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Differential sensitivity to the anxiolytic effects of ethanol and flunitrazepam in PKCγ null mutant mice

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Abstract

Tests of ethanol effects in PKCγ null mutant mice have indicated that PKCγ plays a role in initial sensitivity to ethanol-induced sedation, hypothermia, and $GABA_A$ receptor function and impacts neurochemical pathways mediating anxiety. The present study was undertaken to evaluate whether the decreased sensitivity to ethanol previously observed in these mice generalized to the anxiolytic effects of ethanol. PKCγ null mutant mice and wild-type controls were tested in the elevated-plus maze, the black/white box, and the mirrored chamber after ethanol (0, 1.0, 1.25, 1.5 g/kg) or flunitrazepam (FNZ) (0, 0.015, 0.03, 0.06 mg/kg). Results indicated that although both genotypes exhibited anxiolytic responses to ethanol in the elevated plus-maze, null mutant mice were less sensitive than wild-type control mice; however, in the black/white box, PKCγ null mutants were more sensitive than controls to the anxiolytic effects of FNZ. Neither ethanol nor FNZ produced anxiolytic responses in the mirrored chamber for either genotype. These results suggest that PKCγ differentially mediates anxiolytic responses to ethanol and FNZ and that this relationship interacts with each drug's efficacy in reducing anxiety-related behaviors specific to each of the three mazes.

Keywords: Anxiety; PKCγ; Null mutant mice; Genetics; Ethanol; Benzodiazepines

1. Introduction

Genetic research on anxiety using animal models and human populations has consistently shown that anxiety is a polygenic trait representing a broad range of behaviors. The identification of possible candidate genes mediating anxiety has been made easier using single gene mutation techniques in mouse. For example, null mutations of the serotonin-1A receptor, the corticotropin releasing hormone-1 and -2 receptors, the $GABA_A$-γ2 receptor subunit, glutamic decarboxylase 65-kDa enzyme, and α-calmodulin kinase II enzyme are representative of several mutant lines that have produced mice exhibiting changes in baseline anxiety-related behaviors compared to wild-type control mice (Bale et al., 2000; Chen et al., 1994; Crestani et al., 1999; Heisler et al., 1998; Kash et al., 1999; Kishimoto et al., 2000; Parks et al., 1998; Ramboz et al., 1998; Smith et al., 1998). Recently we have shown that mice lacking the neuronal-specific gamma isoform of protein kinase C (PKCγ) exhibit decreased anxiety in three behavioral tests designed to measure several aspects of anxiety, the elevated plus-maze, the black/white box, and the mirrored chamber aversion test (Bowers et al., 2000).

The relationship between alcohol use and anxiety in humans is complex. It is a common belief that some individuals drink alcohol to relieve anxiety or stress. However, individual differences have been reported concerning alcohol's effects as an anxiolytic. Some experimentally controlled studies suggest that particular personality types, such as aggressive, impulsive, and antisocial individuals or those who have high levels of trait anxiety may experience a greater anxiety/stress reduction effect from alcohol (Eddy, 1979; Kushner et al., 1996; Pohorecky, 1991; Sher and Levenson, 1982) and that these personality types are associated with problem drinking. In contrast, other studies did not find appreciable anxiety reduction after alcohol administration in subjects diagnosed with anxiety disorders (Kushner et al., 1996; Pohorecky, 1991). The genetics of anxiety and alcohol sensitivity most likely explains some of these

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individual differences as well as the possibility that certain types of anxiety and personality disorders are more amenable to alcohol's anxiolytic effects (Kushner et al., 1990). In rodents, alcohol's efficacy as an anxiolytic also appears to be dependent on several factors including the nature of the behavioral task, the dose of ethanol, and genotype (Cao et al., 1993; Durcan and Lister, 1988; Stewart et al., 1993; Stinchcomb et al., 1989). To our knowledge, null mutant mouse lines that exhibit altered baseline anxiety-related behaviors have not been tested for ethanol's anxiolytic effects. Results from such studies could identify genes/proteins that explain some of the variation in alcohol's effects to reduce anxiety in humans and rodents.

To determine whether PKCγ mediates the pharmacological actions of ethanol as an anxiolytic, the present study evaluated the behaviors of PKCγ null mutant mice and wild-type control littermates in the elevated plus-maze, the black/white box, and the mirrored chamber after ethanol administration. These mazes were selected because, although they all represent approach-avoidance tasks, certain variables of the individual tests measure different aspects of anxiety, some of which may be more sensitive to ethanol's anxiolytic effects than others. Principal component and factor analyses of tests commonly used to measure anxiety in mice including the elevated-plus maze and the light/dark choice test, have shown that depending on which aspect of the test is measured, different components of anxiety are represented (Belzung and Le Pape, 1994; File, 1996). For example, the primary fear-related factor in the elevated plus-maze is the animal's behavioral and physiological responses to the open arms that lack side walls; therefore, the fear of open spaces can be considered one variable specific to this maze compared to the black/white box and the mirrored chamber (Lister, 1990). Anxiety-related behaviors in the black/white box arise from the conflict between exploring a novel, brightly lit environment and escaping to a dark, protected environment (Lister, 1990). The mirrored chamber represents a unique test for measuring anxiety-related behaviors. The six-sided mirrored chamber may produce an artificial social stimulus due to the multiple reflections of "additional mice." Therefore, one component of this maze may be anxiety associated with social interaction (Gallup, 1968).

