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# PKCγ is required for ethanol-induced increases in $GABA_A$ receptor α4 subunit expression in cultured cerebral cortical neurons

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## Abstract

Ethanol exposure produces alterations in $GABA_A$ receptor function and expression associated with CNS hyperexcitability, but the mechanisms of these effects are unknown. Ethanol is known to increase both $GABA_A$ receptor α4 subunits and protein kinase C (PKC) isozymes *in vivo* and *in vitro*. Here, we investigated ethanol regulation of $GABA_A$ receptor α4 subunit expression in cultured cortical neurons to delineate the role of protein kinase C. Cultured neurons were prepared from rat pups on post-natal day 0-1 and tested after 18 days. $GABA_A$ receptor α4 subunit surface expression was assessed using P2 fractionation and surface biotinylation following ethanol exposure for 4 hrs. Miniature inhibitory postsynaptic currents (mIPSCs) were measured using whole cell patch clamp recordings. Ethanol increased $GABA_A$ receptor α4 subunit expression in both the P2 and biotinylated fractions, while reducing the decay time constant in mIPSCs, with no effect on γ2 or δ subunits. PKC activation mimicked ethanol effects, while the PKC inhibitor calphostin C prevented ethanol-induced increases in $GABA_A$ receptor α4 subunit expression. PKCγ siRNA knockdown prevented ethanol-induced increases in $GABA_A$ receptor α4 subunit expression, but inhibition of the PKCβ isoform with PKCβ pseudosubstrate had no effect. We conclude that PKCγ regulates ethanol-induced alterations in α4-containing $GABA_A$ receptors.

## Introduction

Ethanol exposure results in neuroadaptive consequences such as tolerance and withdrawal-related hyperexcitability. These adaptations can occur not only after chronic exposure (Cagetti *et al.* 2003, Devaud *et al.* 1995), but also after a single ethanol exposure (Liang *et al.* 2007). Tolerance and withdrawal-related effects are thought to contribute to alcohol abuse and alcoholism, but the molecular mechanism by which the adaptations occur remains

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elusive. By understanding the underlying mechanisms of ethanol's neuroadaptation, we may gain valuable insight of how to treat alcohol abuse and alcoholism.

Ethanol affects numerous targets in the central nervous system. However, γ-aminobutyric acid type A ($GABA_A$) receptors remain one of the most likely candidates. Much evidence suggests that alterations in $GABA_A$ receptor responses may be critical for ethanol adaptations (Kumar *et al.* 2009). Behaviorally, acute and chronic ethanol exposures produce cross-tolerance to certain $GABA_A$ receptor agonists such as benzodiazepines (Mihic *et al.* 1992, Liang *et al.* 2007) and exacerbate bicuculline seizure susceptibility (Devaud *et al.* 1998). Similarly, on a functional level, $GABA_A$ receptors are potentiated by ethanol, and this potentiation is blunted following ethanol exposure (Morrow *et al.* 1988, Liang, 2006 #5074). Also, enhancement of $GABA_A$ receptor electrophysiological responses by benzodiazepines is blunted following an acute or chronic ethanol exposure (Cagetti *et al.* 2003).

$GABA_A$ receptors are pentameric ligand-gated chloride ion channels that mediate the majority of rapid inhibition in the central nervous system. $GABA_A$ receptor subtypes can be assembled from 19 different subunits, however, the most common arrangement found is 2α subunits and 2β subunits, with either a γ or δ subunit (Olsen & Sieghart 2008). Some receptor subtypes are also restricted in their synaptic localization and function. Synaptically localized receptors often contain α1 and γ2 subunits and contribute to phasic inhibition (Farrant & Nusser 2005). Such receptors tend to have low potency for GABA, but high efficacy (Whittemore *et al.* 1996). Conversely, tonic inhibition is mediated by extrasynaptic $GABA_A$ receptors that exhibit high sensitivity to GABA and ethanol and often contain α4 and δ subunits (Santhakumar *et al.* 2006, Wei, 2004 #4791).

Many studies have made it abundantly clear that $GABA_A$ receptor subunit expression is altered following ethanol exposure (Kumar *et al.* 2009). $GABA_A$ receptor α4 subunit expression is consistently increased following chronic ethanol exposure and after a single high dose ethanol exposure *in vivo* (Liang *et al.* 2007). Increases in $GABA_A$ receptor α4 subunit expression have been associated with a change from extrasynaptic to synaptic localization. This increase in synaptic α4-containing receptors is also accompanied with alterations in physiological responses. Chronic ethanol exposure in vivo causes miniature inhibitory post-synaptic currents (mIPSCs) to be shortened thereby reducing the net inhibition of the postsynaptic neurons (Liang *et al.* 2004, Liang *et al.* 2007, Cagetti, 2003 #5064).

