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Ethanol reduces $GABA_A$ α1 subunit receptor surface expression by a PKCγ dependent mechanism in cultured cerebral cortical neurons

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Running title: PKCγ mediates ethanol effects on $GABA_A$ α1 receptor expression

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Abbreviations used in the text: $GABA_A$, γ-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.

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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 $GABA_A$ receptors, comprised of $\alpha1\beta2/3\gamma2$ subunits. However, the mechanisms of ethanol-mediated internalization are unknown. This study explored the effect of ethanol on surface expression of $GABA_A$ $\alpha1$ subunit-containing receptors in cultured cerebral cortical neurons and the role of protein kinase C (PKC) β, γ and ε 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 $GABA_A$ receptor $\alpha1$ 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 PKCγ with $\alpha1$ subunits while PKCβ/$\alpha1$ association and PKCε/$\alpha1$ co-localization were not altered by ethanol exposure. Selective PKCγ inhibition by transfection of selective PKCγ siRNAs blocked ethanol-induced internalization of $GABA_A$ receptor $\alpha1$ subunits whereas PKCβ inhibition using pseudo-PKCβ did not have any effect. These findings suggest that ethanol exposure selectively alters PKCγ translocation to $GABA_A$ receptors and PKCγ regulates $GABA_A$ $\alpha1$ receptor trafficking following ethanol exposure.

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Introduction

Chronic ethanol exposure causes CNS hyperexcitability and tremor that has been partly attributed to a loss of $GABA_A$ α1 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 $GABA_A$ α1 subunit protein has been determined to involve the internalization of $GABA_A$ α1 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 α1 $GABA_A$ 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 α1 subunit – containing $GABA_A$ receptor internalization.

$GABA_A$ receptors are heteropentameric ligand-gated ion channels that mediate inhibition in the central nervous system. The α1 subunit receptors are the most abundant subtype of synaptic $GABA_A$ receptor, expressed widely throughout the brain and are generally composed of two α1, two β(2 or 3) and one γ2 subunit that confers sensitivity to benzodiazepines (Sieghart and Sperk, 2002). Global knockout of the $GABA_A$ receptor α1 subunit results in increased seizure susceptibility and essential-like tremor (Kralic et al., 2005). Previous studies have demonstrated that trafficking of α1 subunit-containing $GABA_A$ receptors is critical for synaptic efficacy and can be regulated by many factors,

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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 α1 subunit-containing $GABA_A$ receptors are not yet elucidated. Ethanol induces $GABA_A$ α1 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 PKCε and PKCγ 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 PKCε are more sensitive to the acute effects of ethanol and the deletion of PKCε attenuates ethanol withdrawal-associated seizures in mice (Olive et al., 2001). In contrast, PKCγ 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 PKCβ, γ and ε are associated with $GABA_A$ receptors (Brandon et al., 1999; Kumar et al., 2002). PKC has been shown to alter surface expression of both recombinant $GABA_A$ 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 $GABA_A$ receptors

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it is critical to determine the role of various PKC isoforms since this knowledge may be useful to modulate trafficking of α1 subunit-containing $GABA_A$ receptors.

The present study focused on the role of PKC isoforms on ethanol-induced altered $GABA_A$ α1 receptor expression in cultured cortical neurons. Trafficking of $GABA_A$ receptors was assessed by determination of α1 $GABA_A$ receptor peptide levels on the cell surface by biotinylation of surface proteins. To establish that internalization of α1 subunit peptides represented internalization of α1 subunit-containing $GABA_A$ receptors, we also explored the effects of ethanol on zolpidem (100 nM) enhancement of GABA evoked Cl^- currents to assess the functional consequences of changes in surface expression of α1 subunit receptors. We explored the effects of ethanol on PKCβ, γ and ε expression and their interactions with $GABA_A$ α1 subunits, by co-immunoprecipitation and dual fluorescence confocal microscopy. Furthermore, using PKCγ specific siRNA and a PKCβ inhibitor, we determined the role of these PKC isoforms on ethanol-induced loss of α1 subunit-containing $GABA_A$ receptors.

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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_2 independent medium and minced into small pieces of 1–2 mm^2. The tissue was incubated at 37^0C 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 1.0–1.5x10^6 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 $GABA_A$ 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

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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 $GABA_A$ receptor subunits using specific antibodies. For immunohistochemistry, cells on coverslips were fixed with 4% paraformaldehyde, washed twice with PBS and stored at 4^0C. 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 PKCγ siRNA and PKCβ 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 PKCγ (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'.

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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 PKCγ or scrambled siRNA was transfected using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Selective PKCγ siRNA or scrambled siRNA were mixed in 200 μl of Opti MEM I low serum media (Gibco) with 6 μl 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 PKCγ was maximal at 72 hours. PKCβ pseudo-substrate was obtained from Tocris (Ellisville, Missouri, USA) and used to block PKCβ activity (0.1 μM, final concentration). PKCβ 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 MΩ and filled with internal solution (mM): KCl 150, $MgCl_2$ 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, $CaCl_2$ 2, $MgCl_2$ 1, sucrose 5 and glucose 10 (adjusted to pH 7.4 with NaOH). The sodium-channel blocker, tetrodotoxin (0.5 μM; Sigma, St. Louis MO),

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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 logEC_{50} for GABA was 0.91 ± 0.08 (8.19 μM) and the Hill coefficient was 1.3 ± 0.3. The EC_{10} was estimated to approximately 1.49 μM. Based on these data, we employed a GABA concentration of 1 μM, 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 1μM GABA for 8 seconds to study enhancement of GABA currents. For each neuron, the % potentiation of 1μM 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 12,000xg for 20 min. (Kumar et al., 2002). The pellet

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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^{0}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°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 µl) 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%

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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 μg of P2 fraction protein was solubilized at 4^0C 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 μl) 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 μl of 0.1 M phosphate buffer. $GABA_A$ receptor α1 subunit specific antibody (rabbit polyclonal, Werner Sieghart) (14.5 μl, 628 μg/μl) 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 4^0C in a final volume of 500 μl 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 μl 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 μl of 5 M DTT, subjected

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

Since the expression of α1 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 α1 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 PKCβ and γ with an equivalent sample of α1 subunit receptors from each experimental group of cells. We confirmed that an equivalent amount of α1 subunits was immunoprecipitated across experimental groups. Furthermore, the signal intensity for PKCβ and γ was normalized to the signal intensity for the α1 subunit in the α1 immunoprecipitate to rule out the possibility of non-equivalent immunoprecipitation efficiency between experimental groups (for explanation see Kumar et al., 2002). Co-immunoprecipitation of PKCε with $GABA_A$ α1 subunit receptors is not detected with this method (Kumar et al., 2002). Therefore, co-localization of PKCε with the $GABA_A$ receptor α1 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 diflouride membranes (Invitrogen, Carlsbad, CA). The membranes with transferred proteins were probed with α1 (ABR, Golden, CO), PKCβ, PKCγ and PKCε

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antibodies (BD Biosciences, San Jose, CA USA). Guinea pig α1 subunit antibody (provided by Jean-Marc Fritschy, University of Zurich, Zurich, Switzerland) was used for western blot analysis of the α1 subunit immunoprecipitate to avoid interference with the rabbit α1 subunit antibody used for immunoprecipitation. Blots were subsequently exposed to a second primary antibody directed against β-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-PKCβ (mouse, 1:75 dilution, BD Biosciences, San Jose, CA), anti-PKCε (mouse, 1:100 dilution, BD Biosciences, San Jose, CA), or anti-PKCγ (mouse, 1:75 dilution, BD Biosciences, San Jose, CA) and $GABA_A$ α1 antibody (rabbit, 1:400

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dilution, Novus Biologicals, Littleton, CO) overnight at 4^0C. 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 $GABA_A$ receptor subunits (α1, 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 $GABA_A$ receptor α1 subunits were identified on each coverslip (5/quandrant). Each cell was then scored for co-localization of the PKC isozyme labeled. The total number of α1 subunit/PKC co-localized cells were normalized to total number of α1 subunit-stained cells on the same coverslip and presented as a ratio. Co-localization of each PKC isozyme with $GABA_A$ α1 subunit receptors was determined in independent experiments from 5 coverslips (approx 30 cells/coverslip).