Earlier studies demonstrated that PKCγ mutants exhibit decreased initial sensitivity to the sedative and hypothermic effects of ethanol (Bowers et al., 1999; Harris et al., 1995). Neurochemical assays indicated that PKCγ's modulation of these ethanol-induced responses most likely occur via the $GABA_A$ receptor (Harris et al., 1995). Microsacs prepared from the cortex and cerebellum in mutant and wild-type control mice were assayed for muscimol-stimulated Cl^- flux in the presence of ethanol. As was observed with the behavioral responses, ethanol stimulation of Cl^- flux was greatly reduced in the mutant mice. The decreased sensitivity was specific to ethanol, as tests of flunitrazepam (FNZ)- and pentobarbital-induced changes in behavior and GABAergic receptor function were not changed in the null mutant mice. Therefore, in the present study a comparison of ethanol's effects with those of FNZ was also done to (1) determine whether the decreased initial sensitivity specific to ethanol that was observed in the previous studies generalizes to the anxiolytic effects of ethanol and (2) to determine if PKCγ regulates the neurochemical pathways mediating the anxiolytic effects of both ethanol and FNZ in a similar manner.

2. Methods

2.1. Animals

Male and female mice were 60–100 days of age at the time of testing and were housed in like-sex groups of two to five. Mice were allowed food (Harlan/Teklad) and water ad libitum and were maintained on a 12 h light/dark cycle (lights on at 0700) in the animal housing facility at the Institute for Behavioral Genetics (IBG), Boulder, CO. All animal use procedures were performed in accordance with the NIH Guide for Care and Use of Laboratory Animals and were approved by the University of Colorado IACUC.

PKCγ null mutant mice were originally derived using gene-targeting and homologous recombination techniques (Abeliovich et al., 1993) and are currently bred on a C57BL/6J × 129/SvEvTac mixed background at IBG as previously described (Bowers et al., 1999). The null mutation is maintained as a heterozygote on a C57BL/6J background. The heterozygotes are mated to 129/SvEvTac inbred mice, producing two populations of F1 mice, one that is heterozygous for the mutation and the other homozygous for the wild-type gene on a C57BL/6J × 129/SvEvTac background. The F1 mice, heterozygous for the null mutation, are then mated to produce an F2 generation consisting of mutant, heterozygote and wild-type littermates. This strategy provides within-litter control mice. Heterozygous littermates were not included in the present study, because their baseline anxiety-related behaviors do not differ from wild-type mice (Bowers et al., 2000). Mice were genotyped prior to testing using isolated tail DNA analyzed by polymerase chain reaction as described previously (Bowers et al., 1999).

2.2. Maze testing

In order to minimize the stress of a change to the testing room, mice were housed overnight in a light-cycle controlled testing room and weighed the day prior to anxiety testing. All testing was done between 0900 and 1200 h. Mazes were cleaned with a 10% ethanol solution between mice. Procedures for testing the mice in the elevated plus-maze, the black/white box, and the mirrored chamber were

done as previously described (Bowers et al., 2000). Briefly, the methods were as follows.

2.2.1. Elevated plus-maze

The maze was designed and patterned after that developed and validated for mice by Lister (1987). Mice were tested and videotaped for 5 min, starting with a 10 s confinement in a cylinder placed in the center platform of the maze to prevent a predetermined choice of arms. Videotapes were scored for the following behaviors: (1) percent of total entrances into the open arms of the maze (open entries/total entries × 100), a score qualified as an entry when all four feet were in the arm; (2) percent of total time in the open or closed arms of the maze (time in the open or closed arms/300 × 100); (3) percent of total time spent on the center platform (100 − percent of open and closed arm time); (4) number of entries into the closed arms of the maze as a measure of locomotor activity (Rodgers and Johnson, 1995); and (5) total number of entries into all the arms of the maze.

2.2.2. Black/White box

The box was adapted from that described by Crawley and Goodwin (1980) and validated for mice by Costall et al. (1989). Illumination in the white box was 230 lx and the black side was illuminated under red light at 12 lx. Mice were videotaped and tested for 5 min. To begin the session mice were placed in a back corner of the light box. The following behaviors were scored: (1) latency to enter the dark box, i.e., the first transition, a transition was counted when all four feet had entered the black side; this has been shown to be a measure of locomotor activity (Chalouff et al., 1997); (2) total number of transitions between the white and black sides of the box; and (3) percent of the total time spent in the light box (time in light chamber/300 × 100).

2.2.3. Mirrored chamber

The chamber was designed and validated for mice according to Toubas et al. (1990). The mirrored chamber consists of a cubical chamber (30 cm^2/side) open on one side to allow entrances by the mouse into the chamber. The interior of this cube is faced with mirrors on all walls, ceiling, and the floor. This cube is placed inside a larger black, square box (40 × 40 × 35.5 cm), which creates 5-cm corridors around the outside of the mirrored chamber. A sixth mirror faces the opening of the mirrored chamber producing a "mirrored passage" between the containing box and the opening to the chamber. Testing was videotaped for 5 min, starting with the placement of the mouse in a back corner of the outside corridor, behind the mirrored chamber. The following behaviors were scored: (1) latency to enter the mirrored chamber; an entrance was counted when all four feet were in the chamber; (2) percent of total time in the mirrored chamber (time in the chamber/300 × 100); (3) number of entrances into the mirrored chamber, and (4) the number of times the mouse entered with all four feet into the mirrored passage.

