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Decreased Ethanol Sensitivity and Tolerance Development in γ-Protein Kinase C Null Mutant Mice Is Dependent on Genetic Background

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Initial sensitivity and tolerance development to the sedative-hypnotic and hypothermic effects of ethanol were investigated in γ-protein kinase C (PKC) null mutant mice. Null mutants from a C57BL/6J × 129/SvJ mixed genetic background demonstrated decreased ethanol sensitivity and failed to develop chronic tolerance after 10 days of ethanol liquid diet. However, when the null mutation was introgressed onto a C57BL/6J background for six generations, the "no tolerance" phenotype for sedative-hypnotic and hypothermic effects of ethanol was no longer apparent. Outcrossing the γ-PKC null mutation to a C57BL/6J × 129/SvEvTac mixed background restored the "no tolerance" phenotype to ethanol-induced sedation after chronic ethanol diet; however, as measured by hypothermia, tolerance was still evident in the null mutant mice. These observations and the results of tests of chronic tolerance in the C57BL/6J, 129/SvJ, and 129/SvEvTac background inbred strains indicate that γ-PKC plays an important role in initial sensitivity and tolerance to ethanol. However, the impact of γ-PKC is modulated by the background genotype. These results stress the importance of including the effect of genetic background when evaluating the effects of single gene mutations on quantitative behavioral traits.

Key Words: Ethanol Tolerance, Initial Sensitivity, γ-Protein Kinase C, Null Mutant Mice, Genetic Background.

THE DEVELOPMENT of tolerance has been considered as one factor in the etiology of alcoholism. Consequently, the identification of neural mechanisms responsible for tolerance has received much attention. In rodents, repeated exposure to ethanol via liquid diet or repeated injections across days has been shown to produce tolerance (Deitrich et al., 1996; Kalant, 1998). Tolerance development involves both metabolic and neural adaptation. The latter type of tolerance can be divided into different forms by its temporal development. Rapid tolerance occurs across treatment sessions, but can often be observed as early as after a single dose of ethanol, whereas chronic functional tolerance occurs over longer time periods after chronic ethanol exposure (weeks to months). Studies of tolerance development have focused on nearly every aspect of brain function, including changes in neurotransmitter systems, ion channel function, and second messenger systems, including protein kinases, after repeated exposure to ethanol (Battaini et al., 1989; Messing et al., 1991; Nevo and Hamon, 1995; Fitzgerald and Nestler, 1995; Coe et al., 1996).

The Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C (PKC), is composed of a family of enzymes that plays an important role in many cellular processes, such as protein receptor function (Snell et al., 1994a; Kitamura et al., 1993; Dildy-Mayfield and Harris, 1994), synaptic plasticity (Akers et al., 1986), and learning and memory (Wehner et al., 1990; Fordyce and Wehner, 1993a,b). Recent evidence has demonstrated that PKC regulates ethanol's acute effects on several neurotransmitter receptors such as NMDA, AMPA/kainate, serotonergic, and M1 muscarinic receptors (Dildy-Mayfield and Harris, 1995; Snell et al., 1994b; Sanna et al., 1994). It has been shown that phosphorylation of the $GABA_A$ receptor subunit, γ-2L, by PKC is necessary for ethanol potentiation of $GABA_A$ receptor function (Wafford et al., 1991). The mechanism by which ethanol influences PKC activity is not well understood. Studies that have investigated the effects of acute ethanol exposure on PKC activity in vitro, have demonstrated decreases (Slater et al., 1993; Dietrich et al., 1989) or no change (Dietrich et al., 1989; Machu et al., 1991) in PKC activity. In vivo studies of ethanol's acute effects on PKC activity indicate that dependent on experimental conditions, membrane-bound PKC activity can be enhanced (Dietrich et al., 1989) or inhibited (Steiner et al., 1997). In contrast to the variability in acute ethanol treatment paradigms, chronic ethanol treatment appears to produce an increase in PKC activity when studied in cell culture systems (Messing et al., 1991; Coe et al., 1996; Gordon et al., 1997) and in vivo (Battaini et al., 1989).

Studies designed to investigate the neuronal mechanisms underlying cellular tolerance in vivo often use one of two approaches: (1) the production of tolerance via repeated ethanol treatment followed by an assessment of changes in brain function or (2) an alteration of brain function produced by genetic, pharmacological, or surgical manipulation, then assessment of changes in tolerance development.

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A unique genetic model for studying PKC's role in ethanol tolerance is the recently developed γ-PKC null mutant mice (Abeliovich et al., 1993a,b). These mice are produced by gene-targeted homologous recombination and lack the neural-specific γ-PKC isotype. Long-term potentiation is impaired in the null mutant mice and in tests of spatial learning ability the mutants demonstrate a slight deficiency in complex learning (Abeliovich et al., 1993a,b). Except for mild ataxia, these mice are normal in appearance. When tested with their heterozygote and wild-type littermates as controls, the γ-PKC null mutants provide an alternative genetic model to evaluate the relationship between γ-PKC and ethanol responses.

Tests of initial sensitivity to ethanol as measured by loss of righting reflex and disruption of the control of body temperature, as well as in vitro assays of $GABA_A$ receptor function as measured by Cl^- -flux, have recently been completed with the γ-PKC null mutants (Harris et al., 1995). The results of these studies demonstrated that the mutant mice exhibit reduced sensitivity to both behavioral measures after a 3.5 g/kg intraperitoneal (IP) injection of ethanol when compared with their wild-type littermates. In addition, ethanol potentiation of muscimol-stimulated Cl^- flux was absent in microsacs prepared from mutant cortical and cerebellar tissue. These responses were specific to ethanol; treatments with pentobarbital and flunitrazepam in the null mutants did not result in a decrease in the duration of loss of righting reflex or drug-induced hypothermia, and muscimol-stimulated Cl^- flux was potentiated by both drugs. Ethanol metabolism was not different between mutants and wild-types (Harris et al., 1995).

Previous studies have suggested that initial sensitivity to ethanol and tolerance development are associated (Crabbe et al., 1982; Gallaher et al., 1996; Waller et al., 1983; Le and Kilanmaa, 1990). Given that the γ-PKC null mutant mice demonstrate reduced initial sensitivity and that PKC appears to be important in ethanol's actions, we have conducted a series of experiments with the γ-PKC null mutants and their control littermates to evaluate possible γ-PKC mechanisms in the development of chronic functional tolerance. When using animal models generated from gene-targeting approaches, it is important to consider possible interactions with background genotypes because most complex phenotypes, including ethanol tolerance, are polygenic traits (Gerlai, 1996; Wehner and Bowers, 1995; Banbury Conference, 1997). Background genotype may be important in ethanol tolerance; several studies have reported significant inbred strain and selected line differences in tolerance development (Crabbe et al., 1982, 1989, 1996; Gallaher et al., 1996; Waller et al., 1983; Luo et al., 1995; Moore and Kakihana, 1978). Therefore we have tested (1) the γ-PKC null mutant genotype on three different genetic backgrounds and (2) the individual inbred strains on which the γ-PKC null mutation was placed to determine the integrity of the ethanol tolerance response in the null mutant mice.