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Results

Ethanol and PKC activator PDBu alter the cell surface expression of $GABA_A$ receptor α1 subunits. Ethanol exposure (50 mM) for 4 hours produced a maximal decrease in expression of $GABA_A$ receptor α1 subunit (40.2 ± 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 $GABA_A$ receptor α1 subunits in the P2 fraction (Figure 1B). Biotinylation of surface proteins confirmed the decrease in cell surface expression of α1 subunits following 4 hours of ethanol exposure (Figure 1C, 38.00 ± 14 %, n=4, $p < 0.05$). Furthermore, unlabeled cytosolic α1 subunit protein levels were increased by 111.1 ± 35% (Figure 1D, n=4, $p < 0.05$,). PKC activators have been shown to internalize $GABA_A$ 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 α1 subunits by 37.3 ± 7% in the P2 fraction (Figure 2A, n=4, $p < 0.05$). Similarly, biotinylation of surface receptors revealed approximately the same decrease in $GABA_A$ receptor α1 subunits (Figure 2B, 44.88 ± 16 %, $p < 0.05$, n=4,) while cytosolic α1 subunit protein levels were increased by 126.5 ± 45% (Figure 2C, n=4, $p < 0.05$, in duplicate). Therefore, ethanol induces the internalization of $GABA_A$ receptor α1 subunits and this effect is mimicked by PKC activation using PDBu.

Ethanol exposure reduces zolpidem enhancement of GABA evoked Cl^- currents.

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The response to application of 1 μM GABA in cortical neurons was not significantly different in neurons treated with vehicle vs. ethanol for 4 hours (vehicle: 133.1 ± 26.50 pA, 4 hr ethanol treated neurons: 187.1 ± 43.94 pA). A low dose of zolpidem (100 nM) selective for alpha-1 subunit-containing $GABA_A$ receptors was chosen to study the effects of ethanol exposure on α1 subunit-containing $GABA_A$ receptors. This dose of zolpidem enhanced responses elicited by 1 μM GABA (~EC_{10}) 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 ± 18.31 (n=14), neurons treated with ethanol for 4 hours: 144.4 ± 14.63 (n=12), *p* value = 0.0052). These data support the conclusion that 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 α1 subunit-containing receptors. Cultured cortical cells were exposed to vehicle, ethanol and ethanol combined with calphostin C (0.3 μM). Calphostin C was added 15 minutes before the start of ethanol exposure. These doses have been shown to inhibit PKC activity and to alter $GABA_A$ receptor function in cortical synaptoneurosomes (Kumar et al., 2005). Calphostin C alone did not alter the expression of α1 subunits in the P2 fraction (Figure 4A). Ethanol exposure for 4 hours decreased the expression of $GABA_A$ receptor α1 subunit expression by 48.2 ± 8 % (n=4, $p < 0.05$,) in the P2 fraction of cultured cortical

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neurons. Calphostin C completely inhibited the ethanol-mediated decrease in $GABA_A$ receptor α1 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^- conductance. First, we examined the effect of Calphostin C alone (0.3 μM, 4 hrs) on currents elicited by application of 1 μM GABA in cortical neurons. Calphostin C alone had no significant effect on the amplitude of 1 μM GABA currents (vehicle: 114.3 ± 20.24 pA, Calphostin C for 4 hours: 152.7 ± 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 μM Calphostin C for 4 hours. We then examined zolpidem (100nM) potentiation of GABA (1 μM) 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 ± 15.50 (n=10), neurons treated with 50 mM Ethanol + 0.3 μM Calphostin C for 4 hours: 191.6 ± 15.58 (n=6), *p* value> 0.5). These data further support the requirement for PKC activity in ethanol-induced internalization of α1 subunit $GABA_A$ benzodiazepine receptors in cultured cortical neurons.

Ethanol increases PKC isoform expression and selectively increases the association of PKCγ with $GABA_A$ receptor α1 subunits. Since the effect of ethanol on $GABA_A$ α1 receptor internalization was mimicked by PKC activation and blocked by PKC inhibition, we investigated whether specific PKC isozymes were involved in these

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effects. Ethanol exposure for 1 hour increased PKCβ, PKCγ and PKCε peptide expression in the P2 membrane fraction of cortical neurons. In contrast, ethanol exposure for 4 hours increased PKCβ and PKCγ, but not PKCε protein levels (Table 1). To determine if these isozymes were translocated and bound to $GABA_A$ α1 subunit receptors, we employed immunoprecipitation studies to determine their association. For this study, intact $GABA_A$ α1 subunit receptors were immunoprecipitated from the P2 fraction using a $GABA_A$ receptor α1 subunit specific antibody. The immunoprecipitate was denatured, separated by SDS PAGE and probed with PKCγ or PKCβ antibodies. Ethanol exposure for 4 hours increased the co-immunoprecipitation of PKCγ with $GABA_A$ receptor α1 subunits by 92.88 ± 24 % ($p < 0.05$, n=4), but did not alter the co-immunoprecipitation of PKCβ with $GABA_A$ receptor α1 subunits (Figure 5A and B). Furthermore, ethanol exposure for 1 hour did not alter association of PKCβ or PKCγ with α1 subunit-containing $GABA_A$ receptors (Table 2).

We also employed dual fluorescence confocal microscopy to visualize the co-localization of PKCβ, γ or ε with $GABA_A$ receptors that contain α1 subunits (Figure 6). In ethanol-naïve cells, $GABA_A$ α1 subunits were co-localized with PKCβ in 44 ± 5% of cells, with PKCγ in 30.34 ± 2% of cells and PKCε in 45.68 ± 2 % of cells (data not shown). Ethanol exposure for 4 hours increased the association of PKCγ with $GABA_A$ receptor α1 subunits by 70.2 ± 15%, ($p < 0.05$, n=5 coverslips/group, 309 α1-labeled cells counted) (Figure 7A) but did not alter the co-localization of PKCβ (n=4 coverslips/group, 377 α1-labeled cells counted) or PKCε (n=5 coverslips/group, 361 α1-labeled cells counted) (Figure 7 B & C) with $GABA_A$ receptor α1 subunits. Furthermore,

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ethanol exposure for 1 hour did not alter the association of PKCβ (n=5 coverslips/group, 306 α1-labeled cells counted), PKCγ (n=5 coverslips/group, 300 α1-labeled cells counted) or PKCε (n=5 coverslips/group, 313 α1-labeled cells counted) with $GABA_A$ receptor αl subunits (Table 3).

