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ETHANOL-INDUCED GABAA RECEPTOR ALPHA4 SUBUNIT PLASTICITY INVOLVES PHOSPHORYLATION AND NEUROACTIVE STEROIDS

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Abstract

GABA_A receptors containing $\alpha 4$ subunits are widely implicated in acute ethanol sensitivity, and their spatial and temporal regulation prominently contributes to ethanol-induced neuroplasticity in hippocampus and cortex. However, it is unknown if $\alpha 4$ -containing GABA_A receptors in the thalamus, an area of high $\alpha 4$ expression, display similar regulatory patterns following ethanol administration, and if so, by which molecular mechanisms. In the current study, thalamic GABA_A receptor $\alpha 4$ subunit levels were increased following a 6-week, but not a 2-week chronic ethanol diet. Following acute high-dose ethanol administration, thalamic GABA_A receptor $\alpha 4$ subunit levels were regulated in a temporal fashion, as a decrease was observed at 2 hours followed by a delayed transient increase. PKC γ and PKC δ levels paralleled $\alpha 4$ temporal expression patterns following ethanol exposure. Initial decreases in $\alpha 4$ subunit expression were associated with reduced serine phosphorylation. Delayed increases in expression were not associated with a change in phosphorylation state, but were prevented by inhibiting neuroactive steroid production with the 5 α -reductase inhibitor finasteride. Overall, these studies indicate that thalamic GABA_A receptor $\alpha 4$ subunit expression following acute and chronic ethanol administration exhibits similar

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regulatory patterns as other regions and that transient expression patterns following acute exposure *in vivo* are likely dependent on both subunit phosphorylation state and neuroactive steroids.

Keywords

Ethanol; Thalamus; GABA Receptors; Alpha4 Subunit; PKC; Neuroactive Steroids

INTRODUCTION

Ethanol exposure impacts a number of neurotransmitter systems within the brain, and the plasticity of such systems likely underlies tolerance, dependence, and potentially alcohol addiction. Much evidence supports a prominent role of γ -aminobutyric acid type A (GABA_A) receptors in ethanol-induced neuroadaptations (see: Kumar *et al.*, 2009). GABA_A receptors are a family of heteropentameric ligand-gated chloride ion channels that mediate the majority of rapid synaptic inhibition within the central nervous system. Although over 19 different subunits exist, most GABA_A receptors contain $\alpha 2$, 2β , and either a γ or δ subunit (Olsen and Sieghart, 2009). Receptors with $\alpha 1$ and $\gamma 2$ subunits tend to be localized synaptically and contribute to phasic inhibition, whereas receptors containing $\alpha 4$ and δ subunits are extrasynaptic and contribute to tonic inhibition (Farrant and Nusser, 2005). GABA, ethanol, and endogenous neuroactive steroids are more potent at extrasynaptic than synaptic receptor subtypes (Santhakumar *et al.*, 2006; Wei *et al.*, 2004). Although $\alpha 4$ -containing receptors are less prevalent than other α subunit types, they exhibit diffuse expression throughout the brain and are particularly enriched in thalamic and hippocampal regions (Chandra *et al.*, 2006).

Multiple studies strongly support the downregulation of ethanol-sensitive extrasynaptic $\alpha 4$ -containing receptors in response to acute and chronic ethanol exposure as well as increases in synaptic $\alpha 4$ -containing GABA_A receptors paired with $\gamma 2$ subunits (Liang *et al.*, 2007; Liang *et al.*, 2006). Increases in $\alpha 4$ - and $\gamma 2$ -containing GABA_A receptors result in reduced net postsynaptic inhibitory responses. Much of our understanding of $\alpha 4$ -containing receptor regulation following ethanol exposure has been conducted in cortical and hippocampal regions, but little is understood regarding thalamic $\alpha 4$ -containing receptors.