2.2.4. Ethanol testing:

Mice were tested in groups of six to eight. Both genotypes were represented in each testing session and within a testing session at least two doses of drug were administered in an overlapping design across sessions. PKCγ null mutant and wild-type mice ($n = 12$/genotype per dose) were injected intraperitoneally (ip) with saline or ethanol (1.0, 1.25, 1.5 g/kg; 20% w/v). Ten minutes after injection mice were tested in the elevated plus-maze. Preliminary experiments indicated that of the three mazes, the elevated plus-maze is the most sensitive to pretest manipulations including prior exposure to other novel mazes. In particular, pharmacological sensitivity to anxiolytic compounds can be altered (Hogg, 1996). Therefore, mice were always tested in the plus-maze first followed by the black/white box and the mirrored chamber in random order. Each mouse was tested sequentially in the three mazes for 5 min each and had completed the third maze by 25 min, 35 min after injection. Rates of ethanol metabolism are not different among the three genotypes (Harris et al., 1995) and Durcan and Lister (1988) have shown that ethanol's anxiolytic effects in the elevated plus-maze are present at 30 and 60 min post injection.

2.2.5. Flunitrazepam testing:

FNZ, a generous gift from Hoffman-La Roche, was dissolved in saline/0.5% Tween 80 (vehicle). Mice were injected ip in a volume of 0.01 ml/g with vehicle or FNZ (0.015, 0.03, 0.06 mg/kg; $n = 12$/genotype for 0.015 and 0.03 mg/kg and 10/genotype for 0.06 mg/kg). This dose range was chosen empirically; preliminary experiments indicated that most mice were sedated at 0.125 mg/kg. Thirty minutes after injection mice were tested in the three mazes as described for ethanol testing. This time point was selected based on the results from inbred strain surveys of the anxiolytic actions (Grieble et al., 2000) and brain levels of benzodiazepines at 30 min postinjection (Crabbe et al., 1998). FNZ metabolism has not been measured in these genotypes.

2.3. Data analysis

For the ethanol experiments tests for outliers were done for some variables; significant outlier data points were removed from the data set. A data point was considered an outlier if the score was two standard deviations from the mean of all data points for that measure (i.e., elevated plus-maze, three scores; black/white box, one score; mirrored chamber, three scores). This was not warranted in the FNZ experiments. Preliminary analyses indicated that male and female mice did not differ in any test for either drug; therefore, data from both sexes were combined for subsequent analyses. Student's t tests were used to evaluate baseline genotype differences in saline- and vehicle-injected mice. For those tests where significant baseline differences

occurred, data were converted to percent of saline in the ethanol administration tests and percent of vehicle in the FNZ administration tests and were analyzed by two-way ANOVAs for genotype and dose effects. Within genotype one-way ANOVAs that included saline/vehicle-treated groups were used to determine dose-dependent effects independently for each genotype. Student–Newman–Keuls post hoc tests were done when indicated to determine genotype and dose order effects. Unconverted data are illustrated in the figures and tables so that baseline values and the absolute values of the means and S.E.M.s could be observed. However, in Table 3 the means and S.E.M.s of converted data are listed.

3. Results

3.1. Elevated plus-maze

3.1.1. Ethanol

Previous results from this laboratory demonstrated significant baseline anxiety differences among the genotypes in naïve mice in this maze (Bowers et al., 2000). Therefore, data from saline-treated mice were analyzed to determine if the same genotypic differences would be observed. A Student's t test comparison of the two genotypes indicated that the percent entrances into, percent time spent in the open arms of the maze, and total number of entries into all arms were significantly different between saline-treated mutant and wild-type mice ($t(21)=5.51$, $P<.0001$; $t(21)=4.05$, $P<.001$; $t(21)=4.04$, $P<.001$; entries and time in the open arms and total entries, respectively) with wild-type mice spending less time in the open arms than mutant mice, whereas, total entries were greater in mutant mice (Fig. 1a, b; Tables 1 and 3). The remaining dependent measures, percent of time in the closed arms, closed arm entrances, and the percent time on the center platform were not different between the genotypes after saline injection. Genotype and the dose effects of ethanol in the plus-maze were analyzed using data converted to percent of saline values to adjust for baseline differences. There was a significant effect of genotype for percent time in the open arms, $F(1,70)=9.68$, $P<.003$, and percent entrances into the open arms, $F(1,70)=11.08$, $P<.001$, as well as a significant Genotype × Dose interaction for percent of time in the open arms, $F(2,70)=8.31$, $P<.001$ (Fig. 1a, b). Within genotype analyses indicated that ethanol produced anxiolytic responses, as measured by