MATERIALS AND METHODS

Mice

Male and female mice, 60 to 90 days of age, were housed in like-sex groups of 2 to 5. All mice were maintained on a 12-hr light/dark cycle (lights on at 0700) and were allowed food (Wayne Lab Blox) and water ad libitum. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME), then bred in the specific pathogen free mouse colony at the Institute for Behavioral Genetics (Boulder, CO). Mice from the 129/SvJ inbred strain were purchased from Jackson Laboratories, and mice from the 129/SvEvTac inbred strain were purchased from Taconic Farm (Germantown, NY) at 3 to 5 weeks of age. All mice were housed in the animal holding facility at the Institute for Behavioral Genetics until testing. Breeding pairs of 129/SvEvTac mice were maintained to generate litters for subsequent testing. Mice of mixed genetic backgrounds and the congenic mice were developed as described.

γ-PKC Null Mutation on C57BL/6J × 129/SvJ Background Null mutant mice were derived using gene-targeting techniques and homologous recombination as previously described (Abelovich et al., 1993a,b). The original clone was isolated from a library of 129/SvJ inbred mice, altered with a neomycin gene insert to create the γ-PKC null mutation, and injected into embryonic stem cells, then placed into blastocysts from the C57BL/6J inbred strain. The test cross for germline transmission was to C57BL/6J; therefore, the resulting mice were a mixed C57BL/6J × 129/SvJ genotype. Mutant mice were produced from heterozygote matings from different families, such that offspring from common grandparents were never crossed. This breeding strategy produced all three genotypes used as experimental and control groups: mutant, heterozygote, and wild-type. Mice were genotyped prior to testing using isolated tail DNA analyzed by polymerase chain reaction methods. DNA was amplified using two sets of primers, one set designed to amplify the neomycin gene insert and one for the γ-PKC gene. Tests of functional tolerance were compared between the null mutants and wild-type controls.

γ-PKC Null Mutation on C57BL/6J Congenic Background Congenic mice were developed to introgress the null mutation onto a pure genetic background: the C57BL/6J inbred strain. The congenic mice were derived by repeated backcrossing of mice carrying the null mutation from the mixed C57BL/6J × 129/SvJ background with C57BL/6J mice. The first generation (N1) was created by backcrossing homozygous mutants with C57BL/6J mice. Subsequent generations were bred using heterozygotes crossed with C57BL/6J mice. In the present study animals were tested for chronic functional tolerance at the N6 generation. Again, mutants were produced by breeding heterozygotes from the N6 generation resulting in the three genotypes. Breeding was continued for 10 generations. At the N7 to N10 generations, the homozygous null mutants did not survive. Therefore, the null mutation was regenerated by outcrossing to the 129/SvEvTac inbred strain.

γ-PKC Null Mutation of C57BL/6J × 129/SvEvTac Background To regenerate the null mutation on a mixed background, homozygotes from the congenic N6 generation were crossed with 129/SvEvTac mice. This strain of 129 mice was chosen instead of the 129/SvJ inbred strain, because the 129/SvEvTac mice are more reliable breeders, reproducing more consistently with larger litters. The resulting cross (F1) between the N6 congenics and the 129/SvEvTac mice consisted of animals heterozygote for the null mutation on a mixed C57BL/6J × 129/SvEvTac background. These heterozygotes were mated to produce an F2 generation that consisted of all three genotypes on the mixed background.

Chronic Ethanol Diet and Tolerance Testing

Chronic ethanol treatment was administered via liquid diet designed to produce reliable blood ethanol concentrations (De Fiebre et al., 1994). Animals were singly housed in the testing room (ambient room temperature is maintained between 22.8° and 23.4°C) before the start of ethanol diet administration. The schedule of treatment for the ethanol group was as follows: on day 1, animals received a diet containing 0% ethanol-derived calories (EDC); followed by 3 days on 18.5% EDC; and 7 days on

27% EDC. This schedule was selected because mutants did not survive a 36% EDC diet in preliminary studies. Control diets contained sucrose to match the ethanol-derived calories in the ethanol diet. In addition, pilot studies showed that mutant mice consumed less diet than wild-type mice; therefore, the littermate controls on ethanol diet as well as the sucrose-matched controls, were "yoked" to the mutants' consumption levels after the first day when all mice received 25 ml of ethanol or control diet. Mutant mice always received 25 ml of the ethanol diet; measurements of their average consumption for each day were used to determine the volume of diets given to the remaining groups. Tests of the inbred strains (C57BL/6J, 129/SvJ, and 129/SvEvTac) required "yoking" of the sucrose-matched controls to the ethanol-treated group. Mice were weighed every 3 days. Analyses indicated that weight loss occurred in both ethanol diet- and control diet-treated groups, which did not differ from each other (data not shown). Tolerance testing was performed 5 hr after removal of the ethanol diet. This time period has been shown to be sufficient for ethanol clearance (De Fiebre et al., 1994; De Fiebre and Collins, 1993). At the time of diet-removal, "yoked" animals had consumed their allotted volume of control and ethanol diet. Mutant mice never consumed all 25 ml of ethanol diet administered each day. Peak blood ethanol concentrations (BECs) were not measured during chronic ethanol diet treatment. Therefore, a concern may arise to the effects of possible differential BECs among the genotypes at the time of tolerance testing on the final tolerance measures. However, as presented in the "Results" section, ethanol-treated wild-type mice did develop tolerance. This outcome is consistent with the suggestion that adequate ethanol consumption had taken place in wild-type mice and that the duration of tolerance was sufficient to be observed behaviorally for at least 5 hr after the removal of the diet from all mice.

Tolerance was evaluated using sleep-time and hypothermia after a 3.8 g/kg (20% w/v) challenge dose of ethanol injected intraperitoneally. Sleep-time and hypothermia were measured simultaneously in each mouse after injection. Sleep-time was performed as described previously (Marley et al., 1986). Briefly, upon reaching ataxia after IP injection, the mice were placed in a V-shaped trough. Sleep-time is defined as the amount of time, rounded to the nearest minute, between the loss and regaining of the righting response. Hypothermia was evaluated as follows: baseline body temperatures were recorded 5 min before ethanol injection; followed by temperature recordings taken at a minimum, 30-min postinjection; however, in some experiments, measurements were taken up to 210 min after injection. Room temperature was monitored throughout the experiments, which ranged from 21.7° to 23.4°C for the majority of experiments; however on three occasions, by the end of the experiment, room temperature had risen to 26.1° to 26.4°C. When compared with additional experiments within the same genotype, the increase in temperature did not appear to alter hypothermic responses. For measurement of waking BECs, blood was collected in 10 μl heparinized capillary tubes from the retro-orbital sinus immediately after mice regained the righting response. Blood ethanol levels were evaluated by enzymatic assay as described previously (Smolen et al., 1986).

