Ethanol activation of protein kinase A regulates GABA<sub>A</sub> receptor subunit expression in the cerebral cortex and contributes to ethanol-induced hypnosis

Sandeep Kumar<sup>1</sup>, Qinglu Ren<sup>1</sup>, Jonathon H. Beckley<sup>1</sup>, Todd K. O’Buckley<sup>1</sup>, Eduardo D. Gigante<sup>2</sup>, Jessica L. Santerre<sup>2</sup>, David F. Werner<sup>1,2</sup> and A. Leslie Morrow<sup>1,3,4,5</sup>*

<sup>1</sup> Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, Chapel Hill, NC, USA
<sup>2</sup> Department of Psychology, Center for Development and Behavioral Neuroscience, Binghamton University – State University of New York, Binghamton, NY, USA
<sup>3</sup> Department of Psychiatry, University of North Carolina School of Medicine, Chapel Hill, NC, USA
<sup>4</sup> Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC, USA
<sup>5</sup> Correspondence: A. Leslie Morrow, Bowles Center for Alcohol Studies, University of North Carolina School of Medicine, 3027 Thurston-Bowles Building, CB # 7178, Chapel Hill, NC 27599, USA. e-mail: morrow@med.unc.edu

Protein kinases are implicated in neuronal cell functions such as modulation of ion channel function, trafficking, and synaptic excitability. Both protein kinase C (PKC) and A (PKA) are involved in regulation of γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors through phosphorylation. However, the role of PKA in regulating GABA<sub>A</sub> receptors (GABA<sub>A</sub>-R) following acute ethanol exposure is not known. The present study investigated the role of PKA in the effects of ethanol on GABA<sub>A</sub>-R α<sub>1</sub> subunit expression in rat cerebral cortical P2 synaptosomal fractions. Additionally, GABA-related behaviors were examined. Rats were administered ethanol (2.0–3.5 g/kg) or saline and PKA, and GABA<sub>A</sub>-R α<sub>1</sub> subunit levels were measured by western blot analysis. Ethanol (3.5 g/kg) transiently increased GABA<sub>A</sub>-R α<sub>1</sub> subunit expression and PKA RIIβ subunit expression at similar time points whereas PKA RIIα was increased at later time points. In contrast, PKC isoform expression remained unchanged. Notably, lower ethanol doses (2.0 g/kg) had no effect on GABA<sub>A</sub>-R α<sub>1</sub> subunit levels, although PKA type II regulatory subunits RIIα and RIIβ were increased at 10 and 60 min when PKC isozymes are also known to be elevated. To determine if PKA activation was responsible for the ethanol-induced elevation of GABA<sub>A</sub>-R α<sub>1</sub> subunits, the PKA antagonist H89 was administered to rats prior to ethanol exposure. H89 administration prevented ethanol-induced increases in GABA<sub>A</sub>-R α<sub>1</sub> subunit expression. Moreover, increasing PKA activity intracerebroventricularly with Sp-cAMP prior to a hypnotic dose of ethanol increased ethanol-induced loss of righting reflex (LORR) duration. This effect appears to be mediated in part by GABA<sub>A</sub>-R as increasing PKA activity also increased the duration of muscimol-induced LORR. Overall, these data suggest that PKA mediates ethanol-induced GABA<sub>A</sub>-R expression and contributes to behavioral effects of ethanol involving GABA<sub>A</sub>-R.

**Keywords:** GABA<sub>A</sub> receptors, ethanol, PKA, PKC, loss of righting reflex

**INTRODUCTION**

Alcohol (ethanol) exposure results in wide ranging neurobehavioral effects including intoxication, hypnosis, tolerance, and dependence, but its mechanism(s) of action are not fully understood. Nonetheless, much evidence clearly implicates γ-aminobutyric acid type A (GABA<sub>A</sub>) receptors as having a major role in the action(s) of ethanol (Kumar et al., 2009). GABA<sub>A</sub> receptors (GABA<sub>A</sub>-R) are heteropentameric ligand-gated ion channels from a family consisting of 19 different subunits; however, the majority of receptors are composed of 2α, 2β and either α γ or δ subunit (Olsen and Sieghart, 2009). The GABA<sub>A</sub>-R α<sub>1</sub> subunit is the most abundant α subunit in the adult brain as it is a component of about 50% of all GABA<sub>A</sub>-R and can be found expressed in most major brain regions (Fritschy and Mohler, 1995; Kralic et al., 2002).

