are the most abundant subtype of synaptic GABA<sub>A</sub> receptor, expressed widely throughout the brain and are generally composed of two  $\alpha$ 1, two  $\beta$ (2 or 3) and one  $\gamma$ 2 subunit that confers sensitivity to benzodiazepines (Sieghart and Sperk, 2002). Global knockout of the GABA<sub>A</sub> receptor  $\alpha$ 1 subunit results in increased seizure susceptibility and essential-like tremor (Kralic et al., 2005). Previous studies have demonstrated that trafficking of  $\alpha$ 1 subunit-containing GABA<sub>A</sub> receptors is critical for synaptic efficacy and can be regulated by many factors,

including PKC mediated phosphorylation (Brandon et al., 2000; Chapell et al., 1998; Connolly et al., 1999; Poisbeau et al., 1999).

The mechanisms by which ethanol promotes the internalization of  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors are not yet elucidated. Ethanol induces GABA<sub>A</sub>  $\alpha 1$  receptor association with adaptor-complex 2 (AP2) and internalization into clathrin-coated vesicles (CCVs) in cerebral cortex (Kumar et al., 2003). Ethanol has also been shown to alter the expression and translocation of various PKC isoforms (Kumar et al., 2006), but the role of these isoforms in receptor internalization is unknown. Studies with PKC $\epsilon$  and PKC $\gamma$  knock-out mice have demonstrated the potential relevance of these PKC isoforms to ethanol mediated plasticity (Bowers et al., 2001; Choi et al., 2008; Hodge et al., 1999; Proctor et al., 2003). Mutant mice lacking PKC $\epsilon$  are more sensitive to the acute effects of ethanol and the deletion of PKC $\epsilon$  attenuates ethanol withdrawal-associated seizures in mice (Olive et al., 2001). In contrast, PKC $\gamma$  knock-out mice are resistant to the acute intoxicating effects of ethanol and fail to develop ethanol tolerance. Therefore, it appears that these isoforms of PKC are important for ethanol-mediated behavioral effects and may be involved in ethanol-induced plasticity.

Twelve isoforms of PKC exhibit cell and region specific distribution in the brain. Recent studies show that PKC $\beta$ ,  $\gamma$  and  $\epsilon$  are associated with GABA<sub>A</sub> receptors (Brandon et al., 1999; Kumar et al., 2002). PKC has been shown to alter surface expression of both recombinant GABA<sub>A</sub> receptors and native receptors in cultured neurons (Chapell et al., 1998) by endocytosis into CCVs (Kittler et al., 2000). However, other evidence suggests that PKC prevents receptor recycling back to the surface (Connolly et al., 1999). To understand the mechanisms of ethanol effects on surface expression of GABA<sub>A</sub> receptors

it is critical to determine the role of various PKC isoforms since this knowledge may be useful to modulate trafficking of  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors.

The present study focused on the role of PKC isoforms on ethanol-induced altered GABA<sub>A</sub>  $\alpha 1$  receptor expression in cultured cortical neurons. Trafficking of GABA<sub>A</sub> receptors was assessed by determination of  $\alpha 1$  GABA<sub>A</sub> receptor peptide levels on the cell surface by biotinylation of surface proteins. To establish that internalization of  $\alpha 1$  subunit peptides represented internalization of  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors, we also explored the effects of ethanol on zolpidem (100 nM) enhancement of GABA evoked Cl<sup>-</sup> currents to assess the functional consequences of changes in surface expression of  $\alpha 1$  subunit receptors. We explored the effects of ethanol on PKC $\beta$ ,  $\gamma$  and  $\epsilon$  expression and their interactions with GABA<sub>A</sub>  $\alpha 1$  subunits, by co-immunoprecipitation and dual fluorescence confocal microscopy. Furthermore, using PKC $\gamma$  specific siRNA and a PKC $\beta$  inhibitor, we determined the role of these PKC isoforms on ethanol-induced loss of  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors.

## Materials and Methods

**Cultured cerebral cortical neurons:** Experiments were conducted in accordance with National Institutes of Health Guidelines under Institutional Animal Care and Use Committee-approved protocols. Rat pups were decapitated and the brains removed on their first postnatal day. The cerebral cortex was isolated from the brain stem, hippocampus, and olfactory bulb and the meninges were removed from the cortical tissue. The tissue was then placed in a solution of papain, L-cysteine and DNase in CO<sub>2</sub> independent medium and minced into small pieces of 1–2 mm<sup>2</sup>. The tissue was incubated at 37<sup>0</sup>C for 30 minutes and then dissociated by trituration into a growth medium consisting of DMEM supplemented with 10% horse serum and Penicillin-Streptomycin (Pen-Strep; 10,000U/ml, Invitrogen, cat # 15140, final conc. in flasks 50 Units). Cells were plated on poly-D-lysine coated flasks (Corning Incorporated, Corning, NY) and glass coverslips (Carolina Biological Supply Company, Burlington, NC) in 12-well plates at a density of 1.0–1.5x10<sup>6</sup> live cells per well. Healthy neurons grew processes within 24 hours after plating. Glial cells were retained in the culture in order to encourage neuron survival and synapse formation. After 3 days in culture, cells were fed with serum free medium (DMEM supplemented with B27) containing Pen-Strep (50 Units) in order to prevent glial cell overgrowth. Pen-Strep was removed from the cultures on day 14. Rat cortical neurons were maintained *in vitro* for at least 18 days prior to experimentation to allow expression of mature subtypes of PKC isozymes and GABA<sub>A</sub> receptors.

**Ethanol and drug exposure of cultured cerebral cortical neurons:** Cultured cells were exposed to ethanol using a vapor chamber for the maintenance of high ethanol

concentrations in the tissue culture medium. At the beginning of the ethanol exposure period, cells were fed by replacing one third of the tissue culture medium with fresh medium containing ethanol (final concentration 50 mM). Stable ethanol levels were maintained up to five days in the medium by placing the cells in a plastic vapor chamber containing a beaker with 200 ml of 50 mM ethanol in distilled water. Control cells were fed with media that did not contain ethanol and were placed in a vapor chamber with a beaker containing water. After ethanol exposure, cells were removed from the vapor chamber and washed twice with cold PBS and the P2 fraction was prepared as described below for western blot analysis of PKC isoforms and GABA<sub>A</sub> receptor subunits using specific antibodies. For immunohistochemistry, cells on coverslips were fixed with 4% paraformaldehyde, washed twice with PBS and stored at 4<sup>0</sup>C. Calphostin C and PDBu were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in 0.1% DMSO.

For inhibition of specific PKC isoforms, we used PKC $\gamma$  siRNA and PKC $\beta$  pseudo-substrate to selectively reduce PKC isozyme expression in cultured cerebral cortical neurons. Three different siRNA sequence pairs were obtained from Invitrogen and used simultaneously to inhibit PKC $\gamma$  (PRKCCRSS332451, PRKCCRSS332452, and PRKCCRSS332453). The sequences are as follows:

Pair 1 : 5'-GGAGGAGGGCGAGUAUUACAAUGUA-3' and

5'-UACAUUGUAAUACUCGCCCUCCUCC-3';

Pair 2: 5'-UCGGCAUGUGUAAAGAGAAUGUCUU-3' and

5'- AAGACAUUCUCUUUACACAUGCCGA-3';

Pair 3: 5'- CCUGCAAUGUCAAGUCUGCAGCUUU-3' and

5'- AAAGCUGCAGACUUGACAUUGCAGG- 3'.



ClustalW alignment indicates the sequences were to nucleotides 1058 – 1082, 1714 – 1738, and 407 – 431, respectively of the rat protein kinase C gamma mRNA sequence (NM\_012628.1). The specificity of all siRNA sequences were BLAST analyzed to make sure no other unwanted targets would be potentially down regulated. For transfection, cerebral cortical neurons were cultured for 14 days as described above and siRNA for PKC $\gamma$  or scrambled siRNA was transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Selective PKC $\gamma$  siRNA or scrambled siRNA were mixed in 200  $\mu$ l of Opti MEM I low serum media (Gibco) with 6  $\mu$ l of lipofectamine reagent. Complexes were allowed to form for 20 minutes then added to cells (concentration 6 pmol). Cells were subjected to gentle rocking by hand and incubated at 37° C for 24-72 hours prior to collection of cells. Experiments were initiated 67-68 hours after transfection since inhibition of PKC $\gamma$  was maximal at 72 hours. PKC $\beta$  pseudo-substrate was obtained from Tocris (Ellisville, Missouri, USA) and used to block PKC $\beta$  activity (0.1  $\mu$ M, final concentration). PKC $\beta$  pseudo-substrate was applied with ethanol at the beginning of experiment and after 2 hours to maintain inhibition for 4 hours of ethanol exposure.