Data Analysis

Analyses of sleep-time data and BECs in experiments that compared chronic tolerance responses among mutant, heterozygote, and wild-type littermates were performed using two-way analysis of variance (ANOVA), with genotype and treatment as independent variables. Hypothermia (differences between ethanol-induced changes in body temperature and baseline temperatures across sampling time) experiments in which body temperatures were recorded at 30 min after ethanol injection were analyzed using ANOVAs or Student's *t* tests to compare groups. Data from experiments with multiple time points were analyzed using repeated-measures ANOVA, with time as the within-subjects factor, and genotype and treatment as the between-subjects factors. Where significant main effects were reported, post-hoc tests (Newman-Keuls) were used to evaluate differences among the experimental groups. Student's *t* tests were used to analyze treatment effects on sleep-time and BECs in the inbred strain chronic tolerance experiments.

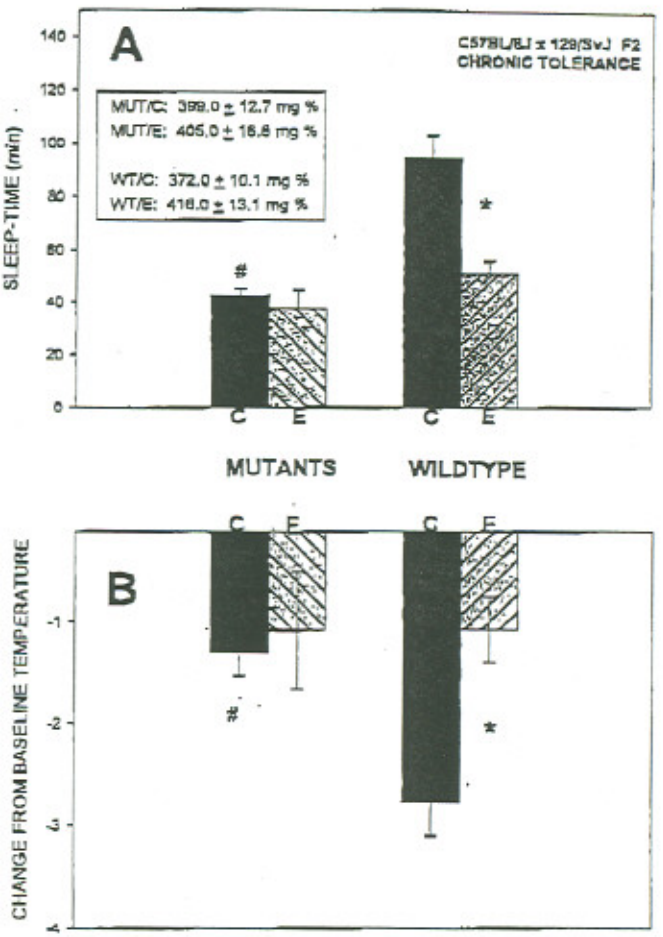

Fig. 1. Development of chronic tolerance after ethanol diet in null mutant (MUT) and wild-type (WT) mice from the C57BL/6J × 129/SvJ F2 generation. Control diet = C; ethanol diet = E. (A) Chronic tolerance did not develop as measured by sleep-time after a 3.8 g/kg challenge dose of ethanol in the ethanol-treated γ-PKC null mutant mice ($n = 11$) when compared with mice on control diet ($n = 14$). Tolerance did develop in the wild-type mice; ethanol-treated mice ($n = 13$) exhibited a reduction in sleep-time, compared with control mice ($n = 15$) ($^{*}p < 0.0001$). Mean waking BECs ± SEM are shown in the figure inset and were greater in the ethanol-treated mice. (B) Chronic hypothermia tolerance measured 30 min after a 3.8 g/kg challenge dose of ethanol did not develop in the null mutant mice ($n = 14$) on ethanol diet when compared with control mice ($n = 15$). However, wild-type mice on ethanol diet ($n = 17$) did develop tolerance demonstrated by an attenuation in the ethanol-induced decrease in body temperature, compared with control-treated mice ($n = 16$) ($^{*}p < 0.001$). Sensitivity to the sedative-hypnotic and hypothermic effects of ethanol in the mutants given control diet was significantly less than wild-type mice on control diet. $^{\#}p < 0.0001$, $^{\#}p < 0.001$, sleep-time and hypothermia, respectively.

RESULTS

C57BL/6J × 129/SvJ F2

Sleep-time Chronic functional tolerance after 10 days of ethanol or control diet was evaluated in γ-PKC null mutant mice by comparing their responses to those of their wild-type littermates in four separate experiments. Sleep-time (Fig. 1A) was performed as described in the "Materials and Methods" section. Both the effects of genotype and ethanol treatment were significant as analyzed by two-way ANOVA ($F_{1,49} = 29.1$, $p < 0.0001$; $F_{1,49} = 15.74$, $p < 0.0001$, respectively). The interaction between genotype and treat-

ment was also significant ($F_{1,49} = 10.15, p < 0.01$). This is primarily due to the lack of tolerance observed in the mutant mice to the sedative-hypnotic effects of a 3.8 g/kg challenge dose of ethanol ($t_{23} = 0.71, p = 0.487$), compared with the tolerance exhibited by the wild-type mice ($t_{26} = 4.45, p < 0.0001$). In addition, the mutants on the control diet were less sensitive to the challenge dose of ethanol than the wild-types on the control diet ($t_{23} = 5.96, p < 0.0001$). Waking BECs in the wild-type mice indicated that ethanol-treated mice had higher BECs consistent with the development of tolerance ($t_{25} = 2.76, p < 0.01$) (see Fig. 1 insert for values).

Hypothermia A comparison of baseline body temperatures before ethanol injection demonstrated that temperatures were lower in ethanol-treated mice ($F_{1,59} = 20.31, p < 0.0001$), but the interaction of genotype and treatment was not significant indicating that basal body temperatures in wild-type and mutant mice were not differentially affected by ethanol diet. A two-way ANOVA comparing the change in baseline body temperature at 30 min after a 3.8 g/kg challenge dose of ethanol (see Fig. 1B) indicated that the main effects of genotype and treatment were significant ($F_{1,60} = 3.93, p < 0.05$; $F_{1,60} = 6.69, p < 0.01$; respectively). The interaction between genotype and treatment was also significant ($F_{1,60} = 8.37, p < 0.05$). As was observed for sleep-time, the interaction was primarily due to the lack of tolerance observed in the mutant mice ($t_{26} = 0.35, p = 0.73$). A comparison of treatment effects within the wild-type group demonstrated that they were tolerant to the challenge dose; the drop in body temperature was greater in mice that had received the control diet ($t_{31} = 3.73, p < 0.001$). Also, like the sleep-time data, within the control groups null mutants mice were less responsive than wild-type mice ($t_{29} = 3.64, p < 0.001$).

