

Protein Kinase C δ Regulates Ethanol Intoxication and Enhancement of GABA-Stimulated Tonic Current

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Ethanol alters the distribution and abundance of PKC δ in neural cell lines. Here we investigated whether PKC δ also regulates behavioral responses to ethanol. PKC $\delta^{-/-}$ mice showed reduced intoxication when administered ethanol and reduced ataxia when administered the nonselective GABA_A receptor agonists pentobarbital and pregnanolone. However, their response to flunitrazepam was not altered, suggesting that PKC δ regulates benzodiazepine-insensitive GABA_A receptors, most of which contain δ subunits and mediate tonic inhibitory currents in neurons. Indeed, the distribution of PKC δ overlapped with GABA_A δ subunits in thalamus and hippocampus, and ethanol failed to enhance tonic GABA currents in PKC $\delta^{-/-}$ thalamic and hippocampal neurons. Moreover, using an ATP analog-sensitive PKC δ mutant in mouse L(tk⁻) fibroblasts that express $\alpha 4\beta 3\delta$ GABA_A receptors, we found that ethanol enhancement of GABA currents was PKC δ -dependent. Thus, PKC δ enhances ethanol intoxication partly through regulation of GABA_A receptors that contain δ subunits and mediate tonic inhibitory currents. These findings indicate that PKC δ contributes to a high level of behavioral response to ethanol, which is negatively associated with risk of developing an alcohol use disorder in humans.

Key words: protein kinase C; ethanol; GABA; tonic current; intoxication; extrasynaptic

Introduction

GABA_A receptors are the main inhibitory neurotransmitter receptors in the brain and are a major target for sedative-hypnotic drugs, including ethanol (Mehta and Ticku, 1999). Although ethanol can enhance the function of GABA_A receptors, the importance of this effect for mediating behavioral responses to ethanol in addition to anesthesia has remained controversial (Criswell and Breese, 2005; Weiner and Valenzuela, 2006). Recently, several laboratories have reported that mild to moderately intoxicating concentrations of ethanol (3–30 mM) enhance tonic GABA currents in neurons (Wei et al., 2004; Fleming et al., 2007; Liang et al., 2008) and in heterologous systems expressing $\alpha 4\beta 3\delta$ receptors (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003), which form extrasynaptic GABA_A receptors that mediate tonic inhibitory currents in neurons (Glykys and Mody, 2007). These results suggest that enhancement of tonic GABA currents con-

tributes to pharmacological effects of ethanol observed during social drinking. However, this conclusion has been challenged by recent studies that could not replicate these findings (Borghese et al., 2006; Yamashita et al., 2006). The basis for this discrepancy is not known.

Recently, we found that phosphorylation plays a critical role in altering the sensitivity of synaptic GABA_A receptors to ethanol. Synaptic receptors mediate phasic inhibitory currents in neurons, and instead of δ subunits, contain $\gamma 2$ subunits, which target the receptors to the synapse (Glykys and Mody, 2007). Using receptors comprised of $\alpha 1\beta 2\gamma 2$ subunits, we found that protein kinase C (PKC) ϵ -mediated phosphorylation of $\gamma 2$ subunits reduces ethanol enhancement of receptor function (Qi et al., 2007). This finding raised the possibility that phosphorylation also regulates GABA-stimulated tonic currents carried by receptors containing δ subunits.

Previous studies using gene-targeted mice have demonstrated that two members of the protein kinase C (PKC) family, PKC γ and PKC ϵ , reciprocally modulate behavioral responses to ethanol. Mice that lack PKC γ show reduced signs of ethanol intoxication and consume more ethanol than wild type mice (Harris et al., 1995; Bowers et al., 1999; Bowers and Wehner, 2001), whereas mice lacking PKC ϵ show increased signs of ethanol intoxication (Hodge et al., 1999) and decreased ethanol self-administration (Hodge et al., 1999; Olive et al., 2000). In NG108–15 neuroblastoma-glioma cells, acute exposure to ethanol alters the subcellular localization of a third PKC isozyyme, PKC δ (Gordon

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et al., 1997), and in PC12 cells, chronic ethanol exposure increases the abundance of this isozyme (Messing et al., 1991). These *in vitro* studies suggest that PKC δ is important for cellular responses to ethanol. It is not yet known, however, whether PKC δ regulates behavioral or cellular responses to ethanol *in vivo*.

Here we investigated whether PKC δ is important for effects of ethanol on behavior and neuronal function using mice that lack PKC δ , which we generated by homologous recombination in embryonic stem cells (Chou et al., 2004). We report a series of behavioral, anatomical, pharmacological, and electrophysiological studies, that together show that PKC δ is required for full expression of ethanol intoxication and for ethanol enhancement of GABA-stimulated tonic inhibitory currents in neurons.

Materials and Methods

PKC $\delta^{-/-}$ mice. PKC $\delta^{-/-}$ mice were generated on a C57BL/6J \times 129X1/SvJ background as described previously (Chou et al., 2004). Chimeric mice were bred with C57BL/6J mice to generate F1 hybrids, which were intercrossed to generate F2 hybrid (~50% C57BL/6J and ~50% 129X1/SvJ) littermates for experiments. Mice were housed in standard Plexiglas cages with rodent chow and water available *ad libitum*. The colony room was maintained on a 12 h light/dark cycle with lights on at 6:00 A.M. Only male mice were used for experiments when they reached ~10 weeks of age. The Gallo Center Institutional Animal Care and Use Committee approved animal care and handling procedures in accord with NIH guidelines and Policies on the Use of Animals and Humans in Neuroscience Research as approved by the Society for Neuroscience.

Animal behavior. Ethanol-induced ataxia was evaluated using a mouse rotarod treadmill (Ugo Basile) set to a fixed speed of 20 rpm (Dar, 1997). Mice received an intraperitoneal injection of ethanol [20% (v/v) with isotonic saline], and then tested for latency to fall from the rotarod every 15 min over a 60 min period. The animals were used only once in this experiment. Ethanol-induced hypothermia was examined by measuring rectal temperature using a type J thermocouple (Barratt, Barrington, IL) at room temperature ($22 \pm 0.5^\circ\text{C}$) before and after an intraperitoneal injection of 4 g/kg ethanol (20% (v/v) with isotonic saline). Rectal temperature was assessed every 30 min up to 120 min after ethanol administration. The duration of the loss of righting reflex (LORR) and plasma ethanol clearance were examined as described (Choi et al., 2002). Other behavioral studies are described in the supplemental Methods available at www.jneurosci.org as supplemental material.

Histology. Mice were anesthetized with pentobarbital (80 mg/kg) and perfused *via* the aorta with 4% paraformaldehyde (Sigma-Aldrich) in phosphate buffer (PB). Brains were removed and postfixed for 2–4 h in the same fixative at 4°C . For immunoperoxidase studies, brains were immersed in 30% sucrose for 24 h, frozen, and cut in 35 μm sections using a sliding microtome (Leica). Free-floating sections were incubated in 3% hydrogen peroxide in PBS for 10 min followed by 50% alcohol for 20 min, and then 10% normal donkey serum in PBS for 30 min, followed by primary goat polyclonal antibody recognizing the C terminus of rat PKC δ (1:1000–1500) (Santa Cruz Biotechnology) overnight. Sections were then incubated in 2% normal donkey serum in PBS for 10 min followed by biotinylated secondary donkey anti-goat antibody (1:300) (Jackson ImmunoResearch) for 2 h and then ExtrAvidin-peroxidase complex (1:3,000) (Sigma-Aldrich) for 2 h. Peroxidase was histochemically visualized with diaminobenzidine. Control experiments omitting primary antibody resulted in lack of immunostaining, and specificity of the primary antibody was confirmed by absence of immunoreactivity in brain sections from PKC $\delta^{-/-}$ mice.

