Conditional Rescue of Protein Kinase C ϵ Regulates Ethanol Preference and Hypnotic Sensitivity in Adult Mice

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Conventional gene targeting is a powerful tool to study the influence of specific genes on behavior. However, conclusions relevant for adult animals are limited by consequences of gene loss during development. Mice lacking protein kinase C ϵ (PKCϵ) consume less alcohol and show greater acute sensitivity to alcohol than do wild-type mice. There are no selective inhibitors of PKCϵ that can be administered systemically and cross the blood–brain barrier to test whether these phenotypes result from loss of PKCϵ during development or in adulthood. Here we used conditional expression of PKCϵ in the basal forebrain, amygdala, and cerebellum to rescue wild-type responses to alcohol in adult *PKCϵ*$^{-/-}$ mice. Subsequent suppression of transgenic PKCϵ restored *PKCϵ*$^{-/-}$ behaviors. These findings establish that PKCϵ signaling in the adult brain regulates alcohol consumption and sensitivity. If this extends to humans, then PKCϵ inhibitors might prove useful as novel therapeutics for alcoholism.

Key words: protein kinase C; alcohol; ethanol; doxycycline; $GABA_A$ receptor; NMDA receptor

Alcoholism is the most common form of substance abuse and is a major public health problem, causing an annual economic burden of approximately $184 billion in the United States (Shalala, 2000). One approach to understanding the neural mechanisms that underlie alcoholism is to identify molecular targets for ethanol in the brain. At the cellular level, ethanol alters the function of specific ion channels, neurotransmitter receptors, membrane transporters, and intracellular enzymes, including several protein kinases (Diamond and Gordon, 1997). One such kinase is protein kinase C ϵ (PKCϵ). In cultured neural cells, ethanol stimulates the translocation of PKCϵ from perinuclear regions to the cytoplasm (Gordon et al., 1997), whereas chronic alcohol exposure increases the abundance of PKCϵ (Messing et al., 1991; Coe et al., 1996). In PC12 cells, chronic ethanol exposure increases the density of N-type voltage-gated calcium channels (McMahon et al., 2000) and enhances NGF-induced differentiation (Hundle et al., 1997) through PKCϵ-dependent mechanisms.

In addition to being regulated by ethanol, PKCϵ also modulates behavioral responses to ethanol, as demonstrated using mutant mice that lack PKCϵ (Hodge et al., 1999; Olive et al., 2000, 2001). These mice are similar in weight to wild-type (WT) littermates, behave normally in their home cage, and show no compensatory changes in the abundance of other PKC isozymes in the nervous system (Hodge et al., 1999; Khasar et al., 1999). Compared with wild-type littermates, *PKCϵ*$^{-/-}$ mice are more sensitive to the low-dose, locomotor stimulatory effects and the high-dose, hypnotic effects of ethanol (Hodge et al., 1999). They also voluntarily consume less alcohol than wild-type mice in a two-bottle choice paradigm and during operant self-administration or after a period of alcohol deprivation that leads to increased ethanol intake (Hodge et al., 1999; Olive et al., 2000). This is associated with markedly blunted increases in extracellular dopamine in the nucleus accumbens after systemic ethanol administration (Olive et al., 2000). Because drug reinforcement is associated with drug-induced increases in nucleus accumbens dopamine (Koob et al., 1998), absence of PKCϵ may decrease alcohol consumption partly by reducing the reinforcing properties of alcohol.

These findings suggest that inhibiting PKCϵ might reduce alcohol consumption and enhance sensitivity to the acute effects of alcohol. However, these studies were conducted using conventional knock-out mice, so it is possible that the phenotypes we observed were attributable to a developmental change rather than absence of PKCϵ in adulthood. This appears to be the case for mice lacking the serotonin 5-HT1A receptor, where absence of the receptor during development is required for expression of increased anxiety-like behavior in adult mice (Gross et al., 2002). Therefore, here we used a tetracycline-regulated system (Gossen and Bujard, 1992) to restore PKCϵ expression in adult *PKCϵ*$^{-/-}$ mice and examined alcohol consumption and sensitivity to the hypnotic effect of alcohol. The findings indicate that absence or presence of PKCϵ in the adult brain regulates alcohol sensitivity and consumption.

MATERIALS AND METHODS

Generation of mouse lines. Mouse PKCϵ cDNA was cloned into the *Bam*HI site of pUHG10-3 (Kistner et al., 1996) to generate a vector-containing mouse PKCϵ driven by *tet* operator sequences coupled to a minimal cytomegalovirus (CMV) promoter. Founder lines were generated by pronuclear injection of BssS1/Ase1 fragments of this vector into C57BL/6J zygotes. Ptet-PKCϵ lines were selected for inducibility by examining PKCϵ expression in primary fibroblast cultures transfected with CMV-tetracycline transactivator (tTA) (Tremblay et al., 1998). To generate bigenic mice expressing *Ptet-PKCϵ* and prion promoter-driven tTA (*Prnp-tTA*) transgenes on the *PKCϵ*$^{-/-}$ background [double knock-

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out (DKO) mice], we crossed a founder from the line exhibiting the highest level of inducibility (Ptet-PKCε/T9) with F_2 generation C57BL/6J × 129SvJae *PKCε*$^{+/-}$ mice to generate *PKCε*$^{+/-}$ mice carrying the *Ptet-PKCε* transgene. We also crossed Prnp-tTA/F959 mice (Tremblay et al., 1998) with *PKCε*$^{+/-}$ mice to generate *PKCε*$^{+/-}$ mice carrying the *Prnp-tTA* transgene. The Prnp-tTA/F959 line had been established in the FVB/N background and backcrossed for four generations with C57BL/6J mice. These two lines of mice were then crossed to generate DKO mice. Therefore, the genetic background of the experimental mice was C57BL/6J (73.4%), 129SvJae (25%), and FVB/N (1.6%). Wild-type littermates and *PKCε*$^{-/-}$ mice singly transgenic for either the *Ptet-PKCε* transgene or the *Prnp-tTA* transgene [single knock-out (SKO) mice] were used as controls.