N6 Congenic F2

Sleep-time In the offspring of the sixth generation (N6) of the congenic line of mice, the phenotype previously associated with the γ-PKC null mutants (i.e., loss of tolerance to the sedative-hypnotic effects of ethanol administered after chronic liquid ethanol diet) was no longer apparent. Figure 2A illustrates the results of a genotype by treatment two-way ANOVA of the sleep-time data from one experiment indicating that all three ethanol-treated genotypes, including the mutant mice, exhibited tolerance to the 3.8 g/kg challenge dose of ethanol ($F_{1,27} = 17.56, p < 0.0001$). The main effect of genotype and the interaction between genotype and treatment were not significant, indicating that within the ethanol-treated and control-treated groups, the genotypes did not differ.

Waking blood ethanols were different between treatments ($F_{1,27} = 38.96, p < 0.0001$) with the ethanol-treated groups waking at higher BECs than control animals (see Fig. 2A insert for values). This suggests that the observed tolerance is functional tolerance.

Hypothermia Baseline body temperatures before injection did not differ among the genotypes ($p = 0.8$) and did not differ after ethanol diet ($p = 0.925$). Body temperatures were recorded 30 min after a 3.8 g/kg challenge dose of ethanol (Fig. 2B). The results of a treatment × genotype ANOVA indicated a significant main effect of treatment ($F_{1,28} = 11.48, p < 0.003$) (i.e., tolerance developed in the ethanol-treated mice). While the heterozygotes' responses were variable, there were no differences among the genotypes, and the treatment × genotype interaction was not significant according to the overall ANOVA.

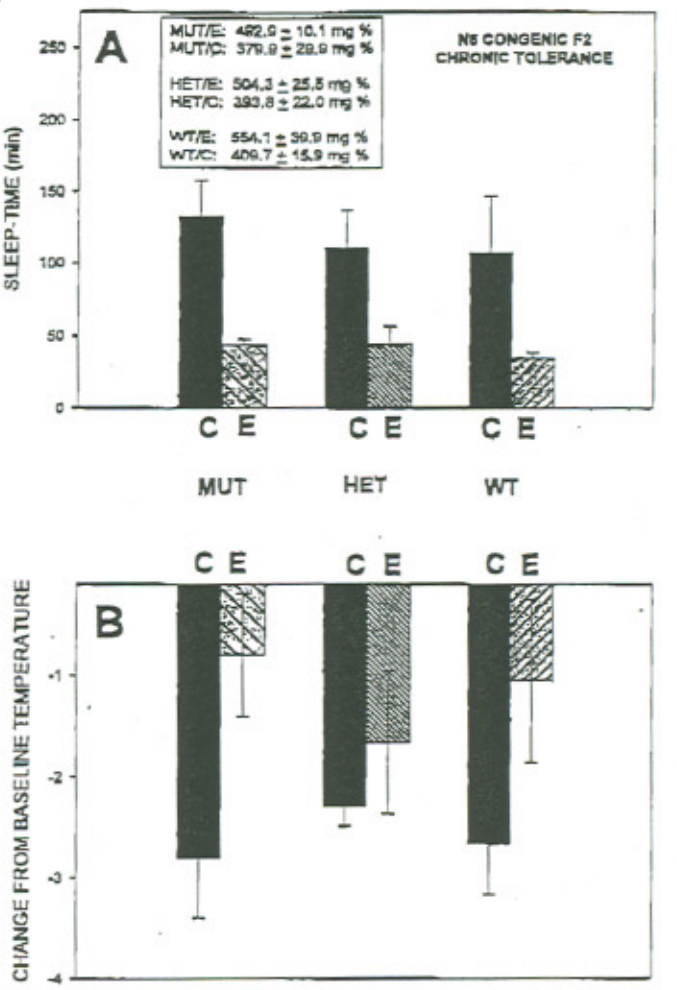

Fig. 2. Development of chronic functional tolerance after 10 days of ethanol diet in γ-PKC null mutant (MUT), heterozygote (HET), and wild-type (WT) mice from the F2 generation of the N6 congenic line. Mice were administered ethanol diet or control diet for 10 days as described in the "Materials and Methods" section. Bars represent the mean ± SEM for treatments within each genotype ($n = 4$–6, genotype by treatment); control diet = C; ethanol diet = E. (A) Chronic tolerance as measured by sleep-time after a 3.8 g/kg challenge dose of ethanol was observed in all three genotypes ($p < 0.0001$). Mean waking BECs ± SEM (shown in the insert) differed between the treatments in all groups; the tolerant, ethanol-treated mice woke with higher BECs than the nontolerant, control-treated mice. (B) Chronic tolerance to ethanol-induced hypothermia was observed at 30-min postinjection in all three genotypes ($p < 0.003$).

C57BL/6J × 129/SvEvTac F2

Sleep-time The results of a two-way ANOVA of data collected from three separate experiments testing for sleep-

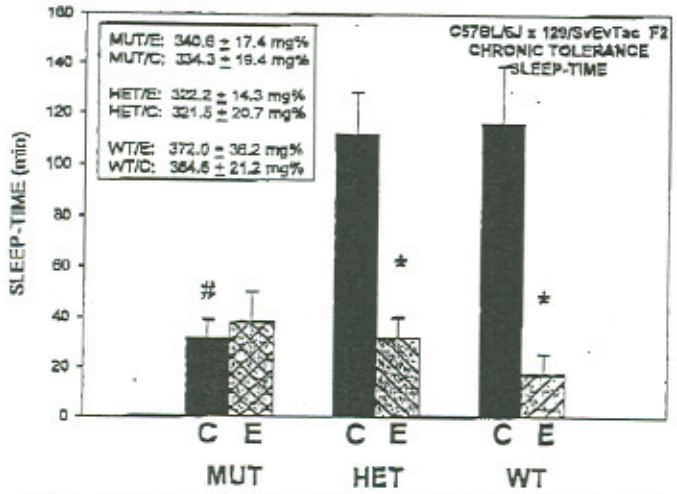

Fig. 3. Tests of chronic functional tolerance to the sedative-hypnotic effects of a 3.8 g/kg challenge dose of ethanol in mice from the C57BL/6J × 129/SvEvTac F2 generation. Null mutant (MUT), heterozygote (HET), and wild-type (WT) mice were administered chronic ethanol diet ($n = 13, n = 17, n = 13$, mutant, heterozygote, and wild-type, respectively) or control diet ($n = 12, n = 14, n = 10$, mutant, heterozygote, and wild-type, respectively) for 10 days as described in the "Materials and Methods" section. Bars represent the mean ± SEM of sleep-time (min) of control- and ethanol-treated mice from each; control diet = C; ethanol diet = E. Tolerance was observed in the heterozygote and wild-type mice ($^*p < 0.0001$), but not in null mutant mice. Waking BECs, shown in the figure insert, did not differ among the groups. Initial sensitivity was reduced in control-treated mutant mice relative to control groups from the remaining genotypes ($\#p < 0.001$).

time differences among the three genotypes after chronic ethanol diet (Fig. 3) indicated that differences in both genotype and ethanol treatment were significant ($F_{2,78} = 4.811, p < 0.01$; $F_{1,78} = 29.387, p < 0.0001$; genotype and treatment, respectively). The interaction between genotype and treatment was also significant ($F_{2,78} = 9.04, p < 0.001$). This is primarily due to the observation that mutant mice on ethanol diet were not tolerant to the effects of a 3.8 g/kg challenge dose of ethanol; whereas the wild-type and heterozygote mice did demonstrate significant tolerance ($t_{21} = 4.47, p < 0.0001$; $t_{29} = 4.47, p < 0.0001$; wild-type and heterozygote, respectively). Therefore, the "no-tolerance" phenotype for sleep-time was restored on this mixed genetic background. Waking BECs did not differ among any of the treatment groups (see Fig. 3 insert for values).