### **Whole cell voltage-clamp recordings**

Standard whole-cell voltage-clamp recordings were made with glass electrodes, fire-polished to a resistance of 3–5 M $\Omega$  and filled with internal solution (mM): KCl 150, MgCl<sub>2</sub> 3.1, HEPES 15, K-ATP 5, EGTA 5 and phosphocreatine 15 (adjusted to pH 7.4 with KOH). The recording chamber was perfused with external solution (mM): NaCl 145, KCl 5, HEPES 10, CaCl<sub>2</sub> 2, MgCl<sub>2</sub> 1, sucrose 5 and glucose 10 (adjusted to pH 7.4 with NaOH). The sodium-channel blocker, tetrodotoxin (0.5  $\mu$ M; Sigma, St. Louis MO),

was included in the perfusion solution. Drugs were diluted in external solution and applied using a U-tube apparatus. This technique allowed a brief cellular application and rapid removal of drugs. The interval between applications was at least 1 min. Recordings were performed at room temperature (22–23°C). The membrane potential was held at –60 mV using a patch-clamp amplifier (Axopatch 1D, Axon Instruments, Union City, CA), and data were collected with Clampex 10.2 software (Axon Instruments).

Concentration-response curves were determined to select the proper concentration of GABA to detect enhancement of currents by 100 nM zolpidem (Sigma, St. Louis MO). The  $\log EC_{50}$  for GABA was  $0.91 \pm 0.08$  (8.19  $\mu$ M) and the Hill coefficient was  $1.3 \pm 0.3$ . The  $EC_{10}$  was estimated to approximately 1.49  $\mu$ M. Based on these data, we employed a GABA concentration of 1  $\mu$ M, a value close to the  $EC_{10}$ , to investigate zolpidem enhancement of GABA responses. For each neuron, GABA was applied 2-3 consecutive times to obtain a stable baseline. 100 nM zolpidem was co-applied with 1 $\mu$ M GABA for 8 seconds to study enhancement of GABA currents. For each neuron, the % potentiation of 1 $\mu$ M GABA current by 100 nM zolpidem was calculated as follows: % potentiation = (Current in presence of GABA + zolpidem / Current in presence of GABA alone)\*100. The % potentiation for each experimental group was compared statistically using the Student's *t*-test or ANOVA as appropriate. A *p* value < 0.05 was considered statistically significant.

**Tissue preparation:** P2 membrane fractions from cultured cortical neurons were prepared by homogenization, low speed centrifugation in 0.32 M sucrose and centrifugation of the supernatant at 12,000 $\times$ g for 20 min. (Kumar et al., 2002). The pellet

was resuspended in phosphate buffered saline (PBS) with phosphatase inhibitor Cocktail-I, (proprietary mixture of microcystin LR, catharidin and bromotetramisole, Sigma-Aldrich, St. Louis, MO) and stored at  $-80^{\circ}\text{C}$ . Protein was quantified using the bicinchoninic acid (BCA) protein assay kit (Pierce Chemical, Rockford, IL).

**Biotinylation of surface receptors:** Surface expression of receptors was determined in cultured cells using a biotin labeling kit (Pierce Chemical, Rockford, IL) as described by the manufacturer. Cerebral cortical cells were cultured on poly-D-lysine coated flasks for 18 days. On day 19, cells were incubated with ethanol and/or PKC modulators or vehicle. The cells were washed twice with ice-cold phosphate-buffered saline followed by addition of sulfosuccinimidobiotin (sulfo-NHS-SS-biotin – 10 ml in each flask) (Pierce Chemical, Rockford, IL) diluted with ice-cold PBS. The cells were gently mixed with biotin reagent on a rocker for 30 minutes at  $4^{\circ}\text{C}$ . Unbound biotin was inactivated with quenching solution (Pierce Chemical, Rockford, IL). The cells were then scraped and transferred to a 50 ml conical tube and washed three times by adding Tris buffered saline (TBS) and centrifuging at 500Xg for 5 minutes. After washing, lysis buffer provided in the kit (500  $\mu\text{l}$ ) was added and cells were sonicated on ice for five 1 second bursts. Biotin-labeled proteins and flow-through (cytosolic proteins) were separated with neutravidin slurry (Pierce, Rockford, IL) as described by the manufacturer. The biotinylated (surface) proteins were eluted from the beads by incubation for 60 min at room temperature with an equal volume of Laemmli SDS-polyacrylamide gel electrophoresis sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05%

mercaptoethanol, and 0.05% bromphenol blue). The samples were then subjected to gel electrophoresis and Western blotting.

### **Immunoprecipitation**

Immunoprecipitation was conducted as previously described (Kumar et al., 2002). Briefly, one hundred thirty-five  $\mu\text{g}$  of P2 fraction protein was solubilized at  $4^{\circ}\text{C}$  in 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) sodium dodecyl sulfate (SDS), 140 mM NaCl and 10 mM Tris-HCl, pH 7.5 and protease inhibitors. Dynabeads (125  $\mu\text{l}$ ) were washed 3 times with 0.5 ml of 0.1 M phosphate buffer with 0.1% BSA (pH 8.1) and resuspended in 100  $\mu\text{l}$  of 0.1 M phosphate buffer. GABA<sub>A</sub> receptor  $\alpha 1$  subunit specific antibody (rabbit polyclonal, Werner Sieghart) (14.5  $\mu\text{l}$ , 628  $\mu\text{g}/\mu\text{l}$ ) was conjugated to Dynabeads by incubation for 1 hour at room temperature with agitation, and then washed once with 0.1 M phosphate buffer/ 0.1 % BSA and twice with 0.2 M triethanolamine (pH 8.2). Antibodies were cross linked with protein A in 1 ml of 0.2 M triethanolamine and 5 mg of dimethyl pimelimidate cross linker (DMP-HCl) to prevent co-elution of antibody with receptor. The reaction was incubated for 30 minutes at room temperature with constant rotation and washed three times with PBS/0.1% BSA.

Solubilized receptors and antibody conjugated to Dynabeads were mixed and incubated overnight at  $4^{\circ}\text{C}$  in a final volume of 500  $\mu\text{l}$  of solubilizing buffer. Receptor-antibody beads were then washed 3 times with PBS. After the final wash, the receptor-antibody-bead complex was resuspended in 50  $\mu\text{l}$  of 1X SDS and boiled for 5 minutes. Beads were separated from the immunoprecipitate by exposure to a magnet for 2 minutes and the immunoprecipitate was collected and denatured with 1  $\mu\text{l}$  of 5 M DTT, subjected

to western blot analysis and immunoblotted with various antibodies using standard immunoblotting procedures.

Since the expression of  $\alpha 1$  subunits is altered in P2 membrane fractions following ethanol (4 hour) exposure, it was necessary to conduct immunoprecipitation experiments under conditions of limited antibody so that identical amounts of  $\alpha 1$  subunit receptors would be isolated from both control and experimental groups. Since the concentration of antibody determines the number of receptors immunoprecipitated, it was possible to determine if ethanol exposure altered the association of PKC $\beta$  and  $\gamma$  with an equivalent sample of  $\alpha 1$  subunit receptors from each experimental group of cells. We confirmed that an equivalent amount of  $\alpha 1$  subunits was immunoprecipitated across experimental groups. Furthermore, the signal intensity for PKC $\beta$  and  $\gamma$  was normalized to the signal intensity for the  $\alpha 1$  subunit in the  $\alpha 1$  immunoprecipitate to rule out the possibility of non-equivalent immunoprecipitation efficiency between experimental groups (for explanation see Kumar et al., 2002). Co-immunoprecipitation of PKC $\epsilon$  with GABA $_A$   $\alpha 1$  subunit receptors is not detected with this method (Kumar et al., 2002). Therefore, co-localization of PKC $\epsilon$  with the GABA $_A$  receptor  $\alpha 1$  subunit was assessed by dual fluorescence confocal microscopy.

**Western blot analysis:** The various subcellular fractions were analyzed by Western blot analysis under conditions of protein linearity (Kumar et al., 2003). P2 fractions were subjected to SDS PAGE using Novex Tris-Glycine gels (8-16%) and transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membranes with transferred proteins were probed with  $\alpha 1$  (ABR, Golden, CO), PKC $\beta$ , PKC $\gamma$  and PKC $\epsilon$

antibodies (BD Biosciences, San Jose, CA USA). Guinea pig  $\alpha 1$  subunit antibody (provided by Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland) was used for western blot analysis of the  $\alpha 1$  subunit immunoprecipitate to avoid interference with the rabbit  $\alpha 1$  subunit antibody used for immunoprecipitation. Blots were subsequently exposed to a second primary antibody directed against  $\beta$ -actin, to verify equivalent protein loading and transfer. Bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL), apposed to x-ray films under non-saturating conditions, and analyzed by densitometric measurements using NIH Image 1.57. All comparisons were made within blots. Statistical analysis was conducted using the student's t test or one-way ANOVA.

