

Ethanol Consumption and Behavioral Impulsivity Are Increased in Protein Kinase C γ Null Mutant Mice

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Etiological factors influencing the development of alcoholism are complex and, at a minimum, include an interaction between polygenic factors and personality and biological traits. Human and animal studies suggest that some genes may regulate both the traits associated with alcohol abuse, such as decreased sensitivity or anxiety, and vulnerability to alcoholism. The identification of these genes could elucidate neurochemical pathways that are important in the development of alcohol abuse. Results from the present study indicate that the gene encoding the neuronal-specific γ subtype of protein kinase C (PKC γ) influences both ethanol consumption and behavioral impulsiv-

ity, a personality characteristic associated with Type II alcoholics, in a pleiotropic manner. Mice lacking PKC γ consume more ethanol in a two-bottle choice paradigm and also demonstrate increased behavioral impulsivity in an appetitive-signaled nose-poke task when compared with wild-type littermate control mice. Therefore, PKC γ may be an important mechanism within the cell that mediates one or more neurochemical pathways relevant to an increased predisposition to alcoholism.

Key words: alcohol drinking; nicotine; impulsivity; PKC γ null mutant mice; genetics; pleiotropy

Several studies of alcoholics and individuals at risk for alcoholism have identified biological and personality trait markers that can be used to predict a vulnerability to alcoholism (Cloninger, 1987; Finn et al., 1992; Schuckit, 1998; Kushner et al., 2000). It is well accepted that alcoholism is genetically influenced and that it is polygenically regulated. Several biological and personality trait markers also appear to be under genetic control (Marks, 1986; Schuckit, 1987; Goldman et al., 1996; Young et al., 2000). Therefore, it may be that some of these markers and the risk for alcoholism are genetically associated or are the end result of pleiotropic influences of genes acting on both behaviors. At-risk populations demonstrate low levels of responses (i.e., initial sensitivity) to alcohol measured by cognitive and psychomotor tasks, hormonal responses, and self-reports of intoxication (for review, see Schuckit, 1987). Also, increased tolerance development in humans has been associated with a risk for alcoholism (Newlin and Thomson, 1991).

In addition, subtypes of alcoholics have been categorized according to personality characteristics. These are best exemplified by Cloninger's (1987) Type I and Type II subgroups, although several other investigators have reported similar findings (Finn et al., 1992; Schuckit, 1998). Type I alcoholics are characterized by late onset of alcohol abuse, increased anxiety, and low novelty-seeking behaviors. In contrast, Type II alcoholics usually start drinking before age 25, demonstrate high novelty-seeking behaviors, are impulsive, and are often socially aggressive. Recently, a genetic study of adolescent twins indicated that behavioral disin-

hibition, a personality trait that encompasses most of the Type II characteristics and drug experimentation, is highly heritable (Young et al., 2000). A genetic association between impulsivity and alcohol drinking has also been shown in mice. Logue et al. (1998) reported a significant genetic correlation between impulsivity and ethanol consumption in 13 inbred strains of mice such that strains of mice that were more impulsive drank more ethanol.

Recent results from this laboratory have indicated that the neuronal-specific γ subtype of protein kinase C (PKC γ) is involved in several responses to ethanol and appears to regulate certain baseline behaviors. Using mice deficient in PKC γ , we have shown that null mutant mice demonstrate decreased initial sensitivity to the sedative-hypnotic and anxiolytic effects of ethanol when compared with wild-type littermate controls (Harris et al., 1995; Bowers et al., 1999, 2000b, 2001). PKC γ null mutants also display decreased tolerance development to the sedative-hypnotic and hypothermic effects of ethanol (Bowers et al., 1999, 2000b). Tests of anxiety-related behaviors in these mice indicated that baseline anxiety is reduced in null mutant mice compared with wild-type control mice (Bowers et al., 2000a).