The significant interaction also reflects differences among the three genotypes within the control groups; the mutants were less sensitive to the challenge dose of ethanol than both the heterozygote and the wild-type mice, which did not differ from each other ($p < 0.05$, Newman-Keuls). Within the ethanol-treated groups the three genotypes did not differ.

Hypothermia Like the effects of the γ-PKC mutation on the N6 congenic background, tolerance to ethanol-induced hypothermia after chronic diet was observed in γ-PKC null mutant mice when the mutation was placed on the C57BL/6J × 129/SvEvTac mixed genetic background (Fig. 4). Thus, the effects of the γ-PKC mutation was different from those in the C57BL/6J × 129/SvJ background (Fig. 1). A

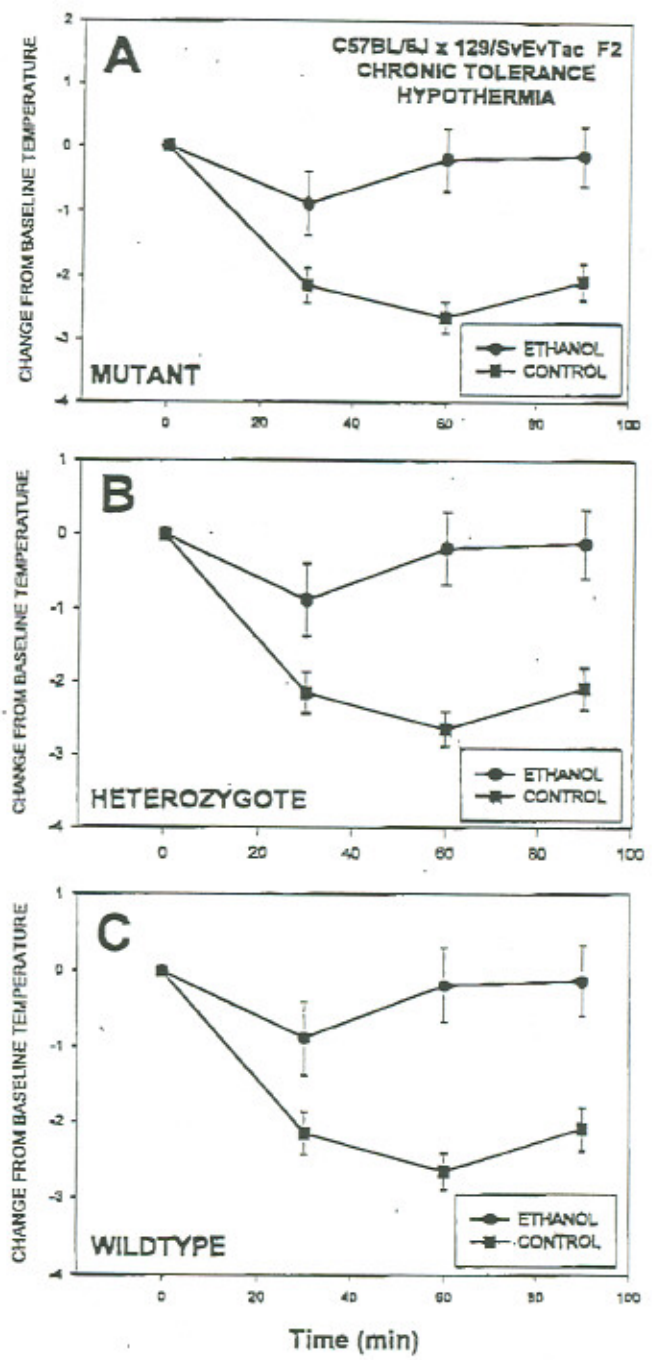

Fig. 4. Development of chronic functional tolerance to the hypothermic effects of a 3.8 g/kg challenge dose of ethanol in (A) mutant, (B) heterozygote, and (C) wild-type mice from the C57BL/6J × 129/SvEvTac F2 generation. Mice were administered ethanol diet ($n = 14, n = 17, n = 12$, mutant, heterozygote, and wild-type, respectively) or control diet ($n = 11, n = 14, n = 10$, mutant, heterozygote, and wild-type, respectively) for 10 days as described in the "Materials and Methods" section. Data points represent the mean ± SEM of the change in baseline body temperatures recorded at 30, 60, and 90-min postinjection. Chronic tolerance was observed in all genotypes ($p < 0.0001$), with no differences among the genotypes.

repeated-measures ANOVA using temperature differences from baseline at 30, 60, and 90 min time points, as the within-subjects factor and genotype (mutant, heterozygote, and wild-type), and treatment as between-subject factors

indicated a significant treatment effect ($F_{1,72} = 33.03, p < 0.0001$). Chronic ethanol treatment produced tolerance in all three genotypes which did not differ from each other. Baseline body temperatures were significantly less in mice from all genotypes on chronic ethanol diet ($F_{1,71} = 37.09, p < 0.0001$). To compare data as accumulated for the γ-PKC mutation on the SvJ background with that from the γ-PKC on the SvEvTac background, a two-way ANOVA comparing genotype and treatment at the 30 min time point was done. Unlike null mutants from the C57BL/6J × 129/SvJ background, at 30 min, the C57BL/6J × 129/SvEvTac null mutants still exhibited tolerance and did not differ from heterozygotes and wild-types ($F_{2,71} = 21.1, p < 0.0001$).

Inbred Strains

The observation that the lack of tolerance demonstrated by the γ-PKC null mutant mice occurred on some genetic backgrounds and not others, and that it appeared to be dependent on the behavioral measure indicated that the background genotypes' responses were important for understanding this difference. Therefore, the development of chronic tolerance was measured in the three background strains used in the generation of mice carrying the null mutation in the congenic strains and the two C57BL/6J × 129 backgrounds.

C57BL/6J Figure 5 shows the results of two chronic tolerance experiments in mice from the C57BL/6J inbred strain. Mice exhibited tolerance to the sedative-hypnotic and hypothermic effects of a challenge dose of 3.8 g/kg ethanol after chronic ethanol diet ($t_{22} = 2.70, p < 0.01$; $F_{1,8,1} = 9.42, p < 0.01$; sleep-time and hypothermia, respectively). Baseline body temperatures in mice treated with chronic ethanol diet did not differ from those in control diet-treated mice ($p = 0.22$). Waking blood ethanols differed between the treatment groups, with the ethanol-treated mice waking at higher BECs than the control groups ($t_{20} = 3.14, p < 0.01$). This supports the observation that the decrease in sleep-time was due to functional tolerance.