The regulation of GABA<sub>A</sub>-R likely contributes to the responses to ethanol exposure. Multiple studies have shown that chronic ethanol exposure regulates GABA<sub>A</sub>-R expression and function (see: Kumar et al., 2009). In many cases, GABA<sub>A</sub>-R α<sub>1</sub> subunit expression is decreased following chronic ethanol exposure (e.g., Devaud et al., 1997). More recently, studies have demonstrated that acute ethanol exposure also yields similar effects. For instance, a single high dose ethanol exposure decreased α<sub>1</sub> subunit expression (Li et al., 2007). Additionally, in vitro studies indicate that GABA<sub>A</sub>-R α<sub>1</sub> subunit is decreased in as little as 4 h following ethanol exposure in cultured cortical neurons (Kumar et al., 2010). Protein kinases have been implicated in regulating GABA<sub>A</sub>-R α<sub>1</sub> subunit homeostasis, most likely through phosphorylation. GABA<sub>A</sub>-R subunits contain consensus sites for both protein kinase A (PKA) and protein kinase C (PKC; Moss et al., 1992a; Brandon

**Abbreviations:** aCSF, artificial cerebrospinal fluid; GABA, γ-aminobutyric acid; PKA, protein kinase A; PKC, protein kinase C.
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et al., 2000) and much attention has focused on PKC regulation of GABA<sub>A</sub>-Rs. Ethanol has been routinely demonstrated to increase PKC activity (Messing et al., 1991), and work by our lab has shown that moderate doses of ethanol (2.0 g/kg) differentially regulate PKCδ, γ, and ε expression and translocation to the P2 synaptosomal fraction in cerebral cortical tissue (Kumar et al., 2006), indicative of increased PKC activity following ethanol exposure. In particular, PKCγ co-localization with α1-containing GABA<sub>A</sub>-R is quickly increased following an acute ethanol exposure in vitro, and is necessary for internalization of α1 subunits in primary cortical neuronal cultures (Kumar et al., 2010). Moreover, PKCγ is also involved in the up-regulation of the surface expression of α4 subunit-containing GABA<sub>A</sub>-Rs following a short term ethanol exposure (Werner et al., 2011).

In addition to activating PKC, ethanol is known to increase intracellular cyclic adenosine monophosphate (cAMP) via adenylyl cyclase, thereby activating PKA (Diamond and Gordon, 1997). PKA is a tetramer composed of a homodimer of regulatory subunits and two catalytic subunits. Four regulatory subunits denoted as RIIα, RIIβ, RIIβ and two catalytic subunits Ca and Cβ exist (McKnight, 1991). PKA activity appears to play a prominent role in ethanol-related behavior, as noted by studies using genetically modified animals and PKA modulators. For instance, RIIβ knockout mice have decreased ethanol-induced loss of righting reflex (LORR), but increased ethanol consumption (Thiele et al., 2000). Additionally, mutant Drosophila with hypomorphic PKA RII also have reduced sensitivity to ethanol’s hypnotic effects (Park et al., 2000). Also, inhibiting PKA decreases the sedative–hypnotic and motor ataxic effects of ethanol (Lai et al., 2007).

However, relatively little is known about the role of PKA in regulating GABA<sub>A</sub>-Rs; although limited studies have hinted that PKA activity can regulate GABA<sub>A</sub>-R expression (Ives et al., 2002; Brandon et al., 2003). Functionally, the effects of PKA on GABA<sub>A</sub>-R responses are not straightforward and many attempts at understanding these effects have yielded mixed results. PKA activation may either increase or decrease GABA<sub>A</sub>-R responses (e.g., Leidenheimer et al., 1991; Kano and Konnerth, 1992). Such discrepancies are thought to be dependent on the brain region, cell type and exposure time of PKA modulators. It is possible that PKA’s effects on GABA-related behaviors may be the net result of these effects. Interestingly, the effects of PKA on GABA<sub>A</sub>-R-related behavioral responses have not been determined. Variations in GABA<sub>A</sub>-R functional responses have been suggested to be due to differences in GABA<sub>A</sub>-R subunit composition (Nusser et al., 1999), and the regulation of GABA<sub>A</sub>-R by PKA likely contributes to the interpretation of these functional responses. Nonetheless, investigation of GABA<sub>A</sub>-R regulation by PKA has been limited, and no studies to date have assessed PKA involvement in regulating GABA<sub>A</sub>-Rs in response to ethanol exposure. Importantly, co-application of a PKA activator and ethanol results in increased GABA<sub>A</sub>-R potentiation over the effects of application of a PKA activator alone (Freund and Palmer, 1997).