On the basis of observations that compared with wild-type control mice PKC γ mutant mice exhibit altered biological and behavioral phenotypes, some of which have been associated with a predisposition to alcoholism in human populations, the present

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study investigated the role of PKC γ in ethanol consumption. PKC γ null mutant and wild-type littermates were tested for ethanol consumption and preference using a 24 hr access, two-bottle choice paradigm for ethanol drinking. Nicotine preference was also measured in these genotypes to test for any generalization of preference behavior to nicotine, a substance frequently used in conjunction with alcohol drinking (Daepfen et al., 2000). In addition, impulsive behavior was tested in these mice to expand on behavioral phenotypes that may be genetically associated with ethanol consumption.

MATERIALS AND METHODS

Animals

Male and female mice were 60–140 d of age at the time of testing and were housed in like-sex groups of two to five. Mice were given food and water *ad libitum* and maintained on a 12 hr light/dark cycle (lights on at 7:00 A.M.). PKC γ null mutant mice were derived using gene-targeting and homologous recombination techniques (Abeliovich et al., 1993) and are currently bred on a mixed (F2) C57BL/6J \times 129/SvEvTac genetic background at the Institute for Behavioral Genetics (Boulder, CO) as described previously (Bowers et al., 1999). Groups of null mutant and wild-type mice used in the following experiments were derived from multiple F2 litters.

Ethanol preference

Data for the ethanol preference study were collected from two experiments. Naïve male and female PKC γ null mutants ($n = 13$) and wild-type littermate control mice ($n = 13$) were housed individually and given food and water *ad libitum* 24 hr before the start of ethanol testing. Ethanol consumption and preference testing were performed using a 24 hr access, two-bottle choice paradigm with mice receiving 4 d each of five increasing concentrations of ethanol [3, 5, 7, 9, and 11% (v/v)] and water. Food was available *ad libitum*. Ethanol and water bottles were weighed and refilled each day. The weights were used to calculate an ethanol preference ratio (volume consumed from the ethanol bottles/total volume consumed from both water and ethanol bottles). The amount of ethanol consumed was converted to grams per kilogram per 24 hr. Body weights were measured every fourth day and did not change as a function of ethanol concentration. In the first ethanol experiment, mice were also tested for saccharin preference 2 d after ethanol testing, using the same two-bottle choice paradigm, and 4 d each of three saccharin solutions (0.05, 0.1, and 0.2%).

Nicotine preference

Data for the nicotine preference study were collected from two experiments. Naïve male and female null mutant ($n = 12$) and wild-type littermate control ($n = 12$) mice were housed individually for 4 d before the start of testing and received food and water *ad libitum*. The experimental paradigm was the same as that used for ethanol preference: mice were given a choice between water and seven increasing concentrations of nicotine (10, 20, 35, 50, 65, 80, and 100 μ g/ml). Bottles were weighed daily, and the positions were rotated; fresh nicotine solutions were placed in the cages every other day. Body weights remained constant throughout the study. Nicotine preference ratios and consumption (milligrams per kilogram per 24 hr) were calculated the same as for ethanol preference and consumption.

Impulsivity testing

Data for the impulsivity testing were collected from three experiments. Male and female PKC γ null mutant ($n = 25$) and wild-type littermate control ($n = 25$) mice were used for testing.

Apparatus. Impulsivity testing was performed in four identical Igloo ice chests (54 cm long \times 30 cm high \times 27 cm deep) adapted for nosepoke training as described previously (Logue et al., 1998). The reward was delivery of a 20 mg sucrose pellet (P. J. Noyes Company, Lancaster, NH) via a MED Associates pellet dispenser (St. Albans, VT). The auditory stimulus was a 3 sec, 80 dB, 6 clicks per second train of clicks. The training chambers were interfaced to an IBM-compatible computer via a MED Associates interface that was controlled with MED-PC software.