Slice electrophysiology. Tonic and phasic inhibitory currents were recorded as previously described (Stell et al., 2003; Wei et al., 2004) in slices prepared from PKC $\delta^{-/-}$ and PKC $\delta^{+/+}$ littermates (40- to 60-d-old). An examiner blinded to the genotype of the slice performed the recordings. The artificial CSF contained (in mM): 126 NaCl, 2.5 KCl, 2 CaCl $_2$, 2 MgCl $_2$, 1.25 NaH $_2$ PO $_4$, 26 NaHCO $_3$, 10–25 D-glucose, 0.005 GABA, and 3 kynurenic acid at a pH = 7.3–7.4 when bubbled with 95% O $_2$ /5% CO $_2$. Whole-cell recordings were made from neurons identified by IR video-

microscopy (Versascope; E. Marton Electronics) using pipettes filled with (in mM) 125 CsCl, 5 NaCl, 2 MgCl $_2$, 10 Hepes, 0.1 EGTA, 2 Na-ATP, 0.5 Na-GTP, and 5 QX-314 (pH: 7.25, 280–290 mOsm). The amount of tonic current was measured as described (Stell et al., 2003) by subtracting the current in the presence of saturating concentrations of SR 95531. IPSCs were detected and analyzed using LabVIEW-based software (National Instruments).

PKC δ assay. PKC δ was expressed and purified as described previously (Allen et al., 2007). Kinase activity was measured by fluorescence polarization as described (Qi et al., 2007) using 4 ng of native PKC δ or 8 ng of as-PKC δ , 2.5 μM ATP, and $0.012\text{--}2.0 \times 10^5$ nM 1-naphthyl-4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (1NaPP1). Reaction mixtures were incubated for 90 min at 27°C .

Electrophysiology in *L(tk $^-$)* cells. Whole-cell patch clamp recordings were made on *L(tk $^-$)* cells that either stably express both as-PKC δ and $\alpha 4\beta 3\delta$ GABA $_A$ receptors (see supplemental Methods available at www.jneurosci.org as supplemental material), or only $\alpha 4\beta 3\delta$ GABA $_A$ receptors (Brown et al., 2002). Cells were plated on a 35 mm Petri dish, which was placed in a recording chamber on the stage of an IX71 Olympus inverted microscope. The recording electrodes were pulled from borosilicate glass capillary tubing (Garner Glass) using a micropipette puller (P-97) (Sutter Instruments). The resistances of the electrodes were 5–8 M Ω when filled with pipette solution containing (in mM): 145 N-methyl-D-glucamine (NMDG)-Cl, 1 MgCl $_2$, 10 HEPES, and 4 Mg $^{2+}$ -ATP, 2 Na $^+$ -ATP (pH 7.3, adjusted with HCl). The external solution used to perfuse the cells continuously during the experiment contained (in mM): 145 NaCl, 3 KCl, 2 CaCl $_2$, 1 MgCl $_2$, 10 HEPES, 10 D-glucose, and 20 D-sucrose (pH 7.4, adjusted with NaOH).

The procedures used to test GABA and ethanol sensitivity were similar to those previously described (Qi et al., 2007). To test the effect of inhibiting PKC δ on GABA $_A$ receptor function, cells were incubated with external solution containing 10 μM 1NaPP1 or vehicle (0.1% DMSO) for 30 min before stimulation with GABA. Cells were then voltage-clamped at -75 mV and the GABA dose–response relationship was determined in the presence or absence of 10 μM 1NaPP1. The response to ethanol was then assessed by coapplication of ethanol with an EC $_{20}$ concentration of GABA using a Perfusion Fast-Step SF-77B system (Warner Instruments) driven by pClamp 9 software (Axon CNS-Molecular Devices). There was a 1-min washout period between each drug application. Whole-cell currents were recorded using an Axopatch 200B patch amplifier (Axon CNS-Molecular Devices), filtered at 2 kHz, and digitized at 5 kHz with a Digidata 1322A interface and pClamp 9 software. The serial resistance was monitored continuously during each experiment, and data from cells that showed a $>30\%$ change in resistance were discarded. All recordings were obtained at 27°C .

Statistical analysis. Unless otherwise noted, results are expressed as mean \pm SEM values for the indicated number of experiments. Data were examined by unpaired, two-tailed *t* tests, or ANOVA, and differences between means were considered significant when $p < 0.05$. ANOVA for repeated measures was used when testing for the effects of drugs pre and postethanol in slice electrophysiology experiments. Chi-square analysis was used to determine whether inheritance of the mutant allele deviated from a Mendelian pattern and to detect a difference in survival between PKC $\delta^{-/-}$ and PKC $\delta^{+/+}$ mice. Nonlinear regression analysis was used to determine IC $_{50}$ values for kinase inhibition by 1NaPP1.

Results

Normal baseline behavior in PKC $\delta^{-/-}$ mice

PKC $\delta^{-/-}$ mice displayed normal reproductive behavior. At weaning, 919 offspring of heterozygous breeding pairs showed a Mendelian pattern of inheritance with 258 (28.0%) PKC $\delta^{+/+}$, 443 (48.2%) PKC $\delta^{+/-}$, and 218 (23.7%) PKC $\delta^{-/-}$ mice ($\chi^2 = 2.247$, $df = 2$, $p = 0.325$). The spontaneous mortality rates were similar for PKC $\delta^{-/-}$ (1.83%) and PKC $\delta^{+/+}$ (1.16%) mice maintained for at least 6 weeks ($\chi^2 = 0.003$, $p = 0.95$). The brains of PKC $\delta^{-/-}$ mice showed no gross anatomical abnormalities, evidence of abnormal neuronal migration, loss or overabundance of neurons, or gliosis (data not shown). Water consumption was

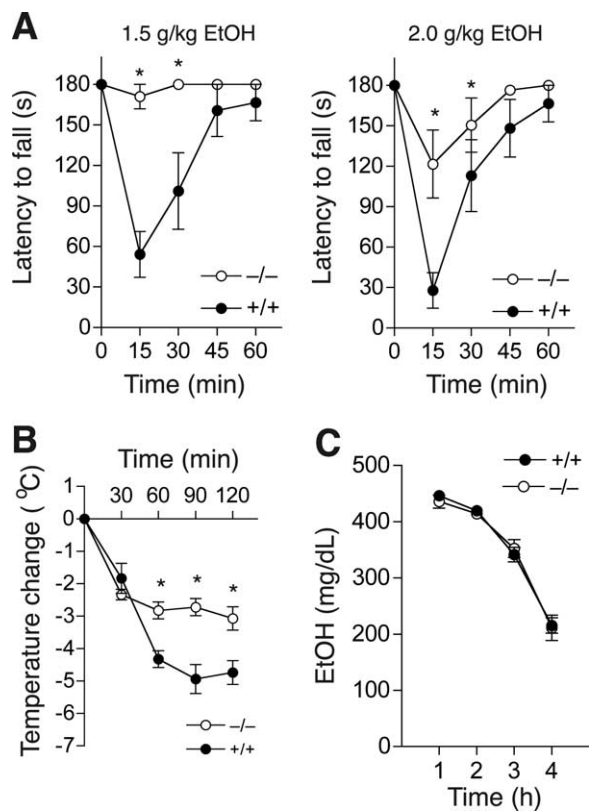


Figure 1. Acute responses to ethanol in PKC $\delta^{-/-}$ mice. **A**, Ethanol induced much more ataxia in PKC $\delta^{+/+}$ mice compared with PKC $\delta^{-/-}$ mice ($n = 8$ for each genotype; $*p < 0.05$ compared with PKC $\delta^{+/+}$ mice at the same time by Tukey's tests). **B**, Ethanol-induced hypothermia was greater in PKC $\delta^{+/+}$ mice compared with PKC $\delta^{-/-}$ mice ($n = 8$ for each genotype; $*p < 0.05$ compared with PKC $\delta^{+/+}$ mice at the same time by Tukey's tests). **C**, Blood ethanol clearance after administration of 4.0 g/kg ethanol was similar in both genotypes ($n = 12$ for each genotype).

similar among wild type and PKC $\delta^{-/-}$ mice (supplemental Fig. 1A, available at www.jneurosci.org as supplemental material). Strength, locomotor activity, and coordination were also similar between the genotypes (supplemental Fig. 1B–F, available at www.jneurosci.org as supplemental material). Therefore baseline motor function was not altered by deletion of PKC δ .