PKCγ, but not PKCβ inhibition, reduces ethanol-mediated internalization of $GABA_A$ receptor α1 subunits. Ethanol exposure for 4 hours increased the association of PKCγ with $GABA_A$ receptor α1 subunits and internalization of $GABA_A$ receptor α1 subunits. Therefore, we hypothesized that the internalization of $GABA_A$ receptor α1 subunit was PKCγ dependent. PKCγ siRNA transfection was used to inhibit the effect of ethanol on PKCγ expression in cultured cortical neurons. Selective PKCγ siRNA reduced PKCγ expression by 74.36 ± 7 % ($P<0.05$, n=3, in duplicate) in the P2 fraction of cortical neurons compared to control. However, PKCγ siRNA transfection of cortical neurons had no effect on expression of PKCβ and PKCε isoforms (Figure 8A.). Similarly, PKCγ siRNA transfection did not alter expression of $GABA_A$ receptor α1 subunits. However, the effect of ethanol (4 hrs) on $GABA_A$ receptor α1 subunit expression was blocked by selective PKCγ siRNA transfection (Figure 8B).

Ethanol exposure did not increase the association of PKCβ with $GABA_A$ α1 subunit receptors but increased PKCβ expression in the P2 fraction. Therefore, to further verify the role of PKCβ in ethanol-mediated internalization of α1 subunits, we used PKCβ pseudo-substrate peptide to block the activity of PKCβ. PDBU exposure was used as a positive control to evaluate the effectiveness of PKCβ pseudo-substrate. PDBu

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increased the expression of PKCβ in P2 fraction by 103.2 ± 27% ($p < 0.05$, ANOVA, n=4). PKCβ pseudo-substrate completely inhibited the effect of PDBu on PKCβ expression, demonstrating inhibition of PKCβ expression in the cells. PKCβ pseudo-substrate alone did not alter PKCβ expression. (Figure 9A). Furthermore, PKCβ pseudo-substrate had no effect on the ethanol-mediated decrease in $GABA_A$ receptor α1 subunits (Figure 9B).

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Discussion:

The present study demonstrates that ethanol exposure *in vitro* increases internalization of $GABA_A$ receptor α1 subunits in a time-dependent manner. The effect of ethanol on $GABA_A$ receptor α1 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 alpha1 containing receptors are significantly reduced following ethanol exposure and this effect is also dependent upon PKC. Ethanol exposure increases PKCβ, ε and γ isoform expression, but ethanol-induced association with $GABA_A$ receptor α1 subunits is selective for PKCγ isoforms. PKCγ, but not PKCβ, inhibition prevented ethanol-induced internalization of $GABA_A$ receptor α1 subunits. Thus, these data suggest that decreased expression of α1 subunits at the cell surface following ethanol exposure is mediated by PKCγ activity. The effect of PKCγ 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 PKCγ siRNA would lack functional significance, since the PKC inhibitor calphostin C altered the effect of ethanol on zolpidem responses.

The regulation of $GABA_A$ receptor α1 subunit surface expression appears to be a conserved mechanism both *in vitro* and *in vivo*. The effects of ethanol exposure on $GABA_A$ α1 subunit receptors in cultured cerebral cortical neurons mimics the effects of chronic ethanol exposure *in vivo*, as α1 subunits are reduced following ethanol exposure in both experimental paradigms. The ethanol-induced reduction in $GABA_A$ receptor α1 subunits are thought to contribute to ethanol dependence. Studies have shown that α1

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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 $GABA_A$ receptor α1 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 α1 subunit receptors. Together, these data suggest that loss of α1 containing $GABA_A$ 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 α1 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^- currents in the cortical cultured neurons supports the conclusion that ethanol reduces the surface expression of synaptic α1 subunit-containing $GABA_A$ 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 $GABA_A$ benzodiazepine (α1,β2/3,γ2) 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 $GABA_A$ receptor surface expression in these studies did not involve withdrawal from ethanol. This is consistent with our studies on the effects of

ethanol on α1 subunit-containing $GABA_A$ receptors in the cerebral cortex (Kumar et al., 2003) and with the effects of ethanol on α1 subunit expression in cerebral cortical and hippocampal cultured neurons (Sheela Rani and Ticku, 2006). Other studies suggest that ethanol-induced internalization of α1 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 $GABA_A$ receptors are needed..

Surface expression of $GABA_A$ 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 $GABA_A$ receptors. In the present study, despite the increases in PKCβ, γ and ε expression, ethanol exposure for 1 hour did not alter the surface expression of $GABA_A$ receptor α1 subunits. This may indicate that there was no endocytosis of α1 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 α1 subunit receptors in CCVs or alter association of α1 subunits with adaptin-α that is required for $GABA_A$ 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 $GABA_A$ receptor α1 subunits. Systematic study of these possibilities is needed to further understand effects of ethanol on $GABA_A$ 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 PKCβ, γ and ε expression in the membrane fraction of cultured cortical neurons without altering their association with $GABA_A$ α1 subunits. Therefore, it is possible that theses PKC isoforms are targeted to other subtypes of $GABA_A$ 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 $GABA_A$ 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 PKCγ co-localization and co-immunoprecipitation with $GABA_A$ receptor α1 subunits. Furthermore, surface expression of $GABA_A$ α1 receptors was decreased after 4 hours of ethanol exposure suggesting that PKCγ is necessary for ethanol regulation of α1 receptor internalization. Previous studies have shown that *in vivo* chronic ethanol exposure (two weeks) increases endocytosis of α1 subunit-containing $GABA_A$ receptors into CCVs of cerebral cortex (Kumar et al.,

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2003), but this effect is accompanied by a decrease in PKCγ co-immunoprecipitation with $GABA_A$ α1 receptors (Kumar et al., 2002). It is possible that ethanol effects on PKCγ association with $GABA_A$ 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 $GABA_A$ receptor function following ethanol exposure *in vivo* and *in vitro*. For example, mice lacking the gene for PKCγ show a significant reduction in ethanol potentiation of muscimol-stimulated Cl^- influx compared to responses in wild type mice (Harris et al., 1995). In contrast, ethanol and flunitrazepam potentiation of muscimol-stimulated Cl^- uptake is greater in microsacs from PKCε null mutant mice compared to wild-type controls (Hodge et al., 1999). In our study, ethanol exposure did not alter PKCβ or ε association with $GABA_A$ α1 receptors following ethanol exposure. While PKCβ and PKCε may not be responsible for ethanol-induced receptor trafficking, these kinases clearly play a role in receptor activity. For example, PKCε has been shown to alter phosphorylation of $GABA_A$ receptor γ2 subunit and receptor function following ethanol exposure (Qi et al., 2007). In addition PKCβ has been shown to phosphorylate $GABA_A$ receptor β1 subunits (Brandon et al., 1999). Thus, ethanol exposure may alter the association of these PKC isoforms with other subunits of $GABA_A$ receptors and thereby regulate the trafficking of distinct receptor subtypes. Further studies are needed to explore these possibilities.

The present work demonstrates that PKCγ plays an essential role in the regulation of the surface expression of α1 subunit-containing $GABA_A$ receptors and highlights the

possibility of modulating GABAergic activity through selective targeting of PKC isoforms. Altered $GABA_A$ receptor α1 subunit expression is implicated in anxiety, alcoholism, epilepsy and many other neurological disorders. Therefore, an understanding of the mechanism of $GABA_A$ receptor α1 subunit trafficking could lead to new therapeutic approaches that aim to restore normal surface expression of $GABA_A$ receptors.