**Dual label confocal fluorescence microscopy:** Staining and microscopy were performed as described by (Essrich et al., 1998) with some modifications. Coverslips with cortical neurons (paraformaldehyde fixed) were used for staining and confocal microscopy. Antibody dilution curves were performed to determine the optimum concentration of primary and secondary antibody for staining. Specific labeling was determined by staining in the absence of primary antibodies and by monitoring fluorescence detection in every channel to exclude bleed-through. Briefly, coverslips were washed with PBS and permeabilized with 0.1% Triton X-100 (w/v) in PBS containing 10% normal horse serum (Vector, Burlingame, CA) for 30 min at room temperature. After washing, the cells were stained with anti-PKC $\beta$  (mouse, 1:75 dilution, BD Biosciences, San Jose, CA), anti-PKC $\epsilon$  (mouse, 1:100 dilution, BD Biosciences, San Jose, CA), or anti-PKC $\gamma$  (mouse, 1:75 dilution, BD Biosciences, San Jose, CA) and GABA $_A$   $\alpha 1$  antibody (rabbit, 1:400

dilution, Novus Biologicals, Littleton, CO) overnight at 4°C. The following morning, coverslips were washed with PBS (containing 0.1% Triton X-100 and 10% serum) followed by incubation with the fluorescent-coupled secondary antibodies, Alexa Fluor 488 goat anti-mouse IgG (1:200 dilution) and Alexa Fluor 594 goat anti-rabbit IgG (1:200 dilution) (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Coverslips were washed and mounted onto glass slides with mounting medium (Fluoromount-G, Southern Biotech, Birmingham, AL) and examined under a confocal laser scanning microscope.

Visualization was performed using a Leica SP2 Laser Scanning Confocal Microscope (Wetzlar, Germany). The microscope was used at 40X magnification to identify relevant GABA<sub>A</sub> receptor subunits ( $\alpha$ 1, 594, Red Color) using the objective lens and 594 filter. Next, the view field was scanned in both 594 (anti-rabbit-red) and 488 (anti-mouse-green). Images were saved for analysis by an observer (KB) blind to the experimental conditions. Approximately, 20-27 cells expressing GABA<sub>A</sub> receptor  $\alpha$ 1 subunits were identified on each coverslip (5/quadrant). Each cell was then scored for co-localization of the PKC isozyme labeled. The total number of  $\alpha$ 1 subunit/PKC co-localized cells were normalized to total number of  $\alpha$ 1 subunit-stained cells on the same coverslip and presented as a ratio. Co-localization of each PKC isozyme with GABA<sub>A</sub>  $\alpha$ 1 subunit receptors was determined in independent experiments from 5 coverslips (approx 30 cells/coverslip).

## Results

**Ethanol and PKC activator PDBu alter the cell surface expression of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits.** Ethanol exposure (50 mM) for 4 hours produced a maximal decrease in expression of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit ( $40.2 \pm 15\%$ ,  $n=5$ ,  $p < 0.05$ ) in the P2 membrane fraction of cerebral cortical neurons compared to control values (Figure 1A). The effect of ethanol was dose-dependent and ethanol (25 mM) for 4 hours did not significantly decrease GABA<sub>A</sub> receptor  $\alpha$ 1 subunits in the P2 fraction (Figure 1B). Biotinylation of surface proteins confirmed the decrease in cell surface expression of  $\alpha$ 1 subunits following 4 hours of ethanol exposure (Figure 1C,  $38.00 \pm 14\%$ ,  $n=4$ ,  $p < 0.05$ ). Furthermore, unlabeled cytosolic  $\alpha$ 1 subunit protein levels were increased by  $111.1 \pm 35\%$  (Figure 1D,  $n=4$ ,  $p < 0.05$ ). PKC activators have been shown to internalize GABA<sub>A</sub> receptors in recombinant cell expression systems (Chapell et al., 1998) and primary cultures (Connolly et al., 1999). We used the PKC activator PDBu to determine if PKC has similar activity in our primary cell culture. PDBu exposure for 1 hour reduced the expression of  $\alpha$ 1 subunits by  $37.3 \pm 7\%$  in the P2 fraction (Figure 2A,  $n=4$ ,  $p < 0.05$ ). Similarly, biotinylation of surface receptors revealed approximately the same decrease in GABA<sub>A</sub> receptor  $\alpha$ 1 subunits (Figure 2B,  $44.88 \pm 16\%$ ,  $p < 0.05$ ,  $n=4$ ), while cytosolic  $\alpha$ 1 subunit protein levels were increased by  $126.5 \pm 45\%$  (Figure 2C,  $n=4$ ,  $p < 0.05$ , in duplicate). Therefore, ethanol induces the internalization of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits and this effect is mimicked by PKC activation using PDBu.

**Ethanol exposure reduces zolpidem enhancement of GABA evoked Cl<sup>-</sup> currents.**



The response to application of 1  $\mu$ M GABA in cortical neurons was not significantly different in neurons treated with vehicle vs. ethanol for 4 hours (vehicle:  $133.1 \pm 26.50$  pA, 4 hr ethanol treated neurons:  $187.1 \pm 43.94$  pA). A low dose of zolpidem (100 nM) selective for alpha-1 subunit-containing GABA<sub>A</sub> receptors was chosen to study the effects of ethanol exposure on  $\alpha$ 1 subunit-containing GABA<sub>A</sub> receptors. This dose of zolpidem enhanced responses elicited by 1  $\mu$ M GABA ( $\sim$ EC<sub>10</sub>) in cultured cortical neurons, consistent with prior studies (Liang et al., 2004). As shown in Figure 3, this enhancement was significantly reduced in neurons exposed to ethanol (50 mM) for 4 hours (% potentiation in vehicle treated neurons:  $218.2 \pm 18.31$  (n=14), neurons treated with ethanol for 4 hours:  $144.4 \pm 14.63$  (n=12),  $p$  value = 0.0052). These data support the conclusion that the fraction of alpha-1 containing receptors at the cell-surface is significantly reduced by ethanol exposure *in vitro*, similar to prior studies on the effects of ethanol exposure *in vivo* (Kumar et al., 2003; Liang et al., 2004).

**PKC inhibitor calphostin C, prevents ethanol-induced internalization of  $\alpha$ 1 subunit-containing receptors.** Cultured cortical cells were exposed to vehicle, ethanol and ethanol combined with calphostin C (0.3  $\mu$ M). Calphostin C was added 15 minutes before the start of ethanol exposure. These doses have been shown to inhibit PKC activity and to alter GABA<sub>A</sub> receptor function in cortical synaptoneurosome (Kumar et al., 2005). Calphostin C alone did not alter the expression of  $\alpha$ 1 subunits in the P2 fraction (Figure 4A). Ethanol exposure for 4 hours decreased the expression of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit expression by  $48.2 \pm 8$  % (n=4,  $p < 0.05$ .) in the P2 fraction of cultured cortical

neurons. Calphostin C completely inhibited the ethanol-mediated decrease in GABA<sub>A</sub> receptor  $\alpha$ 1 subunits (Figure 4B).

We then employed Calphostin C in electrophysiological studies to determine the involvement of PKC in ethanol-induced alterations in zolpidem enhancement of GABA evoked Cl<sup>-</sup> conductance. First, we examined the effect of Calphostin C alone (0.3  $\mu$ M, 4 hrs) on currents elicited by application of 1  $\mu$ M GABA in cortical neurons. Calphostin C alone had no significant effect on the amplitude of 1  $\mu$ M GABA currents (vehicle: 114.3  $\pm$  20.24 pA, Calphostin C for 4 hours: 152.7  $\pm$  25.48 pA, Figure 4C). To investigate the involvement of PKC in the effects of ethanol on activation of benzodiazepine responses, we incubated the cells with 50 mM ethanol in presence or absence of 0.3  $\mu$ M Calphostin C for 4 hours. We then examined zolpidem (100nM) potentiation of GABA (1  $\mu$ M) current in these treatment groups. As shown in Figure 4D, Calphostin C ablated the decrease in zolpidem enhancement that was seen in neurons treated with ethanol alone for 4 hours (% potentiation in vehicle neurons: 211.1  $\pm$  15.50 (n=10), neurons treated with 50 mM Ethanol + 0.3  $\mu$ M Calphostin C for 4 hours: 191.6  $\pm$  15.58 (n=6), *p* value > 0.5). These data further support the requirement for PKC activity in ethanol-induced internalization of  $\alpha$ 1 subunit GABA<sub>A</sub> benzodiazepine receptors in cultured cortical neurons.

**Ethanol increases PKC isoform expression and selectively increases the association of PKC $\gamma$  with GABA<sub>A</sub> receptor  $\alpha$ 1 subunits.** Since the effect of ethanol on GABA<sub>A</sub>  $\alpha$ 1 receptor internalization was mimicked by PKC activation and blocked by PKC inhibition, we investigated whether specific PKC isozymes were involved in these

effects. Ethanol exposure for 1 hour increased PKC $\beta$ , PKC $\gamma$  and PKC $\epsilon$  peptide expression in the P2 membrane fraction of cortical neurons. In contrast, ethanol exposure for 4 hours increased PKC $\beta$  and PKC $\gamma$ , but not PKC $\epsilon$  protein levels (Table 1). To determine if these isozymes were translocated and bound to GABA $_A$   $\alpha$ 1 subunit receptors, we employed immunoprecipitation studies to determine their association. For this study, intact GABA $_A$   $\alpha$ 1 subunit receptors were immunoprecipitated from the P2 fraction using a GABA $_A$  receptor  $\alpha$ 1 subunit specific antibody. The immunoprecipitate was denatured, separated by SDS PAGE and probed with PKC $\gamma$  or PKC $\beta$  antibodies. Ethanol exposure for 4 hours increased the co-immunoprecipitation of PKC $\gamma$  with GABA $_A$  receptor  $\alpha$ 1 subunits by  $92.88 \pm 24\%$  ( $p < 0.05$ ,  $n=4$ ), but did not alter the co-immunoprecipitation of PKC $\beta$  with GABA $_A$  receptor  $\alpha$ 1 subunits (Figure 5A and B). Furthermore, ethanol exposure for 1 hour did not alter association of PKC $\beta$  or PKC $\gamma$  with  $\alpha$ 1 subunit-containing GABA $_A$  receptors (Table 2).