While the biochemical and functional significance of increased $GABA_A$ receptor α4 subunit expression is known, our understanding of the underlying mechanism is restricted. Recent work has investigated transcriptional regulation of *Gabra4*, the gene for the $GABA_A$ receptor α4 subunit. Work by Pignataro *et al.*, (2007) demonstrated that *Gabra4* is rapidly activated by low to moderate concentrations of ethanol, an effect that is dependent on heat shock factor 1. Protein kinase C (PKC) may be involved in post-translational regulation of α4-containing $GABA_A$ receptors as the intracellular loop of the $GABA_A$ receptor α4 subunit contains consensus sites for PKC (Wisden *et al.* 1991, Macdonald 1995). Our lab has also reported PKCγ is associated with $GABA_A$ receptors containing α4 subunits in the cerebral cortex (Kumar *et al.* 2002). In other brain regions, PKCδ has been demonstrated to co-localize with extrasynaptic $GABA_A$ receptors containing α4 subunits and also enhance their responses to ethanol (Choi *et al.* 2008). Interestingly, both PKCγ and δ knockout mouse models exhibit reduced behavioral response to acute ethanol and PKCγ knockouts display reduced tolerance (Bowers *et al.* 1999, Choi *et al.* 2008). Given the association of these PKC isoforms and altered ethanol-induced behavioral effects, it is possible that PKC regulation of

$GABA_A$ receptors containing α4 subunits may contribute to ethanol-related neuroadaptive responses.

Recently, our lab has demonstrated that PKCβ and PKCγ isoform expression are increased following ethanol exposure of cultured cortical neurons, while PKCδ is undetectable (Kumar *et al.* 2010). In the present study, we 1) assessed the effects of physiologically relevant ethanol concentrations on $GABA_A$ receptor α4 subunit expression and function, and 2) determined the role of PKC in $GABA_A$ receptor α4 subunit expression. Surface expression of α4 subunits was assessed using biotinylation techniques, whereas mIPSCs were measured to assess the functional properties of $GABA_A$ receptors. PKC involvement was determined using a general PKC activator and inhibitor, as well as PKCγ-specific siRNAs, and a selective PKCβ inhibitor.

## Material and Methods

### Cultured Cerebral Cortical Neurons

All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee. Cultured cerebral cortical neurons were isolated as described elsewhere (Kumar *et al.* 2010, Fleming, 2009 #6075). Briefly, rat pups from Sprague-Dawley breeding pairs (Harlan, Indianapolis, IN) were decapitated on postnatal day 0-1. Brains were rapidly dissected and the cerebral cortices were isolated. Cortical halves were minced into fine pieces and tissue was incubated in $CO_2$-independent media containing papain (50U/ml, Worthington, Lakewood, NJ), L-cysteine and DNase (both from Sigma, St. Louis, MO) for 30 min at 37°C. Tissue pieces were gently washed followed by gentle trituration in Dulbecco's modified eagle's medium containing 10% horse serum, penicillin-streptomycin (Pen-Strep) and DNase. Cells used for biochemistry were plated onto poly-D-lysine–coated flasks, while cells used for electrophysiology were plated onto poly-D-lysine–coated cover slips. Cells were maintained in a 5% $CO_2$ humidified incubator. After day 3, cells were fed with serum-free medium containing B27 and Pen-Strep (10,000U/ml; final concentration 50U per flask) to discourage glial overgrowth. Media was changed twice per week with no more than one-third of the media being removed during exchanges. For siRNA experiments, Pen-Strep was removed from cultures on day 14. Cultures were maintained for at least 17 days before conducting experiments, since prior studies determined that GABAA receptors expression was stable between 15-19 days in vitro (Kumar *et al.* 2010).

### Ethanol and Drug Exposure

For ethanol exposure, cells were incubated in media containing 50mM ethanol and placed into an enclosed plastic vapor chamber inside the incubator. A beaker of water containing 50mM ethanol was used to maintain stable ethanol concentrations in the chamber. Control cells had media that did not contain ethanol and were placed in a vapor chamber with a beaker containing only water. The 50mM ethanol was used as this concentration may be achieved during binge sessions in the population. The four hour time point was used here was based on preliminary experiments as well as evidence from other reports indicating changes in $GABA_A$ receptor subunits under similar conditions (Pignataro *et al.* 2007, Kumar *et al.* 2010).

To examine PKC involvement, calphostin C (0.3μM final concentration) and phorbol-12,13-dibutyrate (PDBu,, 100nM final concentration) were dissolved in 0.1% dimethyl sulfoxide. Calphostin C was added 15 min prior to ethanol exposure. PKCβ pseudosubstrate (0.1μM final concentration, Tocris, Ellisville, MO) was used to specifically inhibit PKCβ. PKCβ pseudosubstrate was added at the beginning of the experiment and again at 2hrs to maintain

inhibition. To examine the involvement of PKCγ, 3 different pairs of siRNA sequences specific to PKCγ were used simultaneously as described elsewhere (Kumar *et al.* 2010). siRNA sequences were 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′.

Neurons at 14 days *in vitro* were transfected with either PKCγ-specific or scrambled siRNA using Lipofectamine RNAiMAX (Invitrogen) as recommended by the manufacturer's protocol. Briefly, siRNA was mixed in 200μl OptiMEM I low-serum media (Invitrogen, Carlsbad, CA) with 6μl of Lipofectamine reagent. Following a 20 min incubation period for complex formation, and the mixture was added to cells (final concentration: 6pmol). Cells were gently rocked and placed back in the incubator. Cells were then used for experiments 72 h later (D17) due to maximal PKCγ knockdown at this time point.