Work from our lab and elsewhere strongly implicates protein kinase C (PKC) involvement in $\alpha 4$ -containing GABA_A receptor trafficking. In particular, not only does PKC γ co-localize with GABA_A receptor $\alpha 4$ subunits, but their association is increased following chronic ethanol exposure *in vivo* (Kumar *et al.*, 2002). Furthermore, our *in vitro* studies not only demonstrate that PKC activity is necessary for increases in GABA_A receptor $\alpha 4$ subunit expression, but knocking down PKC γ prevents increases in $\alpha 4$ subunit expression (Werner *et al.*, 2011). Despite these outcomes, it remains unknown whether rapid changes in response to acute ethanol exposure *in vivo* involve PKC regulation. PKC δ co-localizes with extrasynaptic receptors and modulates enhanced tonic inhibition by ethanol in thalamic relay neurons (Choi *et al.*, 2008). Furthermore, as GABA_A receptor δ subunits do not appear to contain a PKC substrate (Abramian *et al.*, 2010), and endocytosis of extrasynaptic receptors is independent of $\beta 3$ subunit dephosphorylation (Gonzalez *et al.*, 2012), the phosphorylation

state of $\alpha 4$ -containing receptors may also be a contributing factor. Conversely, PKC-independent factors such as neuroactive steroids may also be involved (Gulinello *et al.*, 2001; Shen *et al.*, 2005).

In the present study, we explored whether thalamic GABA_A receptors are regulated similar to other brain regions following acute and chronic ethanol exposure *in vivo*. Further, we investigated PKC isoform expression in parallel with rapid reductions in GABA_A receptor $\alpha 4$ subunit expression and serine phosphorylation as well as the role of neuroactive steroids in these adaptations.

MATERIALS AND METHODS

Subjects

All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Adult male Sprague-Dawley rats (~10–12 weeks of age) were purchased from Harlan (Indianapolis, IN, USA). For acute ethanol exposure experiments, rats were group housed and maintained with *ad libitum* access to rat chow and water. For chronic ethanol experiments, rats were single-housed and maintained on a liquid diet (see below), but had *ad libitum* access to water. All rats were maintained on a standard 12-hour light-dark schedule with lights on at 7:00 AM.

Drugs

Ethanol was obtained from Pharmco-AAPER (Brookfield, CT) for use in all experiments. The 5 α -reductase inhibitor finasteride (100 mg/kg, Sternaloids, Newport, RI) was dissolved in 20% beta-cyclodextrin (2 ml/kg) and administered 1-hour prior to ethanol administration (Khisti *et al.*, 2003; VanDoren *et al.*, 2000).

Ethanol exposure

For acute ethanol experiments, rats were injected with 3.5 g/kg ethanol (20% v/v in saline, intraperitoneally) and tissue was collected at various time points between 1 and 48 hours. Chronic ethanol exposure was conducted similar to prior studies (Boyd *et al.*, 2010; Kumar *et al.*, 2002). Individually housed rats were given free access to a nutritionally complete liquid diet for 3 days (Custom Stanley Diet, MP Biomedicals, Solon, OH, USA), after which they received 6% v/v ethanol for 1 week and 7.5% ethanol for subsequent weeks. Control rats were fed identical diet, but with dextrose as an isocaloric substitute. All dietary consumption was monitored daily. Rats consumed between 6–10 g/kg ethanol per day. Mean body weights for control and ethanol diet rats did not differ following completion of the experiment ($p > 0.05$). Tissue was collected following either 2- or 6-weeks of chronic ethanol diet. Ethanol-exposed animals had continual access to ethanol diet up until tissue harvesting. All acute ethanol exposures and tissue harvesting were conducted during morning time periods between the beginning of the light cycle (7:00am) and noon.

Tissue and protein preparation

P2 synaptosomal fractions from thalamic and cerebral cortical tissue were prepared as described elsewhere (Werner *et al.*, 2011). Briefly, samples were homogenized in 320 mM sucrose in PBS followed by low-speed centrifugation after which the supernatant was spun at 12,000×g for 20 min. The resulting pellet (P2) was resuspended in phosphate buffered saline with phosphatase inhibitor cocktail (1:100 dilution; a proprietary mixture of microcystin LR, cantharidin, and bromotetramisole; Sigma, St. Louis, MO). For analysis of total lysate, samples were lysed in a homogenization buffer (1% SDS, 1mM EDTA, and 10mM Tris) as noted elsewhere (Grosshans *et al.*, 2002). All protein concentrations were determined through use of a bicinchoninic acid protein assay and stored at -80°C until further use.