We also employed dual fluorescence confocal microscopy to visualize the co-localization of PKC $\beta$ ,  $\gamma$  or  $\epsilon$  with GABA $_A$  receptors that contain  $\alpha$ 1 subunits (Figure 6). In ethanol-naïve cells, GABA $_A$   $\alpha$ 1 subunits were co-localized with PKC $\beta$  in  $44 \pm 5\%$  of cells, with PKC $\gamma$  in  $30.34 \pm 2\%$  of cells and PKC $\epsilon$  in  $45.68 \pm 2\%$  of cells (data not shown). Ethanol exposure for 4 hours increased the association of PKC $\gamma$  with GABA $_A$  receptor  $\alpha$ 1 subunits by  $70.2 \pm 15\%$ , ( $p < 0.05$ ,  $n=5$  coverslips/group, 309  $\alpha$ 1-labeled cells counted) (Figure 7A) but did not alter the co-localization of PKC $\beta$  ( $n=4$  coverslips/group, 377  $\alpha$ 1-labeled cells counted) or PKC $\epsilon$  ( $n=5$  coverslips/group, 361  $\alpha$ 1-labeled cells counted) (Figure 7 B & C) with GABA $_A$  receptor  $\alpha$ 1 subunits. Furthermore,

ethanol exposure for 1 hour did not alter the association of PKC $\beta$  (n=5 coverslips/group, 306  $\alpha$ 1-labeled cells counted), PKC $\gamma$  (n=5 coverslips/group, 300  $\alpha$ 1-labeled cells counted) or PKC $\epsilon$  (n=5 coverslips/group, 313  $\alpha$ 1-labeled cells counted) with GABA $_A$  receptor  $\alpha$ 1 subunits (Table 3).

**PKC $\gamma$ , but not PKC $\beta$  inhibition, reduces ethanol-mediated internalization of GABA $_A$  receptor  $\alpha$ 1 subunits.** Ethanol exposure for 4 hours increased the association of PKC $\gamma$  with GABA $_A$  receptor  $\alpha$ 1 subunits and internalization of GABA $_A$  receptor  $\alpha$ 1 subunits. Therefore, we hypothesized that the internalization of GABA $_A$  receptor  $\alpha$ 1 subunit was PKC $\gamma$  dependent. PKC $\gamma$  siRNA transfection was used to inhibit the effect of ethanol on PKC $\gamma$  expression in cultured cortical neurons. Selective PKC $\gamma$  siRNA reduced PKC $\gamma$  expression by  $74.36 \pm 7\%$  ( $P < 0.05$ , n=3, in duplicate) in the P2 fraction of cortical neurons compared to control. However, PKC $\gamma$  siRNA transfection of cortical neurons had no effect on expression of PKC $\beta$  and PKC $\epsilon$  isoforms (Figure 8A.). Similarly, PKC $\gamma$  siRNA transfection did not alter expression of GABA $_A$  receptor  $\alpha$ 1 subunits. However, the effect of ethanol (4 hrs) on GABA $_A$  receptor  $\alpha$ 1 subunit expression was blocked by selective PKC $\gamma$  siRNA transfection (Figure 8B).

Ethanol exposure did not increase the association of PKC $\beta$  with GABA $_A$   $\alpha$ 1 subunit receptors but increased PKC $\beta$  expression in the P2 fraction. Therefore, to further verify the role of PKC $\beta$  in ethanol-mediated internalization of  $\alpha$ 1 subunits, we used PKC $\beta$  pseudo-substrate peptide to block the activity of PKC $\beta$ . PDBU exposure was used as a positive control to evaluate the effectiveness of PKC $\beta$  pseudo-substrate. PDBU

increased the expression of PKC $\beta$  in P2 fraction by  $103.2 \pm 27\%$  ( $p < 0.05$ , ANOVA,  $n=4$ ). PKC $\beta$  pseudo-substrate completely inhibited the effect of PDBu on PKC $\beta$  expression, demonstrating inhibition of PKC $\beta$  expression in the cells. PKC $\beta$  pseudo-substrate alone did not alter PKC $\beta$  expression. (Figure 9A). Furthermore, PKC $\beta$  pseudo-substrate had no effect on the ethanol-mediated decrease in GABA $_A$  receptor  $\alpha 1$  subunits (Figure 9B).

## Discussion:

The present study demonstrates that ethanol exposure *in vitro* increases internalization of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits in a time-dependent manner. The effect of ethanol on GABA<sub>A</sub> receptor  $\alpha$ 1 subunit surface expression is PKC dependent, since it is inhibited by the PKC inhibitor calphostin C. In addition, electrophysiological measurement of zolpidem enhancement of GABA-mediated currents confirms that surface expression of  $\alpha$ 1 containing receptors are significantly reduced following ethanol exposure and this effect is also dependent upon PKC. Ethanol exposure increases PKC $\beta$ ,  $\epsilon$  and  $\gamma$  isoform expression, but ethanol-induced association with GABA<sub>A</sub> receptor  $\alpha$ 1 subunits is selective for PKC $\gamma$  isoforms. PKC $\gamma$ , but not PKC $\beta$ , inhibition prevented ethanol-induced internalization of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits. Thus, these data suggest that decreased expression of  $\alpha$ 1 subunits at the cell surface following ethanol exposure is mediated by PKC $\gamma$  activity. The effect of PKC $\gamma$  siRNA on zolpidem enhancement of GABA evoked currents could not be determined since individual cells that were transfected could not be identified. However, it seems unlikely that the preservation of surface expression by PKC $\gamma$  siRNA would lack functional significance, since the PKC inhibitor calphostin C altered the effect of ethanol on zolpidem responses.

The regulation of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit surface expression appears to be a conserved mechanism both *in vitro* and *in vivo*. The effects of ethanol exposure on GABA<sub>A</sub>  $\alpha$ 1 subunit receptors in cultured cerebral cortical neurons mimics the effects of chronic ethanol exposure *in vivo*, as  $\alpha$ 1 subunits are reduced following ethanol exposure in both experimental paradigms. The ethanol-induced reduction in GABA<sub>A</sub> receptor  $\alpha$ 1 subunits are thought to contribute to ethanol dependence. Studies have shown that  $\alpha$ 1

knockout mice exhibit hyperexcitability marked by increased seizure susceptibility to bicuculline (Kralic et al., 2002), as well as withdrawal-like tremor (Kralic et al., 2005). Furthermore, other groups have shown that ethanol exposure also reduces GABA<sub>A</sub> receptor  $\alpha$ 1 subunit expression in hippocampus (Liang et al., 2004) and hippocampal cultured neurons (Sanna et al., 2003), where electrophysiological studies have also demonstrated a loss of zolpidem enhancement of GABA responses, suggesting similar effects of ethanol on the trafficking of hippocampal  $\alpha$ 1 subunit receptors. Together, these data suggest that loss of  $\alpha$ 1 containing GABA<sub>A</sub> receptors plays an important role in the etiology of ethanol dependence and withdrawal (Biggio et al., 2007). Therefore, further understanding of the mechanisms that regulate cell surface expression of these  $\alpha$ 1 subunit-containing receptors could have profound therapeutic relevance for treatment of alcohol abuse and alcoholism.

The observation that ethanol exposure diminishes zolpidem potentiation of GABA evoked Cl<sup>-</sup> currents in the cortical cultured neurons supports the conclusion that ethanol reduces the surface expression of synaptic  $\alpha$ 1 subunit-containing GABA<sub>A</sub> benzodiazepine receptors. This result is also consistent with previous *in vivo* studies that found reductions in benzodiazepine sensitivity in ethanol dependence (Buck and Harris, 1990; Kumar et al., 2003; Liang et al., 2004; Sanna et al., 2003), suggesting that type 1 GABA<sub>A</sub> benzodiazepine ( $\alpha$ 1, $\beta$ 2/3, $\gamma$ 2) receptor internalization is the mechanism for ethanol-induced reductions in behavioral benzodiazepine sensitivity (cross tolerance) in alcohol dependence (Cagetti et al., 2003).