## P2 Fractionation

Following completion of experiments, cells were removed from the vapor chambers, washed with ice-cold PBS and cells were scraped, centrifuged at 1000g and stored at −80°C until further use. P2 fractions were isolated as described elsewhere (Kumar *et al.* 2010). Briefly, cell pellets were homogenized in 0.32M sucrose followed by centrifugation at 1000g. The resulting supernatant was then spun twice in PBS at 12,000g. The final pellet was resuspended in PBS with phosphatase inhibitor cocktail I (Sigma, St. Louis, MO), quantified using a bicinchoninic acid method and stored at −80°C until western blot analysis.

## Biotinylation of Cell Surface Proteins

Cell surface expression was conducted using a biotin kit according to the manufacturer's protocol (Pierce, Rockford, IL). Following ethanol exposure, cells were washed with ice-cold PBS and sulfosuccinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate diluted in PBS was immediately added to each flask. Cells were gently rocked for 30 min at 4°C. Following biotinylation, unbound biotin was inactivated using a quenching solution. Cells were scraped and spun at 500g. Cell pellets were then washed with TBS and spun at 500g 3 times. Cells were then lysed using the supplied lysis buffer and sonication. Cell lysates were then incubated with NeutrAvidin slurry to bind biotin-labeled proteins. The cytosolic proteins were isolated by centrifugation and biotinylated cell surface proteins were then eluted by either incubation in Laemmli SDS-PAGE sample buffer at room temperature for 60 min or by incubation at 95°C for 5 min. Samples were then separated by gel electrophoresis and α4 subunits were detected by western blotting.

## Western Blot Analysis

$GABA_A$ receptor subunits in P2 fractions, biotin-labeled surface proteins, and cytosolic fractions were analyzed by western blotting as described elsewhere (Kumar *et al*. 2010). Protein samples were subjected to SDS-PAGE using Novex Tris-Glycine (8-16%) gels and transferred to PVDF membranes (Invitrogen, Carlsbad, CA). Membranes were probed with $GABA_A$ receptor α4 (Millipore, Billerica, MA), γ2 (gifts from Jean-Marc Fritschy, University of Zurich, Zurich Switzerland) or δ (Santa Cruz Biotech, Santa Cruz, CA) antibodies. Blots were then exposed to an antibody for β-actin for normalization. Proteins

were detected with enhanced chemilumnesence (GE Healthcare, Amersham, UK). Membranes were exposed to film under nonsaturating conditions. Densitometric analysis was conducted using NIH Image 1.57. Comparisons were made within blots. Data were analyzed using Student's *t*-test or ANOVA.

### Electrophysiology

Electrophysiological recordings and analysis were conducted as reported elsewhere (Criswell *et al.* 2008, Fleming *et al.* 2009, Kumar et al. 2010). Whole-cell voltage clamp recording was used to assess mIPSCs. Cells were incubated in an external solution (145mM NaCl, 5mM KCl, 10mM HEPES, 2mM $CaCl_2$, 1mM $MgCl_2$, 10mM glucose, and 5mM sucrose, at a pH of 7.4) containing 6-cyno-7-nitroquinoxaline-2,3-dione (CNQX, 10μM, Sigma), D-2-amino-5-phosphonopentanoic acid (AP-5, 40μM, Tocris, Ellisville MO), CGP-54626 (1μM, Tocris) and tetrodotoxin (1μM, Sigma). Large neurons with smooth cell bodies were selected for recordings. Glass electrodes (Drummond Scientific; Broomall, PA) with a resistance of 3-5MΩ were fire-polished and filled with an internal solution (130mM CsCl, 10mM HEPES, 5mM ethylene glycol-bis (2-aminoethylether)-N,N,N′-N′-tetraacetic acid (EGTA), 4mM MgATP, 0.3mM TrisGTP, 10mM phosphocreatine, pH 7.2). Recordings were performed at room temperature. Membrane potential was held at −60mV and currents were recorded with a patch-clamp amplifier (Axopatch ID or 200B, Axon Instruments, Union City, CA). Data were collected using Clampex software (Axon Instruments).

mIPSCs were analyzed using the miniAnalysis software (v5.6.4; Synaptosoft, Decatur, GA). mIPSCs were recorded for a minimum of 90 sec. To assess mIPSC kinetics, the recording trace was visually inspected and only events with a stable baseline, sharp rising phase and single peak were used. Decay time constants were obtained by using a double exponential fit for the average mIPSCs. Numerical data are given as mean ± S.E.M., and *n* represents the number of cells tested. Data were analyzed using Student's *t*-test.