Diminished acute responses to ethanol in PKC $\delta^{-/-}$ mice

To study the role of PKC δ in the acute effects of intoxicating concentrations of ethanol, we examined ethanol-induced ataxia using a constant velocity rotarod (Rustay et al., 2003). PKC $\delta^{-/-}$ mice remained on the rotarod much longer than wild type littermates when administered 1.5 or 2.0 g/kg ethanol (Fig. 1A). Two-way, repeated measures ANOVA showed main effects of genotype ($F_{(1,56)} = 14.58$, $p = 0.002$) and time ($F_{(4,56)} = 12.77$, $p < 0.001$), and an interaction between these factors ($F_{(4,56)} = 9.92$, $p < 0.001$) with 1.5 g/kg ethanol. Similarly, there were main effects of genotype ($F_{(1,56)} = 5.52$, $p < 0.034$), and time ($F_{(4,56)} = 19.98$, $p < 0.001$), and an interaction between these factors ($F_{(4,56)} = 3.47$, $p < 0.013$) at 2.0 g/kg ethanol.

We next examined responses to higher, hypnotic doses of ethanol by measuring ethanol-induced hypothermia and ethanol-induced loss of the righting reflex (LORR). Basal body temperature was similar in PKC $\delta^{-/-}$ ($37.88 \pm 0.17^\circ\text{C}$, $n = 8$) and PKC $\delta^{+/+}$ mice ($37.44 \pm 0.16^\circ\text{C}$, $n = 8$; $p = 0.086$). However, when administered 4.0 g/kg ethanol, PKC $\delta^{-/-}$ mice developed less severe hypothermia than PKC $\delta^{+/+}$ mice (Fig. 1B). Two-way,

repeated measures ANOVA showed effects of genotype ($F_{(1,56)} = 19.68$, $p < 0.001$) and time ($F_{(4,56)} = 66.62$, $p < 0.001$), and an interaction between these factors ($F_{(4,56)} = 8.06$, $p < 0.001$). The duration of the LORR was also reduced ($p = 0.034$) in PKC $\delta^{-/-}$ mice (48.8 ± 5.8 min; $n = 8$) compared with PKC $\delta^{+/+}$ littermates (78.0 ± 11.3 min; $n = 7$) after acute administration of ethanol.

Because differential absorption, distribution, or clearance of ethanol could contribute to altered acute responses, we measured blood ethanol concentrations 1–4 h after intraperitoneal injection of 4.0 g/kg ethanol. We found that blood ethanol concentrations did not differ between the genotypes during this time period (Fig. 1C). Thus, absence of PKC δ diminishes the ataxic, hypothermic, and hypnotic effects of ethanol by altering the nervous system response to ethanol rather than by altering ethanol clearance.

PKC δ expression in mouse brain

The resistance of PKC $\delta^{-/-}$ mice to ethanol-induced ataxia led us to investigate whether PKC δ is expressed in brain regions that control motor function. To determine where in the brain PKC δ is expressed, we identified PKC δ immunoreactivity in brain sections from PKC $\delta^{+/+}$ mice (Fig. 2A, C–I). We examined sections from PKC $\delta^{-/-}$ mice to identify nonspecific immunoreactivity (Fig. 2B). We found that PKC δ was most abundant in thalamic neurons (Fig. 2A, C), including those of ventromedial, ventrolateral, and ventroanterior thalamic nuclei, which receive fibers from the cerebellum, pallidum, and substantia nigra, and send projections to the motor cortex (Paxinos, 1995). Strong PKC δ immunoreactivity was also found in thalamocortical fibers in layers I, IV, and VI of the neocortex (Fig. 2A, D). In the cerebellum, PKC δ immunoreactivity was detected in fibers of inhibitory basket cells contacting Purkinje cells whereas Purkinje cells overall displayed low to medium levels of PKC δ immunoreactivity. Lower levels of PKC δ were present in pyramidal neurons, dentate granule cells, and molecular layer interneurons of the hippocampus (Fig. 2F), in the central amygdala, and, to a lesser extent, the basolateral amygdala (Fig. 2G). There were some areas of light PKC δ immunoreactivity in the caudate–putamen (Fig. 2AA–H), but none in the nucleus accumbens (Fig. 2H) or the ventral tegmental area (Fig. 2I). Therefore, mouse PKC δ is mainly expressed in cerebellar and thalamocortical circuits, and to a lesser extent in the hippocampus and central amygdala.

Diminished ataxic response to GABA $_A$ receptor agonists in PKC $\delta^{-/-}$ mice

To investigate molecular mechanisms that explain why ethanol-induced ataxia is reduced in PKC $\delta^{-/-}$ mice, we used pharmacological agents to examine receptors previously implicated in ethanol-induced incoordination (Lüddens et al., 1990; Fidecka and Langwinski, 1995; Khanna et al., 1995; Grobin et al., 1998; Dar, 2002). We first tested pentobarbital and pregnanolone, which, depending on concentration, act as direct or allosteric agonists of most types of GABA $_A$ receptor. We found that, like ethanol, these drugs produced less ataxia in PKC $\delta^{-/-}$ mice than in wild type littermates (Fig. 3A, B). Two-way, repeated measures ANOVA showed an effect of genotype ($F_{(1,56)} = 7.04$, $p = 0.019$) and time ($F_{(4,56)} = 5.63$, $p = 0.007$) without interaction between these factors ($F_{(4,56)} = 1.74$, NS) for pentobarbital and an effect of genotype ($F_{(1,56)} = 5.76$, $p = 0.031$) and time ($F_{(4,56)} = 13.68$, $p < 0.001$) without an interaction between these factors ($F_{(4,56)} = 1.50$, NS) for pregnanolone. Because pentobarbital and pregnanolone act at many different subtypes of GABA $_A$ receptors, we