The effects of ethanol on GABA<sub>A</sub> receptor surface expression in these studies did not involve withdrawal from ethanol. This is consistent with our studies on the effects of

ethanol on  $\alpha 1$  subunit-containing GABA<sub>A</sub> receptors in the cerebral cortex (Kumar et al., 2003) and with the effects of ethanol on  $\alpha 1$  subunit expression in cerebral cortical and hippocampal cultured neurons (Sheela Rani and Ticku, 2006). Other studies suggest that ethanol-induced internalization of  $\alpha 1$  receptors may occur more rapidly (Liang et al., 2007), less rapidly (Matthews et al., 1998) or may require withdrawal from ethanol (Cagetti et al., 2003). The reason for these discrepancies is unclear but may be related to different ethanol doses, brain regions and the intervals investigated following ethanol exposure. Indeed, ethanol does not produce the same effects on PKC isoform expression in hippocampus as cerebral cortex (Kumar et al., 2006) and this could result in differential effects of ethanol on receptor trafficking in these regions. Further studies on the mechanisms of ethanol trafficking of GABA<sub>A</sub> receptors are needed..

Surface expression of GABA<sub>A</sub> receptors involves a highly regulated process of synthesis, endocytosis, recycling and degradation. PKC has been shown to alter both endocytosis (Terunuma et al., 2008) and recycling of GABA<sub>A</sub> receptors. In the present study, despite the increases in PKC $\beta$ ,  $\gamma$  and  $\epsilon$  expression, ethanol exposure for 1 hour did not alter the surface expression of GABA<sub>A</sub> receptor  $\alpha 1$  subunits. This may indicate that there was no endocytosis of  $\alpha 1$  subunits or that receptor recycling compensated for endocytosis of the receptors. Previous studies demonstrated that ethanol exposure to rats for 1 hour *in vivo* did not alter the expression of  $\alpha 1$  subunit receptors in CCVs or alter association of  $\alpha 1$  subunits with adaptin- $\alpha$  that is required for GABA<sub>A</sub> receptor internalization (Kumar et al., 2003), suggesting that endocytosis does not occur at this point. Alternatively, it is possible that ethanol activation of other kinases such as PKA (Dohrman et al., 2002) or tyrosine kinases (Marutha Ravindran and Ticku, 2006),



influence the surface expression of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits. Systematic study of these possibilities is needed to further understand effects of ethanol on GABA<sub>A</sub> receptor trafficking.

Association of kinases with receptor targets and subsequent phosphorylation may directly alter receptor conformation and channel conductance while indirect actions may produce changes in receptor subunit composition at the membrane surface by altering the normal trafficking of receptors. In the present study, ethanol exposure for 1 hour increased PKC $\beta$ ,  $\gamma$  and  $\epsilon$  expression in the membrane fraction of cultured cortical neurons without altering their association with GABA<sub>A</sub>  $\alpha$ 1 subunits. Therefore, it is possible that these PKC isoforms are targeted to other subtypes of GABA<sub>A</sub> receptors and/or other ion channels. Translocation of PKC from cytosol to the membrane requires distinct mechanisms and intermediary proteins like AKAP and RACK (Mochly-Rosen, 1995; Wong and Scott, 2004), and these proteins are also altered by ethanol exposure (He et al., 2002; Ron, 2004). Therefore, it is likely that ethanol effects on such transporting molecules determine the translocation of PKC to GABA<sub>A</sub> receptors. These intermediary proteins are also affected by ethanol and may determine the localization of specific PKC isoforms on the cell surface following ethanol exposure.

Ethanol exposure for 4hrs selectively increased PKC $\gamma$  co-localization and co-immunoprecipitation with GABA<sub>A</sub> receptor  $\alpha$ 1 subunits. Furthermore, surface expression of GABA<sub>A</sub>  $\alpha$ 1 receptors was decreased after 4 hours of ethanol exposure suggesting that PKC $\gamma$  is necessary for ethanol regulation of  $\alpha$ 1 receptor internalization. Previous studies have shown that *in vivo* chronic ethanol exposure (two weeks) increases endocytosis of  $\alpha$ 1 subunit-containing GABA<sub>A</sub> receptors into CCVs of cerebral cortex (Kumar et al.,

2003), but this effect is accompanied by a decrease in PKC $\gamma$  co-immunoprecipitation with GABA<sub>A</sub>  $\alpha$ 1 receptors (Kumar et al., 2002). It is possible that ethanol effects on PKC $\gamma$  association with GABA<sub>A</sub> receptors are transient, whereas continual ethanol effects on neurons during longer ethanol exposure periods may account for this observed difference. Alternatively, the potential role of other signaling pathways, endogenous molecules and neurocircuitry that differ *in vivo* vs. *in vitro* may account for this difference.

Selective PKC activity has also been suggested to play a role in GABA<sub>A</sub> receptor function following ethanol exposure *in vivo* and *in vitro*. For example, mice lacking the gene for PKC $\gamma$  show a significant reduction in ethanol potentiation of muscimol-stimulated Cl<sup>-</sup> influx compared to responses in wild type mice (Harris et al., 1995). In contrast, ethanol and flunitrazepam potentiation of muscimol-stimulated Cl<sup>-</sup> uptake is greater in microsacs from PKC $\epsilon$  null mutant mice compared to wild-type controls (Hodge et al., 1999). In our study, ethanol exposure did not alter PKC $\beta$  or  $\epsilon$  association with GABA<sub>A</sub>  $\alpha$ 1 receptors following ethanol exposure. While PKC $\beta$  and PKC $\epsilon$  may not be responsible for ethanol-induced receptor trafficking, these kinases clearly play a role in receptor activity. For example, PKC $\epsilon$  has been shown to alter phosphorylation of GABA<sub>A</sub> receptor  $\gamma$ 2 subunit and receptor function following ethanol exposure (Qi et al., 2007). In addition PKC $\beta$  has been shown to phosphorylate GABA<sub>A</sub> receptor  $\beta$ 1 subunits (Brandon et al., 1999). Thus, ethanol exposure may alter the association of these PKC isoforms with other subunits of GABA<sub>A</sub> receptors and thereby regulate the trafficking of distinct receptor subtypes. Further studies are needed to explore these possibilities.

The present work demonstrates that PKC $\gamma$  plays an essential role in the regulation of the surface expression of  $\alpha$ 1 subunit-containing GABA<sub>A</sub> receptors and highlights the

possibility of modulating GABAergic activity through selective targeting of PKC isoforms. Altered GABA<sub>A</sub> receptor  $\alpha$ 1 subunit expression is implicated in anxiety, alcoholism, epilepsy and many other neurological disorders. Therefore, an understanding of the mechanism of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit trafficking could lead to new therapeutic approaches that aim to restore normal surface expression of GABA<sub>A</sub> receptors.

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#### Footnotes

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## Figure Legends

**Figure 1. Ethanol exposure alters GABA<sub>A</sub> receptor  $\alpha$ 1 subunit surface expression in cerebral cortical neurons.** Cortical neurons were exposed to ethanol (50 mM for 1 to 6 hrs) followed by preparation of P2 fractions and biotinylation of surface proteins. Biotin-labeled (surface) and flow-through (cytosolic) proteins were isolated with neutravidin slurry (Pierce). Panel A shows representative western blots of  $\alpha$ 1 subunit in the P2 fraction whereas panel C and D shows representative western blots of  $\alpha$ 1 subunit on surface and cytosol respectively. (A) Ethanol exposure for 1 hour did not alter  $\alpha$ 1 subunit expression while ethanol exposure for 4 hrs produced maximal decrease in expression of subunits in the P2 fraction. (B) Ethanol exposure (25 mM) for 4 hours had no effect on  $\alpha$ 1 subunit expression whereas 50 mM ethanol decreased  $\alpha$ 1 subunit expression by 40% in the P2 fraction of cells. Following biotinylation of surface receptors, ethanol exposure for 4 hours decreased  $\alpha$ 1 subunit level on cell surface (C) and increased  $\alpha$ 1 subunit protein levels in cytosol (D). Data represents mean  $\pm$  SEM. \*  $p < 0.05$

**Figure 2. PDBu alters GABA<sub>A</sub> receptor  $\alpha$ 1 subunit surface expression in cultured cerebral cortical neurons.** Cortical neurons were exposed to PDBu (100 nM for 60 min) followed by preparation of P2 fraction and biotinylation of surface proteins as described in the methods. (A) Western blot analysis of P2 fraction of cortical neurons shows a decrease in  $\alpha$ 1 subunit following PDBu exposure. (B & C) Purification of surface (B) and cytosolic (C) receptors by biotinylation confirms increased internalization of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits. Data represents mean  $\pm$  SEM. \*  $p < 0.05$ , Student's *t* test



**Figure 3. Ethanol exposure reduces zolpidem potentiation of GABA-induced currents in whole cell patch clamp recordings of cerebral cortical neurons.** Cortical neurons were exposed to vehicle or ethanol (50 mM) for 4 hours. Representative electrophysiological responses to the application of 1  $\mu$ M GABA, co-application of 1  $\mu$ M GABA with 100 nM zolpidem and subsequent washout with 1  $\mu$ M GABA following (A) vehicle and (B) ethanol exposure (50mM) for 4 hrs. (C) shows that zolpidem (100nM) potentiation of the 1  $\mu$ M GABA response in cortical neurons was significantly diminished in ethanol-treated vs. vehicle-treated neurons. Data represent the mean  $\pm$  SEM. \*\*  $p < 0.001$ .