## Results

Ethanol exposure increased $GABA_A$ receptor α4 subunit expression in the P2 fractions of cultured cortical neurons (89 ± 14%, n = 3, in duplicate, $p < 0.01$, Fig. 1A). To determine whether this change resulted from an alteration of surface expression of these receptors, cell surface proteins were isolated by biotinylation following exposure to ethanol. Again, ethanol exposure resulted in an increase in $GABA_A$ receptor α4 subunit expression (60.1 ± 24.7%, n = 6, $p < 0.05$; Fig. 1B). Conversely, ethanol exposure did not alter intracellular $GABA_A$ receptor α4 subunit expression (−7 ± 3%, n = 5; Fig. 1C). Synaptic α4 receptors are usually associated with γ2 subunits, but extrasynaptic α4 receptors are associated with δ subunits. Therefore, western blot analysis was performed to determine whether $GABA_A$ receptor γ2 or δ subunit expression were altered in the P2 fraction. No difference was observed in $GABA_A$ receptor γ2 subunit in ethanol treated cells compared to controls (Fig. 2A, 24 ± 25% n = 5). Similarly, no difference was detected for $GABA_A$ receptor δ subunits (Fig. 2B, −26 ± 13% n = 5), although there was a trend towards a small decrease ($p = 0.07$).

Previous *in vivo* studies have suggested that ethanol-induced increases in the expression of α4 subunits are associated with changes in the functional properties of synaptic $GABA_A$ receptors (Liang *et al.* 2006). Further, our lab has previously shown that ethanol exposure in cultured cortical neurons alters the mIPSC decay constant as well (Fleming *et al.* 2009). To investigate potential changes in synaptic α4-containing $GABA_A$ receptors, mIPSC kinetics were investigated. Ethanol exposure produced a pronounced shortening of the decay time constant (Table 1, $p < 0.01$) with no change in frequency or current amplitude. Representative traces illustrating the decay kinetics of mIPSCs from a neuron pre-treated with ethanol for 4 hrs compared to a control neuron are shown in Figure 3. This result is

consistent with the effects of elevated synaptic α4-containing $GABA_A$ receptors in the rat hippocampus (Liang et al. 2006).

PKC activity has been shown to have a major role in regulating $GABA_A$ receptor expression and activity. To determine whether PKC activity alters $GABA_A$ receptor α4 subunit expression, cells were exposed to the PKC activator PDBu. PDBu exposure (100nM) for 1 hr increased $GABA_A$ receptor α4 subunit expression (Fig. 4A, 54.3 ± 7 %, n = 4, $p < 0.05$) in the P2 fraction, similar to the effect of ethanol. Next, the PKC inhibitor calphostin C was used to assess whether PKC activity is required for ethanol-induced increases in $GABA_A$ receptor α4 subunit expression. Calphostin C completely abolished ethanol-induced increases in $GABA_A$ receptor α4 subunit expression in the P2 fraction (Fig. 4B). Calphostin C alone did not have any effect on $GABA_A$ receptor α4 subunit expression.

Although inhibition of PKC activity eliminates ethanol-induced increases in $GABA_A$ receptor α4 subunit expression, several PKC isoforms exist in the brain. Recent work by our lab has demonstrated that PKCγ and PKCβ are elevated in the P2 fraction 1 hour following 50mM ethanol exposure and remain elevated after 4 hours *in vitro* (Kumar et al. 2010). Therefore, we investigated the involvement of both isoforms in ethanol-induced increases in $GABA_A$ receptor α4 subunit expression. To investigate the involvement of PKCγ, cells were transfected with PKCγ-specific or scrambled siRNA sequences. Knockdown of PKCγ prevented ethanol-induced increases in $GABA_A$ receptor α4 subunit expression (Fig. 5A). PKCγ knockdown alone did not alter $GABA_A$ receptor α4 subunit expression. To investigate the involvement of PKCβ, we used a PKCβ pseudosubstrate that blocks PKCβ activity. Prior work established that PKCβ pseudosubstrate can block PDBu-induced increases in PKCβ (Kumar et al. 2010). Administration of PKCβ pseudosubstrate did not alter ethanol-induced increases in $GABA_A$ receptor α4 subunit expression (Fig. 5B), nor did it alter α4 subunit expression alone. Overall, these data suggest that PKCγ is required for ethanol-induced increases in $GABA_A$ receptor α4 subunit expression.

## Discussion

Ethanol exposure causes behavioral adaptations that are the result of molecular changes in the central nervous system. Alterations in $GABA_A$ receptor regulation may underlie many of ethanol's behavioral effects (Kumar et al. 2010). In the current study, we investigated $GABA_A$ receptor α4 subunit expression following exposure to a physiologically relevant ethanol concentration *in vitro* using cultured cerebral cortical neurons. Ethanol exposure increased $GABA_A$ receptor α4 subunit expression and this effect could be detected after biotinylation of surface receptors as well as by measurement of expression in P2 fractions. Increases in α4-containing $GABA_A$ receptors are associated with a significant decrease in mIPSC decay tau measured in electrophysiological studies. The effect of ethanol is dependent on PKC, as activation of PKC also resulted in an increase in $GABA_A$ receptor α4 subunit expression, and inhibition of PKC activity abolished this increase. Furthermore, ethanol-induced increases in $GABA_A$ receptor α4 subunit expression appear to be selectively mediated by PKCγ, and not PKCβ.