129/SvJ Chronic tolerance evaluated in a single experiment was exhibited by the 129/SvJ mice to both the sedative-hypnotic and hypothermic effects of ethanol (Fig. 6). The sleep-time response to a 3.8 g/kg dose of ethanol was significantly reduced in mice who had received chronic ethanol diet ($t_{16} = 5.33, p < 0.0001$) (Fig. 6A). Waking BECs also differed between the groups, with the ethanol-treated mice waking at higher BECs ($t_{13} = 4.58, p < 0.001$; see Fig. insert for values) indicating the development of functional tolerance.

Baseline temperatures in the ethanol-treated group were less than those from mice on control diet ($t_{17} = 7.88, p < 0.0001$). Hypothermia was measured at 30 min after the challenge dose of ethanol (Fig. 6B). Changes from baseline temperatures were significantly less in the ethanol-treated

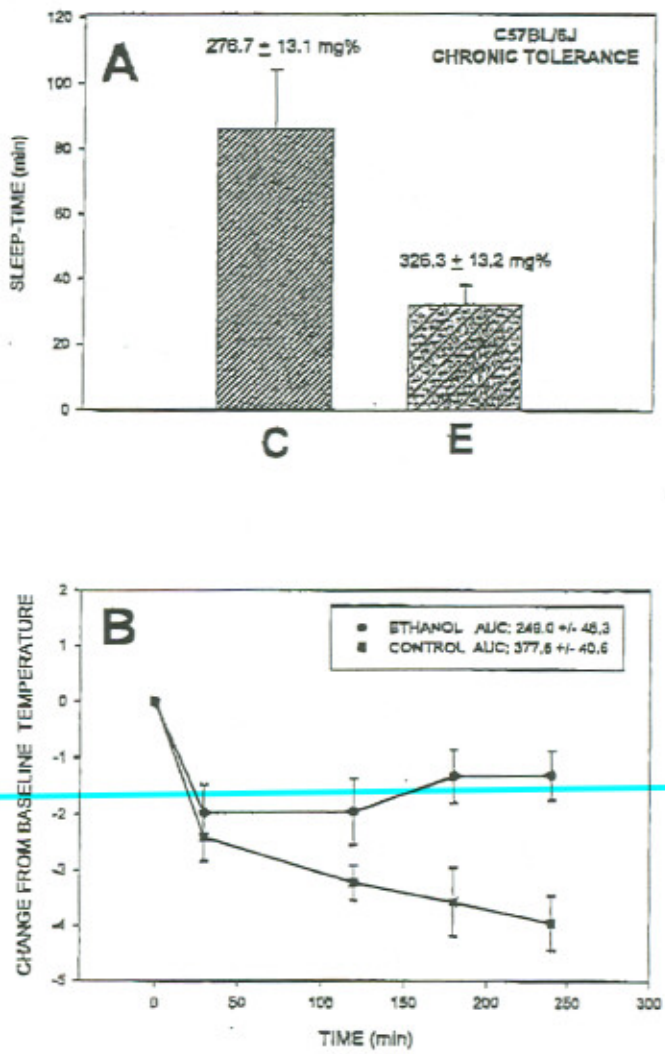

Fig. 5. Development of chronic tolerance to the hypothermic and sedative-hypnotic effects of a 3.8 g/kg challenge dose of ethanol in C57BL/6J inbred mice. Mice were administered ethanol diet or control diet for 10 days as described in the "Materials and Methods" section. (A) Tolerance to ethanol-induced sleep-time is represented by the mean ± SEM ($n = 11, n = 13$, ethanol and control, respectively) of time (min) to regain the loss of righting reflex ($p < 0.01$). (B) Tolerance to ethanol-induced hypothermia is represented by the mean ± SEM ($n = 11, n = 13$, ethanol and control, respectively) of changes in baseline body temperatures recorded at 30, 120, 180, and 240 min postinjection ($p < 0.01$). Control diet = C, ethanol diet = E. Mean waking BECs ± SEM are indicated above the treatment bars and were significantly greater in the tolerant, ethanol-treated mice ($p < 0.005$). AUC, area under the curve.

mice ($t_{17} = 7.38, p < 0.0001$) whose temperatures did not change from baseline values (−0.18 ± 0.54°C).

129/SvEvTac Figures 7A and 7B illustrate that after 10 days of ethanol-liquid diet, 129/SvEvTac mice demonstrated significant tolerance to both the sedative-hypnotic and hypothermic effects of a 3.8 g/kg challenge dose of ethanol ($t_{13} = 4.48, p < 0.001$; $F_{1,13} = 47.0, p < 0.0001$; sleep-time and hypothermia as analyzed by repeated-measures ANOVA for temperature changes from baseline at 30, 60, and 90 min, respectively; data from one experiment). The mean temperature changes in mice from the ethanol treated-group were above baseline values at every

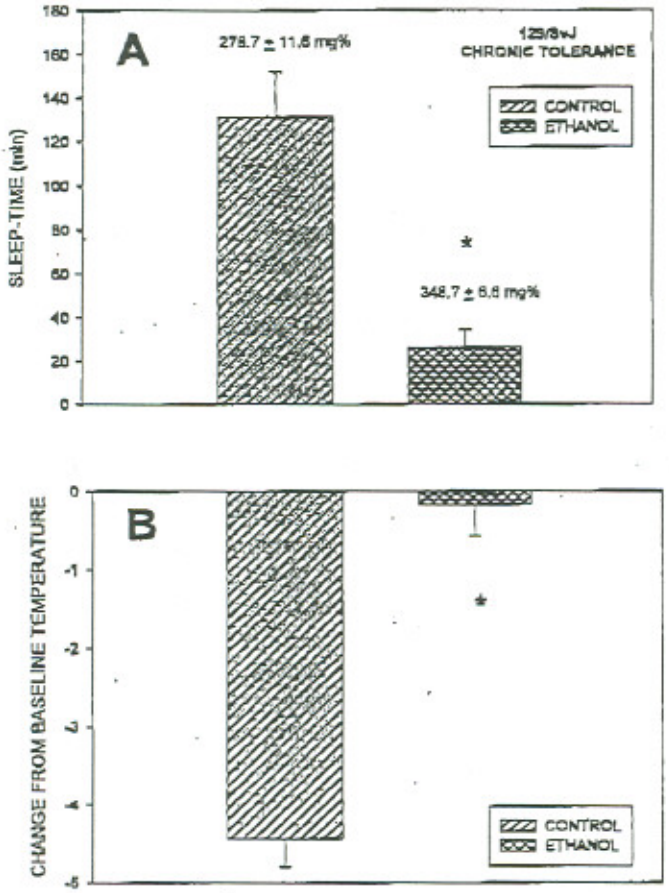

Fig. 6. Development of chronic tolerance to the sedative-hypnotic and hypothermic effects of a 3.8 g/kg challenge dose of ethanol in 129/SvJ inbred mice after 10 days of ethanol or control diet as described in the "Materials and Methods" section. (A) Bars represent the mean ± SEM ($n = 8$, $n = 10$, ethanol and control, respectively) of time (min) to regain the loss of righting reflex in ethanol- and control-treated mice (*$p < 0.0001$). Waking BECs ± SEM, shown above the treatment bars, were significantly greater in the tolerant, ethanol-treated mice ($p < 0.001$). (B) Bars represent the mean ± SEM ($n = 9$, $n = 10$, ethanol and control, respectively) of changes in baseline body temperature recorded 30 min postinjection. *$p < 0.0001$).