**Figure 4. PKC inhibitor calphostin C blocks ethanol-mediated internalization of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit.** Cultured cortical neurons were exposed to vehicle (Veh), ethanol and ethanol with calphostin C (Cal). P2 fractions were prepared and analyzed by Western blot analysis. In a separate set of experiments, whole cell patch clamp recordings were carried out from cultured cortical neurons (A) Effect of Calphostin C alone on expression of GABA<sub>A</sub> receptor  $\alpha$ 1 subunits. (B) Ethanol exposure for four hours decreased the expression of  $\alpha$ 1 subunits and this was blocked by Calphostin C. \*  $p < 0.05$ , Newman-Keuls test, compared to control. Data represents mean  $\pm$  SEM. (C) Calphostin C alone had no significant effect on zolpidem enhancement of 1  $\mu$ M GABA currents in neurons treated with ethanol for 4 hours as compared to vehicle treated neurons. (D) Co-exposure of neurons with Calphostin C and ethanol for 4 hours

prevented the decrease in zolpidem potentiation of 1  $\mu$ M GABA evoked currents that was found in neurons that were exposed to ethanol alone for 4 hours.

**Figure 5. Ethanol exposure for 4 hours increases the association of PKC $\gamma$  with  $\alpha$ 1-containing GABA<sub>A</sub> receptors in cultured cortical neurons.** Receptors in the P2 fraction of control (lane 1) and ethanol-exposed cells (lane 2) were immunoprecipitated with  $\alpha$ 1 subunit antibody. Immunoprecipitated receptors were separated by SDS-PAGE and immunoblotted with PKC $\gamma$  or PKC $\beta$  antibodies. The same blot was probed by  $\alpha$ 1 subunit antibody. PKC $\gamma$  or PKC $\beta$  signal intensity was normalized to GABA<sub>A</sub> receptor  $\alpha$ 1 subunit signal intensity from the western blots of the immunoprecipitate. (A) Association of PKC $\gamma$  with  $\alpha$ 1-containing GABA<sub>A</sub> receptors was increased  $92.88 \pm 23.60$  % in cultured neurons after 4 hour ethanol exposure (B) There was no change in PKC $\beta$  association with  $\alpha$ 1-containing receptors after 4 hour ethanol exposure. Data represents mean  $\pm$  SEM. \*  $p < 0.05$ , Student's  $t$  test.

**Figure 6. PKC isoforms are co-localized with GABA<sub>A</sub> receptor  $\alpha$ 1 subunits in ethanol naïve cells.** Cortical neurons were stained with anti- $\alpha$ 1 and PKC $\beta$ , PKC $\gamma$  or PKC $\epsilon$  antibodies for confocal microscopy. Images A, D, G are  $\alpha$ 1 labeled cells while, B, E and H are PKC $\beta$ ,  $\gamma$  and  $\epsilon$  labeled cells respectively. Images C, F, and I are merged images of dual immuno-staining of  $\alpha$ 1 subunit and the PKC isoform. The yellow color in merged panels (arrow) indicates co-localization of the two proteins.

**Figure 7. Ethanol increases PKC $\gamma$ , but not PKC $\beta$  or PKC $\epsilon$ , colocalization with GABA $_A$  receptor  $\alpha$ 1 subunit in cultured cortical neurons:** Cells were exposed to ethanol for 4 hours and stained with anti- $\alpha$ 1 and PKC antibodies. Immunostaining of  $\alpha$ 1 and PKC labeled cells were examined under confocal microscope. The total number of  $\alpha$ 1/PKC co-localized cells were normalized to total number of  $\alpha$ 1- stained cells on the same coverslip and presented as percent control values. Ethanol exposure for 4 hours increased PKC $\gamma$  association with GABA $_A$  receptor  $\alpha$ 1 subunit by  $70.17 \pm 15\%$  (A). In contrast, ethanol exposure did not alter the association of PKC $\beta$  or PKC $\epsilon$  with GABA $_A$  receptor  $\alpha$ 1 subunit compared to controls (B& C). Data represents mean  $\pm$  SEM. \*  $p < 0.05$ , Student's  $t$  test.

**Figure 8. PKC $\gamma$  RNAi transfection inhibits ethanol-induced internalization of GABA $_A$  receptor  $\alpha$ 1 subunit in cultured cortical cells.** Cerebral cortical neurons were transfected with scrambled or PKC $\gamma$  siRNA. The P2 fraction was analyzed by SDS-Page analysis and probed with PKC $\gamma$ ,  $\beta$  and  $\epsilon$  antibodies. (A) PKC $\gamma$  specific, but not scrambled, siRNA decreased the expression of PKC $\gamma$  by  $76.09 \pm 1.087\%$ , whereas the expression of PKC $\beta$  and  $\epsilon$  were not altered. (B) Selective PKC $\gamma$  siRNA inhibits ethanol (50 mM)-induced internalization of GABA $_A$  receptor  $\alpha$ 1 subunit. \*  $p < 0.05$  compared to vehicle, Newman-Keuls test.

**Figure 9. PKC $\beta$  inhibition does not prevent ethanol-induced internalization of GABA $_A$  receptor  $\alpha$ 1 subunit.** Cultured cortical cells were exposed to PDBu and or

PKC $\beta$  pseudo substrate. (A) PDBu increased the PKC $\beta$  expression in P2 fraction by 203  $\pm$  26%. The PDBu-induced increase in PKC $\beta$  expression was blocked by PKC $\beta$  pseudosubstrate. PKC $\beta$  pseudosubstrate alone did not affect PKC $\beta$  expression. (B) Ethanol exposure for 4 hours reduced the expression of GABA $_A$  receptor  $\alpha$ 1 subunit and PKC $\beta$  pseudo substrate had no effect on GABA $_A$  receptor  $\alpha$ 1 subunit expression following ethanol exposure. \*  $p < 0.05$  compared to vehicle, Newman-Keuls test.

**Table 1. PKC isoforms expression in P2 fraction of cultured cortical neurons**  
(Percent change)

	PKC $\beta$	PKC $\gamma$	PKC $\epsilon$
EtOH 1 hour	$\uparrow 60.8 \pm 24^*$	$\uparrow 78.1 \pm 27\% *$	$\uparrow 24.3 \pm 10\% *$
EtOH 4 hours	$\uparrow 51.1 \pm 22^*$	$\uparrow 52.3 \pm 19\% *$	$\downarrow 13.6 \pm 29\%$

EtOH: 50 mM, final concentration

The values are mean  $\pm$  SEM from 4 experiments performed in duplicate.

\* p 0.05 < compared to control

**Table 2.**  
**Co-immunoprecipitation of PKC isoforms with GABA<sub>A</sub>  $\alpha$ 1 subunit receptors following ethanol (50 mM) exposure for 1 hour**

Association of	Vehicle	EtOH
PKC $\beta$	100.0 $\pm$ 9	102.9 $\pm$ 9
PKC $\gamma$	100.0 $\pm$ 11	104.8 $\pm$ 13

The values are mean  $\pm$  SEM from 4 experiments performed in duplicate.

**Table 3**

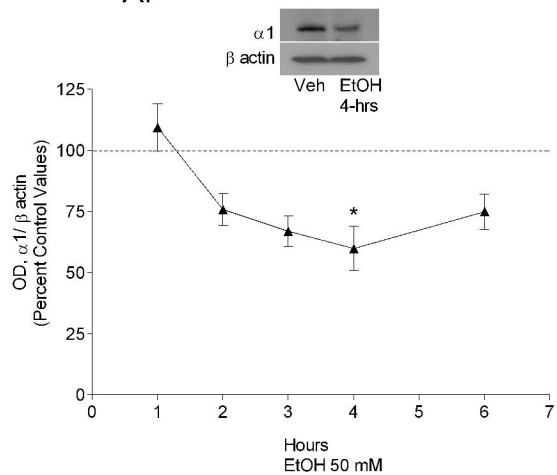
**Co-localization of PKC isoforms with GABA<sub>A</sub> receptor  $\alpha$ 1 subunit following 1 hour ethanol (50mM) exposure**

Association with GABA <sub>A</sub> $\alpha$ 1	Vehicle 1 hour	EtOH 1 hour
PKC $\beta$	100.0 $\pm$ 11	95.4 $\pm$ 16
PKC $\gamma$	100.0 $\pm$ 11	112.4 $\pm$ 16
PKC $\epsilon$	100.0 $\pm$ 8	116.2 $\pm$ 4

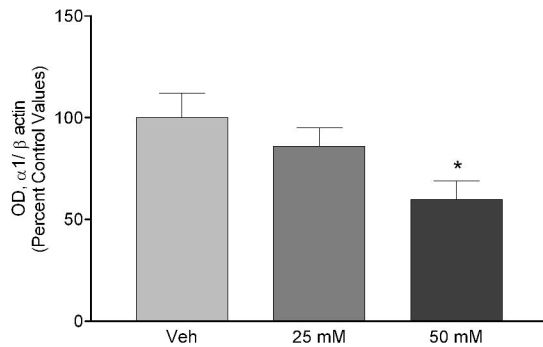
Data represent the percent change in the ratio of the number of  $\alpha$ 1/PKC co-localized cells to the total number of GABA<sub>A</sub> receptor  $\alpha$ 1 subunit-stained cells.