The *in vitro* results presented here are in line with *in vivo* studies. Recent work by Liang *et al*., (2007) has shown that a single high-dose ethanol exposure *in vivo* resulted in an increase in $GABA_A$ receptor α4 subunit surface expression that is accompanied by increases in γ2 and decreases in δ subunits in the hippocampus. These changes were associated with functional adaptations in synaptic $GABA_A$ receptor responses similar to those observed in the present study. Furthermore, the ability of short-term ethanol exposure to increase the expression of α4 subunit receptors, both *in vitro* and *in vivo* is consistent with effects of

long-term ethanol exposure to increase the expression of these receptors (Devaud *et al.* 1997, Cagetti et al. 2003).

Synaptic α4 $GABA_A$ receptors are usually assembled with γ2 subunits while extrasynaptic α4 $GABA_A$ receptors are assembled with δ subunits (Wei *et al.* 2003, Hsu *et al.* 2003, Liang *et al.* 2006). We reasoned that an increase in extrasynaptic α4δ receptors should result in an increase in δ subunit expression, but this effect was not observed. Since we previously found that alcohol exposure produced internalization of benzodiazepine sensitive α1 $GABA_A$ receptors (Kumar *et al.* 2010), we further reasoned that an increase in synaptic receptors containing α4 and γ2 subunits would not produce a change in overall γ2 subunit expression due to the bi-directional trafficking of synaptic α1 and α4 receptors. Indeed, we found no change in γ2 subunit expression, consistent with the idea that ethanol simultaneously increases surface expression of α4γ2-containing receptors and decreases surface expression of α1γ2-containing receptors. The change in mIPSC decay tau is also consistent with this interpretation. Alternatively, it is possible that the increased α4 subunit expression is not co-localized with γ2 subunits. A significant population of α4β receptors has been reported that are devoid of either γ2 or ∂ subunits (Bencsits *et al.* 1999). Further detailed pharmacological characterization of synaptic and extra-synaptic GABA responses are needed to clarify the nature of the α4 receptors that are regulated by ethanol in cerebral cortical cultured neurons.

Apart from the expression of other $GABA_A$ receptor subunits, it is possible that the increases in $GABA_A$ receptor α4 receptors are synaptic in localization due to the decreased mIPSC time decay constants. Because the mIPSCs are the result of spontaneous release of GABA release from presynaptic terminals, changes in the decay of mIPSCs is most likely the result of adaptations to $GABA_A$ receptors in synaptic or perisynaptic receptors. Previous work has demonstrated that α4 containing $GABA_A$ receptors in recombinant systems display higher GABA affinity and faster desensitization rates (Whittemore *et al.* 1996; Brown *et al.* 2002). Furthermore, α4 knockout mice display increased decay time constants compared to wildtype littermates (Chandra *et al.* 2006). Moreover, this alteration in decay tau is consistent with other reports that indicate increased synaptic α4-containing $GABA_A$ receptors by decreased mIPSC decay (Cagetti *et al.* 2003, Liang *et al.* 2007, Liang *et al.* 2006). However, further pharmacologic characterization of mISC responses with the inverse-agonist Ro15-4513 and the agonist gaboxadol will aid in characterizing the electrophysiologic responses.

Since increases in γ2 subunits are most likely the result of increased transcriptional or translational processes, assessment or RNA levels or pulse-chase analysis of newly formed proteins could also help to address this issue. Additionally, it is likely that the effect of ethanol at the 4 hour time point is only a snapshot in the adaptation of α4-containing $GABA_A$ receptors and may not represent maximal expression. Indeed, since we did not observe differences in α4 subunit expression in the intracellular faction, it is possible that newly synthesized receptors are formed at an earlier time point and integrated into surface receptors at the time of analysis. This idea is consistent with previous mRNA analysis showing robust increases in α4 subunit mRNA levels only 1 hour following ethanol exposure (Pignataro *et al.* 2007). Examining $GABA_A$ receptor subunit expression at different time points may illuminate such effects.

Given the high degree of similarity of altered $GABA_A$ receptor expression and kinetics following acute or chronic exposure, it's tempting to speculate that the cellular mechanisms regulating $GABA_A$ receptors overlap under both conditions. Indeed, the decreased mIPSC decay observed in the present study (4 hr exposure) is similar to decreased mIPSC decay observed after a longer ethanol exposure (24h), as noted in previous studies by our lab (Fleming *et al.* 2009). In contrast, Fleming (2009), found that decreased mIPSC decay was

not observed after lengthier ethanol exposures (2-7 days). It is possible that such changes are transient or require ethanol withdrawal to persist. In support of this, increased *Gabra4* transcripts were also not observed in hippocampal or cerebellar granule cells immediately following a 5 day ethanol exposure – but transcripts were increased during ethanol withdrawal (Follesa *et al.* 2003, Sanna *et al.* 2003). This may hint at additional neuroadaptive mechanisms to stabilize GABAergic function/expression occurring *during* ethanol exposure and *following* ethanol withdrawal. It remains to be determined whether these neuroadaptations are similar to those investigated here. Thus, cultured neurons represent an ideal system with which to further study the cellular mechanisms governing ethanol-induced neuroadaptation. However, it should be cautioned that the maturation state of cortical neurons in culture is not clear and may coincide with critical developmental periods *in vivo*. Therefore, it is possible that the regulation $GABA_A$ receptors may vary during other developmental stages and aging.