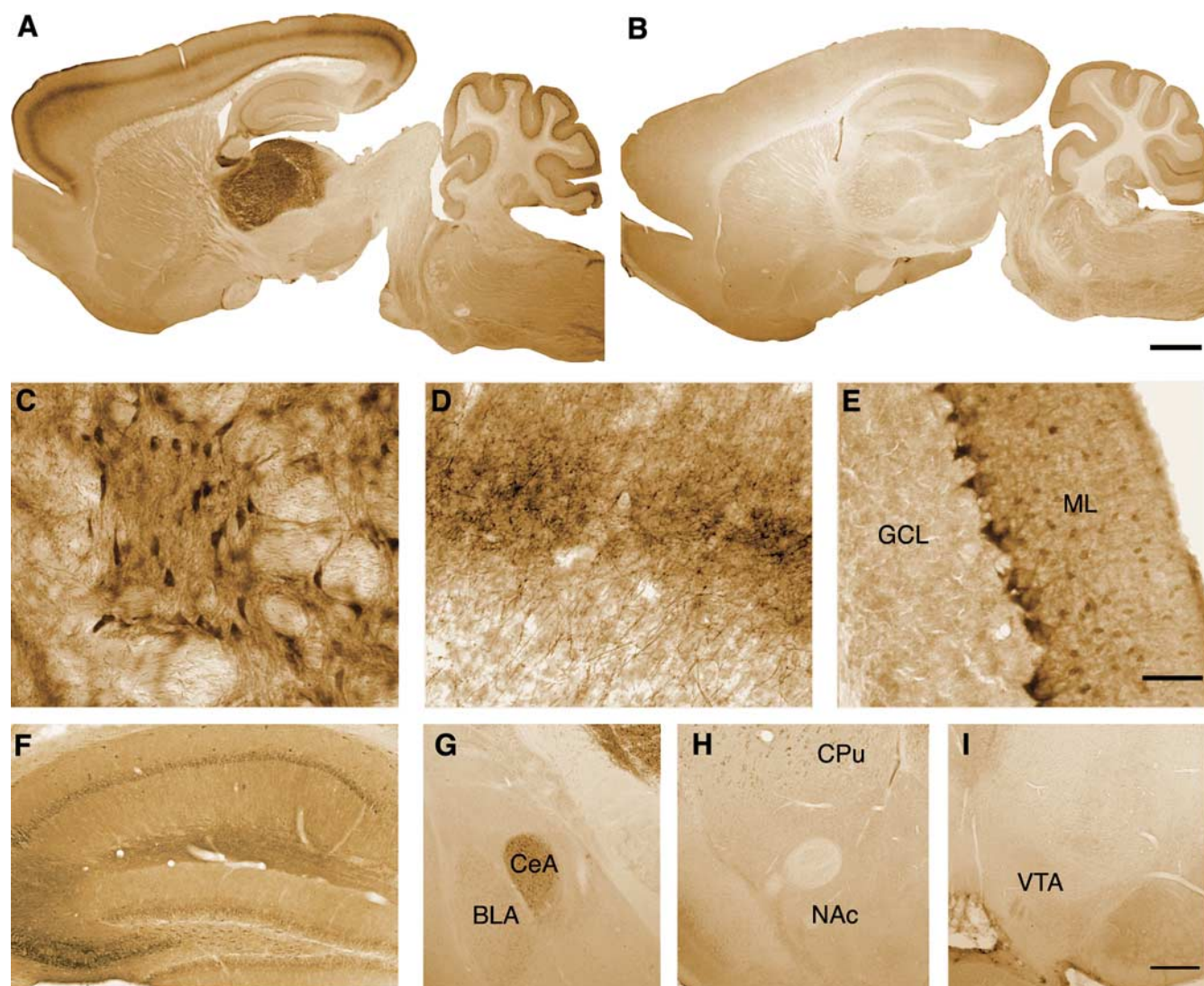


Figure 2. Expression of PKC δ in mouse brain. **A, B**, Immunoperoxidase staining for PKC δ immunoreactivity in sagittal brain sections from PKC $\delta^{+/+}$ (**A**) and PKC $\delta^{-/-}$ (**B**) mice. **C–E**, High-power micrographs of (**C**) thalamus, (**D**) thalamocortical fibers in layer IV of cerebral cortex, and (**E**) cerebellar cortex from a PKC $\delta^{+/+}$ mouse. ML, Molecular layer; GCL, granule cell layer. **F, G**, PKC δ immunoreactivity was moderate in cell bodies of pyramidal neurons, dentate gyrus granule cells and molecular layer interneurons of the hippocampus (**F**), and in the central amygdala (CeA) (**G**). **H, I**, Scattered PKC δ immunoreactivity was observed in the caudate–putamen (CPu), but none was observed in the nucleus accumbens (NAc) or the ventral tegmental area (VTA). Scale bars: (in **B**) **A, B**, 1 mm; (in **E**) **C–E**, 50 μ m; (in **I**) **F–I**, 250 μ m.

also examined the response to the classical benzodiazepine flunitrazepam, which acts as a positive allosteric modulator only at receptors containing $\gamma 2$ subunits together with $\alpha 1$, $\alpha 2$, $\alpha 3$ or $\alpha 5$ subunits (Mehta and Ticku, 1999). In contrast to pentobarbital, pregnanolone and ethanol, 2 mg/kg flunitrazepam (Fig. 3C) produced similar responses in both genotypes [$F_{(1,120)}$ (genotype) = 0.005, NS; $F_{(4,120)}$ (time) = 50.25, $p < 0.0001$; $F_{(4,120)}$ (genotype \times time) = 0.249, NS]. This pattern of responses suggests that PKC δ regulates a subset of GABA $_A$ receptors that are not benzodiazepine-sensitive.

Ethanol inhibition of NMDA receptors has also been implicated in ethanol-induced ataxia (Khanna et al., 1995; Dar, 2002). Therefore, we investigated whether PKC $\delta^{-/-}$ mice show less ataxia in response to NMDA receptor antagonists. We found that the response to the noncompetitive NMDA receptor antagonist ketamine (50 mg/kg; Fig. 3D) was similar in both genotypes [$F_{(1,56)}$ (genotype) = 0.895, NS; $F_{(4,56)}$ (time) = 63.97, $p < 0.001$; $F_{(4,56)}$ (genotype \times time) = 2.47, NS]. Because ketamine also modulates GABA $_A$ receptors, we tested a second noncompetitive

NMDA receptor antagonist, MK801 which does not alter GABA $_A$ receptor function (Hevers et al., 2008). Ataxia induced by MK801 (0.5 mg/kg; Fig. 3E), was also similar between the genotypes [$F_{(1,52)}$ (genotype) = 0.002, NS; $F_{(4,52)}$ (time) = 24.63, $p < 0.001$; $F_{(4,52)}$ (genotype \times time) = 1.29, NS].

Colocalization of PKC δ with $\alpha 4$ and δ subunits of GABA $_A$ receptors

Because PKC $\delta^{-/-}$ mice showed a diminished response to pentobarbital and pregnanolone, which act at most GABA $_A$ receptors, but not to the benzodiazepine flunitrazepam which acts at the majority of receptors that contain $\gamma 2$ subunits, we postulated that PKC δ regulates GABA $_A$ receptors that lack $\gamma 2$ subunits. The most abundant of these receptors are those that contain δ subunits, which combine mainly with $\alpha 4$ or $\alpha 6$ subunits to form receptors that mediate tonic inhibitory currents in neurons (Farrant and Nusser, 2005). Analysis of expression patterns for PKC δ in mice (Fig. 2) and for $\alpha 4$ and δ GABA $_A$ receptor subunits in the rat (Pirker et al., 2000) suggested that these proteins should overlap

in the mouse thalamus and hippocampus. We confirmed this prediction by incubating sagittal brain sections with antibodies against PKC δ and $\alpha 4$ (Fig. 4A) or δ (Fig. 4B) GABA $_A$ receptor subunits. In contrast, in the cerebellum, PKC δ immunoreactivity was present in the molecular and Purkinje cell layers of the cortex and did not overlap with immunoreactivity to GABA $_A$ δ (Fig. 4C) or GABA $_A$ $\alpha 6$ (data not shown) subunits, which were expressed instead in the granule cell layer.