As ethanol alters PKA and PKC activity, and both kinase families regulate GABA<sub>A</sub>-R expression and function, it is quite likely that ethanol-induced regulation of GABA<sub>A</sub>-R expression is the net result of both PKA and PKC effects. In the present study, we report that GABA<sub>A</sub>-R α1 subunit expression is altered following acute ethanol exposure in a dose-dependent manner in vivo. We further examined the contribution of PKA to ethanol-induced changes in GABA<sub>A</sub>-R α1 subunit expression as well as behavioral responses induced by ethanol and direct activation of GABA<sub>A</sub>-Rs.

MATERIALS AND METHODS

ANIMALS

Experiments were conducted in accordance with the National Institute of Health Guidelines under Institutional Animal Care and Use Committee-approved protocols at the University of North Carolina at Chapel Hill and at the State University of New York – Binghamton. Adult male Sprague-Dawley rats (190–220 g, approximate age 10–12 weeks) were purchased from Harlan (Indianapolis, IN, USA) or Taconic (Germantown, NY, USA). Animals from Harlan were used for experiments in Figures 1, 2, 4, and 5. Animals from Taconic were used in experiments in Figures 3 and 6.

![FIGURE 1](image) Ethanol (3.5 g/kg) temporally alters GABA<sub>A</sub> receptor α1 subunit expression. Rats were injected with vehicle (V) or ethanol (EtOH, E) and cerebral cortex was collected at various timepoints. P2 fractions were isolated and analyzed by western blot analysis. (A) GABA<sub>A</sub> receptor α1 subunit expression was increased by 3.5 g/kg ethanol compared to controls 60 min following ethanol exposure, but not at lower doses. Inset: representative western blot image is shown for 3.5 g/kg ethanol. (B) GABA<sub>A</sub> receptor α1 subunit expression following 3.5 g/kg ethanol administration. Data represent mean ± SEM. *p < 0.05, compared to controls; #p < 0.01, compared to 0.75 g/kg EtOH. Two-way ANOVA, with Bonferroni post-test, n = 3–6 per group, in duplicate. For clarity, matched control groups are shown by a dotted black line at 100%.
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FIGURE 2 | Ethanol (3.5 g/kg) temporally alters PKA subunit expression in the P2 fraction of cerebral cortex. Rats were injected with vehicle (V) or ethanol (E) and cerebral cortex was collected after 10, 60, 120, and 180 min. P2 fraction was analyzed by western blot analysis. (A) PKA RIIα expression was increased at 120 and 180 min following 3.5 g/kg ethanol exposure. (B) PKA RIIβ expression following 3.5 g/kg ethanol exposure. Representative western blot images are shown at 60 min following ethanol exposure. Data are compared to matched controls for each time point. Data represent mean ± SEM. *p < 0.05, compared to controls; #p < 0.01, compared to 10 min. Two-way ANOVA, with Bonferroni post-test, n = 3–4 per group, in duplicate. For clarity, matched control groups are shown by a dotted black line at 100%.

Rats were group-housed for most experiments, with the exception of animals that underwent intracerebroventricular (i.c.v.) surgery. Rats were maintained on a standard 12 h light–dark schedule with ad libitum access to rat chow and water.

DRUG EXPOSURE

Rats were injected with ethanol (20% v/v in saline, intraperitoneally (i.p.); Pharmco, Brookfield, CT, USA) or saline, and sacrificed after intervals between 10 min and 24 h. Ethanol doses ranged from 0.75 to 3.5 g/kg. This method of ethanol administration was chosen to produce consistent blood ethanol concentrations. H89 (10 mg/kg, subcutaneously (s.c.); Sigma-Aldrich, St. Louis, MO, USA), a PKA inhibitor, was injected 30 min prior to saline or ethanol injection. Sp-adenosine 3',5'-cyclic monophosphate triethylammonium salt (Sp-cAMP; 100 nmol/rat, i.c.v.; Sigma-Aldrich, St. Louis, MO, USA), a PKA activator, was administered 15 min prior to ethanol or muscimol (5 mg/kg, i.p.; Sigma-Aldrich, St. Louis, MO, USA).