Figure 1

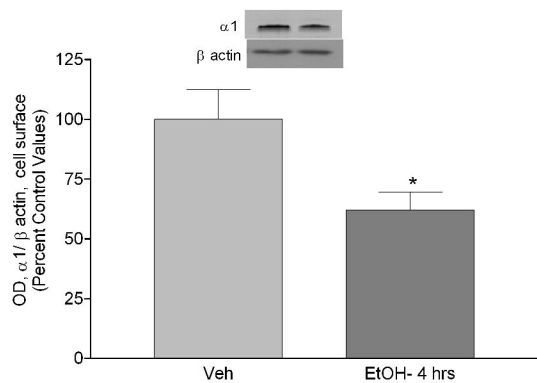
A.



B.



C.



D.

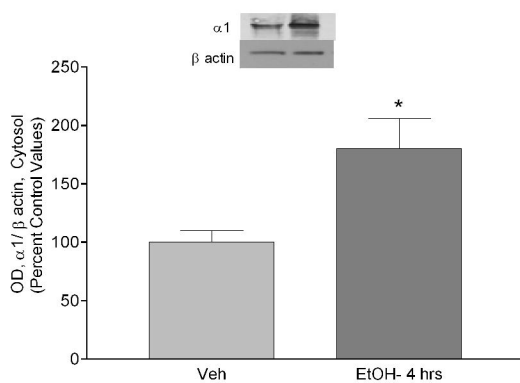
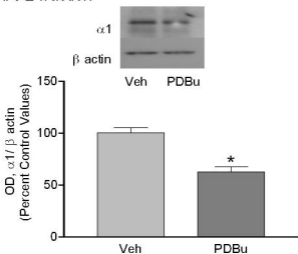


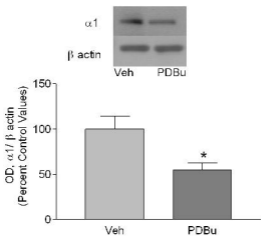


Figure 2

A. P2 fraction



B. Cell surface



C. Cytosol

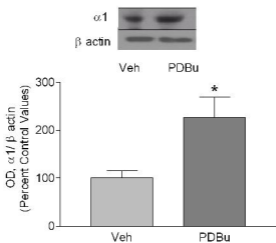
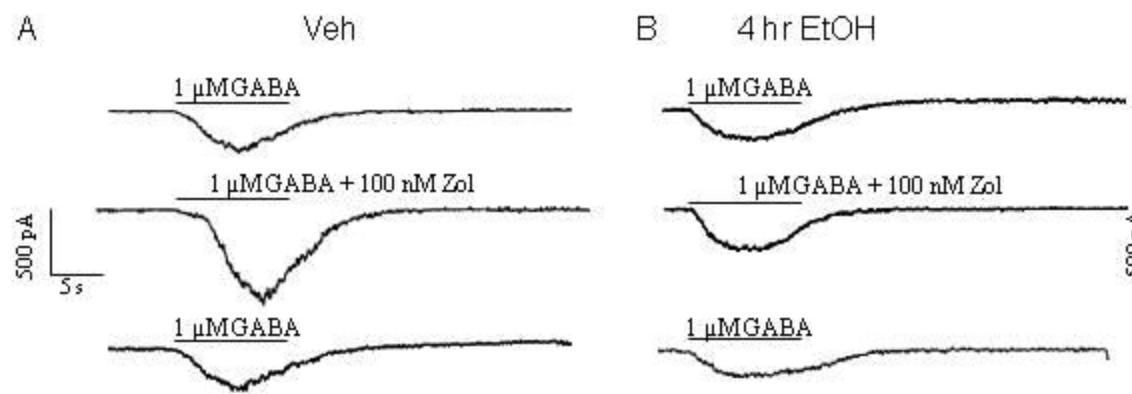


Figure 3



C

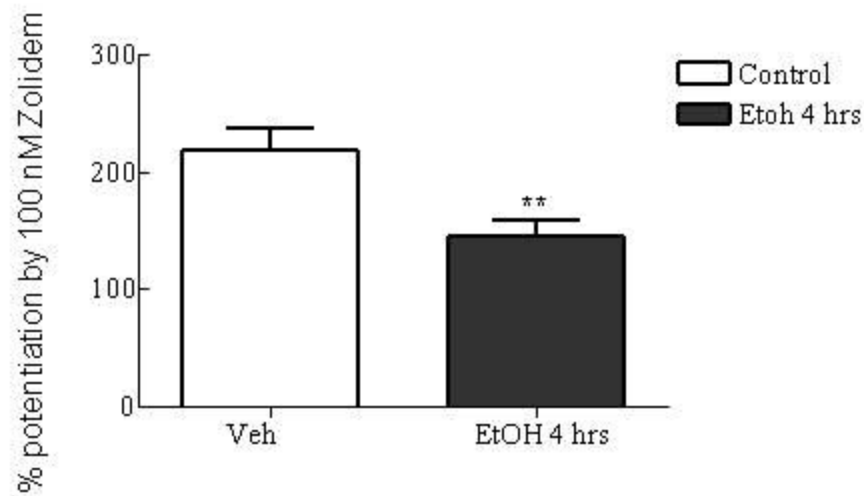


Figure 4

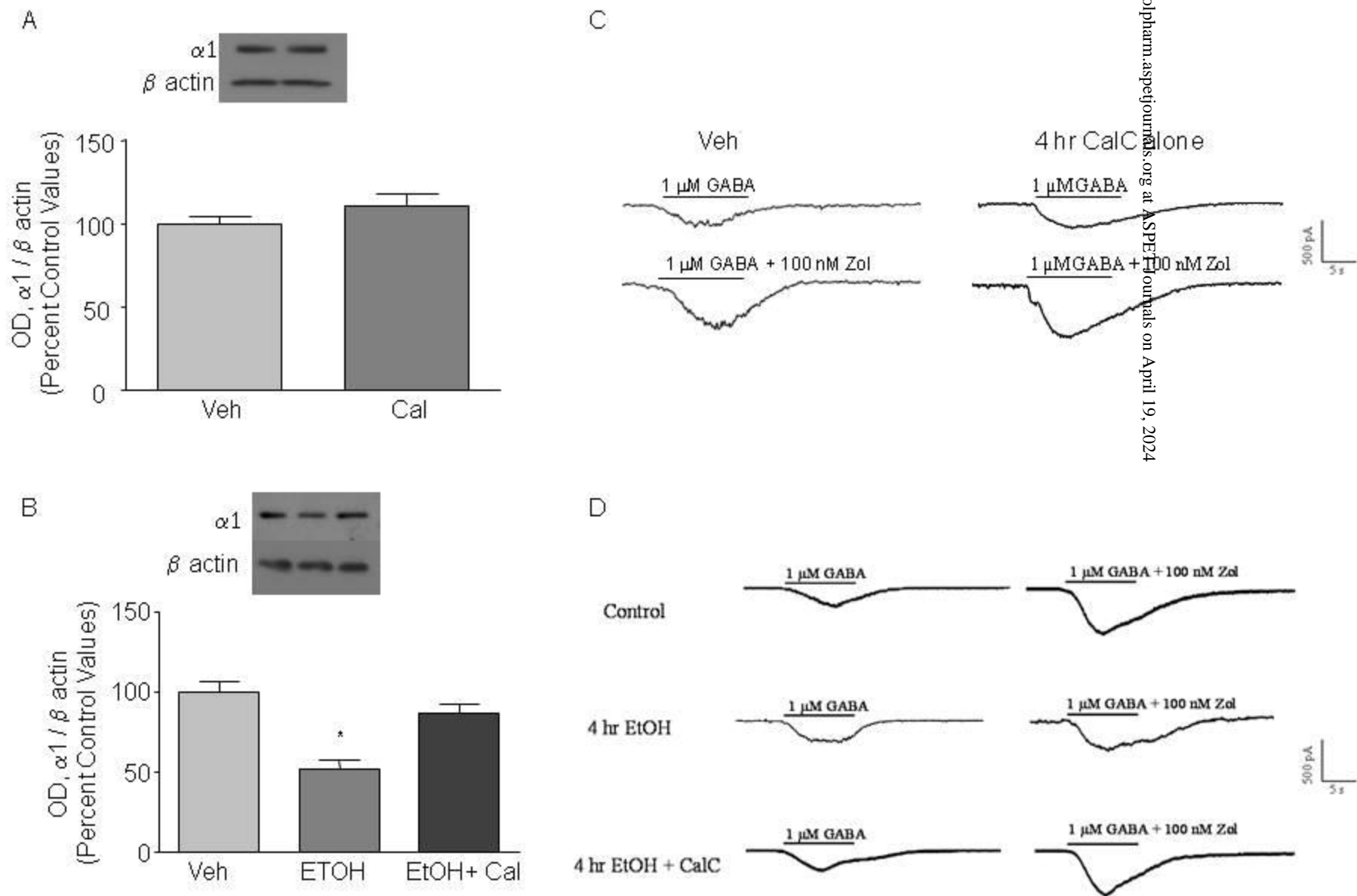
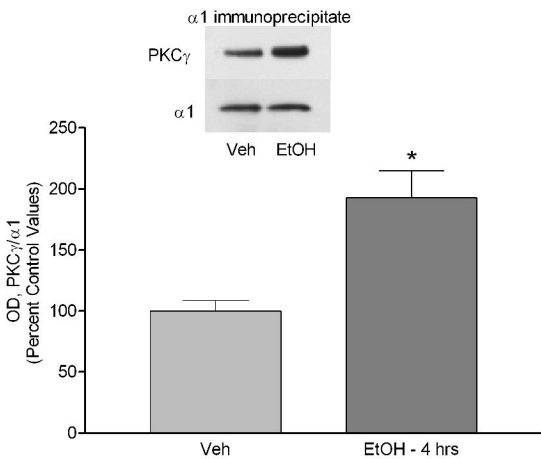