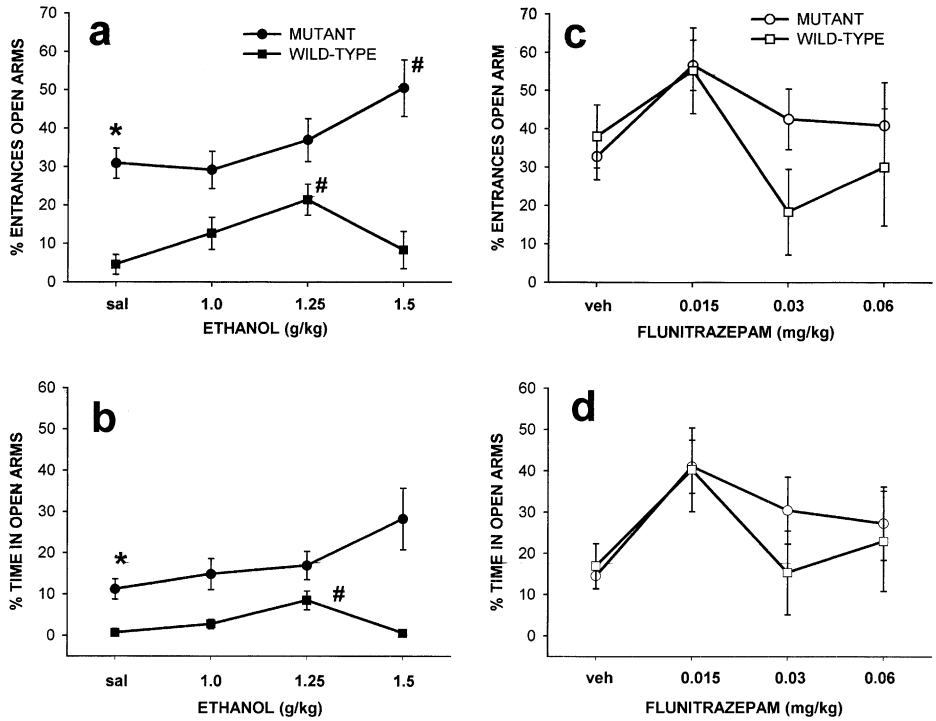

Fig. 1. Behavior in the open arms of the elevated plus-maze after ip injections of ethanol (0, 1.0, 1.25, 1.5 g/kg) or FNZ (0, 0.015, 0.03, 0.06 mg/kg). (a) Saline-treated mutant mice entered the open arms significantly more often than saline-injected wild-type controls (*$P<.0001$). Sensitivity to ethanol-induced anxiolysis was decreased in mutant mice; the percent entrances into the open arms of the maze increased after 1.25 g/kg ethanol in wild-type mice ($^{\#}P<.05$) and after 1.5 g/kg in mutant mice ($^{\#}P<.05$). (b) Saline-treated mutant mice spent more time in the open arms compared to saline-injected wild-type mice (*$P<.001$) and ethanol significantly increased the percent of total time spent in the open arms of the maze in wild-type mice at 1.25 g/kg ($^{\#}P<.05$). (c) Vehicle-treated mice did not differ in percent entrances into the open arms and both genotypes exhibited nonsignificant anxiolytic responses after FNZ at 0.015 mg/kg. (d) The genotypes did not differ in the percent time spent in the open arms of the maze after vehicle injection and both mutant and wild-type mice increased their percent times nonsignificantly at 0.015 mg/kg FNZ. Data is expressed as the means ± S.E.M. at each drug concentration.

Table 1
The effects of ethanol on maze-related behaviors

	Ethanol dose (g/kg)			
	Saline	1.0	1.25	1.5
Elevated plus-maze				
Close arm entries				
MUT	7.67 ± 0.7	11.92 ± 0.7#	10.83 ± 1.4#	5.67 ± 0.9
WT	5.09 ± 1.1	8.40 ± 1.2#	7.5 ± 1.2#	3.17 ± 1.1
Total entries				
MUT	11.08 ± 0.9 *	17.08 ± 1.8#	17.08 ± 1.5#	9.67 ± 1.2
WT	5.64 ± 1.1	9.20 ± 1.6#	10.08 ± 1.7#	3.42 ± 1.1
% Time in center				
MUT	27.63 ± 3.6	34.08 ± 3.0	42.57 ± 3.1	39.65 ± 5.6
WT	38.48 ± 10.8	32.61 ± 4.5	38.37 ± 8.2	63.03 ± 10.3
% Time in closed arms				
MUT	61.21 ± 4.5	51.09 ± 4.7	40.52 ± 4.7#	32.33 ± 6.5#
WT	60.85 ± 10.8	64.62 ± 4.9	53.17 ± 8.2	36.41 ± 10.2
Black/White box				
Latency into dark box				
MUT	8.25 + 0.61	6.25 + 2.5	5.83 + 1.6	6.5 + 1.3
WT	12.91 + 3.6	8.33 + 1.9	10.00 + 3.5	59.83 + 32.5
Mirrored chamber				
Entrances into chamber				
MUT	5.08 ± 0.9**	6.27 ± 1.2	3.58 ± 1.1	3.67 ± 0.9
WT	0.25 ± 0.2	0.58 ± 0.3	0.33 ± 0.2	0.08 ± 0.08
No. of times in passage				
MUT	8.25 ± 0.8**	11.00 ± 1.0#	7.33 ± 0.8	7.33 ± 1.1
WT	1.83 ± 0.6	2.58 ± 0.7	5.00 ± 1.0#	0.67 ± 0.4