Mouse genotyping. Genotypes were identified by Southern blot or by PCR using DNA extracts from tail biopsies (Tremblay et al., 1998; Hodge et al., 1999). For Southern blot analysis of *Ptet-PKCε*, a 470 bp *Xho*I fragment probe was hybridized with *Hin*dIII-digested genomic DNA. The following primer sets were used for PCR: for Prnp-tTA, 5′-GGTGTAGAGCAGCCTACATT-3′ and 5′-TTCTGTAGGCCGTGTACCT-3′; for Ptet-PKCε, 5′-CCATCCACGCTGTTTTGACCTC-3′ and 5′-ATGTTGACGCTGAACCGTTGGG-3′; and for PKCε, 5′-ATATTGCTGAAGAGCTTGGCGGC-3′ and 5′-CCTAACTGAATGCTGCTCCTAC-3′. These primers generated fragments of the following sizes: 200 bp for *Prnp-tTA*, 1150 bp for *Ptet-PKCε*, and 840 bp for *PKCε*.

Animal care. Mice were housed in standard Plexiglas cages with rodent chow and water available *ad libitum*. The colony room was maintained on a 12 hr light/dark cycle with lights on at 6:00 A.M. Mice were used for experiments when they reached ~10 weeks of age. Doxycycline (Dox) was administered using chow containing 200 mg of doxycycline per killigram (Dox Diet; Bioserve, Frenchtown, NJ). Animal care and handling procedures were approved by the Institutional Animal Care and Use Committees of the University of California San Francisco and Gallo Center in accordance with National Institutes of Health guidelines.

Western blot analysis and immunohistochemistry. PKCε and β-actin were detected by Western blot analysis (Khasar et al., 1999). For immunohistochemistry, an affinity-purified anti-PKCε polyclonal antibody SN134 was generated in rabbits (SynPep, Dublin, CA) against the peptide sequence NQEEFKGFSYFGEDLMP, which is identical to the last 17 aa of mouse PKCε (Kiley and Parker, 1995). Mice were perfused with PBS, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. Brains were removed and postfixed overnight in 4% PFA, equilibrated for 48 hr in 30% sucrose in PBS at 4°C, embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA), and frozen in liquid nitrogen. PKCε immunoreactivity was identified in 10 μm frozen sections using SN134 (0.4 μg/ml) followed by detection with biotinylated anti-rabbit IgG and avidin peroxidase (Vector Laboratories, Burlingame, CA). For immunofluorescence, sections were incubated with 1.2 μg/ml SN134 and 2 μg/ml mouse anti-neuronal-specific nuclear protein (NeuN) monoclonal antibody (Chemicon International, Temecula, CA) in PBS containing 0.2% BSA and 0.2% Triton X-100 (PBS-T) for 16 hr at 4°C. This was followed by incubation with donkey anti-rabbit FITC-conjugated antibody and donkey anti-mouse Texas Red-conjugated antibody (Jackson ImmunoResearch, West Grove, PA) in PBS-T for 2 hr at 27°C. Sections were visualized and photographed using a Leica (Wetzlar, Germany) DMRB microscope with epifluorescence and Nomarski optics.

Behavioral studies. Voluntary ethanol consumption and preference were measured in three consecutive 12 d trials using the two-bottle choice procedure (Hodge et al., 1999). One month later, we examined two-bottle choice consumption and preference for saccharin and quinine (Hodge et al., 1999). Data were analyzed by three-way ANOVA with a between-subjects factor for genotype and within-subjects factors for ethanol concentration and doxycycline treatment. After completion of the preference drinking studies, mice were administered ethanol (3.6 gm/kg, i.p.), pentobarbital (50 mg/kg, i.p.), or ketamine (150 mg/kg, i.p.) and tested for duration of the loss of the righting reflex (LORR) (Hodge et al., 1999). Data were analyzed by two-way ANOVA with between-subjects factors for genotype and doxycycline treatment.

RESULTS

Brain region-specific restoration of PKCε in *PKCε*$^{-/-}$ mice

Tissue-specific expression of PKCε was established using two transgenes, one encoding mouse PKCε downstream of an array of seven *tet* operator sequences coupled to a minimal human cytomegalovirus promoter (*Ptet*) and the other encoding a tTA driven by the prion protein (*Prnp*) promoter (Fig. 1*A*) to direct expression of PKCε in the brain (Tremblay et al., 1998). Both were placed on the *PKCε*$^{-/-}$ background, generating *PKCε*$^{-/-}$, doubly transgenic mice (DKO mice). In the absence of the tetracycline analog Dox, transgenic PKCε was expressed in the brain (Fig. 1*B*,*D*) but not in other tissues (data not shown) of DKO mice. In the forebrain, the level of PKCε-like immunoreactivity was ~50% of that observed in wild-type mice (Fig. 1*B*), whereas in the cerebellum, it was ~75% of that observed in WT mice (data not shown). We were especially interested in the regional distribution of PKCε in DKO mice, because specific regions in the brainstem and forebrain are involved in drug reinforcement and motivational aspects of drug dependence, including the ventral tegmental area, nucleus accumbens, bed nucleus of the stria terminalis, lateral hypothalamus, and amygdala (Koob et al., 1998). In DKO mice, the pattern of PKCε-like immunoreactivity resembled that observed in wild-type mice in the nucleus accumbens, caudate putamen, and cerebellum (Fig. 1*D*). In contrast to wild-type mice, DKO mice showed patchy PKCε-like immunoreactivity in the central amygdala and ventral pallidum. Unlike wild-type mice, DKO mice showed minimal PKCε-like immunoreactivity in the hippocampus and cingulate cortex and no PKCε-like immunoreactivity in the ventral tegmental area, the bed nucleus of the stria terminalis, or the lateral hypothalamus. Within brain regions where PKCε-like immunoreactivity was observed, it was found primarily in neurons (Fig. 1*C*). The addition of doxycycline to the diet for 14 d completely suppressed PKCε-like immunoreactivity in DKO mice (Fig. 1*B*,*D*) but did not alter it in wild-type mice (data not shown).

Transgenic PKCε restores wild-type drinking behavior in adult *PKCε*$^{-/-}$ mice

To examine whether restoration of PKCε alters ethanol self-administration, we reared DKO mice without doxycycline and gave them continuous access to two drinking bottles, one containing water and the other containing ascending concentrations (3, 6, and 10%) of ethanol with 4 d of access at each concentration. Ethanol was then removed, and mice were administered doxycycline for 2 weeks. This was followed by a second course of ethanol access, during which time the mice were maintained on doxycycline. After this second trial, mice were fed normal chow for 2 weeks and then administered a third trial of preference drinking. Drinking behavior was examined in parallel in wild-type and singly transgenic, PKCε-deficient mice (SKO mice).