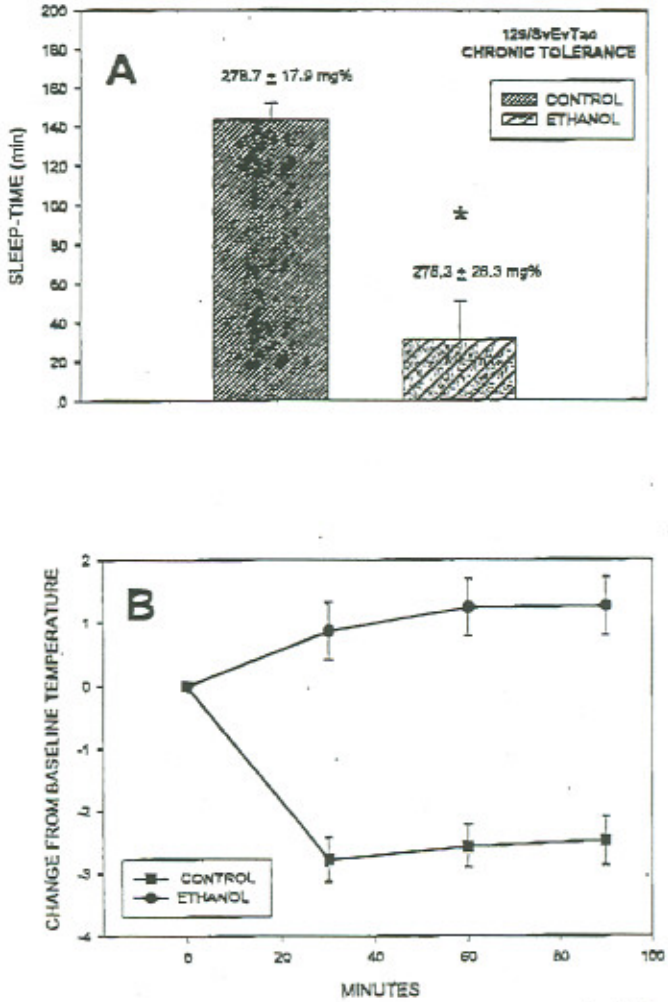

Fig. 7. Development of chronic tolerance to the hypothermic and sedative-hypnotic effects of a 3.8 g/kg challenge dose of ethanol in 129/SvEvTac inbred mice. Mice were treated with ethanol diet or control diet for 10 days as described in the "Materials and Methods" section. (A) Mice from the tolerant, ethanol-treated group demonstrated reduced sleep-time (min) represented by the mean ± SEM ($n = 9$), compared with the control mice ($n = 8$) (*$p < 0.001$). Mean waking BECs ± SEMs are shown above the treatment bars and did not differ between the treatment groups. (B) Hypothermic tolerance developed in the ethanol-treated mice ($p < 0.0001$). Data represents the mean ± SEM ($n = 9$, $n = 6$, ethanol and control, respectively) of changes in baseline body temperatures recorded at 30, 60, and 90 min postinjection from control- and ethanol-treated mice.

time point, compared with the control mice whose temperatures decreased by ~2.5°. Baseline body temperatures were significantly less in ethanol-treated mice ($t_{15} = 6.41$, $p < 0.0001$). Waking BECs did not differ between the two treatment groups (see Fig. 7A for values).

DISCUSSION

The results from the present series of experiments confirm those reported in a previous study that demonstrated significant decreased initial sensitivity to ethanol in mice lacking the γ-PKC gene (Harris et al., 1995). The present study extends these findings to include data demonstrating that, on some genetic backgrounds, γ-PKC null mutant mice do not develop tolerance to the hypothermic and sedative-hypnotic effects of ethanol. This suggests that γ-PKC plays a major role in both initial sensitivity and tolerance to some effects of ethanol. However, the impact of γ-PKC on these responses is influenced by the background genotype and may depend on the specific measure of sensitivity or tolerance. The "no tolerance" phenotype was observed when the null mutation was maintained on a mixed background that is commonly used in mice generated from targeted mutagenesis, C57BL/6J × 129/SvJ. Unexpectedly, when the γ-PKC null mutation was backcrossed for six generations to the C57BL/6J inbred strain the development of chronic tolerance was not different in null mutant congenic mice compared with wild-type mice. When the null mutants from this same N6 generation were outcrossed to 129/SvEvTac mice, tolerance to the hypothermic, but not the sedative-hypnotic, effects of ethanol was exhibited by the null mutant mice. These results serve to

substantiate the relevance of including the effects of genetic background when evaluating the effects of single gene mutations on complex behavioral phenotypes (Gerlai, 1996; Wehner and Bowers, 1995; Banbury Conference, 1997; Homanics et al., 1998). In addition, initial sensitivity to the hypothermia-inducing effects of ethanol was not decreased in these populations.

Recently, Kelly et al. (1998) reported that background strain effects contributed to locomotor responses in D2 dopamine receptor-deficient mice. In their study, the phenotype of the parental 129/SvEvTac inbred strain was virtually identical to the D2 receptor-deficient mice, suggesting that cosegregation of 129 alleles around the D2 allele were responsible for the locomotor impairments in the null mutant mice. In studies of ethanol-induced sedation, tolerance, and withdrawal responses in $GABA_A$ receptor α_6 null mutant mice, Homanics et al. (1997, 1998) also reported that the background alleles from the parental strains, C57BL/6J and 129/SvJ, appeared to mediate the lack of ethanol responses in the null mutant mice. However, the explanation of differences in ethanol tolerance observed in the present study is more complicated. Whereas null mutant mice from the C57BL/6J × 129/SvJ F2 generation did not exhibit tolerance in any paradigm tested, both parental strains did develop tolerance. Epistatic interactions between the targeted γ-PKC null mutation and multiple genes from the parental strains most likely explains the lack of tolerance observed in null mutants from this mixed background. On the other hand, γ-PKC null mutants from the C57BL/6J × 129/SvEvTac F2 generation did exhibit chronic tolerance to the hypothermic effects of ethanol, like their parental strains (129/SvEvTac and C57BL/6J), in contrast, to the lack of chronic tolerance development to the sedative-hypnotic effects in null mutants from this same background. Therefore, direct influence from 129/SvEvTac and C57BL/6J background genes may be occurring for the hypothermia responses; however, the sedative-hypnotic effects may be the result of epistatic interactions in this cross.