Figure 5

A



B

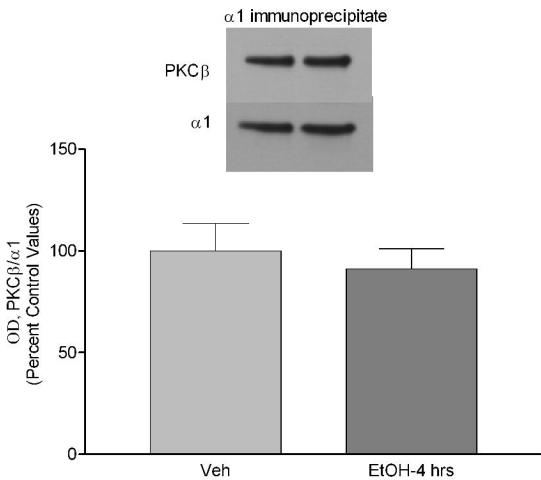


Figure 6

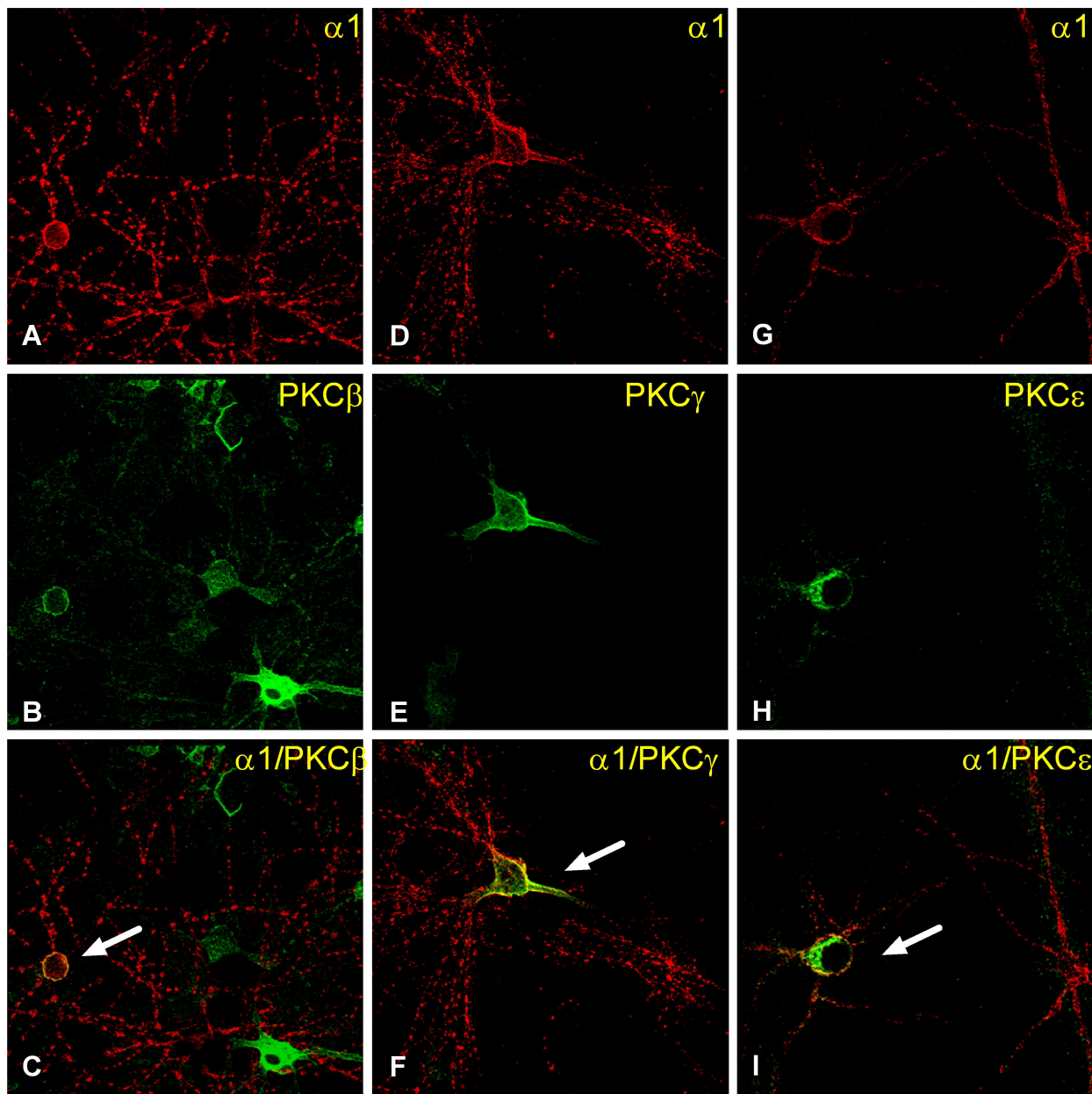
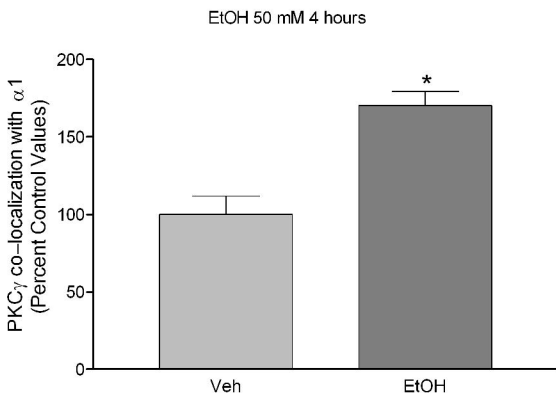
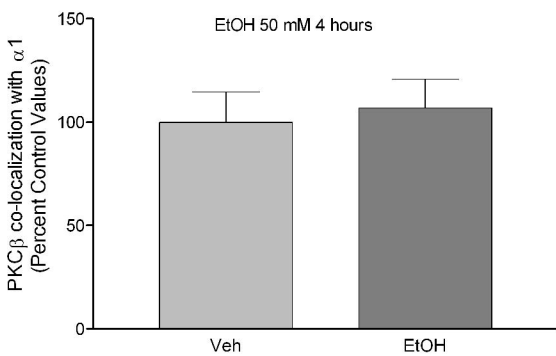


Figure 7

A



B



C

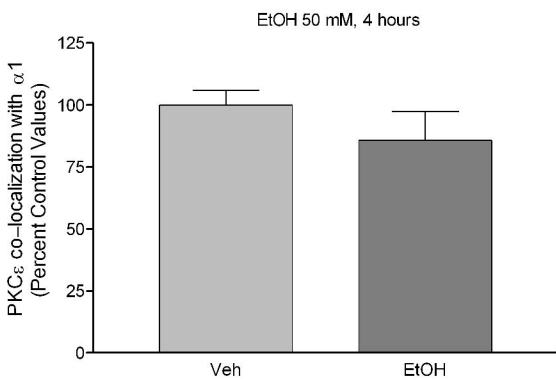
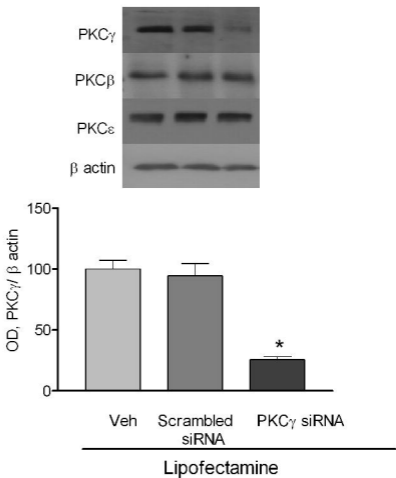


Figure 8

A.



B.

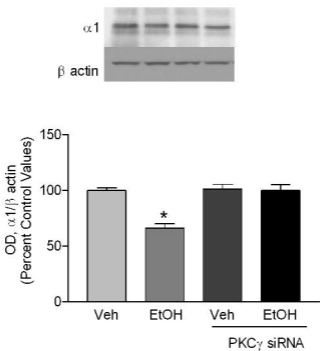
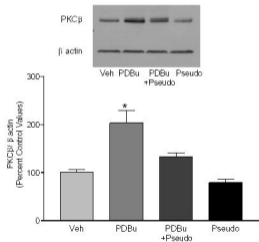


Figure 9

A



B