Doxycycline had a specific effect on ethanol consumption in DKO mice. During the first trial, ethanol consumption was similar in DKO and wild-type mice, whereas SKO mice drank less ethanol than wild-type mice (Fig. 2*A*). Doxycycline suppressed ethanol drinking in DKO mice during the second trial to levels observed in SKO mice (Fig. 2*B*). Removal of doxycycline increased ethanol consumption in DKO mice during the third trial to levels observed during the first trial (Fig. 2*C*). Three-way ANOVA revealed a significant interaction between genotype and doxycycline treatment ($F_{(4,70)} = 3.62$; $p = 0.0097$), such that ethanol consumption was greater in wild-type and DKO mice compared with SKO mice in trials 1 and 3, whereas consumption was greater in wild-type mice compared with DKO and SKO mice in trial 2 ($p < 0.05$; Tukey's test).

Doxycycline also selectively modulated ethanol preference in DKO mice (Fig. 2*D–F*). Three-way ANOVA revealed a signifi-

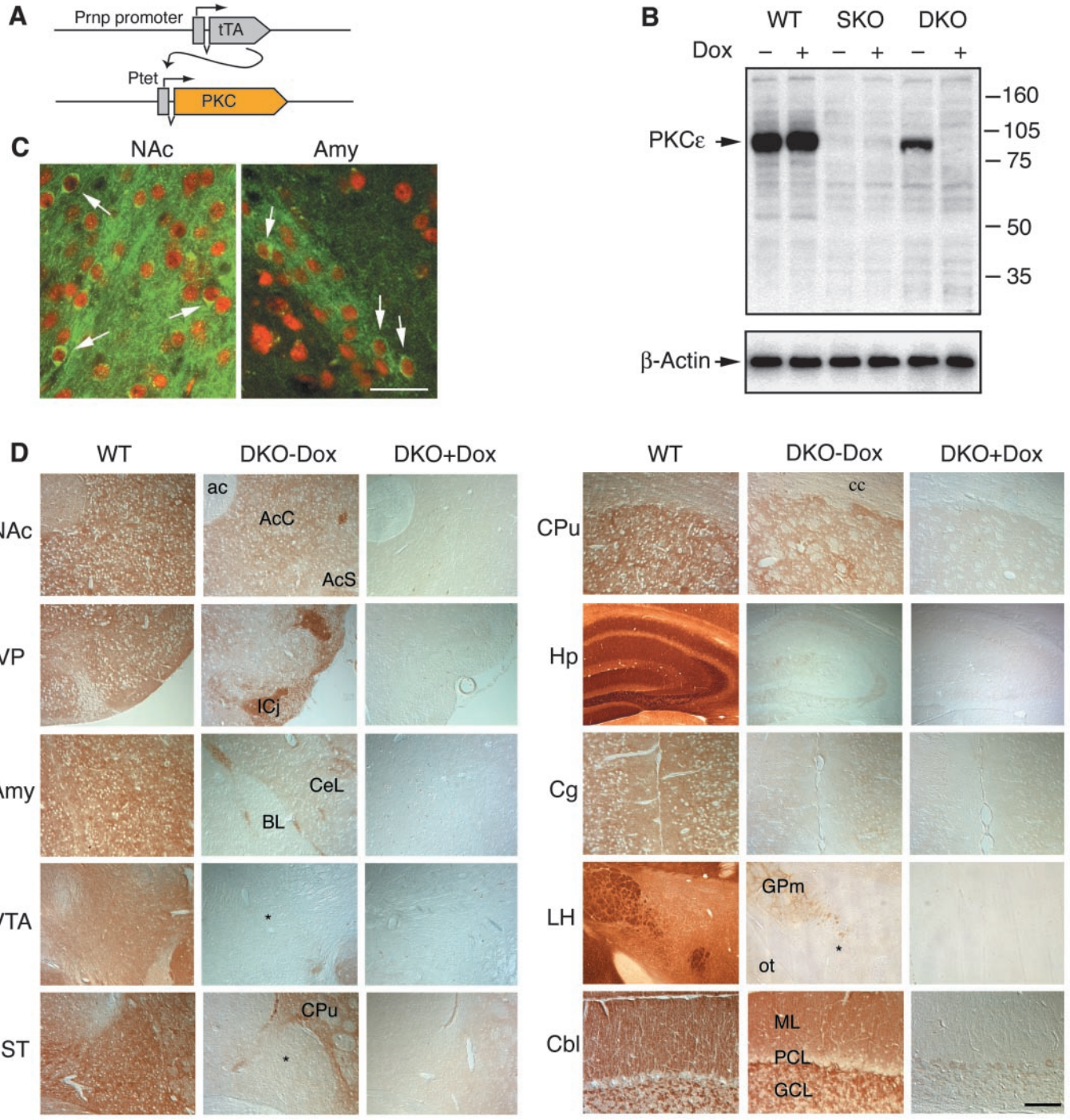

Figure 1. Regulated expression of PKCε. *A*, Schematic drawing of the *Prnp-tTA* transgene of line $Tg^{Prnp\text{-}tTA/F959}$ and the *Ptet-PKCε* transgene of line $Tg^{Ptet\text{-}PKCε/T9}$. *B*, Western blot of brain homogenate (40 μg per *lane*) from WT mice and PKCε-deficient mice carrying only the *Ptet-PKCε* transgene (*SKO*) or both *Ptet-PKCε* and *Prnp-tTA* transgenes (*DKO*), before (−) or during (+) treatment with Dox. *C*, Immunofluorescence detection of the neuronal marker NeuN (*red*) and of PKCε (*green*) in the nucleus accumbens (*NAc*) and amygdala (*Amy*). *White arrows*, Several PKCε-expressing neurons with NeuN immunoreactive nuclei surrounded by PKCε immunoreactivity in the cell soma. Scale bar, 50 μm. *D*, Immunohistochemical staining of brain tissue from WT and DKO mice fed normal chow (−*Dox*) or chow containing doxycycline (+*Dox*) for 2 weeks. Sections shown are from the nucleus accumbens (*NAc*), ventral pallidum (*VP*), amygdala (*Amy*), ventral tegmental area (*VTA*), bed nucleus of the stria terminalis (*BST*), caudate putamen (*CPu*), hippocampus (*Hp*), cingulate cortex (*Cg*), lateral hypothalamic area (*LH*), and cerebellum (*Cbl*). Labeled subregions are the nucleus accumbens core (*AcC*), nucleus accumbens shell (*AcS*), islands of Calleja (*ICj*), lateral division of the central amygdaloid nucleus (*CeL*), anterior portion of the basolateral amygdaloid nucleus (*BL*), caudate putamen (*CPu*), anterior commissure (*ac*), corpus callosum (*cc*), medial globus pallidus (*GPm*), optic tract (*ot*), and the molecular layer (*ML*), Purkinje cell layer (*PCL*), and granule cell layer (*GCL*) of the cerebellum. *Asterisks* indicate the locations of the VTA, BST, and LH. Scale bar: Cbl, 50 μm; Hp, LH, 200 μm; other sections, 100 μm. Images are from representative experiments, each repeated two times using different mice, with similar results.