Procedure. Mice were trained in the nosepoke task as described previously (Logue et al., 1998). Before training, all mice were deprived of food to 85% of their *ad libitum* body weight (>8 d) and were maintained

at this weight throughout training. Training consisted of four phases. (1) Mice were reinforced for every nosepoke on a preassigned left or right side (FR1). Phase 1 continued until 25 reinforcements had been made in 30 min. (2) Mice were reinforced for every third correct nosepoke (FR3) until 25 reinforcements had been made in 30 min. (3) In phase 3, the auditory stimulus was introduced and consisted of 50 3 sec stimulus presentations separated by an intertrial interval (ITI) of 30 sec. Reinforcement occurred only on the first nosepoke on the reinforced side during the auditory stimulus; however, all nosepokes during the session were recorded. The nosepoke to the auditory stimulus was the conditioned response (CR), and when 10 CRs were made in the 30 min session, the mice moved to the final phase. (4) Each mouse received 10 daily, 30 min training sessions in phase 4. This phase was similar to phase 3 except that the ITI was 20 sec followed by a 1–8 sec preauditory stimulus period. If the mouse nosepoked during this pseudorandomly varied preauditory stimulus period, the clock was reset; this sequence continued until the mouse withheld responding for the duration of this period. The next trial was then initiated immediately. Withholding a response during this 1–8 sec period was important for learning the auditory signal to the CR and for the mouse's ability to control its nosepoke behavior. The dependent variables measured in this task include the number of days to reach criterion for phases 1–3, the percentage of conditioned responses (%CR) (the number of tone trials on which a nosepoke was rewarded/total number of tone trials in a session) in each session of phase 4, the efficiency ratio (the number of reinforcements/total number of nosepokes in a session) in each of the 10 sessions of phase 4, and the slopes of the efficiency ratio curves. The efficiency ratios and slopes were used as the measures of impulsivity.

Data analysis

Data from the ethanol and nicotine experiments were analyzed using repeated measures ANOVAs with drug concentration as the within subjects factor and genotype and gender (ethanol experiment) as the between subjects factors. Gender was not included in the nicotine analyses because only 6 of 24 mice were female. Efficiency ratio and percentage of conditioned response data from the signaled nosepoke task were also analyzed using repeated measures ANOVAs with trial number as the within subjects factor and genotype and gender as between subjects factors. Student's *t* tests were used to compare genotypes for days to criterion and to compare slopes of the efficiency ratio curves between mutant and wild-type groups.

RESULTS

Ethanol consumption

PKC γ null mutant mice consumed significantly more ethanol (grams per kilogram per 24 hr) than their wild-type littermate controls (Fig. 1C,D) ($F_{(1,22)} = 9.63$; $p < 0.005$). A statistical comparison of total fluid consumption (in milliliters) indicated that mutant and wild-type mice did not differ in the volume of liquid consumed (data not shown), indicating that the increased ethanol consumption by the null mutants was not caused by an overall increase in total amount of fluid consumed. There was also a significant effect of gender, with female mice consuming more ethanol than male mice ($F_{(1,22)} = 11.13$; $p < 0.003$) (Fig. 1C); however, the genotype \times gender interaction term was not significant, indicating that both female and male mutant mice drank more than their wild-type counterparts. Increased ethanol drinking by female mice has been reported in selected lines of mice (Grahame et al., 1999), recombinant inbreds (Rodriguez et al., 1994), and C57BL/6 mice (Middaugh et al., 1999) and may involve sex-specific loci that influence alcohol drinking (Peirce et al., 1998). Preference ratios were also significantly greater in the null mutant mice (Fig. 1A,B) ($F_{(1,22)} = 9.76$; $p < 0.005$), with a significant gender effect ($F_{(1,22)} = 6.89$; $p < 0.015$). Female mutant mice exhibited the greatest preference for ethanol over water at all concentrations (preference $>50\%$), whereas male wild-type littermate controls demonstrated no preference for ethanol over water at any concentration (preference $<36\%$) (Fig. 1A,B). Ethanol consumption levels in the wild-type female mice