Ethanol enhancement of tonic inhibitory currents in thalamic neurons and dentate gyrus granule cells

These histological findings raised the possibility that PKC δ regulates tonic GABA currents in brain regions that regulate motor function in which PKC δ expression overlaps with GABA $_A$ $\alpha 4$ and δ subunits. To investigate this hypothesis, we performed whole cell voltage clamp recordings on thalamic relay neurons because they express high levels of PKC δ and GABA $_A$ $\alpha 4$ and δ subunits and because the thalamus participates in neuronal circuits that control coordinated movement. The input resistances were similar ($p < 0.05$) in PKC $\delta^{+/+}$ (168.4 ± 8.8 M Ω) and PKC $\delta^{-/-}$ (164.1 ± 12.1 M Ω) neurons. In mice, intraperitoneal injection of 1.5 g/kg ethanol yields a peak blood ethanol concentration of 200–240 mg/dl (43–52 mM) within 10 min which falls to ~ 150 mg/dl (32 mM) after 60 min (Gentry et al., 1983). Therefore, to conservatively approximate the blood ethanol concentrations achieved during our studies of ethanol-induced ataxia, we examine the effects of 30 mM ethanol on tonic inhibitory currents. Addition of 30 mM ethanol enhanced the tonic GABA current in thalamic relay neurons from wild type mice, but not from PKC $\delta^{-/-}$ mice [$F_{(1,16)}$ (genotype) = 0.1, NS; $F_{(1,16)}$ (treatment) = 51.23, $p < 0.0001$; $F_{(1,16)}$ (genotype \times treatment) = 21.49, $p = 0.0003$] (Fig. 5A–C). To confirm this result, we examined neurons in another brain region, the hippocampal dentate gyrus, at which we have previously identified ethanol-sensitive tonic currents (Wei et al., 2004; Glykys et al., 2007). As in thalamic neurons, input resistances were similar ($p < 0.05$) in PKC $\delta^{+/+}$ (151.2 ± 13.3 M Ω) and PKC $\delta^{-/-}$ (155.1 ± 15.2 M Ω) dentate gyrus granule cells. Similar to thalamic relay neurons, ethanol enhanced the tonic GABA current in hippocampal dentate gyrus granule cells from PKC $\delta^{+/+}$ mice, but not from PKC $\delta^{-/-}$ mice (Fig. 5D) [$F_{(1,14)}$ (genotype) = 1.35, NS; $F_{(1,14)}$ (treatment) = 30.43, $p < 0.0001$; $F_{(1,14)}$ (genotype \times treatment) = 19.85, $p = 0.0005$]. This concentration of ethanol did not alter phasic GABA inhibitory postsynaptic currents in thalamic relay neurons (supplemental Fig. 2, supplemental Table 1, available at www.jneurosci.org as supplemental material) or hippocampal neurons (Wei et al., 2004; Glykys et al., 2007).

Recently we found that ethanol increases tonic currents in interneurons that reside within the molecular layer (ML) of the dentate gyrus (Glykys et al., 2007). The tonic inhibitory current in these neurons is mediated by GABA $_A$ receptors that contain δ subunits and $\alpha 1$ rather than $\alpha 4$ subunits, indicating that δ subunits are critical for ethanol enhancement of the tonic inhibitory current. To determine whether only δ subunits are required for

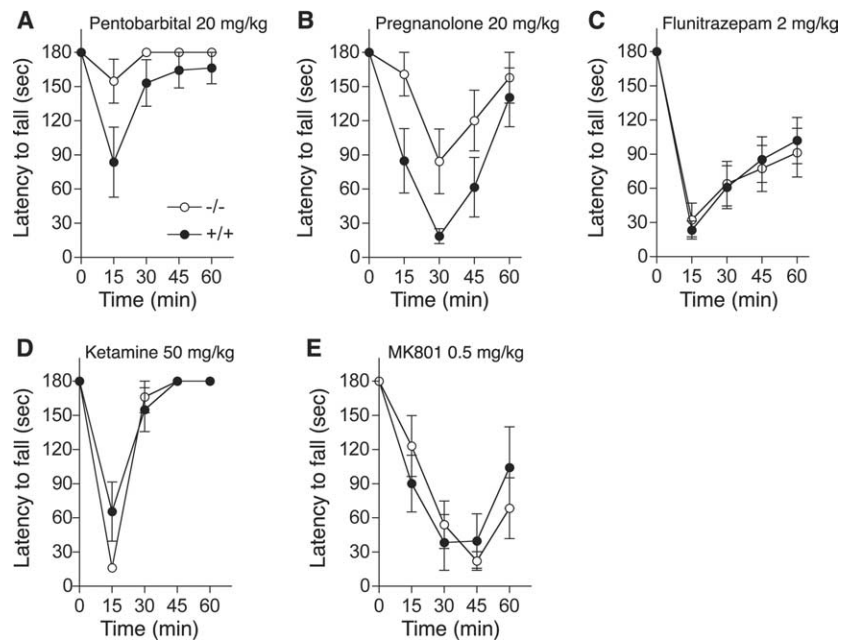


Figure 3. A–E, Ataxia induced by drugs that act at GABA $_A$ and NMDA receptors. Mice were tested for their ability to remain for 3 min on a rotarod treadmill rotating at a constant velocity of 20 rpm before and after intraperitoneal injection of pentobarbital (A), pregnanolone (B), flunitrazepam (C), ketamine (D), or MK-801 (E). $n = 8$ (A, B, D) and $n = 16$ (C) for each genotype. In E, $n = 7$ for PKC $\delta^{+/+}$ and $n = 8$ for PKC $\delta^{-/-}$ mice.

the permissive effect of PKC δ on the response to ethanol, we examined tonic currents in ML interneurons from wild type and PKC $\delta^{-/-}$ mice. Again, ethanol enhanced the tonic current (Fig. 5E) in ML interneurons from wild type mice but not from PKC $\delta^{-/-}$ mice [$F_{(1,12)}$ (genotype) = 0.48, NS; $F_{(1,12)}$ (treatment) = 13.39, $p = 0.0033$; $F_{(1,14)}$ (genotype \times treatment) = 7.20, $p = 0.0199$]. This result indicates that PKC δ modulation of ethanol sensitivity requires the presence of GABA $_A$ δ subunits but not of $\alpha 4$ subunits.

PKC δ regulates ethanol enhancement of GABA-stimulated current carried by $\alpha 4\beta 3\delta$ receptors

Because PKC δ appeared necessary for ethanol enhancement of tonic GABA currents, we investigated whether PKC δ acts directly on GABA $_A$ receptors that contain δ subunits by using mouse L(tk $^-$) fibroblasts that stably express $\alpha 4\beta 3\delta$ receptors (Brown et al., 2002). GABA-stimulated currents in these cells have been reported to be insensitive to ethanol at concentrations of 100 mM or less (Borghese et al., 2006). Suspecting that the abundance of PKC δ might be low in $\alpha 4\beta 3\delta$ -expressing L(tk $^-$) cells, we measured PKC δ levels relative to other cell lines. We found that PKC δ immunoreactivity was $>50\%$ lower in L(tk $^-$) cells compared with CHO or HEK-293 cells (Fig. 6A).

We next investigated whether the low abundance of PKC δ might limit the response to ethanol in $\alpha 4\beta 3\delta$ -expressing L(tk $^-$) cells. To test this hypothesis we increased the abundance of PKC δ in these cells by stably transfecting them with as-PKC δ , which is an ATP analog-sensitive mutant of PKC δ (Allen et al., 2007). The advantage of expressing as-PKC δ instead of native PKC δ is that as-PKC δ can be inhibited with high specificity by compounds that do not inhibit native kinases. The resulting stable cell line expressed PKC δ immunoreactivity that was >2 -fold greater than the parent cell line (Fig. 6B). We then compared the effect of ethanol on GABA $_A$ currents in the parent $\alpha 4\beta 3\delta$ -expressing cell line and the cell line stably transfected with as-PKC δ . In the parent cell line, ethanol increased currents evoked by an EC $_{20}$ con-