Western blot analysis

P2 synaptosomal fractions were denatured and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene-difluoride membranes (Life Technologies, Carlsbad, CA). Membranes were probed with antibodies for the following proteins: GABA_A receptor $\alpha 4$ and $\alpha 1$ subunits (Millipore, Billerica, MA; 1:250 and 1:500, respectively); GABA_A receptor δ subunit (Santa Cruz Biotechnology, Santa Cruz, CA, 1:250); GABA_A receptor $\gamma 2$ subunit (kind gift from Jean-Marc Fritschy, University of Zurich, Zurich Switzerland 1:1000); and PKC β , γ , δ , and ϵ (BD Biosciences, San Jose, CA, 1:500). Bands were visualized using enhanced chemiluminescence (GE Healthcare, Amersham, UK) under nonsaturating conditions. Blots were then exposed to an actin specific antibody (Millipore, Billerica, MA, 1:1000) for normalization. Densitometric analysis was conducted using NIH Image J. Data were analyzed using Student's t-test or ANOVA with Newman-Keul's post hoc where appropriate.

Phosphoserine Immunoprecipitation

P2 membrane fractions (650 μ g) were solubilized and denatured in modified radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich) with PMSF (1 mM), leupeptin (1 μ g/ μ L), sodium fluoride (50 mM), sodium vanadate (200 μ M) and EDTA (2 mM) to prevent protein degradation and dephosphorylation as described elsewhere (Kumar *et al.*, 2006). Solubilized protein was centrifuged at 10,000×g for 30 min. The resulting supernatant was incubated overnight with 100 μ L of anti-phosphoserine specific antibody (Abcam, Cambridge, MA) linked to magnetized Dynabeads (Life Technologies). The receptor-antibody-bead solution was washed with PBS three times followed by boiling in SDS. Beads were separated from the immunoprecipitate by magnetic exposure. Immunoprecipitated serine phosphorylated protein was analyzed by SDS-PAGE and western blot analysis using the GABA_A receptor $\alpha 4$ subunit antibody and normalized to total $\alpha 4$ expression in the P2 fraction. Data were analyzed using Student's t-test.

3 α ,5 α -THP measurements

3 α ,5 α -THP [(3 α ,5 α)-3-hydroxypregnan-20-one or allopregnanolone] was measured using gas chromatography-mass spectrometry as described elsewhere (Porcu *et al.*, 2009). Serum samples (300 μ L) were spiked with 400 pg/ml of deuterated internal standard (d4–

17,21,21,21-3 α ,5 α -THP, Cambridge Isotope Laboratories, Inc., Andover, MA, USA) and applied to C18 solid phase extraction columns (RPN1910, 500 mg, GE Healthcare, UK) preconditioned with methanol and distilled water. The samples were washed with distilled water to eliminate polar impurities. 3 α ,5 α -THP was eluted with methanol, and subsequent extracts were dried. Dry residues were resuspended in ethyl acetate/methanol (80/20 v/v) and then filtered through a NH₂ column (Supelclean LC-NH₂, 500 mg, Supelco, Bellefonte, Pa, USA) previously conditioned with ethyl acetate and ethyl acetate/methanol (80/20 v/v). 3 α ,5 α -THP passed unretained through the sorbent. The percent accuracy for 3 α ,5 α -THP by combining C18 and NH₂ column purification is approximately 90% as noted elsewhere (Porcu *et al.*, 2009). Samples were derivatized with heptafluorobutyric anhydride (Pierce, Rockford, IL, USA) then resuspended in 10 μ L of heptane of which 2 μ L were injected in duplicate into an Agilent 6890 gas chromatograph coupled to a 5975 mass selective detector (Agilent Technologies, Inc., Santa Clara, CA, USA) operated in negative chemical ionization mode. Data were analyzed using ANOVA as above.

RESULTS

GABA_A receptor α 4 subunit upregulation in the thalamus following chronic ethanol diet

Ethanol exposure is known to regulate GABA_A receptor α 4 subunit expression, (Grobin *et al.*, 2000; Kumar *et al.*, 2002; Matthews *et al.*, 1998) but it is not known if such effects occur in all regions, such as the thalamus, where α 4 expression is abundant. Therefore, we initially investigated whether a 2-week chronic ethanol exposure would alter thalamic GABA_A receptor α 4 subunit expression. Thalamic GABA_A receptor α 4 expression in animals exposed to the ethanol diet did not differ from those given a calorically equivalent control diet (Figure 1A). To determine whether the ethanol diet was effective, cortical GABA_A receptor α 1 and α 4 levels were assessed which we previously have shown are changed by 2-week chronic ethanol diet (Grobin *et al.*, 2000; Kumar *et al.*, 2002). Consistent with prior reports, ethanol increased cortical α 4 subunit expression by $27.4 \pm 10.2\%$ compared to controls ($p < 0.05$, $n = 5$ –6/group, not shown) whereas α 1 subunit expression was decreased by $35.0 \pm 5.5\%$ compared to controls ($p < 0.05$, $n = 4$ /group, not shown). We next determined whether a lengthened ethanol exposure period would alter thalamic GABA_A receptor α 4 subunit expression, as noted for hippocampal tissue (Matthews *et al.*, 1998). Following a 6-week ethanol exposure, α 4 subunit expression was increased by $39.5 \pm 13.7\%$ as compared to controls ($p < 0.05$, Figure 1B).