Elevated plus-maze: Total arm entries were significantly greater in saline-treated mutant mice compared to controls. Analysis of percent of saline values indicated a significant effect of dose for all dependent variables except percent entrances into open arm.
Mirrored chamber: The number of entrances into the mirrored chamber and number of entrances into the passage were significantly greater in saline-treated mutant mice. Entrances into the passage increased at 1.0 and 1.25 g/kg (mutants and wild types, respectively).
Black/White box: There were no differences in the saline treated groups for latency to enter the dark box. The increased latency observed in the wild-type mice at 1.5 g/kg represents sedation at this dose.
MUT, mutant; WT, wild type.
# $P < .05$, significantly different from saline.
* $P < .001$. ** $P < .0001$, mutant significantly different than wild type.

percent entrances or time, in both mutant, $F(3,47) = 2.99$, $P < .05$; $F(3,47) = 2.48$, $P = .07$; entrances and time, respectively, and wild-type control mice, $F(3,44) = 3.34$, $P < .03$, $F(3,44) = 8.06$, $P < .0001$; entrances and time, respectively. For wild-type mice anxiolytic effects were greatest at 1.25 g/kg (percent time in and percent entrances into the open arms; $P < .05$), whereas the maximum anxiolytic effect occurred at 1.5 g/kg for the null mutants (percent entrances into the open arms $P < .05$); suggesting a shift to the right in the dose–response curve. As a measure of locomotor activity, increases and decreases in the number of closed arm entries were dose dependent in both wild-type and mutant groups, $F(3,47) = 3.31$, $P < .03$; $F(3,47) = 6.06$, $P < .01$, respectively. Post hoc analyses indicated that both wild-type and mutant mice significantly increased their closed arm entries at 1.0 and 1.25 g/kg; however, for the mutants at 1.5 g/kg, closed arm entries were not different from saline, suggesting that the anxiolytic effect of ethanol at this dose was not confounded by increased locomotion. On the other hand, increased locomotion cannot be ruled out as a factor in the anxiolytic response of wild-type mice at 1.25 g/kg.

The overall main effect of dose, collapsed across genotype, was significant for all dependent measures except the percent of entrances into the open arms, $F(2,70) = 6.82$, $P < .002$; $F(2,70) = 5.56$, $P < .006$; $F(2,70) = 4.29$, $P < .02$; $F(2,70) = 11.92$, $P < .0001$; $F(2,70) = 12.25$, $P < .0001$; percent time in the open arms, which increased as a function of dose; percent time in the closed arms, which correspondingly decreased with dose; percent time in the center

Table 2
The effects of flunitrazepam on maze-related behaviors

	Flunitrazepam dose (mg/kg)			
	Vehicle	0.015	0.03	0.06
Elevated plus-maze				
Closed arm entries				
MUT	6.91 ± 1.0	5.50 ± 1.2	7.41 ± 1.7	4.25 ± 1.4
WT	4.58 ± 1.0	3.67 ± 1.4	1.17 ± 0.3#	0.70 ± 0.3#
Total entries				
MUT	10.41 ± 1.3	10.67 ± 1.6	10.91 ± 1.7	6.08 ± 1.6
WT	7.58 ± 1.8	6.75 ± 1.4	1.41 ± 0.3#	1.00 ± 0.2#
% Time in center				
MUT	27.91 ± 4.9	24.57 ± 2.6	33.03 ± 5.4	36.55 ± 8.9
WT	29.72 ± 7.5	30.84 ± 7.9	12.47 ± 4.0	36.07 ± 12.6
% Time in closed arms				
MUT	57.60 ± 6.6	33.58 ± 6.3	32.41 ± 7.2	36.22 ± 10.4
WT	53.77 ± 9.5	28.91 ± 10.1	72.18 ± 10.5	41.89 ± 15.1
Black/White box				
Latency into dark box				
MUT	6.16 ± 0.9	13.5 ± 6.4	44.08 ± 27.8	112.3 ± 40.6
WT	25.66 ± 16.8	128.3 ± 43.8	201.0 ± 40.0#	196.4 ± 42.9#
Mirrored chamber				
Entrances into chamber				
MUT	4.41 ± 1.3 *	5.58 ± 1.2	3.66 ± 0.9#	2.08 ± 0.7#
WT	0.92 ± 0.3	0.67 ± 0.4	0.17 ± 0.2	0.0 ± 0.0
No. of times in passage				
MUT	7.50 ± 1.4	7.67 ± 1.6	5.41 ± 1.2	2.25 ± 0.6
WT	3.91 ± 1.1	2.00 ± 0.08	0.41 ± 0.3#	0.30 ± 0.30#

Elevated plus-maze: No differences in vehicle-treated mice. The dose–response for FNZ was not different between the genotypes for most variables; however, closed arm and total entrances decreased at 1.25 and 1.5 g/kg for both genotypes.
Mirrored chamber: Entrances into the mirrored chamber were greater in vehicle-treated mutant mice. Analysis of percent of vehicle conversions indicated no overall effect of FNZ dose on any dependent variable; however, within-genotype analyses showed a decrease in passage entrances in wild-type mice at the higher doses.
Black/White box: No differences in vehicle-treated groups. Latencies increased in both groups as a function of dose, representing sedation at the higher doses.
# $P < .05$ significantly different than saline.
* $P < .004$; mutant versus wild-type.