TISSUE AND PROTEIN PREPARATIONS

Rats were immediately sacrificed following ethanol exposure at predetermined time points. The brain was rapidly removed from the skull and the cerebral cortex was dissected out. Tissue was flash frozen and stored at −80°C, until further use. P2 synaptosomal fractions from individual cerebral cortices were prepared by homogenization, low speed centrifugation in 0.32 M sucrose, and centrifugation of the supernatant at 12,000 × g for 20 min. The pellet (P2 fraction) was resuspended in phosphate buffered saline (PBS) with phosphatase inhibitor cocktail (1:100 dilution, proprietary mixture of microcystin LR, cantharidin, and bromotetramisole, Sigma-Aldrich, St. Louis, MO, USA) and stored at −80°C. Protein concentrations were quantified using a bicinchoninic acid method.

WESTERN BLOT ANALYSIS

P2 synaptosomal fractions were subjected to sodium-dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using Novex Tris–Glycine gels (8–16%) and transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA). Membranes were probed with antibodies for the following proteins: GABAA-Rα1 subunit (Novus, Lake Placid, NY, USA); PKCβ, PKCγ, PKCδ, and PKCε (BD Biosciences, San Jose, CA, USA); and PKA RIIα and RIIβ (BD Biosciences). 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 (GE Healthcare, Piscataway, NJ, USA), exposed to X-ray films under non-saturating conditions, and analyzed by densitometric measurements using NIH Image 1.57.

INTRACEREBROVENTRICULAR SURGERY

Stereotactic surgery was performed to implant guide cannula directed toward the lateral ventricles. Briefly, rats were anesthetized with 3.0% isoflurane and subsequently restrained in a stereotactic frame. Guide cannula (Plastics One, Roanoke, VA, USA) were implanted unilaterally into the lateral cerebral ventricle at coordinates AP -0.8 mm, L+ or -1.2 mm from bregma, and DV -2.5 mm (Paxinos and Watson, 2007). The cannula was secured to the skull using three stainless steel screws and dental cement and the cannula patency was maintained and protected with an internal guide and cap. The skin surrounding the surgical site was sutured to prevent infection. Animals were allowed a 1-week recovery period prior to behavioral testing. Rats were sacrificed 24 h following the completion of testing. Brains were rapidly removed and stored at −80°C until further use. India ink was used to determine i.c.v. cannula placements. Only animals with a positive indication of ink in their ventricles (43/44) were used for subsequent analysis.
LOSS OF RIGHTING REFLEX
To determine the effect of PKA activators on ethanol- or muscimol-induced hypnotic responses, rats were randomly selected to receive either Sp-cAMP (100 nmol/rat) or artificial cerebrospinal fluid (aCSF) 15 min prior to ethanol or muscimol administration. Two microliters of Sp-cAMP or aCSF was infused i.c.v. at a flow rate of 1 μL/min. Injector needles were left in place for an additional minute to ensure proper distribution and prevent backflow of the drug into the cannula. Rats were subsequently administered a hypnotic dose of ethanol (3.5 g/kg, i.p.) or muscimol (5 mg/kg, i.p.) 15 min later. Rats were observed and repeatedly tested until they were unable to right themselves from a supine position. The length of time from ethanol administration until onset of LORR was recorded (LORR Onset). Animals remained in a supine position in v-shaped troughs (90° angle, 12.5 cm × 25.5 cm) until they regained their righting reflex. An animal was deemed to have regained their righting reflex if they were able to right themselves three times in a 60-s period. The duration of LORR was calculated by subtracting the time of onset of LORR from the time at which the animal regained the righting reflex. Rats that did not lose the righting reflex were excluded from the study (n = 9).

STATISTICAL ANALYSIS
For western blots, all comparisons were made within blots. For ethanol dose and time dependent studies, each group was compared to saline controls run in parallel. Analyses were conducted using Student’s t-test, one-way ANOVA with Newman–Keuls post hoc test or two-way ANOVA with Bonferroni post hoc test. For data in Figures 1, 2, and 4, each respective dose or time point had a matched saline control group. Therefore, two-way ANOVAs were used for analysis. LORR data were assessed using Student’s t-test. For all experiments, p < 0.05 was considered significant.