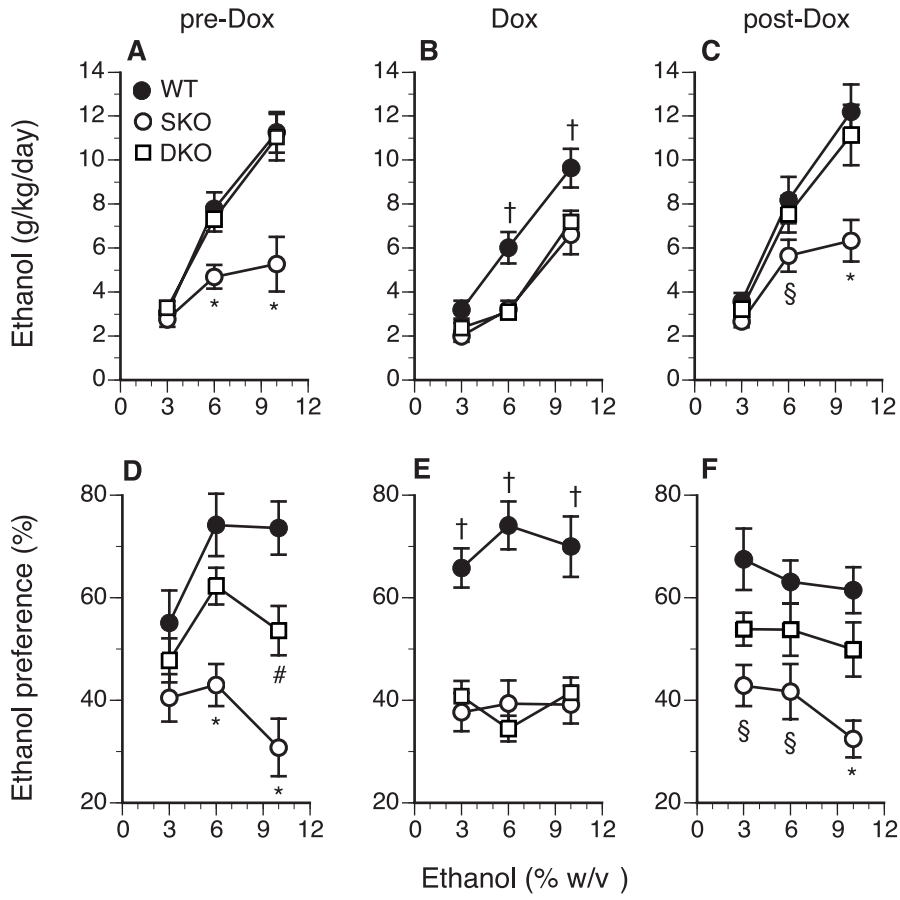

Figure 2. Doxycycline-regulated ethanol consumption and preference (mean ± SEM values) examined in 10 WT (●), 13 SKO (○), and 15 DKO (□) mice before (*pre-Dox*, *A*, *D*), during (*Dox*, *B*, *E*), and after (*post-Dox*, *C*, *F*) treatment with doxycycline. All mice were experimentally naive at the beginning of the experiment. *A–C*, Doxycycline altered ethanol consumption in a genotype and ethanol concentration-dependent manner [$F_{(8,140)}$ (genotype × doxycycline treatment × ethanol concentration) = 3.29; $p = 0.0018$]. *D–F*, Doxycycline also altered ethanol preference (100 × milliliters of ethanol per total milliliters consumed) in a genotype and ethanol concentration-dependent manner [$F_{(8,140)}$ (genotype × doxycycline treatment × ethanol concentration) = 2.27; $p = 0.026$]. Shown are the results of Tukey's tests for the three-way interaction between genotype, doxycycline treatment, and ethanol concentration (*$p < 0.05$ relative to wild-type and DKO mice in trials 1 and 3; §$p < 0.05$ relative to wild-type mice only in trial 3; †$p < 0.05$ relative to SKO and DKO mice in trial 2; #$p < 0.05$ relative to WT mice in trial 1). Results for the two-way interaction between genotype and doxycycline treatment are given in Results.

cant interaction between genotype and doxycycline treatment ($F_{(4,70)} = 3.98$; $p = 0.0058$), with ethanol preference greater in wild-type and DKO mice compared with SKO mice in trials 1 and 3 and greater in wild-type mice compared with DKO and SKO mice in trial 2 ($p < 0.05$; Tukey's test). Doxycycline treatment did not alter preference for saccharin or quinine in any of the genotypes (Fig. 3*A*,*B*), indicating that differences in ethanol intake were not related to specific taste neophobias.

Because altered ethanol intake can result from changes in appetite or fluid balance, we recorded daily water intake and body weights in these mice. There was no significant difference in average daily water intake between the genotypes, which was 2.7 ± 0.3 ml in WT ($n = 7$), 2.9 ± 0.8 ml in SKO ($n = 5$), and 3.6 ± 0.6 ml in DKO ($n = 6$) mice ($F_{(2,18)} = 0.8$; NS). During the two-bottle choice drinking study, doxycycline did not alter water consumption in DKO mice, which was 2.7 ± 0.2 ml/d during trial 1 (before doxycycline), 2.7 ± 0.1 ml/d during trail 2 (doxycycline present), and 3 ± 0.2 ml/d during the third trial (after doxycycline) ($F_{(2,132)} = 1.26$; NS). Body weight increased by 4.4 ± 0.8% over the 53 d study among mice of all three genotypes [$F_{(2,70)}$ (study day) = 9.68; $p < 0.0002$; $F_{(4,70)}$ (genotype × study day); NS]. These findings indicate that absence or presence of PKCε does not produce changes in food or water consumption that could account for the decreased ethanol consumption we observed when PKCε was absent.