A single high dose ethanol exposure regulates thalamic GABA_A receptor α 4 subunit expression

Increased α 4 subunit expression following chronic ethanol exposure is associated with increased transcription/translation (see: Kumar *et al.*, 2009); however, recent *in vitro* and *in vivo* studies demonstrate that acute high dose ethanol exposure rapidly regulates GABA_A receptor α 4 subunit expression (Liang *et al.*, 2007; Pignataro *et al.*, 2007; Werner *et al.*, 2011). Therefore, we next assessed whether thalamic α 4 subunit expression displayed a similar rapid adaptation following a single high dose ethanol exposure. Analysis revealed transient temporal changes. Specifically, P2 synaptosomal GABA_A receptor α 4 subunit expression was reduced 2-hours post ethanol exposure (Figure 2 A, B: ethanol, 72.4 ± 7.4 ,

control, 100 ± 5.4 , $p < 0.05$). GABA_A receptor $\alpha 4$ subunit expression returned to basal levels by 3-hours, but by 4-hours post ethanol, expression was increased (ethanol, 125.1 ± 6.9 , control, 100 ± 6.9 , $p < 0.05$). GABA_A receptor $\alpha 4$ subunit expression returned to baseline levels by 8-hours and did not differ at later time points.

We next examined total thalamic GABA_A receptor $\alpha 4$ subunit expression in whole cell lysates to determine whether increased synaptosomal GABA_A receptor $\alpha 4$ subunit expression following acute ethanol exposure was due to transcription/translation-related processes or limited to synaptic regions. Total GABA_A receptor $\alpha 4$ subunit expression did not differ between controls and ethanol-exposed subjects (100.0 ± 10.3 and 99.4 ± 19.3 , respectively, $n = 4/\text{group}$; not shown), thereby suggesting that synaptosomal changes are likely the result of post-translational receptor regulation. To gain some insight as to which GABA_A receptor subtypes may be changing at the 2- and 4-hour time points, we next assessed GABA_A receptor $\gamma 2$ and δ subunit expression. GABA_A receptor δ subunit expression displayed a trend towards a reduction 2 hours following acute ethanol exposure (Figure 3A, B: $72.6 \pm 12.7\%$, $p = 0.055$), whereas δ subunit expression was statistically decreased at 4 hours (61.8 ± 9.2 , $p < 0.05$). Conversely, $\gamma 2$ did not significantly differ at either time point (Figure 3A, B). Taken together, this data suggests that $\alpha 4/\delta$ -containing GABA_A receptor subtypes were initially decreased following acute ethanol exposure, whereas $\alpha 4$ -containing receptors independent of δ are likely transiently increased.

Reduced phosphorylation state associates with immediate decreases in thalamic GABA_A receptor $\alpha 4$ subunit expression following acute ethanol exposure

We next determined whether PKC isoform regulation associated with changes in GABA_A receptor $\alpha 4$ subunit expression. Similar to GABA_A receptor $\alpha 4$ subunits, we noted that both PKC γ and PKC δ expression were decreased at 2-hours post ethanol exposure by 27.3 ± 9.7 and $30.8 \pm 6.9\%$, respectively (Figure 4A, B). At 4-hours, however, both PKC γ and PKC δ had returned to baseline levels. Conversely, PKC β expression was increased at both time points (44.8 ± 8.3 and $50.0 \pm 14.5\%$, respectively), whereas PKC ϵ was unaffected at either time point (Figure 4A, B). Finally, because of the similar directional changes in P2 synaptosomal GABA_A receptor $\alpha 4$ as well as PKC γ and PKC δ expression, we assessed whether GABA_A receptor $\alpha 4$ subunit serine residue phosphorylation was impacted. 2-hours post ethanol exposure, serine phosphorylated $\alpha 4$ subunits were reduced by $56.8 \pm 16.3\%$ compared to controls ($p < 0.05$, Figure 5A, B), but no difference was detected between groups at 4-hours.