Differential responsiveness exhibited by null mutant mice from the two mixed 129 backgrounds was not entirely unexpected in light of recent reports that the lineages of the 129 strains have diverged due to deliberate and accidental outcrossings since their original derivation in 1928 (Simpson et al., 1997). Using simple sequence length polymorphism markers, it has been shown that between any two 129 strains, as many as 25 polymorphic alleles may exist (Simpson et al., 1997). For example, there are 16 polymorphic alleles between the SvJ and SvEvTac strains that primarily occurred as a result of outcrossing or genetic contamination. In general, behaviors have not been well characterized in the 129 strains, including behaviors measured in response to treatment with drugs of abuse. However, recently, Belknap et al. (1993) included 129/J mice in a 15 inbred strain comparison of voluntary ethanol consumption. Their results showed that the consumption of the 129 strain was intermediate to the high ethanol-consuming C57BL/6J and low-consuming DBA/2J inbred strains. Also, Homanics et al. (1998) recently tested 129/SvJ mice for ethanol-induced acute tolerance as part of a study of background parental strains for the $GABA_A$ receptor α_6 subunit null mutant mice. Similar to the results presented herein, they reported that 129/SvJ mice developed acute functional tolerance after a single 1.75 g/kg IP injection of ethanol. In contrast to the rapid and chronic tolerance demonstrated by the 129/SvEvTac inbred strain to ethanol in the present study, Kolesnikov et al. (1998) have shown that neither rapid *nor* chronic tolerance to the analgesic effects of morphine developed in this 129 strain. To our knowledge nonpharmacological behavioral differences due to allelic polymorphisms among the 129 strains have not been well defined. We have recently shown using a strain survey of spatial learning behavior that 129/SvJ mice are severely impaired in the Morris water maze, whereas the performance scores of the 129/SvEvTac strain were among the highest, suggesting that at least a subset of genes must determine behavioral differences between 129 strains (Owen et al., 1997).

The significance of genetic background was apparent in the γ-PKC/C57BL/6J congenic line in two ways: (1) the "no tolerance" phenotype observed in null mutant mice up to the N5 generation was unexpectedly lost in the N6 generation and (2) null mutant homozygotes did not survive to adulthood in the N7 generation. This lack of survival of homozygotic mutants has continued into the N8, N9, and N10 generations; however the mutation is maintained on a heterozygote cross. The reduced viability of a mutation may not be unique to the γ-PKC line as reduced fitness of inbreds is well known (Banbury Conference, 1997). The unexpected development of chronic tolerance in the N6 generation is presumably due to the increase in homozygosity at C57BL/6J loci. The change in percentage of C57BL/6J background from the N5 to N6 generation would be estimated to be 96.9% in the N5 generation to 98.4% in the N6 generation, a relative small increase that appears to be sufficient to mask and/or mediate the impact of the γ-PKC null mutation on ethanol tolerance.

One concern that has been raised in respect to the interpretation of phenotypic differences between wild-type and null mutants is the fact that in the typical derivation of such lines of mice, the wild-type form of the targeted gene is derived from the test cross background (i.e., C57BL/6J in the present case and not of 129 origin) (Smithies and Maeda, 1995). Functional strain polymorphisms might exist in the γ-PKC gene from these two strains, but the existence of such a functional polymorphism would not explain our data. Simple sequence length polymorphisms were used in a small subset of mice from the C57BL/6J congenic line at the N6 generation to distinguish 129 from C57BL/6J sequences flanking the γ-PKC gene (data not shown). No relationship was observed between strain-specific simple sequence length polymorphisms in or around the γ-PKC gene and initial sensitivity. More importantly, outcrossing

of homozygous null mutants from the C57BL/6J congenics (which did not have the altered ethanol phenotype) with 129/SvEvTac mice produced F1 hybrid wild-type mice that were heterozygous for the wild-type gene (i.e., they had one copy of the 129-derived normal γ-PKC gene and one copy of the 129-targeted γ-PKC gene). The F2 wild-types with 129-derived γ-PKC genes that resulted from crossing such F1 heterozygotes showed clear tolerance to the sedative-hypnotic effects of ethanol while the null mutants did not. This indicates that the source of the wild-type PKC was not of consequence to the phenotype for tolerance to the sedative-hypnotic effects of ethanol. Moreover, each of the inbred backgrounds presented herein all developed tolerance.

Several studies have indicated that initial sensitivity to ethanol and tolerance development are correlated behaviors (Le and Kiianmaa, 1990; San-Marina et al., 1989; Crabbe et al., 1981) that may share common genetic regulation (Gallaher et al., 1996). Kalant et al. (1971) suggested that the greater the degree of physiological disturbance due to a drug's effects, the more rapid would be the development of tolerance. In the present study, the qualitative assessment of initial sensitivity in the majority of the populations for a given response appears to be positively associated with tolerance development to that response. Mutant mice from either of the 129 mixed backgrounds did not develop chronic sedative-hypnotic tolerance and null mutant mice from the C57BL/6J × 129/SvJ cross did not develop chronic tolerance to hypothermia. These responses were positively associated with decreased initial sensitivity in these groups. In addition, mutant mice from the N6 congenic line and the C57BL/6J × 129/SvEvTac F2 line did develop chronic tolerance that was also positively associated with an increased initial sensitivity matched to the specific behavior. Therefore, γ-PKC may be responsible for pleiotropic effects on both initial sensitivity to and the development of chronic tolerance to ethanol. These findings suggest a mechanism for the correlated responses, but do not attempt to explain the confound of decreased initial sensitivity and decreased tolerance development.

Previous reports have indicated that there are differential rates of tolerance development to different effects of ethanol. Sedative-hypnotic or motor impairment tolerance after repeated ethanol injections appears to develop over slower time courses than hypothermia tolerance (Melchior and Tabakoff, 1981; Le et al., 1979). Acute tolerance develops to ethanol-induced motor impairment effects; however, acute tolerance to the anticonvulsant effects of ethanol does not develop within the same time period (Le et al., 1992). In the present study, the γ-PKC null mutants from the C57BL/6J × 129/SvEvTac F2 generation demonstrated a dissociation of hypothermia tolerance and sedative-hypnotic tolerance after chronic ethanol diet. This may be due to a differential rate of tolerance development; however, even after 10 days of ethanol diet, the null mutant mice failed to exhibit tolerance as measured by sleep time. An alternative interpretation that takes into account the brain regional localization of PKC may explain this dissociation. In the cerebellum, γ-PKC is found only in Purkinje cells (Hashimoto et al., 1988; Tanaka and Saito, 1992); furthermore, γ-PKC null mutants have undetectable levels of γ-PKC in cerebellar Purkinje cells (Harris et al., 1995). Using selected lines and inbred strains of mice, Spuhler et al. (1982) and Sorensen et al. (1980) reported high genetic correlations between ethanol-induced sedation and ethanol-induced depression of Purkinje cell firing. Therefore, the decrease in initial sensitivity and lack of sedative-hypnotic tolerance development in the null mutant mice may be due to impaired function of γ-PKC deficient-Purkinje cells. However, as previously discussed, genetic background can profoundly influence this phenotype because the null mutants from the N6 generation of the congenic line did exhibit hypnotic tolerance after chronic diet.

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