Rescue of wild-type hypnotic sensitivity to ethanol in adult *PKCε*$^{-/-}$ mice

In addition to drinking less alcohol, *PKCε*$^{-/-}$ mice show heightened sensitivity to the acute, low-dose, locomotor activating and high-dose, hypnotic effect of ethanol (Hodge et al., 1999). To examine whether acute sensitivity to ethanol is also regulated by the expression of PKCε in the adult brain, we examined the ethanol-induced loss of the righting reflex in DKO mice before and after doxycycline treatment. In the absence of doxycycline, the time required to regain the righting reflex after injection of ethanol was not statistically different in DKO mice compared with wild-type mice (Fig. 4*A*). After DKO mice received doxycycline for 14 d to suppress expression of PKCε, they showed a significant increase in the duration of the ethanol-induced LORR to a level similar to the duration observed in SKO mice. After intraperitoneal administration of ethanol, blood ethanol concentrations rose and fell similarly in all three genotypes (Fig. 4*B*). Therefore, differential sensitivity of WT, SKO, and DKO mice to ethanol does not result from altered ethanol clearance but instead correlates with the presence or absence of PKCε in the adult brain.

PKCε$^{-/-}$ mice also show heightened sensitivity to the acute hypnotic effects of pentobarbital and diazepam, which, like ethanol, act as positive allosteric modulators of $GABA_A$ receptors (Hodge et al., 1999). To examine whether the response to another allosteric $GABA_A$ receptor agonist is also regulated by expression of PKCε in the adult brain, we examined pentobarbital-induced LORR in DKO mice before and after doxycycline treatment. Without doxycycline treatment, the duration of the pentobarbital-induced LORR was significantly longer in SKO mice compared with WT or DKO mice, whereas doxycycline treatment selectively increased the LORR duration in DKO mice to that observed in SKO mice (Fig. 4*C*). Because NMDA receptors also mediate acute responses to ethanol (Lovinger et al.,

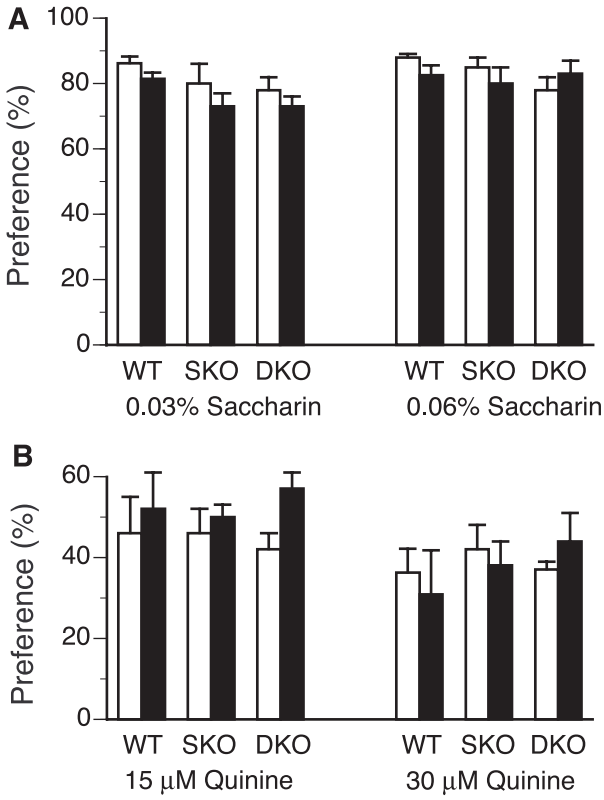

Figure 3. Preference for saccharin and quinine and total fluid intake. Data in *A* and *B* are mean ± SEM values from 10 WT, 13 SKO, and 15 DKO mice. Shown are preference ratios for solutions containing 0.03 and 0.06% saccharin (*A*) and 0.015 and 0.030 mM quinine (*B*) during 2 d of access to each solution before (*white bars*) and during (*black bars*) treatment with doxycycline.