Neuroactive steroids contribute to delayed increases in thalamic GABA_A receptor $\alpha 4$ subunit expression

While the pattern of PKC γ and PKC δ expression changes are parallel with GABA_A receptor $\alpha 4$ subunit expression, these PKC isoforms may not account for the changes since the level of serine phosphorylation was not increased compared to controls at 4-hours. Higher ethanol doses are known to increase systemic neuroactive steroids, which are potent modulators of $\alpha 4$ -containing receptors (Gulinello *et al.*, 2001; Shen *et al.*, 2005); therefore we assessed whether ethanol-induced elevations in neuroactive steroids contributed to GABA_A receptor $\alpha 4$ subunit regulation by inhibition of ethanol-induced elevations in these steroids using

finasteride. Initial assessment of finasteride treatment indicated an effect of treatment ($F_{2,14} = 200.0$, $p < 0.0001$). Further analysis indicated that $3\alpha,5\alpha$ -THP was higher 2-hours post ethanol exposure compared to controls, but that finasteride pretreated animals exposed to ethanol did not differ from controls, indicating that ethanol-induced increases in $3\alpha,5\alpha$ -THP were abolished (Figure 6A). Analysis of GABA_A receptor $\alpha 4$ expression at 2 hours post ethanol exposure revealed an effect of finasteride treatment ($F_{2,15} = 5.69$, $p < 0.05$). Further analysis indicated a highly suggestive reduction in $\alpha 4$ by ethanol alone ($80.0 \pm 10.3\%$ compared to controls, $p = 0.06$), similar to our initial 2-hour results (Figure 6B). Similarly, finasteride pre-treatment also reduced $\alpha 4$ expression ($70.1 \pm 4.2\%$ compared to controls, $p < 0.05$). At 4 hours post ethanol exposure, an effect of finasteride treatment was found ($F_{2,33} = 3.39$, $p < 0.05$; Figure 6C). Further analyses indicated that although ethanol alone increased $\alpha 4$ subunit expression by $38.0 \pm 12.6\%$, animals pretreated with finasteride prior to ethanol exposure did not differ from controls. Finasteride exposure alone did not alter basal levels of $3\alpha,5\alpha$ -THP (vehicle control – 99.9 ± 7.1 pg/mL; finasteride – 97.1 ± 7.4 pg/mL, $p > 0.05$), nor did it affect basal levels of GABA_A receptor $\alpha 4$ subunit expression (vehicle control, $100.0 \pm 13.0\%$; finasteride, $97.1 \pm 13.9\%$, $p > 0.05$). Taken together, these results suggest that the neuroactive steroids such as $3\alpha,5\alpha$ -THP contribute to increases in GABA_A receptor $\alpha 4$ subunit expression.

DISCUSSION

The present study demonstrates that thalamic GABA_A receptor $\alpha 4$ subunit expression is regulated following acute and chronic ethanol exposure. Chronic ethanol exposure of 6-weeks, but not 2-weeks, increased P2 synaptosomal GABA_A receptor $\alpha 4$ subunit expression. Following acute ethanol exposure, P2 synaptosomal $\alpha 4$ subunit expression was more dynamic, with levels decreased at 2-hours followed by a transient increase at 4-hours before returning to baseline levels by 8-hours. Early reductions in GABA_A receptor $\alpha 4$ subunit expression following acute ethanol exposure were paralleled by reductions in both PKC γ and PKC δ isoforms as well as serine phosphorylation of GABA_A receptor $\alpha 4$ subunits. In contrast, the delayed increase was associated with a restoration of both PKC isoforms and phosphorylation. Further, the transient increase in GABA_A receptor $\alpha 4$ subunit expression at 4-hours was dependent on 5α -reductase derived neuroactive steroids, as it was ablated by finasteride pretreatment.