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Footnotes

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Figure Legends

Figure 1. Ethanol exposure alters $GABA_A$ receptor α1 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 α1 subunit in the P2 fraction whereas panel C and D shows representative western blots of α1 subunit on surface and cytosol respectively. (A) Ethanol exposure for 1 hour did not alter α1 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 α1 subunit expression whereas 50 mM ethanol decreased α1 subunit expression by 40% in the P2 fraction of cells. Following biotinylation of surface receptors, ethanol exposure for 4 hours decreased α1 subunit level on cell surface (C) and increased α1 subunit protein levels in cytosol (D). Data represents mean ± SEM. * $p < 0.05$

Figure 2. PDBu alters $GABA_A$ receptor α1 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 α1 subunit following PDBu exposure. (B & C) Purification of surface (B) and cytosolic (C) receptors by biotinylation confirms increased internalization of $GABA_A$ receptor α1 subunits. Data represents mean ± SEM. * $p < 0.05$, Student's *t* test

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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 μM GABA, co-application of 1 μM GABA with 100 nM zolpidem and subsequent washout with 1 μM GABA following (A) vehicle and (B) ethanol exposure (50mM) for 4 hrs. (C) shows that zolpidem (100nM) potentiation of the 1 μM GABA response in cortical neurons was significantly diminished in ethanol-treated vs. vehicle-treated neurons. Data represent the mean ± SEM. ** $p < 0.001$.

Figure 4. **PKC inhibitor calphostin C blocks ethanol-mediated internalization of $GABA_A$ receptor α1 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 $GABA_A$ receptor α1 subunits. (B) Ethanol exposure for four hours decreased the expression of α1 subunits and this was blocked by Calphostin C. * $p < 0.05$, Newman-Keuls test, compared to control. Data represents mean ± SEM. (C) Calphostin C alone had no significant effect on zolpidem enhancement of 1 μM 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

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prevented the decrease in zolpidem potentiation of 1 μM 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 PKCγ with α1-containing $GABA_A$ receptors in cultured cortical neurons. Receptors in the P2 fraction of control (lane 1) and ethanol-exposed cells (lane 2) were immunoprecipitated with α1 subunit antibody. Immunoprecipitated receptors were separated by SDS-PAGE and immunoblotted with PKCγ or PKCβ antibodies. The same blot was probed by α1 subunit antibody. PKCγ or PKCβ signal intensity was normalized to $GABA_A$ receptor α1 subunit signal intensity from the western blots of the immunoprecipitate. (A) Association of PKCγ with α1-containing $GABA_A$ receptors was increased 92.88 ± 23.60 % in cultured neurons after 4 hour ethanol exposure (B) There was no change in PKCβ association with α1-containing receptors after 4 hour ethanol exposure. Data represents mean ± SEM. * $p < 0.05$, Student's *t* test.

Figure 6. PKC isoforms are co-localized with $GABA_A$ receptor α1 subunits in ethanol naïve cells. Cortical neurons were stained with anti-α1 and PKCβ, PKCγ or PKCε antibodies for confocal microscopy. Images A, D, G are α1 labeled cells while, B, E and H are PKCβ, γ and ε labeled cells respectively. Images C, F, and I are merged images of dual immuno-staining of α1 subunit and the PKC isoform. The yellow color in merged panels (arrow) indicates co-localization of the two proteins.

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Figure 7. Ethanol increases PKCγ, but not PKCβ or PKCε, colocalization with $GABA_A$ receptor α1 subunit in cultured cortical neurons: Cell were exposed to ethanol for 4 hours and stained with anti-α1 and PKC antibodies. Immunostaining of α1 and PKC labeled cells were examined under confocal microscope. The total number of α1/PKC co-localized cells were normalized to total number of α1- stained cells on the same coverslip and presented as percent control values. Ethanol exposure for 4 hours increased PKCγ association with $GABA_A$ receptor α1 subunit by 70.17 ± 15 % (A). In contrast, ethanol exposure did not alter the association of PKCβ or PKCε with $GABA_A$ receptor α1 subunit compared to controls (B& C). Data represents mean ± SEM. * $p < 0.05$, Student's *t* test.

Figure 8. PKCγ RNAi transfection inhibits ethanol-induced internalization of $GABA_A$ receptor α1 subunit in cultured cortical cells. Cerebral cortical neurons were transfected with scrambled or PKCγ siRNA. The P2 fraction was analyzed by SDS-Page analysis and probed with PKCγ, β and ε antibodies. **(A)** PKCγ specific, but not scrambled, siRNA decreased the expression of PKCγ by 76.09 ± 1.087%, whereas the expression of PKCβ and ε were not altered. (B) Selective PKCγ siRNA inhibits ethanol (50 mM)-induced internalization of $GABA_A$ receptor α1 subunit. * $p < 0.05$ compared to vehicle, Newman-Keuls test.

Figure 9. PKCβ inhibition does not prevent ethanol-induced internalization of $GABA_A$ receptor α1 subunit. Cultured cortical cells were exposed to PDBu and or

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PKCβ pseudo substrate. (A) PDBu increased the PKCβ expression in P2 fraction by 203 ± 26%. The PDBu-induced increase in PKCβ expression was blocked by PKCβ pseudosubstrate. PKCβ pseudosubstrate alone did not affect PKCβ expression. (B) Ethanol exposure for 4 hours reduced the expression of $GABA_A$ receptor α1 subunit and PKCβ pseudo substrate had no effect on $GABA_A$ receptor α1 subunit expression following ethanol exposure. * $p < 0.05$ compared to vehicle, Newman-Keuls test.

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

	PKCβ	PKCγ	PKCε
EtOH 1 hour	↑ 60.8 ± 24*	↑ 78.1 ± 27% *	↑ 24.3 ± 10%*
EtOH 4 hours	↑ 51.1 ± 22*	↑ 52.3 ±19% *	↓ 13.6 ± 29%

EtOH: 50 mM, final concentration
The values are mean ± SEM from 4 experiments performed in duplicate.
* p 0.05< compared to control

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Table 2.
Co-immunoprecipitation of PKC isoforms with $GABA_A$ α1 subunit receptors following ethanol (50 mM) exposure for 1 hour

Association of	Vehicle	EtOH
PKCβ	100.0 ± 9	102.9 ± 9
PKCγ	100.0 ± 11	104.8 ± 13

The values are mean ± SEM from 4 experiments performed in duplicate.

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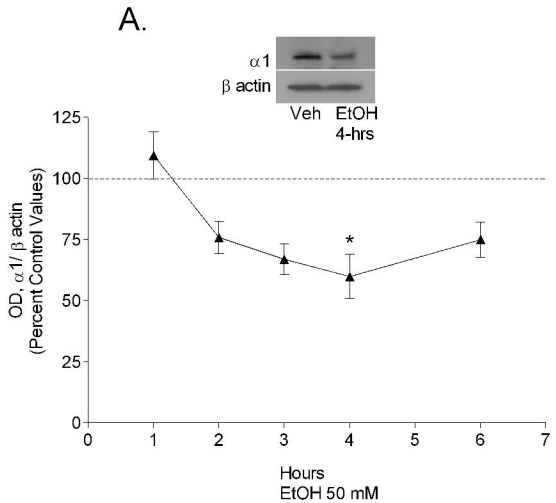
Table 3

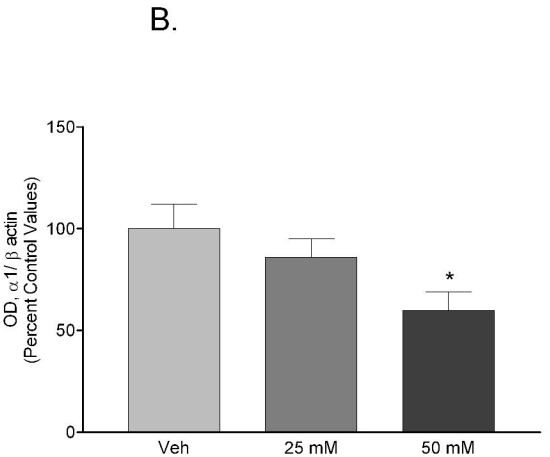
Co-localization of PKC isoforms with $GABA_A$ receptor α1 subunit following 1 hour ethanol (50mM) exposure