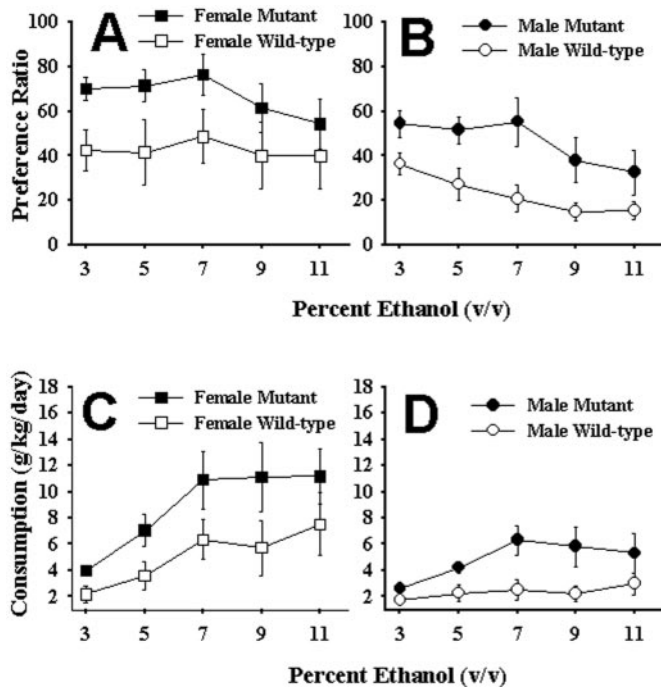


Figure 1. Ethanol preference and consumption were significantly greater in PKC γ null mutant mice ($n = 13$) compared with wild-type control mice ($n = 13$) ($p < 0.005$, $p < 0.005$; preference and consumption, respectively). *A* and *C* illustrate that preference and consumption were greater in female mice of both genotypes ($p < 0.015$, $p < 0.003$; preference and consumption, respectively) compared with male mice of both genotypes (*B*, *D*).

are consistent with previous reports of consumption in female C57BL/6 mice and are greater than that reported for combined sexes of 129/SvEvTac mice. On the other hand, consumption by male wild-type mice is less than that reported for C57BL/6 male mice but is more consistent with intake levels in the 129/SvEvTac inbreds (Belknap et al., 1993; Logue et al., 1998; Middaugh et al., 1999).

Saccharin preferences for the three concentrations ranged from 85 to 98% but did not differ between the genotypes, suggesting that preference for sweet solutions was not a factor in the increased ethanol preference demonstrated by PKC γ null mutant mice (data not shown). Results from the analyses of the nicotine data suggested that PKC γ does not mediate nicotine consumption (milligrams per kilogram per 24 hr) or nicotine preference (data not shown). Both genotypes increased their consumption of nicotine as the concentration of nicotine increased ($F_{(6,31)} = 36.45$; $p < 0.0001$), with no difference between PKC γ null mutant and wild-type control mice. These results indicate that the two genotypes also respond similarly to the bitter taste of the nicotine, further supporting the specificity of PKC γ on ethanol consumption.

Nosepoke test

Preliminary analyses of efficiency ratio and conditioned response data indicated that there were no significant effects of gender. Therefore, subsequent analyses were performed on data collapsed across gender. The mean number of days to criterion for phases 1–3 are listed in Table 1. There were no significant differences between the genotypes for these variables, indicating that the rate of acquisition of the nosepoke task for reward was equivalent in both genotypes. Although the ability to withhold

Table 1. Days to criterion (mean \pm SEM) for PKC γ null mutant mice and wild-type control mice