The finding that GABA_A receptor $\alpha 4$ subunit was increased following chronic ethanol exposure is not surprising as this effect has been demonstrated by several other groups in various brain regions (Devaud *et al.*, 1997; Liang *et al.*, 2006). Further, the lack of change in $\alpha 4$ subunit following 2-weeks of chronic ethanol diet is consistent with observations in hippocampus, where 2-week exposure does not alter GABA_A receptor $\alpha 4$ subunit levels (Matthews, 1998) but 40 day exposure induces a significant elevation. In agreement, increased hippocampal P2 fraction GABA_A receptor $\alpha 4$ subunit expression was also found following chronic intermittent ethanol exposure for 60 days (Cagetti *et al.*, 2004) and this change is driven by elevation of synaptic $\alpha 4\gamma$ receptors that mediate hippocampal mIPSPs (Liang *et al.*, 2006). Similar results are found in cortical neurons where elevations in P2 synaptosomal $\alpha 4$ receptors are driven by elevations in synaptic $\alpha 4\gamma$ receptors that alter mIPSC decay tau (Werner *et al.*, 2011). It is possible that distinct molecular mechanisms

account for the temporal delay in GABA_A receptor $\alpha 4$ subunit adaptations observed in hippocampus and thalamus as opposed to cortex where increases in $\alpha 4$ are seen much earlier. Our current results indicate that neuroactive steroid levels may contribute to acute increases in $\alpha 4$ subunit expression in the thalamus.; however, neuroactive steroid levels are reduced following chronic ethanol exposure (Janis et al., 1998). Speculatively, such mechanisms may involve the spatial and temporal activation of heat shock factors that alter the transcription of ethanol responsive genes such as $\alpha 4$ (Pignataro et al., 2007). Alternatively, it is possible that neuroactive steroids modify phosphorylation of GABA_A receptors and this contributes to the effects on GABA_A $\alpha 4$ subunit expression (Adams paper). Further studies are required to explain the regional differences in response to ethanol exposure for 2-weeks. Additionally, as rodents are known to display higher consumption during the beginning of the dark period of their light cycle, we cannot rule out that elevated $\alpha 4$ expression may be due to ethanol withdrawal.

Following acute ethanol exposure, our data suggest that P2 synaptosomal GABA_A receptor $\alpha 4$ subunits are transiently regulated with a decrease at 2-hours and an increase at 4-hours. Although the regulation of specific GABA_A receptor subtypes is not definitive, it is likely that δ -containing receptors are decreased at both time points as there was a suggestive reduction in the δ subunit at 2-hours that persisted at 4-hours. The transient fluctuations in $\alpha 4$ subunits, coupled with $\gamma 2$ subunit levels not differing following ethanol exposure further supports the idea that perisynaptic trafficking of $\alpha 4\delta$ receptors occurs in response to ethanol exposure and extends previous reports (Liang et al., 2007). In support of this interpretation, recent work strongly suggests that $\alpha 4$ subunits are necessary for intracellular trafficking of δ subunits and membrane expression (Peng et al., 2013; Sabaliauskas et al., 2012). Potentially, increased pairing of $\alpha 4$ -GABA_A receptor subunits, potentially with $\gamma 2$, would lead to extended suppression of δ subunit expression. Future studies will more definitively address this issue. Nonetheless, these results confirm and extend studies examining $\alpha 4\delta$ subunit-containing GABA_A receptors following acute ethanol exposure (Gonzalez et al., 2012; Liang et al., 2007; Suryanarayanan et al., 2011).

The parallel reductions in $\alpha 4$ -containing GABA_A receptors with PKC γ and PKC δ 2-hours following ethanol exposure suggest potential mechanisms of GABA_A $\alpha 4$ receptor regulation. As PKC δ has been shown to co-localize with δ -containing GABA_A receptors in the hippocampus and thalamus (Choi et al., 2008), it is likely that the decrement of PKC δ contributes to thalamic $\alpha 4\delta$ receptor trafficking. Since PKC γ co-immunoprecipitates with $\alpha 4$ receptors (Kumar et al., 2002) and is required for $\alpha 4$ receptor upregulation in cortex following ethanol exposure (Werner et al., 2011), the decrease in PKC γ at 2-hours may prevent up-regulation of $\alpha 4$ receptors at this time point. Decrements in phosphorylation may allow for PKC-independent mechanisms, such as PKA, to more readily internalize $\alpha 4$ -containing GABA_A receptors (Carlson et al., 2014), as knockdown of PKC γ and blocking PKC activity did not reduce basal levels of $\alpha 4$ expression (Werner et al., 2011). Thalamic PKC γ expression is also highly consistent with GABA_A receptor $\alpha 4$ subunit expression across multiple thalamic nuclei, with the exception of the reticular nuclei, which is devoid of both (Ding et al., 2005; Jia et al., 2005). Further work using PKC γ and PKC δ knockouts or thalamic specific knockdown may help elucidate the respective contributions of each PKC isoform.