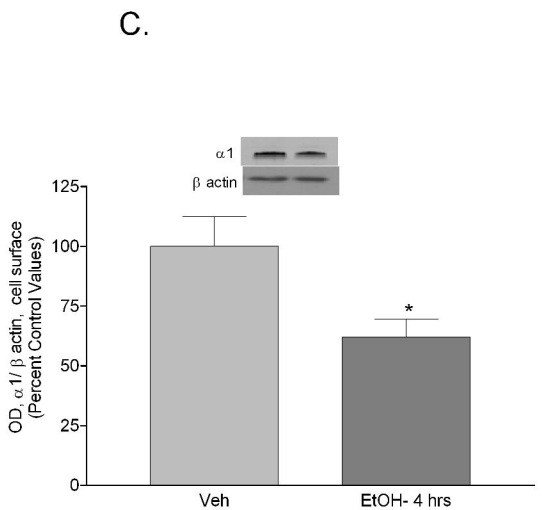
Association with $GABA_A$ α1	Vehicle 1 hour	EtOH 1 hour
PKCβ	100.0 ± 11	95.4 ± 16
PKCγ	100.0 ± 11	112.4 ± 16
PKCε	100.0 ± 8	116.2 ± 4

Data represent the percent change in the ratio of the number of α1/PKC co-localized cells to the total number of $GABA_A$ receptor α1 subunit-stained cells.

Figure 1


A.
α1
β actin
Veh
EtOH
4-hrs
OD, α1/ β actin
(Percent Control Values)
125
100
75
50
25
0
0
1
2
3
4
5
6
7
*
Hours
EtOH 50 mM


B.
OD, α1/ β actin
(Percent Control Values)
150
100
50
0
*
Veh
25 mM
50 mM


C.
α1
β actin
OD, α1/ β actin, cell surface
(Percent Control Values)
125
100
75
50
25
0
*
Veh
EtOH- 4 hrs

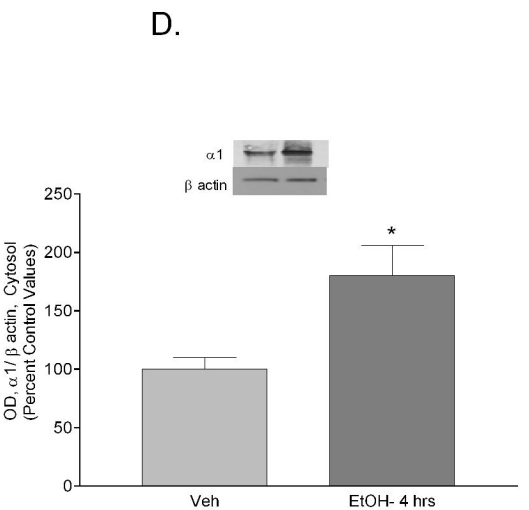

D.
α1
β actin
OD, α1/ β actin, Cytosol
(Percent Control Values)
250
200
150
100
50
0
*
Veh
EtOH- 4 hrs

Figure 2

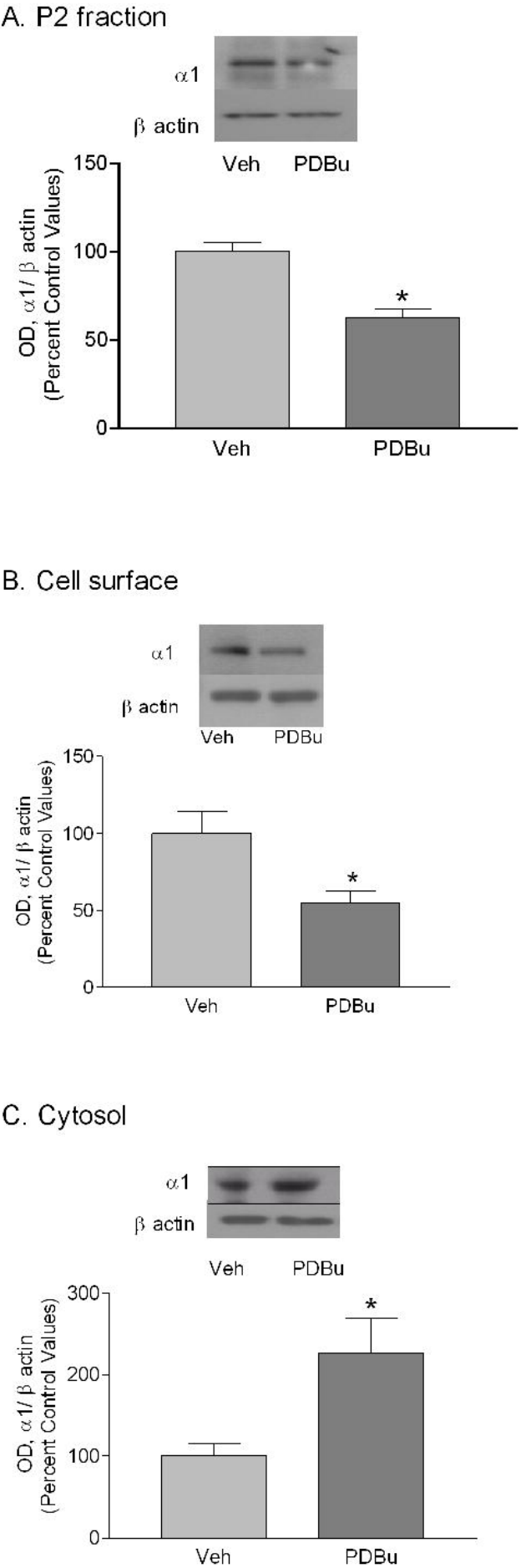

A. P2 fraction
α1
β actin
Veh
PDBu
150
100
50
0
OD, α1/ β actin
(Percent Control Values)
*
Veh
PDBu
B. Cell surface
α1
β actin
Veh
PDBu
150
100
50
0
OD, α1/ β actin
(Percent Control Values)
*
Veh
PDBu
C. Cytosol
α1
β actin
Veh
PDBu
300
200
100
0
OD, α1/ β actin
(Percent Control Values)
*
Veh
PDBu