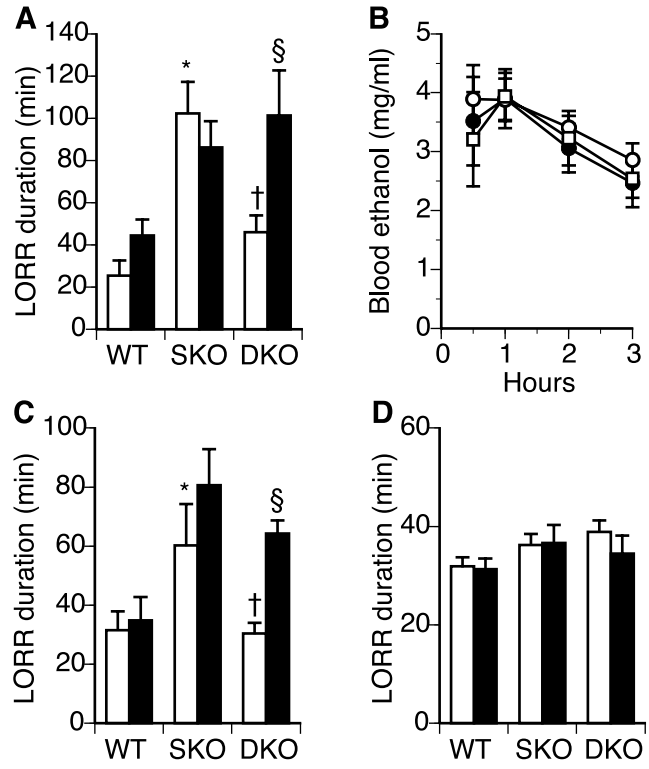

Figure 4. Drug-induced LORR. Duration of drug-induced LORR was examined before (*white bars*) or during (*black bars*) treatment with doxycycline. All data are mean ± SEM values. *A*, Ethanol-induced LORR in nine WT, 11 SKO, and 11 DKO mice not exposed to doxycycline and in 10 WT, 12 SKO, and 12 DKO mice treated with doxycycline for 2 weeks. Doxycycline altered LORR duration in a genotype-specific manner [$F_{(2,59)}$ (genotype) = 9.05, $p = 0.0004$; $F_{(1,59)}$ (doxycycline treatment), NS; $F_{(2,59)}$ (genotype × doxycycline treatment) = 3.56, $p = 0.034$]. *B*, Plasma ethanol concentrations measured using the ethanol diagnostic kit 332-UV (Sigma, St Louis, MO) did not differ among three WT, five SKO, and five DKO mice after acute administration of ethanol (3.6 gm/kg, i.p.). *C*, Pentobarbital-induced LORR examined in seven WT, seven SKO, and 11 DKO mice not exposed to doxycycline and in seven WT, nine SKO, and nine DKO mice treated with doxycycline for 2 weeks. Doxycycline altered LORR duration in a genotype-specific manner [$F_{(2,45)}$ (genotype) = 8.66, $p < 0.001$; $F_{(1,45)}$ (doxycycline treatment) = 7.08, $p = 0.011$; $F_{(2,45)}$ (genotype × doxycycline treatment) = 3.56, $p = 0.034$]. *D*, Ketamine-induced LORR duration examined in seven WT, seven SKO, and eight DKO mice not exposed to doxycycline and in seven WT, eight SKO, and eight DKO mice treated with doxycycline was similar in all genotypes before and during doxycycline treatment. Data are mean ± SEM values. $^{*}p < 0.05$ compared with WT in the absence of doxycycline and DKO in the absence of doxycycline; $^{\dagger}p < 0.05$ compared with DKO plus doxycycline; $^{\S}p < 0.05$ compared with WT plus doxycycline (Newman–Keuls tests).

1989) and are phosphorylated by PKC (Tingley et al., 1997), we examined the LORR induced by the NMDA receptor antagonist ketamine. In contrast to the ethanol- or pentobarbital-induced LORR, the duration of the ketamine-induced LORR was similar in wild-type, SKO, and DKO mice and was not altered by doxycycline treatment (Fig. 4*D*). Together, these results suggest that the presence or absence of PKCε in the adult brain modulates $GABA_A$ receptor but not NMDA receptor function in mice.

DISCUSSION

By conditionally restoring PKCε in PKCε-deficient mice, we have demonstrated that PKCε signaling in the adult brain regulates ethanol sensitivity and consumption in mice. This is the first demonstration of inducible gene expression regulating drug self-administration in animals. When taken together with other evidence indicating decreased operant self-administration and decreased deprivation-induced alcohol drinking in PKCε-deficient mice (Hodge et al., 1999; Olive et al., 2000), our results provide additional support for developing PKCε inhibitors as drugs to reduce alcohol consumption in adults.

A potential limitation with this study is that we used a prion promoter rather than a PKCε promoter to drive expression of transgenic PKCε in DKO mice. It is remotely possible that the abnormal pattern of expression we obtained led to the behavioral contingency on transgenic PKCε expression that we observed. However, we consider this unlikely, particularly because we did not observe ectopic PKCε immunoreactivity in brain regions of DKO mice that do not express PKCε in WT mice. Instead, we found PKCε immunoreactivity in a subset of neurons in DKO mice that normally express PKCε in WT animals, suggesting that findings in DKO mice are relevant for functions attributable to endogenous PKCε.

Ethanol preference ratios in DKO mice fed normal chow appeared intermediate between those observed for WT and SKO mice (Fig. 2*D*,*F*), unlike corresponding values for ethanol consumption (Fig. 2*A*,*C*). This may have been partly attributable to WT mice being slightly heavier (31.0 ± 1.2 gm) than DKO mice (27.9 ± 1.2 gm), although this difference was not statistically significant ($p = 0.099$; two-tailed, unpaired *t* test). This may have also reflected a gene dose effect, because in DKO mice fed normal chow, the level of PKCε in the forebrain was approximately half

of that observed in WT mice (Fig. 1*B*). Moreover, in DKO mice, PKCε was not restored in two regions of the mesolimbic dopaminergic system associated with drug reinforcement and reward, the ventral tegmental area and the bed nucleus of the stria terminalis. Thus, partial rescue of PKCε may have contributed to the intermediate level of alcohol preference observed in DKO mice fed normal chow.

Alcohol preference and sensitivity are quantitative traits influenced by several genes. Interestingly, PKCε has been mapped to human chromosome 2p21 (Chen et al., 1998), which overlaps a region for which modest evidence suggests a susceptibility locus for alcohol dependence (Reich et al., 1998). In contrast, no known quantitative trait loci for LORR duration or alcohol preference (Crabbe et al., 1999; Whatley et al., 1999; Browman and Crabbe, 2000; Radcliffe et al., 2000; Vadasz et al., 2000) map to mouse chromosome 17, region E4, where the gene for PKCε resides (R. Messing, unpublished observations). This does not eliminate the possibility that PKCε polymorphisms contribute to these responses in mice, because loci mapped in one set of recombinant inbred strains may not be identified in another derived from different mouse lines. Additional study is warranted to determine whether PKCε polymorphisms are associated with altered responses to alcohol in mice or with alcoholism in humans.

Our findings suggest that signaling cascades involving PKCε regulate drinking behavior and acute responses to ethanol. Receptor-mediated stimulation of phospholipase C is a major mechanism for activating diacylglycerol-sensitive PKC isozymes, such as PKCε (Nishizuka, 1992). Pharmacological or genetic manipulation of serotonin 5-HT1B (Crabbe et al., 1996), μ-opioid (Johnson and Ait-Daoud, 2000), or dopamine D1 (El-Ghundi et al., 1998) or D2 (Phillips et al., 1998) receptors alters ethanol consumption in rodents. Activation of these receptors can also stimulate phospholipase C or induce PKC translocation (Dickenson and Hill, 1998; Kramer and Simon, 1999; Xie et al., 1999; Nowicki et al., 2000; Gordon et al., 2001). However, it is not yet known whether any of these receptors regulate alcohol responses by coupling specifically to PKCε.