Table 3
Data converted to percent of baseline

	Ethanol (g/kg), % saline			FNZ (mg/kg), % vehicle		
	1.0	1.25	1.5	0.015	0.03	0.06
Plus maze open arm						
% Entrances						
MUT***	94.4 ± 15.0	119.4 ± 17.9	163.2 ± 23.9	n.d	n.d	n.d
WT	277.1 ± 90.9	467.43 ± 88.2	181.9 ± 105.0	n.d.	n.d.	n.d.
% Time						
MUT**	133.0 ± 33.9	168.3 ± 28.7	32.5 ± 13.6	n.d.	n.d.	n.d.
WT	423.0 ± 175.1	1300.0 ± 350.3	84.6 ± 45.7	n.d.	n.d.	n.d.
Black/White box						
Transitions						
MUT	91.1 ± 13.8	98.8 ± 10.3	91.7 ± 11.6	n.d.	n.d.	n.d.
WT	132.2 ± 40.3	105.7 ± 25.8	60.6 ± 24.4	n.d.	n.d.	n.d.
% Time in light						
MUT *	80.1 ± 13.0	81.5 ± 12.9	103.7 ± 17.7	n.d.	n.d.	n.d.
WT	97.2 ± 24.4	92.6 ± 20.5	293.8 ± 112.7	n.d.	n.d.	n.d.
Mirrored chamber						
Latency						
MUT	19.1 ± 5.4	139.4 ± 44.1	124.6 ± 39.4	124.6 ± 43.6	114.6 ± 43.7	170.2 ± 44.9
WT	82.4 ± 14.7	96.2 ± 11.0	111.9 ± 4.9	120.1 ± 11.7	131.2 ± 10.7	141.9 ± 0
% Time in chamber						
MUT	113.2 ± 29.9	60.7 ± 19.1	54.0 ± 15.2	74.6 ± 18.5	112.6 ± 36.1	149.3 ± 48.2
WT	28.8 ± 20.3	45.5 ± 32.9	1.4 ± 1.4	112.9 ± 64.9	63.4 ± 63.4	0 ± 0

n.d., not done. There were no baseline differences in these groups; see text, Figs. 1c, d and 2c, d, and Table 2.
*** $P < .001$. ** $P < .01$. * $P < .05$ Mutants significantly different from wild-type mice; overall ANOVA.

platform, which increased with dose; closed arm entries, which increased at 1.0 and 1.25 g/kg, but decreased at 1.5 g/kg; and total entries, which also exhibited a biphasic curve, respectively.

3.1.2. Flunitrazepam

In contrast to the genotype differences in saline-treated mice from the ethanol study, vehicle-treated (saline/0.5% Tween 80) mutant and wild-type mice did not differ in baseline dependent variables (Fig. 1c, d; Table 2). This appeared to be due to a decrease in anxiety in vehicle-treated wild-type mice when compared to saline-treated wild-type mice from the ethanol experiment. The cause of this change in wild-type mice is unclear, but may be due to a nonspecific anxiolytic effect of the Tween 80 (Table 2). Therefore, data were not converted to percent of vehicle for analysis in this test. The results of a two-way ANOVA of genotype and dose indicated that the percent of time in the open arms of the maze, collapsed across genotypes, was dose dependent, $F(1,94) = 3.23$, $P < .05$ (Fig. 1d), and the effect of dose on the percent of entries into the open arms approached significance (P=.053) (Fig. 1c). FNZ dose–responses were not different between the genotypes and appeared to reach a maximum effect at 0.015 mg/kg. The number of closed arm entries did not change at this dose; therefore, the anxiolytic responses were not due to increased locomotor activity.

3.2. Black/white box

3.2.1. Ethanol

The results of a Student's t test evaluating genotypic baseline differences in saline-injected mice indicated an overall genotype effect for the total number of transitions and the percent of time in the light box, $t(21) = 3.69$, $P < .001$; $t(21) = 2.37$, $P < .03$, respectively (Fig. 2a, c; Tables 1 and 3). Again this effect was primarily due to increased transitions and greater time in the light side exhibited by null mutant mice compared to wild-type control mice. An overall two-way ANOVA (Genotype × Dose) of data converted to percent of saline indicated that ethanol lacked anxiolytic effects as measured by total transitions between the black and white boxes or percent time in the light box. Neither genotype, dose, nor the Genotype × Dose interaction were significant for total transitions. The main effect of dose was significant for percent time spent in the light side and latency to the first transition to the dark box, $F(2,93) = 6,31$, $P < .003$; $F(2,93) = 4.98$, $P < .01$; however, this does not reflect a dose effect for anxiolysis, but rather an effect of