Figure 3

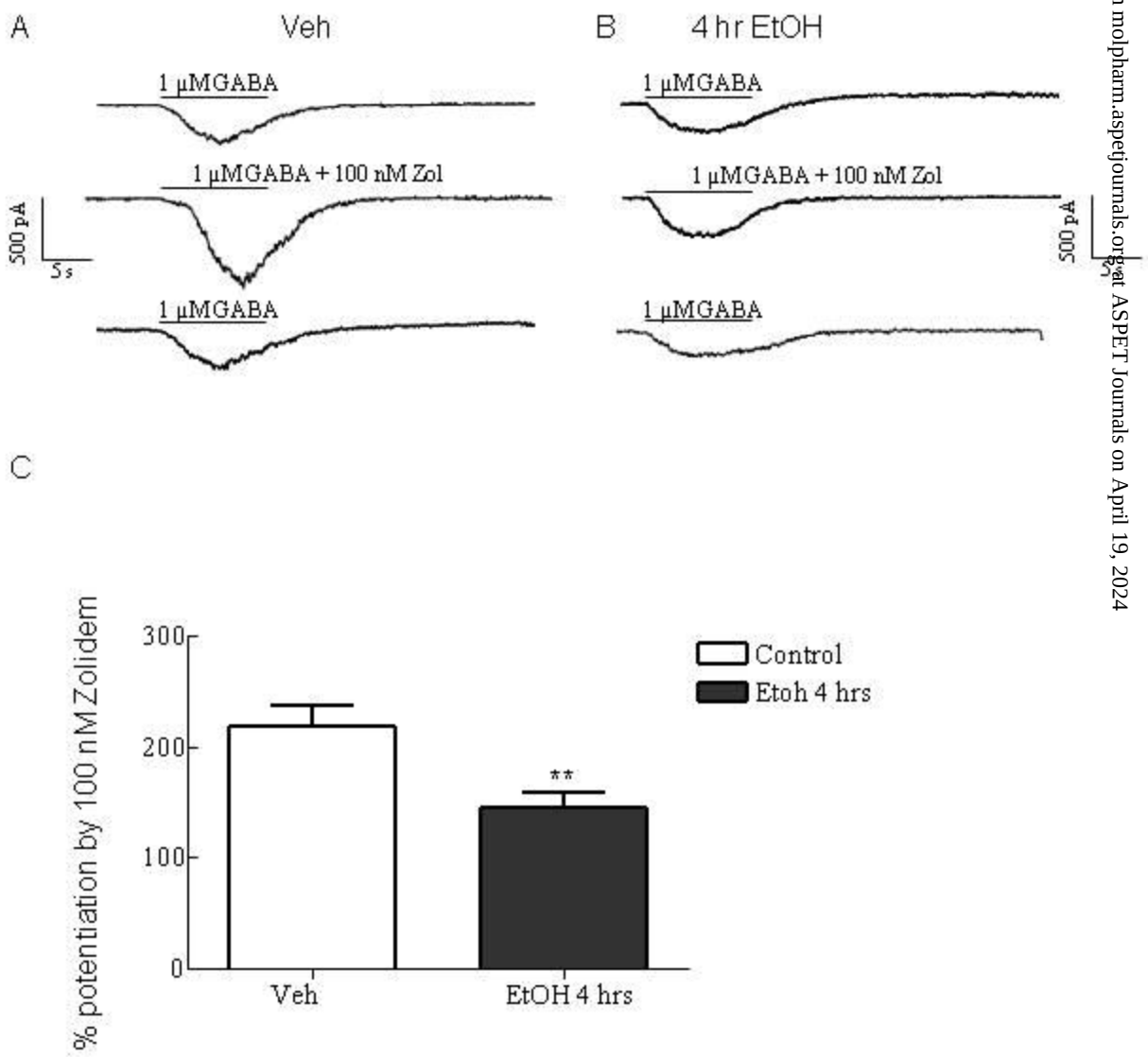


Figure 4

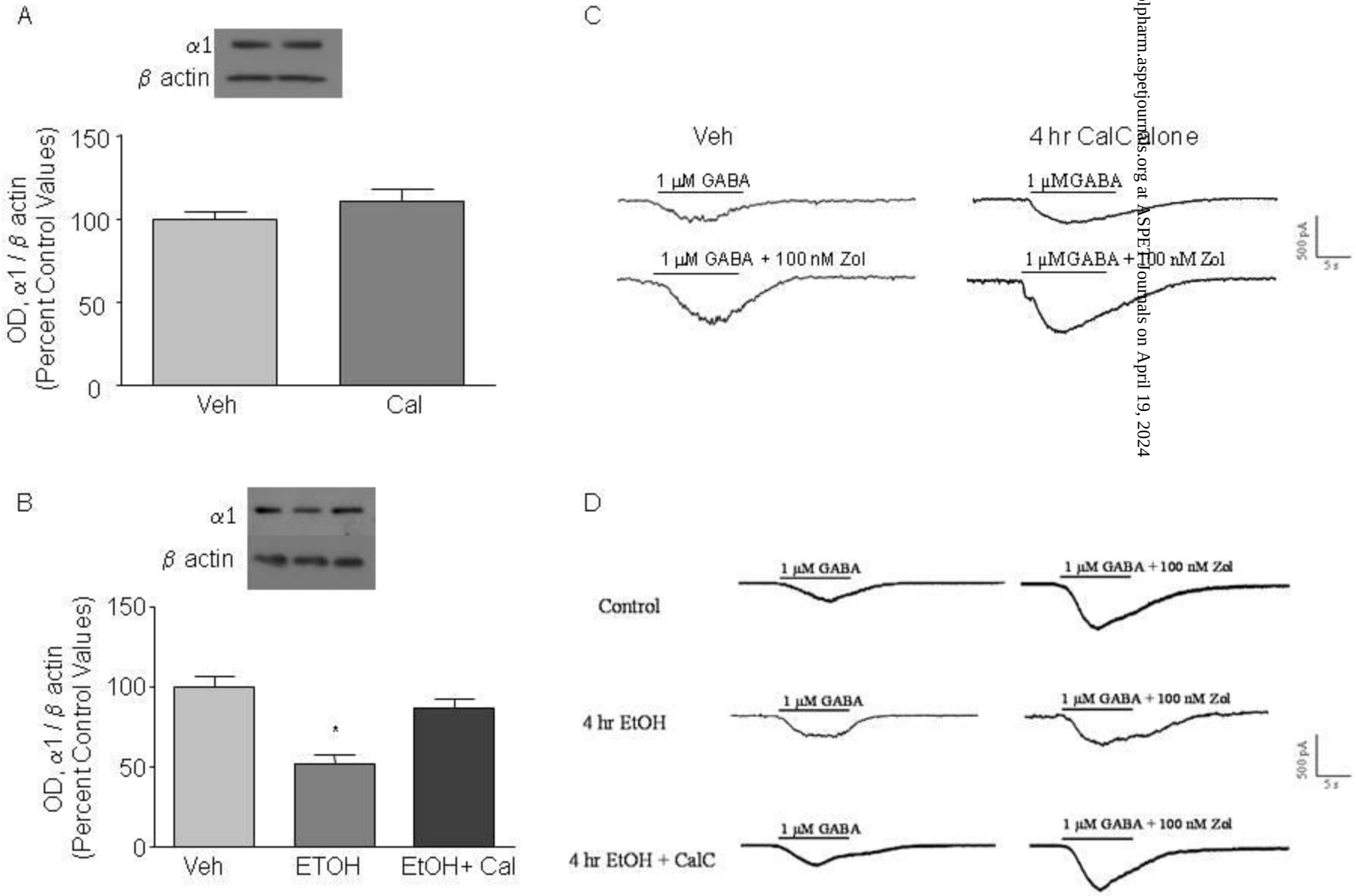
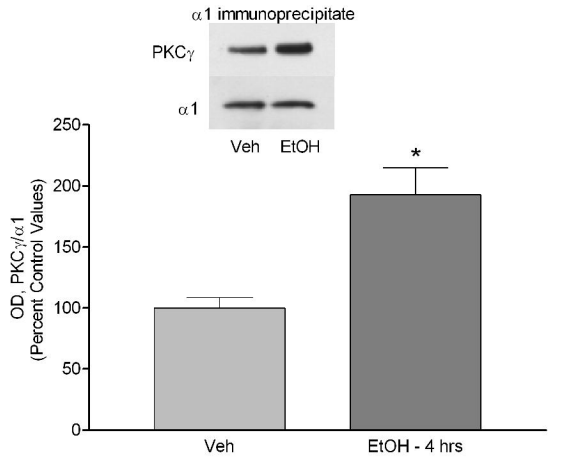