A number of $GABA_A$ receptor subunits contain phosphorylation sites that post-translationally regulate the receptors (Brandon *et al.* 2000, Kumar *et al.* 2005, Kumar *et al.* 2006, Macdonald 1995, Wisden *et al.* 1991). Therefore, it is not surprising that ethanol-induced increases in $GABA_A$ receptor α4 subunit expression are mediated by PKC. Previous work from our lab has shown that PKCγ and $GABA_A$ receptor α4 subunit co-immunoprecipitate using the antibody for either protein (Kumar *et al.* 2002). While PKCβ inhibition did not block ethanol's action on α4 subunit expression, we cannot rule out the possibility that PKCβ has other effects on these receptors in the absence of ethanol. Indeed, the effect of PKCβ pseudosubstrate alone did not differ from the effect of ethanol alone. This result hints at the possibility that inhibition of PKCβ activity may contribute to the modulation of α4 subunit expression by unmasking a secondary pathway. However, since the effect of PKCβ pseudosubstrate alone did not differ from the effect of vehicle alone, further studies are warranted to explore potential effects of PKCβ in the absence of ethanol. The possibility also exists that PKC isoforms may be associating with other subunits that comprise α4 subunit receptors. For instance, while no direct interaction was observed for PKCδ with $GABA_A$ receptor α4 subunits in cerebral cortex (Kumar *et al.* 2002), recent studies have demonstrated that PKCδ co-localizes with α4/δ-containing $GABA_A$ receptors (Choi *et al.* 2008) and PKCε can also regulate $GABA_A$ receptor trafficking through N-ethylmaleimide sensitive factor (Chou *et al.* 2010). We also cannot exclude the possibility that other PKC associated proteins such as RACK1 may play a role (Ron *et al.* 2000). Lastly, we also cannot exclude the possibility that other kinases may also be involved. Given that the intracellular loop of the $GABA_A$ receptor α4 subunit also contains consensus sites for PKA, such involvement should be explored in detail.

While many studies suggest that regulation of $GABA_A$ receptors occurs at the posttranslational level, there is also evidence for transcriptional regulation. Chronic ethanol exposure was shown to alter *Gabra4* mRNA levels at the same time points when effects on protein expression were found (Devaud *et al.* 1997, Devaud *et al.* 1995). The observation that a short exposure to high concentrations of ethanol achieved during a single binge session increases $GABA_A$ receptor α4 subunit expression was also noted. Recently, Pignataro *et al*., (2007) have demonstrated that similar concentrations of ethanol result in increases in *Gabra4* mRNA levels after only 1 hr. Notably, this study also observed increases in *Gabra4* transcripts at concentrations as low as 10mM. Lower ethanol concentrations may result in increases in $GABA_A$ receptor α4 subunit expression, but further studies would have to be conducted. Apart from ethanol, studies by Roberts *et al.* (2006, 2005) have demonstrated that brain derived neurotrophic factor increases *Gabra4* through increases in inducible early growth factor 3 by a PKC pathway (Roberts *et al.* 2006, Roberts *et al.* 2005). While this study is not directly linked to ethanol action, these results hint at a similar mechanism of action to increase $GABA_A$ receptor subunit expression. Microarray

studies following chronic ethanol exposure in PKCγ knockout mice have suggested the PKCγ may play a role in alterations in a select number of genes related to ethanol tolerance (Bowers *et al.* 2006). Interestingly, genes such as BDNF – noted above – as well *Hsp70.2* were identified. The latter is of particular interest given that heat shock proteins have been implicated in ethanol-induced increases in *Gabra4* (Pignataro *et al.* 2007). It is possible that specific PKC isoforms such as PKCγ may play a role in Hsp-mediated regulation of ethanol-induced increases in *Gabra4*; but such an interaction will have to be tested experimentally. Nonetheless, it is clear that PKC regulates $GABA_A$ receptor α4 subunit expression.

Even though much evidence suggests that $GABA_A$ receptor α4 subunit expression is increased in response to ethanol exposure, its contribution to ethanol-related behavior is not clear. Although work has shown that knockdown of $GABA_A$ receptor α4 subunit prevents progesterone withdrawal properties (Smith *et al.* 1998a, Smith *et al.* 1998b), that are similar to ethanol withdrawal properties, no studies have definitively linked $GABA_A$ receptor α4 subunits to altered behavioral responses from ethanol exposure. Studies conducted in $GABA_A$ receptor α4 subunit knockout mice did not indicate any alteration of ethanol-related behavioral responses (Chandra *et al.* 2008), likely due to compensatory mechanisms masking any potential effects (Liang *et al.* 2008). Nonetheless, PKCγ mice fail to develop tolerance following ethanol exposure (Bowers *et al.* 1999). It is possible that the interaction between PKCγ and $GABA_A$ receptors may influence the development of ethanol tolerance and /or withdrawal, but additional strategies are needed to further investigate this relationship.

Overall, the present work suggests that PKCγ plays a critical role in the ethanol-induced regulation of α4-containing $GABA_A$ receptors. By further understanding the regulation of $GABA_A$ receptor α4 subunits, newer therapeutic approaches may be identified that could generate valuable insight into various disorders including alcoholism, anxiety, epilepsy, and premenstrual dysphoric disorder.