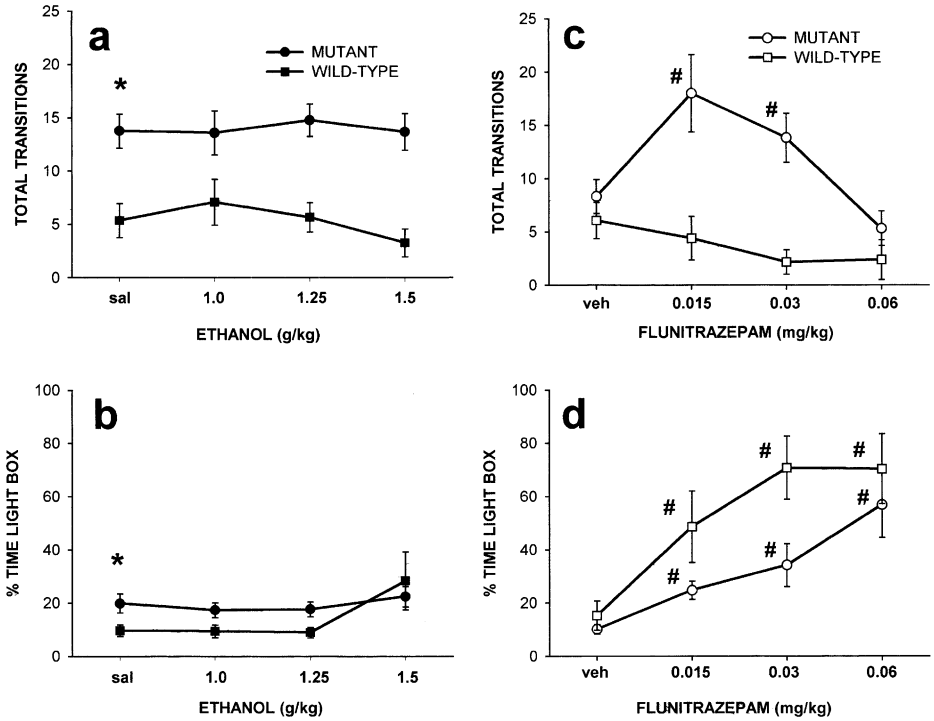

Fig. 2. Behavior in the black/white box after ip injections of ethanol (0, 1.0, 1.25, 1.5 g/kg) or FNZ (0, 0.015, 0.03, 0.06 mg/kg). (a) The baseline number of transitions were greater in mutant mice compared to wild-type controls after saline injection (*$P<.001$). However, ethanol had no effect on transitions for either genotype. (b) Saline-treated mutant mice spent more time in the light side of the box compared to wild-type mice (*$P<.03$). Ethanol had no anxiolytic effect in either mutant or wild-type mice. (c) Vehicle-treated mice did not differ in the number of total transitions between the light and dark sides of the box. However, mutant mice significantly increased their total transitions at 0.015 and 0.03 mg/kg ($^{\#}P<.05$). (d) The genotypes did not differ in the percent time spent in the light box after vehicle injection and both mutant and wild-type mice increased their percent times in the light side after FNZ injection in a nonsignificant manner. Data is expressed as the means ± S.E.M. at each drug concentration.

increased sedation at 1.5 g/kg, specifically in wild-type mice. Mutant mice appeared to be insensitive to the sedative effects of ethanol at this dose. Wild-type mice who were sedated sat in the light side of the box, with low levels of locomotion (experimenter's observations). Percent time in the light box differed between the genotypes, $F(1,70)=4.72$, $P<.05$. and a significant Genotype × Dose interaction was observed for this measure, $F(2,70)=3.21$, $P<.05$, reflecting the increased time in the light box by wild-type mice after 1.5 g/kg ethanol.

3.2.2. Flunitrazepam

Similar to the baseline results in the elevated plus-maze, no significant differences among the genotypes in baseline behaviors were observed in the black/white box after vehicle treatment. Compared to saline-treated null mutant mice, the vehicle-treated mutants decreased their baseline total transitions to match that of the wild-type mice. Again, the cause of this change in baseline transitions is not clear, particularly since in this test mutant, not wild-type mice, were affected after vehicle treatment. Therefore, data were not converted to percent of vehicle for analyses of genotype and dose (Fig. 2c, d; Table 2). The main effect of genotype was highly significant for total transitions, the percent time in the light box and latency to the first transition to the dark, $F(1,94)=24.74$, $P<.0001$; $F(1,94)=8.47$, $P<.005$; $F(1,94)=17.88$, $P<.0001$, respectively. The main effect of dose was also significant for these variables, $F(3,94)=3.21$, $P<.03$; $F(3,94)=10.51$, $P<.0001$; $F(3,94)=7.40$, $P<.0001$, respectively. The genotype × dose interaction term was significant for total transitions, $F(2,94)=3.25$, $P<.03$. Within-genotype analyses indicated that only null mutant mice responded to the anxiolytic effects of FNZ with a twofold increase in the number of transitions between the light and dark sides of the box, significant at the 0.015 and 0.03 mg/kg doses ($P<.05$), as well as increased time spent in the light side (Fig. 1c, d). At the highest doses of FNZ, both genotypes had increased latencies to the first transition, which was possibly due to sedative effects. As was observed after higher doses of ethanol, at 0.03 and 0.06 mg/kg FNZ, most wild-type mice and some mutant mice decreased their activity, remaining in the light box for longer periods of time. Although increased time in the light box suggests decreased anxiety in the wild-type mice, the low number of transitions in these mice indicates that sedation produced an inability to leave the light side of the box, artifactually increasing time spent in the light box.