Ethanol modulates the function of specific voltage-gated and receptor-operated ion channels (Diamond and Gordon, 1997). Some of these channels and some of the receptors noted above that modulate ethanol consumption are PKC substrates (Hell et al., 1993; Stea et al., 1995; Moss and Smart, 1996; Zhang et al., 1996; Tingley et al., 1997; Wecker et al., 2001). Our previous work indicates that $GABA_A$ receptors are an important target for PKCε (Hodge et al., 1999). In that study, we found that ethanol and flunitrazepam caused much greater enhancement of muscimol (1 μM)-stimulated ^{36}Cl uptake in cortical membranes isolated from PKCε null mice compared with tissue from wild-type littermates. This enhanced sensitivity could be reproduced in membranes from wild-type mice after preincubation with the peptide εV1–2, which specifically inhibits PKCε (Johnson et al., 1996). This peptide had no effect on uptake in microsacs from PKCε null mice. These findings indicate that PKCε regulates $GABA_A$ receptor sensitivity to the positive allosteric actions of ethanol and benzodiazepines.

The mechanism by which PKCε regulates $GABA_A$ receptors is not yet known, but it does not appear to involve direct phosphorylation of a receptor subunit (R. Messing, unpublished observations). Increased $GABA_A$ receptor sensitivity to ethanol could contribute to decreased ethanol preference in PKCε-deficient mice, because $GABA_A$ receptors modulate voluntary alcohol consumption in rodents. For example, deletion of the δ subunit reduces ethanol preference drinking in mice (Mihalek et al., 2001). Moreover, in rats, microinjection of the direct $GABA_A$ receptor agonist muscimol into the nucleus accumbens or amygdala substitutes for ethanol in drug discrimination studies (Hodge and Cox, 1998). In addition, microinjection of $GABA_A$ receptor antagonists into the anterior ventral tegmental area (Nowak et al., 1998), or into the central nucleus of the amygdala, the bed nucleus of the stria terminalis, or the shell of the nucleus accumbens (Hyytiä and Koob, 1995), decreases ethanol self-administration. These microinjection studies suggest that actions of ethanol at $GABA_A$ receptors in specific limbic brain regions regulate ethanol discrimination and intake. If true, then considering the pattern of PKCε expression in DKO mice (Fig. 1*D*), PKCε in the nucleus accumbens or the central amygdala may be particularly important for modulating ethanol consumption.

REFERENCES

Browman KE, Crabbe JC (2000) Quantitative trait loci affecting ethanol sensitivity in BXD recombinant inbred mice. Alcohol Clin Exp Res 24:17–23.

Chen X-N, Knauf JA, Gonsky R, Wang M, Lai EH, Chissoe S, Fagin JA, Korenberg JR (1998) From amplification to gene in thyroid cancer: a high-resolution mapped bacterial-artificial-chromosome resource for cancer chromosome aberrations guides gene discovery after comparative genome hybridization. Am J Hum Genet 63:625–637.

Coe IR, Yao L, Diamond I, Gordon AS (1996) The role of protein kinase C in cellular tolerance to ethanol. J Biol Chem 271:29468–29472.

Crabbe JC, Phillips TJ, Feller DJ, Hen R, Wenger CD, Lessov CN, Schafer GL (1996) Elevated alcohol consumption in null mutant mice lacking $5\text{-}HT_{1B}$ serotonin receptors. Nat Genet 14:98–101.

Crabbe JC, Phillips TJ, Buck KJ, Cunningham CL, Belknap JK (1999) Identifying genes for alcohol and drug sensitivity: recent progress and future directions. Trends Neurosci 22:173–179.

Diamond I, Gordon AS (1997) Cellular and molecular neuroscience of alcoholism. Physiol Rev 77:1–20.

Dickenson JM, Hill SJ (1998) Human 5-HT1B receptor stimulated inositol phospholipid hydrolysis in CHO cells: synergy with Gq-coupled receptors. Eur J Pharmacol 348:279–285.

El-Ghundi M, George SR, Drago J, Fletcher PJ, Fan T, Nguyen T, Liu C, Sibley DR, Westphal H, O'Dowd BF (1998) Disruption of dopamine D1 receptor gene expression attenuates alcohol-seeking behavior. Eur J Pharmacol 353:149–158.

Gordon AS, Yao L, Wu ZL, Coe IR, Diamond I (1997) Ethanol alters the subcellular localization of delta- and epsilon protein kinase C in NG108-15 cells. Mol Pharmacol 52:554–559.

Gordon AS, Yao L, Jiang Z, Fishburn CS, Fuchs S, Diamond I (2001) Ethanol acts synergistically with a D2 dopamine agonist to cause translocation of protein kinase C. Mol Pharmacol 59:153–160.

Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci USA 89:5547–5551.

Gross C, Zhuang X, Stark K, Ramboz S, Oosting R, Kirby L, Santarelli L, Beck S, Hen R (2002) Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. Nature 416:396–400.

Hell JW, Yokoyama CT, Wong ST, Warner C, Snutch TP, Catterall WA (1993) Differential phosphorylation of two size forms of the neuronal class C L-type calcium channel alpha 1 subunit. J Biol Chem 268:19451–19457.

Hodge CW, Cox AA (1998) The discriminative stimulus effects of ethanol are mediated by NMDA and GABA(A) receptors in specific limbic brain regions. Psychopharmacology 139:95–107.

Hodge CW, Mehmert KK, Kelley SP, McMahon T, Haywood A, Olive MF, Wang D, Sanchez-Perez AM, Messing RO (1999) Supersensitivity to allosteric $GABA_A$ receptor modulators and alcohol in mice lacking PKCε. Nat Neurosci 2:997–1002.

Hundle B, McMahon T, Dadgar J, Chen C-H, Mochly-Rosen D, Messing RO (1997) An inhibitory fragment derived from protein kinase C ε prevents enhancement of nerve growth factor responses by ethanol and phorbol esters. J Biol Chem 272:15028–15035.

Hyytiä P, Koob GF (1995) $GABA_A$ receptor antagonism in the extended amygdala decreases ethanol self-administration in rats. Eur J Pharmacol 283:151–159.

Johnson BA, Ait-Daoud N (2000) Neuropharmacological treatments for alcoholism: scientific basis and clinical findings. Psychopharmacology 149:327–344.