Figure 5

A


α1 immunoprecipitate
PKCγ
α1
Veh
EtOH
250
200
150
100
50
0
OD, PKCγ/α1
(Percent Control Values)
*
Veh
EtOH - 4 hrs

B

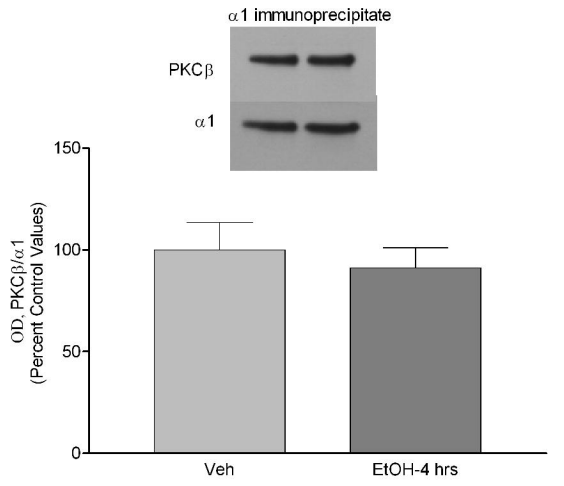

α1 immunoprecipitate
PKCβ
α1
150
100
50
0
OD, PKCβ/α1
(Percent Control Values)
Veh
EtOH-4 hrs

Figure 6

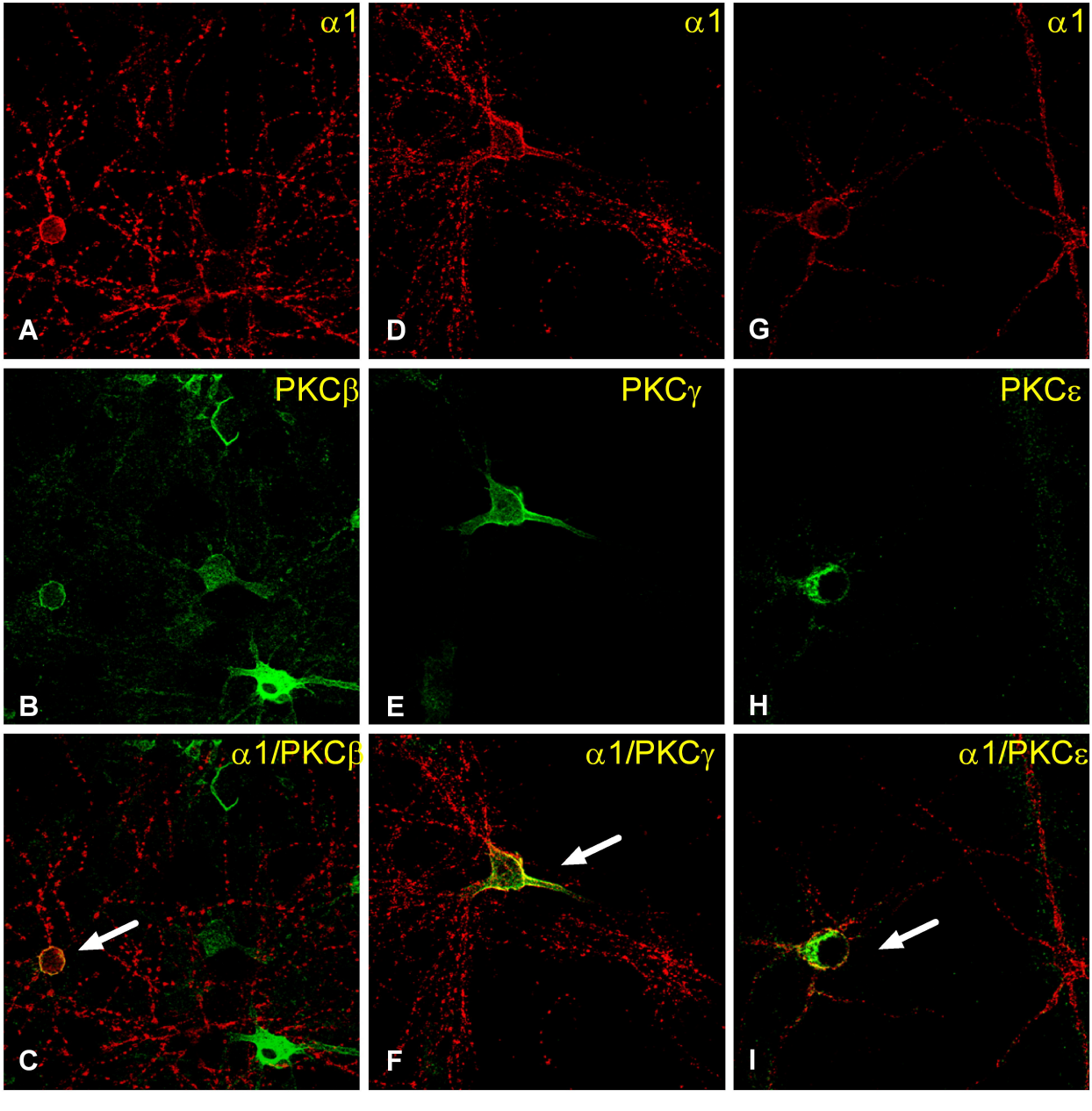
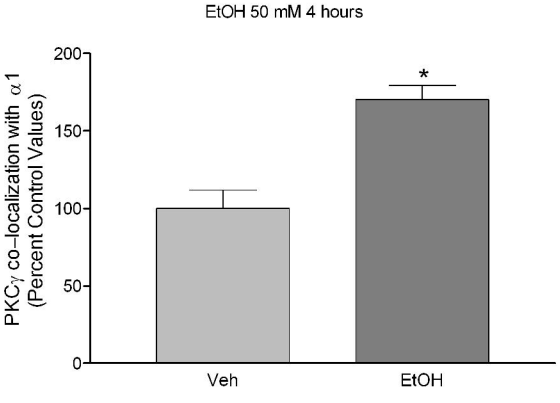
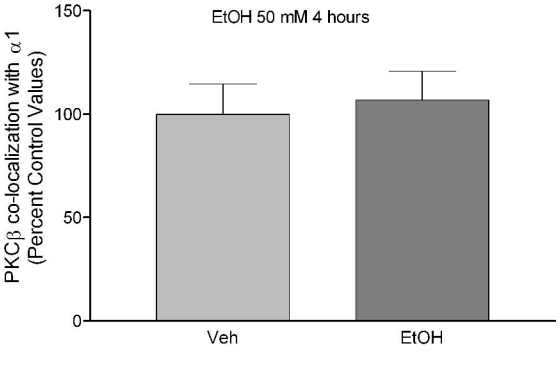


Figure 7

A


EtOH 50 mM 4 hours
PKCγ co-localization with α1
(Percent Control Values)
200
150
100
50
0
*
Veh
EtOH

B


EtOH 50 mM 4 hours
PKCβ co-localization with α1
(Percent Control Values)
150
100
50
0
Veh
EtOH