RESULTS
EFFECTS OF ACUTE ETHANOL EXPOSURE ON GABAA RECEPTOR α1 SUBUNIT EXPRESSION
To assess the effects of ethanol exposure on GABAA-A-R α1 subunit expression, we examined the effects of multiple doses over 1 h of ethanol exposure. Ethanol caused a dose-dependent increase in GABAA-A-R α1 subunit expression 1 h following ethanol exposure in P2 fractions (Figure 1A). Two-way ANOVA indicated an overall main effect of ethanol dose [F_{4,33} = 2.71, p < 0.05], treatment [saline vs. ethanol, F_{1,33} = 9.49, p < 0.01], and an interaction of the two [F_{4,33} = 2.71, p < 0.05]. Notably, 3.5 g/kg ethanol increased GABAA-A-R α1 subunit expression (52.6 ± 19%, p < 0.05, compared to controls), but lower ethanol doses were ineffective. To assess the temporal effects of ethanol administration, 3.5 g/kg was administered and rats were sacrificed at various time points. An overall main effect was only observed for treatment [saline vs. ethanol, F_{1,23} = 9.46, p < 0.01], but not time or an interaction of time × treatment. This suggests that 3.5 g/kg ethanol increased GABAA-A-R α1 subunit expression irrespective of time (Figure 1B).

EFFECTS OF ETHANOL EXPOSURE ON PROTEIN KINASE EXPRESSION
Previous studies have indicated that PKC isoform expression is regulated following 2.0 g/kg ethanol exposure (Kumar et al., 2006) and that PKC plays a role in regulating GABAA-A-R α1 subunit expression (Kumar et al., 2006, 2010). Therefore, we investigated whether PKC isoform expression was altered following 3.5 g/kg ethanol exposure. With the exception of PKCβ [main effect of treatment, F_{1,24} = 8.39, p < 0.05], ethanol exposure failed to modulate most PKC isoform expression (Table 1). Because PKC and PKA are known to regulate GABAA-A-R expression and function, we investigated whether PKA regulatory subunit expression was altered following ethanol exposure. Notably, ethanol exposure (3.5 g/kg) resulted in increases in both PKA RIα and RIβ in P2 membranes. For RIα, overall main effects of time [F_{5,24} = 5.41, p < 0.01], treatment [saline vs. ethanol, F_{1,24} = 5.99, p < 0.05], and their interaction [F_{1,24} = 5.42, 0 < 0.05] were observed. Further analysis revealed that PKA RIα was increased 120 and 180 min following ethanol exposure (Figure 2A). For RIβ, an overall effect of treatment [saline vs. ethanol, F_{1,24} = 15.56, p < 0.001], but no effect of time or interaction was observed, thereby suggesting that 3.5 g/kg ethanol exposure alone increases PKA RIβ expression irrespective of time. Interestingly, PKA RIβ expression was elevated 24 h following ethanol exposure while a trend toward increased expression was observed for RIα (Figures 3A,B). Because of the dose-dependent effects of ethanol on PKC, we determined if changes in PKA expression were also dose-dependent. One hour following a 2.0 g/kg ethanol exposure, an overall main effect of treatment [saline vs. ethanol, F_{1,20} = 35.65, p < 0.0001], time [F_{1,20} = 9.65, p < 0.01] and their interaction [F_{1,20} = 9.65, p < 0.01] were observed for PKA RIα. Post-test revealed that ethanol increased PKA RIα at both time points, but the effect of ethanol was lower at 60 min compared to 10 min of ethanol exposure (Figure 4A). For RIβ, only a main effect of treatment [saline vs. ethanol, F_{1,20} = 26.97, p < 0.0001] was observed after 2.0 g/kg ethanol, but no main effect of time or interaction of time × treatment. This suggests that 2.0 g/kg ethanol increased PKA RIβ subunit expression irrespective of time (Figure 4B).

EFFECTS OF PKA ACTIVITY ON GABAA RECEPTOR α1 SUBUNIT EXPRESSION
Since PKA RIα and RIβ expression were altered following 3.5 g/kg ethanol exposure, we investigated whether PKA activation was required for the effect of ethanol on GABAA-A-R α1 subunits. Administration of the PKA inhibitor H89 prior to ethanol abolished ethanol-induced increases in GABAA-A-R α1 subunit expression (Figures 5A,B). Initial analysis indicated a main effect of treatment [saline vs. ethanol, F_{2,22} = 5.02, p < 0.05], H89 [F_{1,22} = 17.42, Table 1 | PKC isoform expression following 3.5 g/kg ethanol exposure.