Johnson JA, Gray MO, Chen C-H, Mochly-Rosen D (1996) A protein

kinase C translocation inhibitor as an isozyme-selective antagonist of cardiac function. J Biol Chem 271:24962–24966.
Khasar SG, Lin Y-H, Martin A, Dadgar J, McMahon T, Wang D, Hundle B, Aley KO, Isenberg W, McCarter G, Green PG, Hodge CW, Levine JD, Messing RO (1999) A novel nociceptor signaling pathway revealed in protein kinase C ε mutant mice. Neuron 24:253–260.
Kiley SC, Parker PJ (1995) Differential localization of protein kinase C isozymes in U937 cells: evidence for distinct isozyme functions during monocyte differentiation. J Cell Sci 108:1003–1016.
Kistner A, Gossen M, Zimmermann F, Jerecic J, Ullmer C, Lübbert H, Bujard H (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc Natl Acad Sci USA 93:10933–10938.
Koob GF, Sanna PP, Bloom FE (1998) Neuroscience of addiction. Neuron 21:467–476.
Kramer HK, Simon EJ (1999) Role of protein kinase C (PKC) in agonist-induced mu-opioid receptor down-regulation. I. PKC translocation to the membrane of SH-SY5Y neuroblastoma cells is induced by mu-opioid agonists. J Neurochem 72:585–593.
Lovinger DM, White G, Weight FF (1989) Ethanol inhibits NMDA-activated ion current in hippocampal neurons. Science 243:1721–1724.
McMahon T, Andersen R, Metten P, Crabbe JC, Messing RO (2000) Protein kinase C epsilon mediates up-regulation of N-type calcium channels by ethanol. Mol Pharmacol 57:53–58.
Messing RO, Petersen PJ, Henrich CJ (1991) Chronic ethanol exposure increases levels of protein kinase C δ and ε and protein kinase C-mediated phosphorylation in cultured neural cells. J Biol Chem 266:23428–23432.
Mihalek RM, Bowers BJ, Wehner JM, Kralic JE, VanDoren MJ, Morrow AL, Homanics GE (2001) GABA(A)-receptor delta subunit knockout mice have multiple defects in behavioral responses to ethanol. Alcohol Clin Exp Res 25:1708–1718.
Moss SJ, Smart TG (1996) Modulation of amino acid-gated ion channels by protein phosphorylation. Int Rev Neurobiol 39:1–52.
Nishizuka Y (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258:607–614.
Nowak KL, McBride WJ, Lumeng L, Li TK, Murphy JM (1998) Blocking GABA(A) receptors in the anterior ventral tegmental area attenuates ethanol intake of the alcohol-preferring P rat. Psychopharmacology 139:108–116.
Nowicki S, Kruse MS, Brismar H, Aperia A (2000) Dopamine-induced translocation of protein kinase C isoforms visualized in renal epithelial cells. Am J Physiol 279:C1812–C1818.
Olive MF, Mehmert KK, Messing RO, Hodge CW (2000) Reduced operant ethanol self-administration and in vivo mesolimbic dopamine responses to ethanol in PKCε-deficient mice. Eur J Neurosci 12:4131–4140.
Olive MF, Mehmert KK, Nannini MA, Camarini R, Messing RO, Hodge CW (2001) Reduced ethanol withdrawal severity and altered withdrawal-induced c-fos expression in various brain regions of mice lacking protein kinase C-epsilon. Neuroscience 103:171–179.
Phillips TJ, Brown KJ, Burkhart-Kasch S, Wenger CD, Kelly MA, Rubinstein M, Grandy DK, Low MJ (1998) Alcohol preference and sensitivity are markedly reduced in mice lacking dopamine D2 receptors. Nat Neurosci 1:610–615.
Radcliffe RA, Bohl ML, Lowe MV, Cycowski CS, Wehner JM (2000) Mapping of quantitative trait loci for hypnotic sensitivity to ethanol in crosses derived from the C57BL/6 and DBA/2 mouse strains. Alcohol Clin Exp Res 24:1335–1342.
Reich T, Edenberg HJ, Goate A, Williams JT, Rice JP, Van Eerdewegh P, Foroud T, Hesselbrock V, Schuckit MA, Bucholz K, Porjesz B, Li TK, Conneally PM, Nurnberger Jr JI, Tischfield JA, Crowe RR, Cloninger CR, Wu W, Shears S, Carr K, et al (1998) Genome-wide search for genes affecting the risk for alcohol dependence. Am J Med Genet 81:207–215.
Shalala DE (2000) Tenth Special Report to the U. S. Congress on Alcohol and Health. Rockville, MD: United States Department of Health and Human Services.
Stea A, Soong TW, Snutch TP (1995) Determinants of PKC-dependent modulation of a family of neuronal calcium channels. Neuron 15:929–940.
Tingley WG, Ehlers MD, Kameyama K, Doherty C, Ptak JB, Riley CT, Huganir RL (1997) Characterization of protein kinase A and protein kinase C phosphorylation of the *N*-methyl-D-aspartate receptor NR1 subunit using phosphorylation site-specific antibodies. J Biol Chem 272:5157–5166.
Tremblay P, Meiner Z, Galou M, Heinrich C, Petromilli C, Lisse T, Cayetano J, Torchia M, Mobley W, Bujard H, DeArmond SJ, Prusiner SB (1998) Doxycycline control of prion protein transgene expression modulates prion disease in mice. Proc Natl Acad Sci USA 95:12580–12585.
Vadasz C, Saito M, Balla A, Kiraly I, Vadasz II C, Gyetvai B, Mikics E, Pierson D, Brown D, Nelson JC (2000) Mapping of quantitative trait loci for ethanol preference in quasi-congenic strains. Alcohol 20:161–171.
Wecker L, Guo X, Rycerz AM, Edwards SC (2001) Cyclic AMP-dependent protein kinase (PKA) and protein kinase C phosphorylate sites in the amino acid sequence corresponding to the M3/M4 cytoplasmic domain of alpha4 neuronal nicotinic receptor subunits. J Neurochem 76:711–720.
Whatley VJ, Johnson TE, Erwin VG (1999) Identification and confirmation of quantitative trait loci regulating alcohol consumption in congenic strains of mice. Alcohol Clin Exp Res 23:1262–1271.
Xie W, Samoriski GM, McLaughlin JP, Romoser VA, Smrcka A, Hinkle PM, Bidlack JM, Gross RA, Jiang H, Wu D (1999) Genetic alteration of phospholipase C beta3 expression modulates behavioral and cellular responses to mu opioids. Proc Natl Acad Sci USA 96:10385–10390.
Zhang L, Yu Y, Mackin S, Weight FF, Uhl GR, Wang JB (1996) Differential mu opiate receptor phosphorylation and desensitization induced by agonists and phorbol esters. J Biol Chem 271:11449–11454.