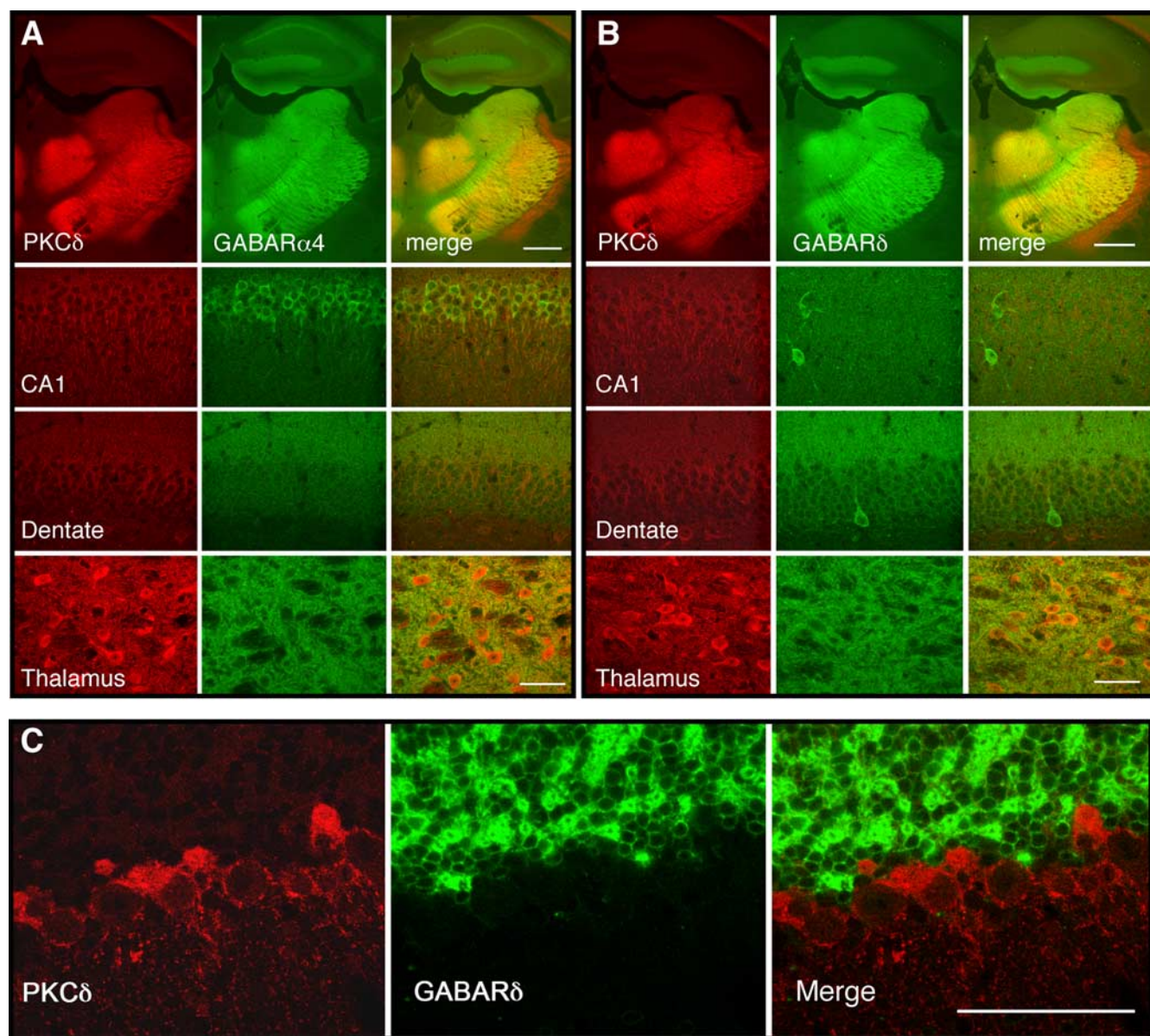


Figure 4. Colocalization of GABA $_A$ $\alpha 4$ and δ subunit immunoreactivity with PKC δ in mouse brain. **A**, Coronal sections through the hippocampus and thalamus showing immunoreactivity for PKC δ (red), $\alpha 4$ subunits (green), and their colocalization (yellow) in hippocampus and thalamus. **B**, Similar sections showing immunoreactivity for PKC δ (red), δ subunits (green), and their colocalization (yellow) in hippocampus and thalamus. **C**, Sections of cerebellar cortex showing PKC δ immunoreactivity in the Purkinje cell and molecular layers, which did not colocalize with δ subunit immunoreactivity present in the granule cell layer. Scale bars: **A**, **B**, 500 μ m for low-power images, 50 μ m for high-power images; **C**, 50 μ m.

centration (0.1 μ M) of GABA (Fig. 6C,D) ($F_{(5,60)} = 28.16$; $p < 0.0001$). This effect was significant at 3–100 mM ethanol compared with current evoked by GABA alone ($p < 0.05$ by Dunnett's test). Enhancement of GABA currents by ethanol was significantly greater in as-PKC δ -transfected cells than in the parent cell line (Fig. 6C,D). Two-way ANOVA revealed main effects of cell line ($F_{(1,97)} = 40.77$, $p < 0.0001$) and ethanol concentration ($F_{(5,97)} = 26.39$, $p < 0.0001$) with a significant interaction between these factors ($F_{(5,97)} = 2.76$, $p < 0.0226$).

Based on experience with other ATP analog-sensitive kinase mutants, we predicted that as-PKC δ would be sensitive to analogs of the general kinase inhibitor 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP1). *In vitro* kinase assays with enzyme expressed and purified from COS-7 cells showed that, unlike wild-type PKC δ , as-PKC δ is inhibited by nanomolar concentrations of 1-naphthyl-PP1

(1NaPP1) (Fig. 6E). In cells expressing as-PKC δ , 30 mM ethanol (Fig. 6F) reversibly enhanced the GABA current by $47 \pm 6\%$ ($n = 10$), whereas treatment with 10 μ M 1NaPP1 significantly reduced enhancement by ethanol to $21 \pm 5\%$ ($n = 4$; $p = 0.019$ by two-tailed, *t* test). 1NaPP1 did not alter the EC $_{50}$ for GABA in as-PKC δ -transfected cells treated with GABA alone (data not shown). These findings demonstrate that PKC δ increases the sensitivity of $\alpha 4\beta 3\delta$ GABA $_A$ receptors to ethanol.

Discussion

In this study we found that PKC $\delta^{-/-}$ mice show reduced signs of acute ethanol intoxication compared with wild type littermates. Particularly striking was the resistance of PKC $\delta^{-/-}$ mice to ethanol-induced ataxia, suggesting that PKC δ residing in neural systems that regulate coordination is important for the motor-impairing effects of ethanol. This conclusion is supported by the

finding that PKC δ is highly expressed in neurons of the ventral thalamus and cerebellar cortex, and in thalamocortical efferents to the cerebral cortex, which all contribute to circuits that control movement.

Because GABA_A (Lüddens et al., 1990; Fidecka and Langwinski, 1995; Grobin et al., 1998) and NMDA (Khanna et al., 1995; Dar, 2002) receptors are implicated in ethanol-induced ataxia, we examined whether responses to drugs that act at these receptors are altered in PKC $\delta^{-/-}$ mice. We found a decrease in the ataxic response to pentobarbital and pregnanolone, suggesting that PKC δ modulates ethanol's effects at GABA_A receptors in central motor circuits that control coordination. Our finding that ataxia induced by flunitrazepam was not altered in PKC $\delta^{-/-}$ mice suggested that PKC δ selectively modulates GABA_A receptors that are benzodiazepine insensitive, the majority of which contain δ subunits and are extrasynaptic. In contrast, we found no difference between PKC $\delta^{+/+}$ and PKC $\delta^{-/-}$ mice in their ataxic response to administration of ketamine or MK-801, suggesting that PKC δ does not regulate the effect of ethanol at NMDA receptors.

Our analysis of brain expression patterns for PKC δ , and $\alpha 4$, $\alpha 6$ and δ GABA_A subunits revealed that PKC δ overlaps with expression of $\alpha 4$ and δ subunits. This overlap was most striking in the thalamus, lesions of which can produce ataxia in humans (Melo et al., 1992). Because recent evidence indicates that GABA_A receptors containing $\alpha 4$ and δ subunits mediate tonic inhibition in thalamic relay neurons (Cope et al., 2005; Jia et al., 2005), we investigated whether ethanol enhancement of tonic GABA current was diminished in thalamic relay neurons from PKC $\delta^{-/-}$ mice. We found that tonic GABA currents in thalamic relay neurons are increased by ethanol to a much greater extent in cells from wild type mice compared with PKC $\delta^{-/-}$ mice. We also examined hippocampal dentate gyrus granule cells because they too exhibit ethanol-sensitive tonic currents that are carried by $\alpha 4/\delta$ -containing GABA_A receptors (Mchedlishvili and Kapur, 2006). Again, ethanol (30 mM) increased tonic GABA currents in cells from wild type but not from PKC $\delta^{-/-}$ mice. Finally, we examined ML interneurons because they express ethanol-sensitive tonic currents mediated by $\alpha 1/\delta$ - rather than $\alpha 4/\delta$ -containing GABA_A receptors (Glykys et al., 2007). Again, we found that ethanol enhanced the tonic current in neurons from wild type, but not from PKC $\delta^{-/-}$ mice. Together, these results indicate that in several populations of neurons, PKC δ is necessary for ethanol enhancement of tonic GABA currents mediated by extrasynaptic receptors that contain GABA_A δ subunits.