| PKCβ | PKCy | PKCd | PKCe |
|------|------|------|------|
| 10 min | 114 ± 14 | 88 ± 9 | 123 ± 18 | 121 ± 6 |
| 60 min | 108 ± 17 | 82 ± 13 | 128 ± 23 | 95 ± 13 |
| 120 min | 122 ± 19 | 103 ± 20 | 171 ± 17 | 94 ± 16 |
| 180 min | 91 ± 7 | 95 ± 25 | 120 ± 9 | 126 ± 12 |

Values are expressed as percent control. Data are presented as mean ± SEM. n = 4 per group, in duplicate.
PKA subunit expression is increased in cerebral cortical P2 fractions 24 h after a single 3.5 g/kg ethanol exposure. Rats were injected with vehicle (Veh, V) or ethanol (EtOH, E) and cerebral cortex was collected and analyzed by P2 fraction and western blot analysis. (A) A trend toward increased PKA RIIα was observed following ethanol exposure. (B) PKA RIIβ was increased following ethanol exposure. Data are presented as mean ± SEM. Representative western blots are shown in insets for both. *p < 0.05, Student’s t-test, n = 8 per group.

EFFECTS OF PKA ACTIVITY ON ETHANOL- AND MUSCIMOL-INDUCED HYPNOTIC RESPONSES

Lastly, because PKA activity regulates GABA_A-R α1 subunit expression at hypnotic doses, we determined if PKA activity would alter ethanol-induced behavioral responses. We found that rats administered the PKA activator Sp-cAMP prior to ethanol exposure had ~50% longer duration of LORR than control rats given aCSF (288.1 ± 25.3 and 191.2 ± 31.8 min, respectively, p < 0.05, Figure 6A). No differences were detected in LORR onset (Figure 6B). To further determine if ethanol’s effects were related to PKA activity on GABA_A-R, we investigated the effects of PKA activity on muscimol-induced LORR. Sp-cAMP increased the LORR duration by ~15% compared to aCSF-treated animals (242.1 ± 9.4 and 210.7 ± 9.9 min, respectively, p < 0.05, Figure 6C). Again, no differences were noted in LORR onset (Figure 6D). Overall, these data suggest that PKA activity contributes to ethanol-induced regulation of GABA_A-R α1 subunits and that PKA’s effects on GABA_A-R contribute to the behavioral effects of ethanol.

DISCUSSION

GABA_A receptors, particularly those containing α1 subunits, have been heavily implicated in alcohol’s actions and alcohol dependence. In the current study, we investigated in vivo cerebral cortical GABA_A-R α1 subunit expression following acute ethanol exposure and found that high doses of ethanol (3.5 g/kg) increased GABA_A-R α1 subunit expression in the P2 membrane fraction, but lower doses were ineffective. The effect appears to be mediated by PKA. PKA RIIα and RIIβ subunit
expression were increased following ethanol exposure, and prior administration of a PKA inhibitor prevented ethanol-induced increases in GABA<sub>A</sub>-R α1 subunit expression. Importantly, activating PKA enhanced the duration of the ethanol-induced LORR. The LORR behavioral effect appears to be mediated, in part, by GABA<sub>A</sub>-Rs as Sp-cAMP also increased muscimol-induced LORR.

The increase in GABA<sub>A</sub>-R α1 subunit expression in vivo appears to contradict previous studies that indicate α1 subunits are decreased following ethanol exposure (reviewed in Kumar et al., 2009). However, we show here that the effect of ethanol is dependent on dose and time, and is likely due to underlying effects on PKA and PKC. Our previous results indicate that PKC<sub>γ</sub> plays a major role in decreasing GABA<sub>A</sub>-R α1 subunit expression (Kumar et al., 2010). However, PKC<sub>γ</sub> expression was not altered in the cerebral cortex following this ethanol dose. Previous results in cultured murine cerebellar granule cells indicate that activation of PKA results in a post-transcriptional increase in GABAA-R α1 subunit surface expression (Ives et al., 2002). Therefore, it is quite likely that the transient increases in GABA<sub>A</sub>-R α1 subunit expression are likely due to PKA activity.

The mechanism by which PKA increases GABA<sub>A</sub>-R α1 subunit expression in P2 synaptosomes is unknown. However, the most likely explanation involves trafficking of α1-containing GABA<sub>A</sub>-R from intracellular stores or extrasynaptic sites (Thomas et al., 2005; Bogdanov et al., 2006). Transcription/translation-related processes are less likely as longer ethanol exposures result in eventual decreases in α1 (Devaud et al., 1997; Cagetti et al., 2003).