The present results indicate that the state of GABA_A receptor $\alpha 4$ subunit serine phosphorylation is altered in conjunction with GABA_A $\alpha 4$ subunit expression. However, it is unknown which serine residue is necessary for GABA_A receptor $\alpha 4$ subunit regulation. Serine 443 is the most likely residue involved given that its phosphorylation increases cell surface stability of $\alpha 4$ -containing receptors (Abramian *et al.*, 2010). However, the same study also indicated that basal endocytosis of $\alpha 4$ receptors with the serine 443 site mutated did not differ from wildtype receptors. Thus, while our data highly suggest $\alpha 4$ subunit phosphorylation may be a contributing factor following ethanol exposure, we cannot exclude that downregulation of $\alpha 4\delta$ receptors may also involve phosphorylation of GABA_A receptor β subunits (Bright and Smart, 2013); however, interactions between clathrin adaptor proteins and the δ subunit in response to ethanol exposure lessens this possibility (Gonzeles *et al.*, 2012).

It is unclear at present how decrements in $\alpha 4$ -containing GABA_A receptors and phosphorylation in the thalamic P2 fraction contribute to acute ethanol's phenotypic responses. It is unlikely that transient effects from a single exposure contribute to withdrawal effects, as synaptosomal changes occurred when blood ethanol are known to be greater than 300 mg/dL as we have reported elsewhere (see: Carter *et al.*, *in press*). GABA_A receptor $\alpha 4$ knockout mice displayed normal behavioral responsivity to acute ethanol exposure (Chandra *et al.*, 2008); however, viral-mediated knockdown of $\alpha 4$ subunit levels in the nucleus accumbens shell reveals that $\alpha 4$ expression impacts ethanol operant responding (Rewal *et al.*, 2012). Given that thalamic extrasynaptic $\alpha 4$ -containing GABA_A receptors mediate tonic inhibitory currents that are potentiated at socially relevant ethanol concentrations (Chandra *et al.*, 2008; Jia *et al.*, 2008), reducing the number of ethanol sensitive receptors is likely a rapid mechanism to ameliorate ethanol's increased central nervous system inhibition and behavioral responses. Such rapid adaptation in the thalamus is particularly important given its relevance to cortical circuitry (e.g., Vijayan *et al.*, 2013). Furthermore, it is possible that rapid adaptations may be in response to ethanol-induced elevations in synaptic vesicle proteins and vesicular GABA release (Varodayan and Harrison, 2013; Weiner and Valenzuela, 2006), but further work would need to be done to critically assess such effects.

Neuroactive steroids, including $3\alpha,5\alpha$ -THP, are potent modulators of extrasynaptic receptor function and expression (Belelli *et al.*, 2002; Brown *et al.*, 2002; Shen *et al.*, 2007; Wohlfarth *et al.*, 2002), particularly related to ovarian cycles (Maguire *et al.*, 2005). We found that initial reductions in GABA_A receptor $\alpha 4$ subunit expression *in vivo* were independent of neuroactive steroids; however, delayed increases following ethanol exposure were dependent on 5α -reductase derived neuroactive steroids. Although acute administration of $3\alpha,5\alpha$ -THP has been shown to increase $\alpha 4$ -containing GABA_A receptors (Shen *et al.*, 2005), Neuroactive steroids alone are likely not sufficient for ethanol-induced increases in $\alpha 4$, as $3\alpha,5\alpha$ -THP and THDOC are rapidly increased in serum following ethanol exposure (VanDoren *et al.*, 2000; Porcu *et al.*, 2010) when $\alpha 4$ levels are initially decreased. More likely, the increases in GABA_A receptor $\alpha 4$ subunit expression are the result of multimodal molecular events. Recent evidence indicating that neuroactive steroids may work in concert with PKC activity to regulate $\alpha 4$ -containing receptor trafficking and functioning (Abramian *et al.*, 2014; Adams *et al.*, 2015) further supports this idea.

In summary, the present results suggest that initial decreases in thalamic GABA_A receptor $\alpha 4$ subunit following acute ethanol exposure are associated with decreases in serine phosphorylation and correlate with decreases in PKC γ and PKC δ isoforms, whereas delayed increases are dependent on 5 α -derived neuroactive steroids and restoration of $\alpha 4$ phosphorylation *in vivo*. Further work is required to determine the subtypes of $\alpha 4$ receptors that are affected by these changes.