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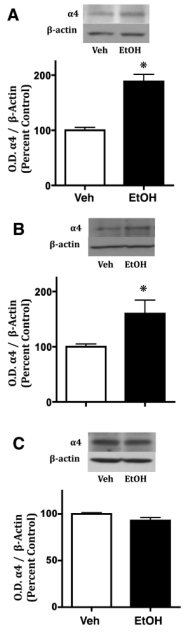


**Figure 1.**

Ethanol exposure alters $GABA_A$ receptor α4 subunit expression in cultured cortical neurons. Neurons were exposed to ethanol (50mM) for 4 hours followed by preparation of P2 fractions as well as biotin-labeling of surface proteins followed by western blotting. Representative Western blots are shown for α4 subunit from P2 fractions (A), biotin-labeled surface proteins (B), or cytosolic fractions (C). Data represent mean ± S.E.M., Data were analyzed using Student's *t*-test. *, (A) $t = 6.534$, (B) $t = 2.426$, *$p < 0.05$ compared to control, n=3-6/group.

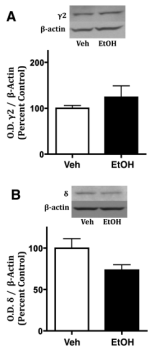


**Figure 2.**

Effects of ethanol exposure on $GABA_A$ receptor γ2 and δ subunits. Neurons were exposed to ethanol (50mM) for 4 hours followed by preparation of P2 fractions. Representative western blots are shown for γ2 (A) and δ subunits (B). A. Ethanol exposure did not alter γ2 subunit expression. B. Ethanol exposure did not alter δ subunit expression, but a trend towards a decrease was observed ($p < 0.07$). Data represent mean ± S.E.M., Student's *t*-test. n = 4-5 per group.

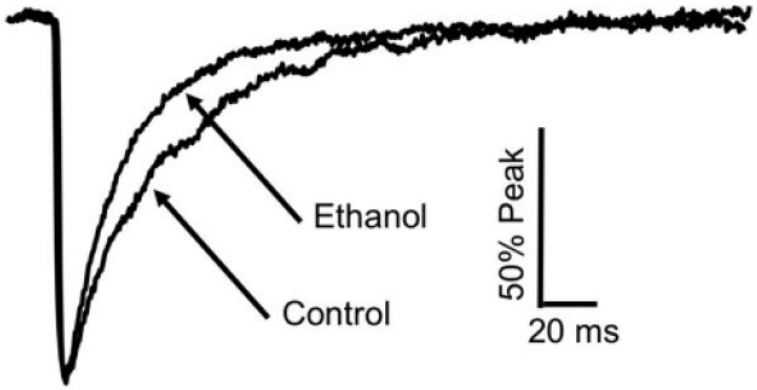


**Figure 3.**

Representative trace showing the effect of ethanol exposure on mIPSC decay time. Averaged mIPSCs are shown from a neuron exposed to 50 mM ethanol for 4 hours (Ethanol) and a control neuron (Control) for which the ethanol was replaced by standard culture media. Current amplitudes from the two neurons were standardized and expressed as a percent of the peak response for each neuron to illustrate the decrease in decay time following a 4 hour ethanol exposure. Details of the decay-tau, amplitude and rate parameters averaged across several neurons are shown in Table 1.