The data presented here coupled with our previous studies suggest that PKC and PKA may have antagonistic effects in regulating GABA<sub>A</sub>-R α1 subunit expression. Indeed, both PKA (presented here) and PKC (Kumar et al., 2006) are increased in P2 synaptosomal fractions at 2.0 g/kg ethanol where GABA<sub>A</sub>-R α1 subunit expression is unaltered. Further experiments will need to be conducted to further determine this antagonistic role of PKA and PKC on GABA<sub>A</sub>-R subunit expression. Other studies have suggested that the phosphorylation state of GABA<sub>A</sub>-R is dependent on both PKA and PKC activity. For instance, Brandon et al. (2000) indicated that PKA activators could only phosphorylate GABA<sub>A</sub>-R β3 subunits in the presence of PKC inhibitors. It should be noted that antagonistic effects of PKA and PKC have been
proposed for other receptor systems. Vaello et al. (1994) have reported that glycine receptors can be phosphorylated in vivo in response to activation of either PKC or PKA with opposite functional consequences. And, more recently, PKC and PKA have been shown to have differential involvement in ghrelin-induced growth hormone and gonadotrophin release (Grey and Chang, 2011). Therefore, our studies lend further support to broader implications that PKC and PKA possibly have opposing roles in the central nervous system. Clearly, more studies need to be conducted to further evaluate this generalization. The role of phosphatase activity also cannot be excluded, but we have previously shown that ethanol exposure does not alter PP1 phosphatase expression in vivo (Kumar et al., 2006) or in vitro (Kumar et al., 2010).

It is unknown if the expression of other GABA<sub>A</sub>-R subunits is altered in response to fluctuations in PKA activity. However, Ives et al. (2002) reported that β2 and β3 subunit surface expression was increased and decreased, respectively, while α6 subunits and R<sub>O</sub>15-4513 (a GABA<sub>A</sub>-R inverse agonist) binding were unchanged. Given that α1-containing GABA<sub>A</sub>-Rs are increased following ethanol treatment, it is likely that other GABA<sub>A</sub>-R subtypes are also affected. This possibility should be investigated further.

Behaviorally, rats that were pretreated with the PKA activator Sp-cAMP exhibited increased sensitivity to ethanol’s sedative-hypnotic effects. This effect is in agreement with other studies. For instance, rats pretreated with a PKA inhibitor display reduced sensitivity to ethanol’s motor ataxic and sedative-hypnotic effects (Lai et al., 2007) as well as ethanol withdrawal-related anxiety (Pandey et al., 2003). Multiple knockout studies also indicate that decreases in the cAMP signaling pathway reduce sensitivity to ethanol. Aside from PKA RII<sub>β</sub> knockout mice (Thiele et al., 2000), ethanol behavioral responses are also reduced in knockouts of adenosine A2a (El Yacoubi et al., 2001; Naassila et al., 2002) and adenylyl cyclase 5 (Kim et al., 2010). However, it should be noted that not all studies are in agreement with this effect (Wand et al., 2001; Yang et al., 2003; Maas et al., 2005). Nonetheless, it is clear that PKA activity contributes to ethanol-induced behavior.