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Highlights

- Thalamic GABA_A receptor alpha4 subunit expression is increased following chronic ethanol exposure
- Thalamic GABA_A receptor alpha4 subunit expression is regulated following a single high dose ethanol exposure.
- Decreases in GABA_A receptor alpha4 subunit expression is accompanied by reduced phosphorylation states.
- Neuroactive steroids contribute to delayed increases in thalamic GABA_A receptor alpha4 subunit expression.

Figure 1. Six weeks, but not two weeks chronic ethanol diet increases thalamic GABA_A receptor $\alpha 4$ subunit expression

Rats were given either a chronic ethanol or isocaloric diet for 2-weeks (A) or 6-weeks (B).

Thalamic tissue was isolated followed by preparations of P2 synaptosomal fractions.

Western blot analysis revealed that A) GABA_A receptor $\alpha 4$ subunit expression was unaffected after 2-weeks, B) GABA_A receptor $\alpha 4$ subunit expression was increased by $39.5 \pm 13.7\%$ after 6-weeks. Graphs show the mean $\pm$ SEM of percent control values normalized to β -actin levels (n=7–8 per group). * p < 0.05 compared to control diet (Student's *t*-test).

Figure 2. Acute ethanol administration transiently alters thalamic GABA_A receptor α4 subunit expression

Graph denotes time course for GABA_A receptor α4 subunit levels at various time points following ethanol administration (A). α4 subunit expression is decreased by $27.6 \pm 7.4\%$ at 2-hours, and is increased by $25.1 \pm 6.9\%$ at 4-hours. Representative blots are shown for both the 2- and 4-hour time points (B). Data are presented as mean $\pm$ SEM. $n = 7-10$ per group, in duplicate. * $p < 0.05$ compared to vehicle controls (Student's t -test).

Figure 3. Acute ethanol administration reduces GABA_A receptor δ subunit expression

Graph denotes time course for GABA_A receptor $\gamma 2$ and δ subunit levels at time points related to changes in $\alpha 4$ subunit expression following ethanol administration (A). GABA_A receptor δ subunit expression was decreased at 4 hours by $38.2 \pm 9.2\%$, and a trend for a reduction was noted at 2 hours ($27.4 \pm 12.7\%$). Representative blots are shown for both subunits (B). Data are presented as mean $\pm$ SEM. $n = 5-8$ per group, in duplicate. * $p < 0.05$ compared to vehicle controls (Student's t -test).

Figure 4. Acute ethanol administration selectively alters thalamic PKC isoform expression in a temporal specific fashion

PKC β , γ , δ and ϵ isoforms were examined at time points related to changes in GABA_A receptor $\alpha 4$ subunit expression (A). Both PKC γ and PKC δ were decreased at 2-hours, but were similar to controls at 4-hours. PKC β remained elevated at both time points. Representative blots are shown for each PKC isoform at 2- and 4-hours (B). Data are presented as mean $\pm$ SEM. $n = 8-10$ per group, in duplicate. * $p < 0.05$ compared to vehicle controls (Student's t -test).

Figure 5. Acute ethanol administration modulates thalamic GABA_A receptor $\alpha 4$ subunits serine phosphorylation

Acute ethanol administration reduced phosphoserine immunoprecipitation of GABA_A receptor $\alpha 4$ subunit levels at 2-hours, but not 4-hours, following ethanol exposure (A).

Representative blots are shown for each time point (B). Data are presented as mean $\pm$ SEM. n = 4–5 per group. * p < 0.05 compared to vehicle controls (Student's *t*-test).

Figure 6. 3α,5α-THP modulates GABA_A receptor α4 subunit expression following acute ethanol administration

Administration of the 5α-reductase inhibitor finasteride prevents ethanol-induced increases in peripheral 3α,5α-THP (A). Finasteride does not alter ethanol-induced changes in GABA_A receptor α4 subunit expression at 2-hours (B), but reverses increases at 4-hours (C). Data are presented as mean ± SEM. Representative blots are shown for 2- and 4-hours. n = 6–12 per group. * p < 0.05, and *** p < 0.001 compared to vehicle controls (one-way ANOVA with Newman-Keuls posthoc).