Tonic currents are stimulated by extracellular concentrations of GABA, which are regulated by the activity of GABA transporters on neurons and glia, and by spillover from GABA released at synapses (Farrant and Nusser, 2005). Therefore, PKC δ could al-

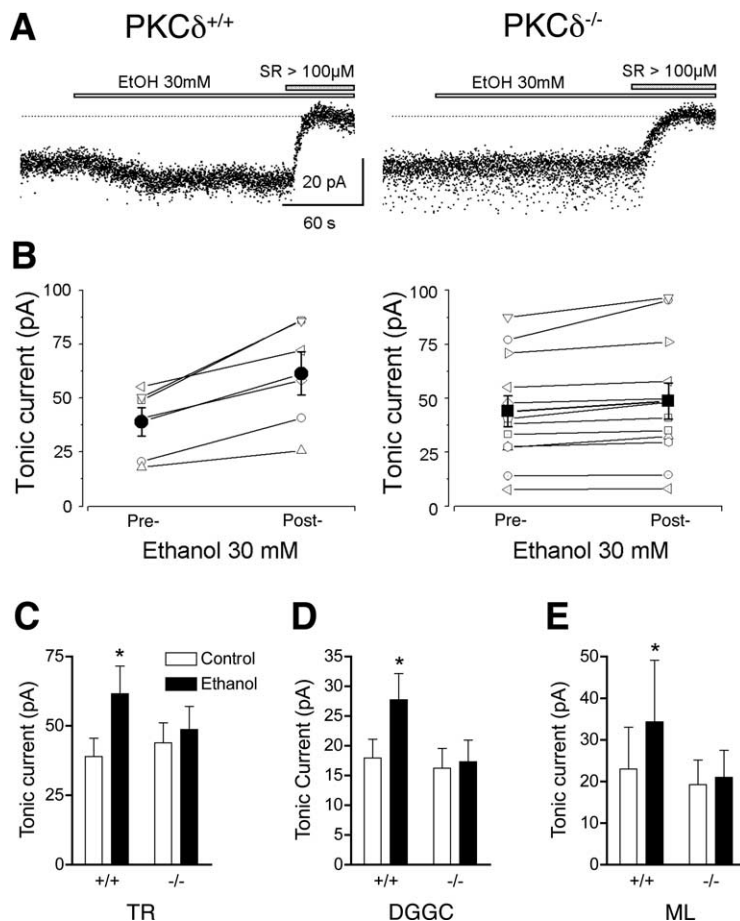


Figure 5. Ethanol enhances tonic inhibitory GABA currents in neurons from PKC $\delta^{+/+}$ but not PKC $\delta^{-/-}$ mice. **A**, Sample tonic current traces recorded in a thalamic relay neuron from a PKC $\delta^{+/+}$ (left) and a PKC $\delta^{-/-}$ (right) mouse. These currents were completely blocked by the nonselective GABA_A receptor antagonist SR95531 (SR). **B**, Paired plots of tonic current amplitudes before and after the addition of 30 mM ethanol to the perfusate of thalamic neurons from PKC $\delta^{+/+}$ (left) and PKC $\delta^{-/-}$ (right) mice. Filled circles (left) and squares (right) with error bars indicate mean \pm SEM current values. **C**, Mean \pm SEM values for tonic current amplitudes in PKC $\delta^{+/+}$ ($n = 6$) and PKC $\delta^{-/-}$ ($n = 12$) thalamic relay (TR) neurons before (control) and after addition of 30 mM ethanol. **D**, Tonic current in PKC $\delta^{+/+}$ ($n = 9$) and PKC $\delta^{-/-}$ ($n = 7$) dentate gyrus granule cell (DGGC) neurons and **E**, in PKC $\delta^{+/+}$ ($n = 5$) and PKC $\delta^{-/-}$ ($n = 9$) ML interneurons (ML) before and after 30 mM ethanol. **C–E**, * $p < 0.05$ compared with the control condition in PKC $\delta^{+/+}$ neurons by Bonferroni tests.

ter tonic GABA currents by regulating the ethanol sensitivity of GABA_A δ -containing receptors, or by altering GABA transport. Our studies with L(tk⁻) cells that express $\alpha 4\beta 3\delta$ receptors, however, showed that overexpression of PKC δ facilitates ethanol enhancement of GABA-stimulated current whereas inhibition of PKC δ diminishes this response. These findings clearly demonstrate that PKC δ regulates the response of δ subunit-containing receptors to ethanol and suggest that PKC δ phosphorylates GABA_A receptor δ subunits or another protein that regulates receptor function. Our findings do not eliminate the possibility that PKC δ also regulates GABA transport, but we do not think this could be important for the response of tonic currents to ethanol, because we still observe ethanol enhancement of tonic current in the presence of the GABA transport inhibitor NO-711 (Glykys et al., 2007).

Several laboratories report that intoxicating concentrations of ethanol ≤ 50 mM enhance GABA_A currents carried by receptors containing δ subunits (Sundstrom-Poromaa et al., 2002; Wallner et al., 2003; Wei et al., 2004; Jia et al., 2008; Liang et al., 2008). However, some investigators cannot reproduce these findings (Borghese et al., 2006; Yamashita et al., 2006) or find that ethanol