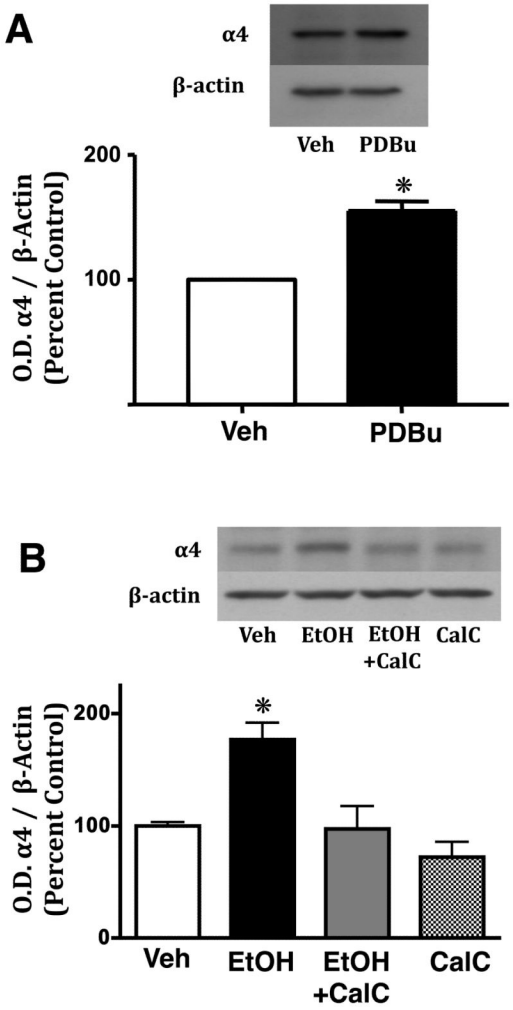


**Figure 4.**

Effects of PKC on $GABA_A$ receptor α4 subunit expression. A. Cultured cortical neurons were exposed to PDBu (100nM) for 1hr and P2 fractions were isolated and analyzed by western blot analysis. PDBu increased α4 expression similar to ethanol. Data represent mean ± S.E.M. (Student's t-test, t = 7.954, * $p < 0.05$, n=4/group). B. Neurons were exposed to ethanol to 4 hrs in the presence or absence of the PKC inhibitor calphostin C (0.3μM). Calphostin C reversed ethanol-induced increases in α4 subunit expression. Calphostin C alone had no effect of α4 subunit expression. Data represent mean ± S.E.M. (ANOVA, F = 9.882, $p < 0.01$, Newman-Keuls posthoc test * $p < 0.05$,), n=6/group. Representative western blot images are shown.

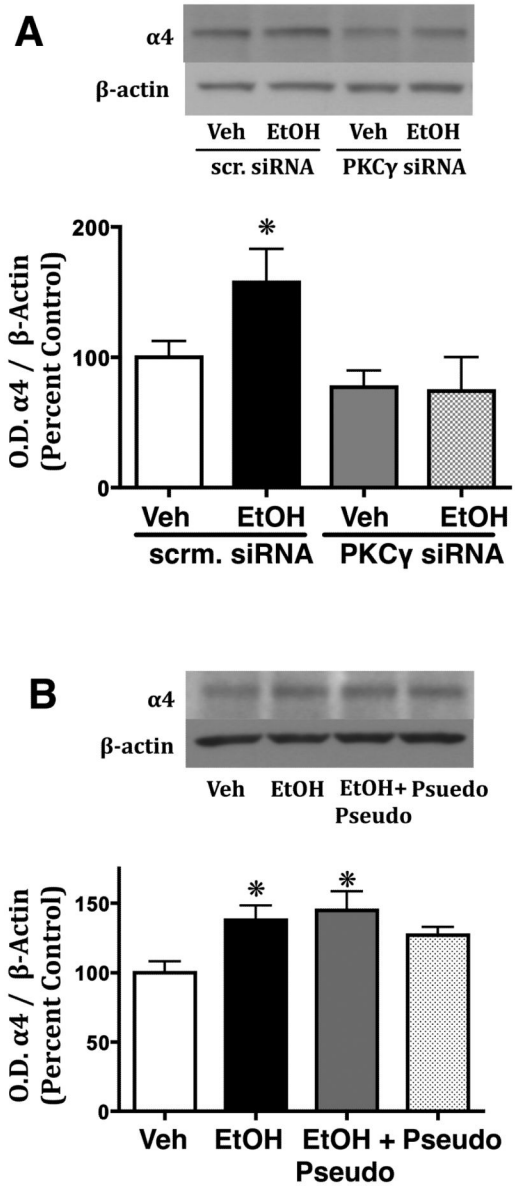


**Figure 5.**

Effects of specific PKC isoforms on $GABA_A$ receptor α4 subunit expression in the P2 fraction. A. Cultured cortical neurons were transfected with scrambled siRNA or siRNA specific for PKCγ. PKCγ siRNA inhibited ethanol-induced increases in α4 subunit expression. PKCγ siRNA alone had no effect on α4 subunit expression. Data represent mean ± S.E.M. *, $p < 0.05$ compared to vehicle, ($_{ANOVA}$, $F = 4.265$, $p < 0.05$, Newman-Keuls posthoc test), n = 6-7/group. B. Cultured neurons were exposed to PKCβ inhibitor PKCβ pseudosubstrate (pseudo) alone or in the presence of ethanol. Blocking PKCβ had no effect on ethanol-induced increases in α4 subunit expression. PKCβ pseudosubstrate alone had no effect on α4 subunit expression. Data represent mean ± S.E.M. *, $p < 0.05$ vs. Vehicle ($_{ANOVA}$, $F = 4.015$, Newman-Keuls posthoc test), n = 4-6/group. Representative western blot images are shown.

## Table 1

$GABA_A$ receptor mIPSC kinetics. Data are from cells exposed to vehicle or 50mM ethanol for 4 hrs. Data are presented as mean ± S.E.M.

| Measure | Vehicle | EtOH |
|---|---|---|
| Frequency (Hz) | 1.07 ± 0.26 | 0.73 ± 0.15 |
| Rise Time (ms) | 2.765 ± 0.432 | 2.319 ± 0.455 |
| Amplitude (pA) | 17.27 ± 3.12 | 14.80 ± 4.59 |
| Decay 90-37 (ms) | 21.38 ± 1.54 | 16.14 ± 1.27* |
| Half-Width (ms) | 18.33 ± 1.98 | 12.72 ± 1.33* |
| Decay $\tau_1$ (ms) | 19.12 ± 1.81 | 13.36 ± 0.66** |
| Decay $\tau_2$ (ms) | 32.52 ± 2.84 | 30.78 ± 3.74 |
| *n* | 12 | 11 |

* $p < 0.05$

** $p < 0.01$ compared to vehicle treated cells, Student's *t*-test (Decay 90-37, t = 2.590; Half-Width, t = 2.311; Decay $\tau_1$, t = 2.885).