Days to criterion	Genotype	
	PKC γ mutants	Wild type
Phase 1 (FR1)	1.76 \pm 0.009	1.92 \pm 0.17
Phase 2 (FR3)	1.72 \pm 0.12	1.72 \pm 0.22
Phase 3 (ITI 30 sec)	3.13 \pm 0.37	3.20 \pm 0.36

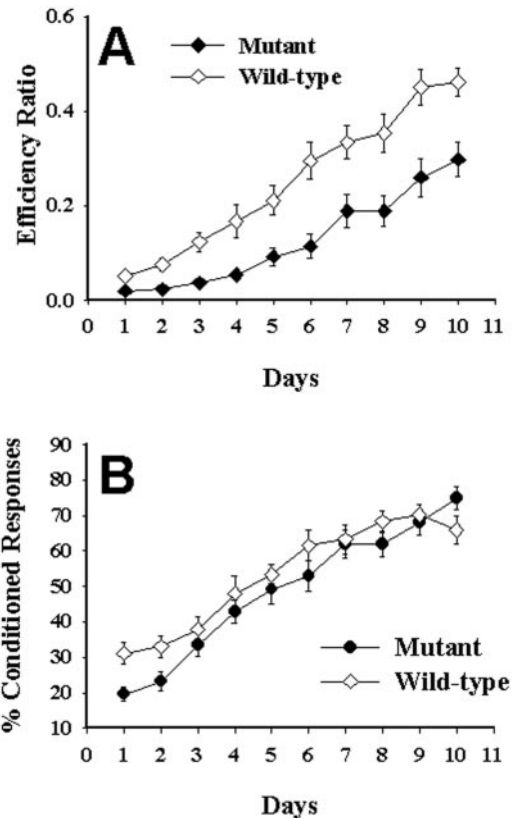


Figure 2. Impulsivity as measured in a signaled-nosepoke task was greater in PKC γ null mutant mice ($n = 25$) compared with wild-type control mice ($n = 25$). *A*, The efficiency ratio, a measure of impulsivity, was significantly decreased in mutant mice over 10 trials ($p < 0.0001$), demonstrating their inability to withhold their nosepoke responses for a food reward. Slopes of the efficiency ratio curves were also significantly different between the genotypes [i.e., mutants: 0.0325 ± 0.005 ; wild-type: 0.048 ± 0.004 ($p < 0.01$)]. *B*, The ability to learn to respond to the auditory signal was not different between PKC γ null mutant mice and wild-type controls, indicating that performance by the mutant mice was not caused by a learning deficit. This is represented by the %CR across the 10 trials.

responses as indicated by the curves of the efficiency ratios for both null mutant and wild-type mice increased across the 10 sessions ($F_{(1,45)} = 69.55$; $p < 0.0001$) (Fig. 2*A*), there was a significant main effect of genotype ($F_{(1,45)} = 15.39$; $p < 0.0001$) and a significant genotype by efficiency ratio interaction ($F_{(1,45)} = 3.16$; $p < 0.001$). This interaction is explained by the significant difference in the slopes of the efficiency ratios of null mutant (0.0325 ± 0.005) and wild-type mice (0.048 ± 0.004) ($t_{48} = 2.609$; $p < 0.01$). The slope of the efficiency ratio in the mutant mice was less than that of the wild-type mice, and the efficiency ratio mean score on day 10 was also lower in the mutant mice, reflecting the

reduction in behavioral control in PKC γ null mutant mice. A repeated measures ANOVA of the percentage of conditioned responses across the 10 trials demonstrated that the %CR curves did not differ between null mutant and wild-type mice ($p = 0.46$) (Fig. 2B). Because this variable is a measure of the ability of the mice to learn to respond to the auditory signal, this result indicates that the impulsive behavior demonstrated by PKC γ mutant mice was not caused by an inability to learn the conditioned response but resulted from an inability to withhold their responses during the preauditory time period.