We further investigated whether PKA’s ethanol-related effects were mediated through GABA<sub>A</sub>-R, and observed that Sp-cAMP also increased muscimol-induced LORR. However, the magnitude of increase in muscimol-induced LORR by Sp-cAMP was less than Sp-cAMP’s effects on ethanol-induced LORR. It is likely that the ethanol-related effects represent a combination of all GABA<sub>A</sub>-R subtypes and distribution, presynaptic GABA release, and PKA effects at other ion channels. GABA<sub>A</sub>-R may only partly mediate ethanol hypnosis. Indeed, PKA is known to phosphorylate and regulate NMDA receptors (Ferrani-Kile et al., 2003; Lau et al., 2004). A second possibility for the difference in magnitude may be the overall net effect of GABA<sub>A</sub>-R inhibition. Multiple electrophysiological studies have noted that PKA activity has differential effects on GABA<sub>A</sub>-R function. For instance, increasing PKA activity has increased GABA<sub>A</sub>-R responses in Purkinje neurons (Kano and Konnerth, 1992; Freund and Palmer, 1997), cerebellar interneurons (Nusser et al., 1999), hippocampal dentate granule cells (Kapur and Macdonald, 1996), CA1 neurons (Shew et al., 2000), hypoglossal motoneurons (Saywell and Feldman, 2004) as well as recombinant receptors (Angelotti et al., 1993). Conversely, PKA has been shown to reduce GABA<sub>A</sub>-R function in microsacs (Leidenheimer et al., 1991), cortical neurons (Tehrani et al., 1989), dorsal root ganglion neurons (White et al., 1992), recombinant receptors as well as primary neuronal cultures (Moss et al., 1992b), cerebellar granule cells (Robello et al., 1993), and hippocampal CA1 neurons (Poisbeau et al., 1999). It should be pointed out that the latter group observed a decrease in mIPSC amplitude, but not an increase in decay; therefore it is possible that even if there is a reduction in receptor numbers, the receptors that are remaining are sensitive to PKA’s potentiating effects. It is also quite possible that PKA’s effects on GABA<sub>A</sub>-R inhibition depends on GABA<sub>A</sub>-<sub>β</sub> subunit composition. Recently Tang et al. (2010) demonstrated that increasing PKA activity increased spontaneous tonic currents. Importantly, it was discovered that this effect was dependent on the concentration of GABA. Nonetheless, further studies need to be conducted to determine PKA activity on specific GABA<sub>A</sub>-R subtypes. Lastly, the difference in ethanol and muscimol-stimulated effects may be related to ethanol or PKA effects on presynaptic GABA release. Ethanol is well documented to increase GABA release (e.g., Criswell et al., 2008), but recent work has indicated that blocking adenylyl cyclase or PKA activity prevents ethanol from increasing GABA release (Kelm et al., 2008).

While PKA RI<sub>α</sub> and RI<sub>β</sub> are up-regulated in response to ethanol exposure, it remains unknown which of these subunits are necessary for GABA<sub>A</sub>-R regulation and ethanol-related behavior. Given that PKA RI<sub>α</sub> and RI<sub>β</sub> are up-regulated following multiple ethanol doses, it is possible that both subunits may contribute to ethanol-related behavior. RI<sub>α</sub> and RI<sub>β</sub> were investigated as they may be localized at cell membranes whereas RI<sub>α</sub> and RI<sub>β</sub> tend to be found in the cytoplasm (discussed in Dohrmann et al., 1996). Because we observed that only PKA RI<sub>β</sub> was increased at the same time we observed increases in GABA<sub>A</sub>-R α1 subunit expression, it is quite possible that PKA RI<sub>β</sub> might be mediating this effect. However, additional studies, potentially using PKA RI<sub>β</sub> knockout mice, may be necessary to further define this effect. Alternatively, it may also be important to further determine whether A kinase associated proteins (AKAPs), such as AKAP150, that co-localize with PKA RI<sub>α</sub> and RI<sub>β</sub> are responsible for placing specific PKA (Glantz et al., 1992). Interestingly, both PKA RI<sub>α</sub> and RI<sub>β</sub> displayed changes in expression that were dependent upon ethanol dose. At moderate doses, both PKA RI<sub>α</sub> and RI<sub>β</sub> were up-regulated. However, at higher ethanol doses, PKA RI<sub>β</sub> exhibited increased expression immediately following ethanol exposure whereas PKA RI<sub>α</sub> had a latent increase in expression in P2 synaptosomes. However, both were up-regulated 24 h following high dose ethanol exposure. Although the reasons for the initial and latent increases in PKA RII expression are not completely understood, it is possible that both may be independent effects in response to ethanol exposure. Previous work has suggested that ethanol results in translocation of PKA in two separate phases. Ethanol has been shown to alter translocation at time points earlier than 30 min that returned to baseline after 60 min in vitro (Dohrmann et al., 2002). However, a second translocation phase was noted after 12 h that did not require adenosine A2 receptor subtypes or cAMP, and is thought to be due to transcriptional and/or translational processes. It remains
unknown whether increases in PKA RII in our results are simply due to activity following ethanol exposure or due to increased production of PKA through transcription and translation-related effects.

In summary, we demonstrate that protein kinases are dose dependently altered in response to ethanol exposure and exhibit temporal differences in expression. Importantly, the pattern of PKA expression likely contributes to the differential regulation of GABA_A-R subtypes as well as ethanol and GABA_A-R-mediated behavior.

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