C

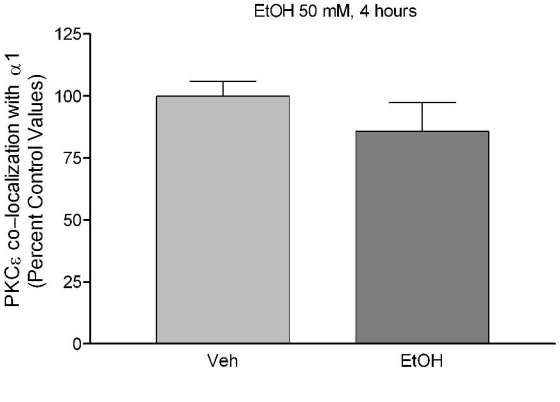

EtOH 50 mM, 4 hours
PKCε co-localization with α1
(Percent Control Values)
125
100
75
50
25
0
Veh
EtOH

Figure 8

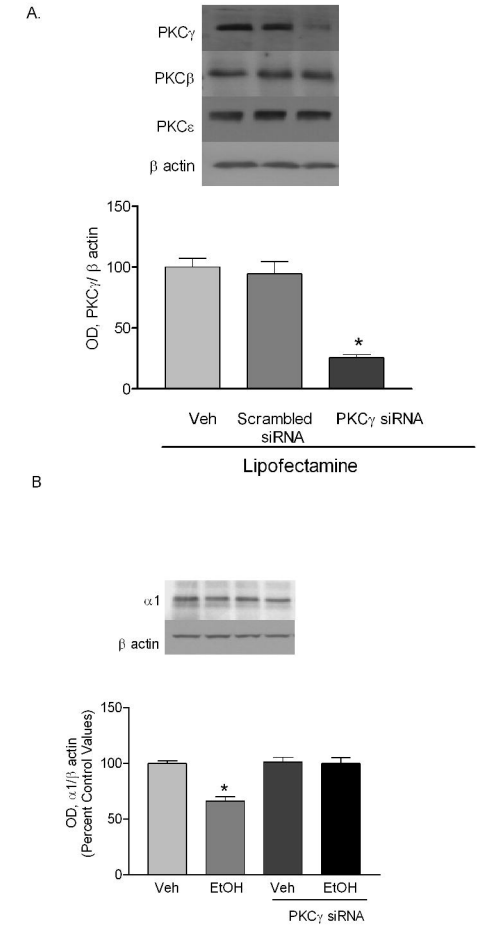
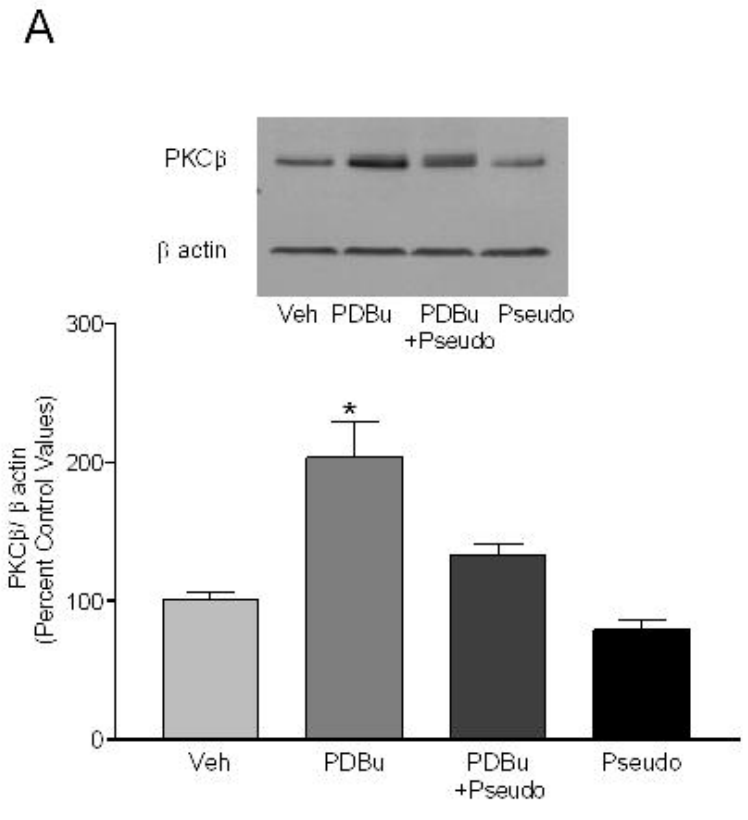
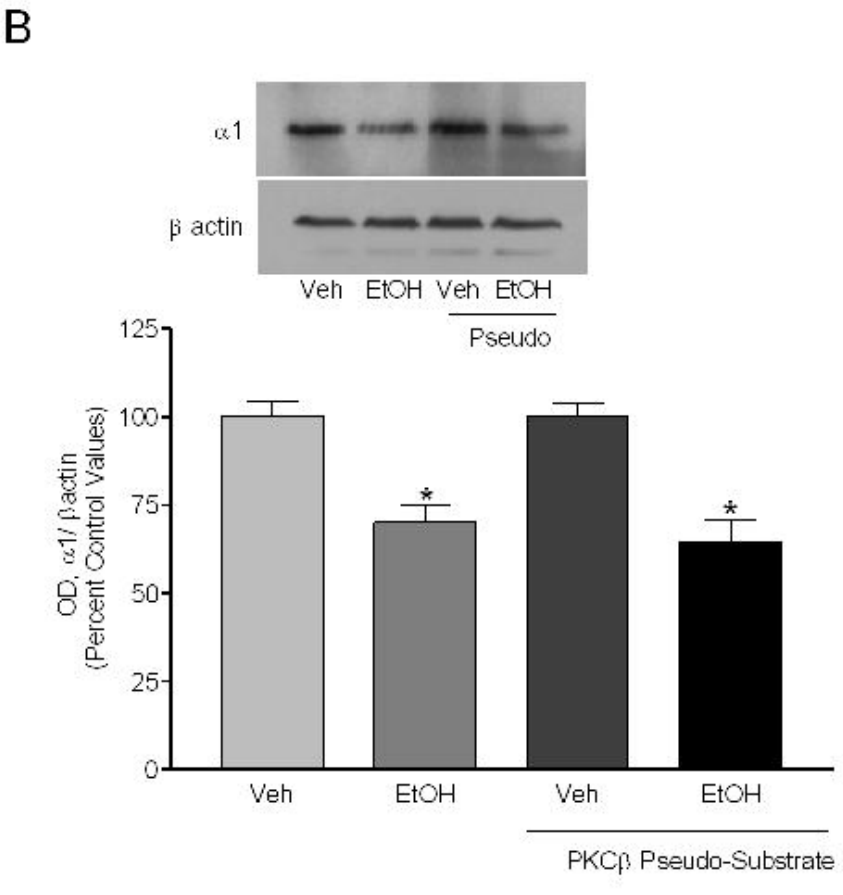

A.
PKCγ
PKCβ
PKCε
β actin
OD, PKCγ/ β actin
150
100
50
0
*
Veh
Scrambled siRNA
PKCγ siRNA
Lipofectamine
B
α1
β actin
OD, α1/β actin (Percent Control Values)
150
100
50
0
*
Veh
EtOH
Veh
EtOH
PKCγ siRNA

Figure 9


A
PKCβ
β actin
Veh PDBu PDBu +Pseudo Pseudo
PKCβ/ β actin (Percent Control Values)
300
200
100
0
*
Veh
PDBu
PDBu +Pseudo
Pseudo


B
α1
β actin
Veh EtOH Veh EtOH
Pseudo
OD, α1/ βactin (Percent Control Values)
125
100
75
50
25
0
*
*
Veh
EtOH
Veh
EtOH
PKCβ Pseudo-Substrate