DISCUSSION

The relationship between impulsivity and a susceptibility to high levels of alcohol consumption has been reported in both animal (Poulos et al., 1995; Logue et al., 1998) and human studies (Cloninger, 1987; Heinz et al., 2001). Recently, Logue et al. (1998) reported a significant genetic correlation in 13 inbred strains of mice between impulsivity and ethanol consumption, suggesting that the same genetic mechanisms regulate both behaviors. The results of the present study indicate that PKC γ may be one genetic factor influencing the two behaviors. Multiple effects of PKC isotypes might be expected because of the role of the PKC gene family as a central regulator of numerous intracellular functions, including phosphorylation of ligand-gated and voltage-dependent ion channels, transcription factors, adenylate cyclase, and calmodulin binding proteins (Mahoney and Huang, 1994). This assumption is supported by recent investigations of ethanol-related behaviors in mice lacking another PKC isotype, the PKC ϵ null mutants, which in contrast to the PKC γ mutant mice have increased initial sensitivity to ethanol and consume less ethanol (Hodge et al., 1999).

The pleiotropic effects of PKC γ on impulsivity and ethanol consumption as well as its effects on initial sensitivity and tolerance to ethanol, and baseline anxiety as reported previously (Harris et al., 1995; Bowers et al., 1999, 2000a,b, 2001), may be caused by direct effects of the enzyme but more likely results from the downstream effects of PKC γ on other proteins that are important in the expression of these behaviors. For example, ethanol potentiation of GABA $_A$ receptor function is significantly decreased in cerebellar tissue from PKC γ null mutant mice (Harris et al., 1995), a brain region associated with the sedative effects of ethanol (Spuhler et al., 1982). Null mutant mice also demonstrate decreased initial sensitivity to the sedative effects of ethanol compared with wild-type controls (Harris et al., 1995; Bowers et al., 1999, 2000b). This implies that PKC γ indirectly regulates initial sensitivity via phosphorylation of the GABA $_A$ receptor. The interaction of PKC γ and the GABA $_A$ receptor may also regulate increased ethanol consumption and impulsivity observed in the present study; however, the interaction of PKC with other neurotransmitter systems cannot be ruled out. Studies of neurochemical pathways in rodent models of increased ethanol consumption as well as numerous studies of alcohol abuse in humans have shown that both increased alcohol consumption and impulsivity may be the result of decreased serotonergic function (LeMarquand et al., 1994a,b; Leyton et al., 2001). Specifically, PKC is involved in the pathway directing agonist-induced down-regulation of 5HT $_2$ receptors (for review, see Roth et al., 1998) and appears to be important for serotonin reuptake (Sakai et al., 2000).

Inbred strain differences in mice have been reported for nicotine preference/consumption, indicating that nicotine consumption is genetically determined (Robinson et al., 1996). In addition,

a genetic relationship has been reported between alcohol and nicotine in humans (Madden et al., 1997; True et al., 1999) and in rodents (de Fiebre and Collins, 1993; Luo et al., 1994), suggesting that sensitivity to both substances may be under the same genetic control. However, in the present study, the increased preference for ethanol exhibited by the null mutant mice did not generalize to nicotine. Therefore, PKC γ does not appear to be a shared genetic factor in oral consumption of both ethanol and nicotine. However, an evaluation of operant responding for nicotine in these genotypes would more clearly address the issue of the role of PKC γ in nicotine reinforcement.

In summary, PKC γ null mutant mice consume more ethanol and are more impulsive than wild-type littermate controls, suggesting a pleiotropic effect of the γ isotype on these two behaviors. However, it should be noted that genetic background can influence responses in these null mutant mice (Bowers et al., 1999); therefore, the results of the present study are in the context of the C57BL/6 \times 129/SvEvTac mixed background. In addition to the increase in impulsivity observed in the null mutant mice, their decreased initial sensitivity to ethanol suggests that these mice may be a relevant model for elucidating genetic regulation as well as neurochemical pathways involved in the predisposition to alcoholism in some individuals.

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