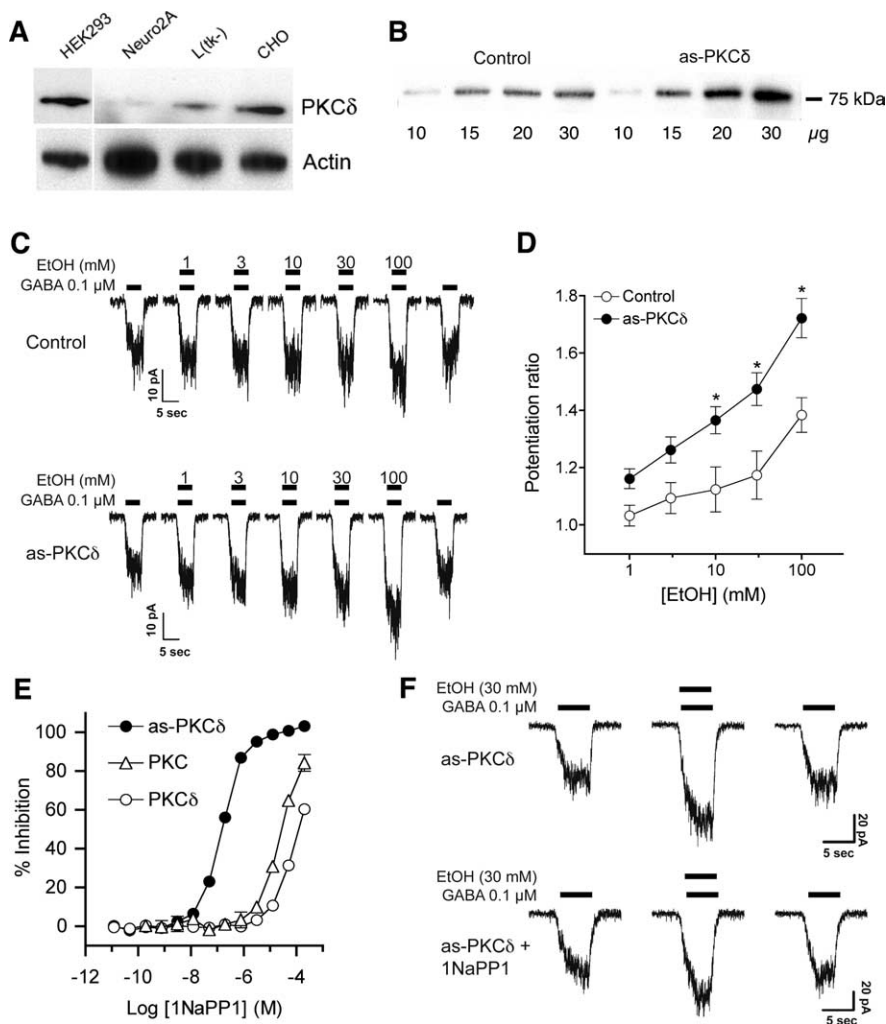


Figure 6. PKC δ regulates ethanol sensitivity of $\alpha 4\beta 3\delta$ GABA $_A$ receptors expressed in L(tk $^-$) cells. **A**, Western blot analysis of PKC δ and actin immunoreactivity in HEK293, Neuro2A, L(tk $^-$), and CHO cells showing less PKC δ abundance in L(tk $^-$) cells relative to actin when compared with CHO and HEK293 cells. **B**, Western blot showing overexpression of PKC δ in L(tk $^-$) cells after transfection with as-PKC δ . **C, D**, Ethanol enhancement of EC $_{20}$ GABA-stimulated currents in L(tk $^-$) cells that express $\alpha 4\beta 3\delta$ receptors. * p < 0.05 compared with cells not transfected with as-PKC δ at the same concentration of ethanol by Bonferroni tests. **E**, Inhibition of as-PKC δ , a commercial mixture of PKC isozymes (PKC) (Calbiochem) and wild-type PKC δ by the PP1 analog 1NaPP1. Nonlinear regression analysis showed significant differences (< 0.0001) between log EC $_{50}$ values (M) for 1NaPP1 inhibition of as-PKC δ (-6.81 ± 0.02) versus wild type PKC δ (-4.06 ± 0.04) or the mixture of PKC isozymes (-4.59 ± 0.04). **F**, L(tk $^-$) cells that express $\alpha 4\beta 3\delta$ receptors and as-PKC δ were treated with 0.1 μ M GABA and 30 mM ethanol. Ethanol reversibly enhanced the GABA-stimulated current and addition of 10 μ M 1NaPP1 reduced this effect of ethanol.

modulation of tonic GABA currents is indirect and occurs *via* stimulation of GABA release rather than by a direct effect on extrasynaptic receptors (Carta et al., 2004). These discrepant results may be due to differences in expression systems, experimental technique, or the age of the animals tested. However, our present results suggest that negative results can also be due to absent or decreased PKC δ activity in some model systems.

Our findings add to a growing body of literature that describes the role of PKC in regulating GABA signaling. Two other PKC isozymes, PKC γ and PKC ϵ , modify responses to ethanol in mice through actions at GABA $_A$ receptors. PKC $\gamma^{-/-}$ mice show reduced ethanol-induced LORR and hypothermia (Harris et al., 1995), whereas PKC $\epsilon^{-/-}$ mice show an increased duration of the ethanol-induced LORR (Hodge et al., 1999; Choi et al., 2002). Likewise, ethanol enhancement of GABA $_A$ receptor function is reduced in PKC $\gamma^{-/-}$ mice but increased in PKC $\epsilon^{-/-}$ mice (Harris et al., 1995; Hodge et al., 1999; Proctor et al., 2003). We re-

cently found that PKC ϵ regulates the ethanol sensitivity of synaptic GABA $_A$ receptors through phosphorylation of GABA $_A$ $\gamma 2$ subunits at S327 (Qi et al., 2007). The mechanism by which PKC γ regulates GABA $_A$ sensitivity to ethanol is not yet known. Despite the widespread distribution of $\gamma 2$ subunits in the brain, PKC ϵ regulation of GABA $_A$ receptors is not evident in all brain regions. In the central amygdala, for example, we recently found that ethanol enhances GABA release rather than GABA $_A$ receptor function through a process that requires PKC ϵ (Bajo et al., 2008). Thus PKC ϵ can regulate ethanol's effects on GABA systems in different brain regions through modulation of ethanol-induced GABA release or ethanol enhancement of GABA $_A$ receptor function.

Given the importance of PKC γ and PKC ϵ in regulating ethanol's effects on GABA systems, we considered whether the phenotypes we observed in PKC $\delta^{-/-}$ mice are due to altered expression of PKC γ or PKC ϵ . We found that the levels of PKC γ and PKC ϵ immunoreactivity are not different in brain samples from PKC $\delta^{-/-}$ and PKC $\delta^{+/+}$ littermates (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). We also measured the abundance of GABA $_A$ $\alpha 4$ and δ subunits because tonic GABA currents in PKC $\delta^{-/-}$ mice were not enhanced by ethanol. We found their abundance to be similar in cortex, thalamus, cerebellum, and hippocampus of both genotypes (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Finally, we measured the abundance of other PKC isozymes present in brain and found no differences between the genotypes (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Therefore, it is unlikely that the present results are due to indirect effects of

other PKC isozymes or altered expression of GABA $_A$ $\alpha 4$ and δ subunits; instead they reflect an effect of PKC δ on GABA $_A$ receptor function.

PKC $\delta^{-/-}$ mice did not show abnormalities in gross neuroanatomy, brain histology or baseline coordination, strength, or open field activity. The lack of altered brain structure and baseline motor function makes it unlikely that the diminished acute response to ethanol in PKC $\delta^{-/-}$ mice is due to major developmental changes resulting from gene deletion. There is a possibility that differences in behavior between hybrid C57BL/6J \times 129X1/SvJ wild type and PKC $\delta^{-/-}$ mice could be due to effects of 129X1/SvJ alleles surrounding the PKC δ locus that are present in the knock-out but not in the wild type population. However, C57BL/6J and 129X1/SvJ mice do not differ in their ataxic response to volatile anesthetics, and the duration of the LORR induced by 3.5 g/kg ethanol is 25% longer in 129X1/SvJ mice than in C57BL/6J mice (Homans et al., 1999). In contrast, in

PKC $\delta^{-/-}$ mice the LORR duration in response to ethanol was decreased compared with wild type littermates. Therefore, the altered behavioral responses we observed in PKC $\delta^{-/-}$ mice treated with ethanol and other drugs that modulate GABA_A receptors are unlikely due to effects of 129X1/SvJ alleles neighboring the mutated PKC δ locus.

In summary, our study provides novel evidence for a PKC δ signal transduction pathway that enhances behavioral responses to intoxicating concentrations of ethanol achieved during social drinking in humans. This regulation occurs at least in part through PKC δ modulation of tonic inhibitory currents carried by extrasynaptic GABA receptors that contain δ subunits. The level of response to acute administration of ethanol has been proposed as a useful endophenotype for genetic studies of alcohol use disorders in humans (Schuckit, 1998; Schuckit et al., 2004). In young adult subjects with a family history of an alcohol use disorder, a low level of response to a three-drink alcohol challenge, as measured by subjective response ratings and ataxia, is associated with increased risk of developing an alcohol use disorder later in life. Our findings in PKC $\delta^{-/-}$ mice, therefore, raise the possibility that identification of proteins within neuronal PKC δ signaling pathways that regulate behavioral sensitivity to ethanol and ethanol sensitivity of tonic GABA-stimulated inhibitory currents may reveal candidate genes that contribute to risk of alcohol use disorders in humans.

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