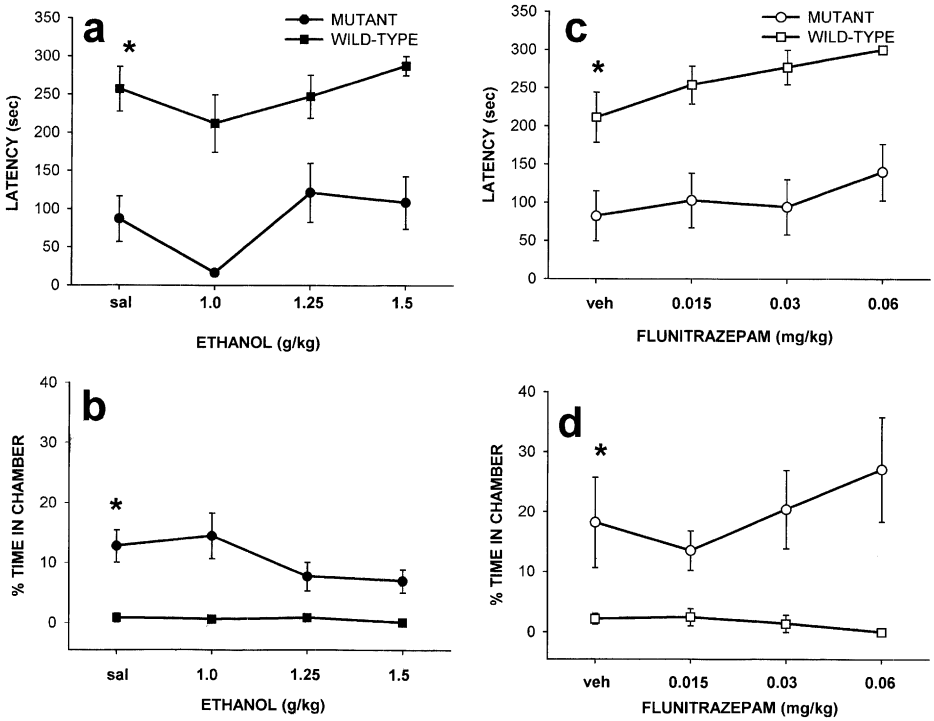

Fig. 3. Behavior in the mirrored chamber after ip injections of ethanol (0, 1.0, 1.25, 1.5 g/kg) or FNZ (0, 0.015, 0.03, 0.06 mg/kg). (a) Baseline latencies to enter the chamber were less in saline-treated mutant mice compared to wild-type controls (*$P<.0001$). Ethanol did not reduce latencies in wild-type mice, but at 1.0 g/kg, mutant mice exhibited a nonsignificant decrease in the time to enter the chamber. (b) Saline-treated mutant mice spent more time in the chamber compared to wild-type mice (*$P<.0001$); however, ethanol had no anxiolytic effect in either mutant or wild-type mice. (c) After vehicle injection mutant mice exhibited decreased baseline latencies compared to wild-type controls (*$P<.02$); however, FNZ treatment did not reduce latencies in either genotype. (d) The genotypes differed in the percent time spent in the mirrored chamber after vehicle injection (*$P<.05$) but neither mutant nor wild-type mice increased their percent time in the chamber after FNZ injection. Data is expressed as the means ± S.E.M. at each drug concentration.

3.3. Mirrored chamber

3.3.1. Ethanol

Highly significant differences between the genotypes were observed in saline-injected mice for all dependent measures in the mirrored chamber test ($t(22)=4.08$, $P<.0001$; $t(22)=6.32$, $P<.0001$; $t(22)=4.29$, $P<.0001$; $t(22)=5.00$, $P<.0001$; latency to enter the mirrored chamber, number of times in the mirrored passage, the percent of time in and total number of entrances into the chamber, respectively) (Fig. 3a, c; Tables 1 and 3). Post hoc tests revealed that the baseline differences were exclusively due to overall decreased anxiety in saline-treated null mutant mice ($P<.05$) compared to wild-type control mice. To control for baseline differences percent of saline values was analyzed for genotype and ethanol dose effects. In this maze, the primary indices of anxiety are latency to enter, the number of entrances into, and the percent time spent in the mirrored chamber. The number of times a mouse enters the mirrored passage is a secondary measure of anxiety, but also reflects movement within the maze. The results of a two-way ANOVA of the converted data demonstrated a significant effect of genotype for the percent time spent in the mirrored chamber, $F(1,70)=7.92$, $P<.01$, and entrances into the mirrored passage, $F(1,70)=18.37$, $P<.0001$. Overall, mutant mice were more active in the maze and ethanol significantly increased entrances into the mirrored passage in both genotypes, $F(2,70)=8.00$, $P<.001$. The Genotype × Dose interaction term was also significant for this measure, $F(2,70)=8.20$, $P<.001$; the number of entrances into the mirrored passage were increased after 1.25 g/kg ethanol in wild-type mice ($P<.05$); and after 1.0 g/kg in mutant mice ($P<.05$) suggesting that the mice were moving about the maze at these doses, even though the lack of a dose effect on the number of entrances into the chamber and the time spent in the chamber might suggest sedation. Latency to enter the chamber was also significantly affected by dose, $F(2,70)=4.45$, $P<.02$; however, within-genotype analyses did not reveal a dose effect for either group, even though mutant mice exhibited a nonsignificant decreased latency at 1.0 g/kg (saline = 87.0 ± 29.8 s and 1.0 g/kg = 16.6 ± 4.67 s; $P=.09$). The lack of significance may be due to the variation in latency in the saline-treated mice; latencies ranged from 9 to 300 s; however, tests to detect outliers did not identify any scores to remove.

3.3.2. Flunitrazepam

Significant genotype differences were seen in vehicle-treated mice ($t(22)=2.79$, $P<.01$; $t(22)=2.65$, $P<.02$; $t(22)=2.12$, $P<.05$; latency to enter the chamber, total number of entrances, and the percent time spent in the chamber, respectively), with vehicle-treated mutant mice displaying decreased anxiety compared to wild-type mice (Fig. 3c, d; Tables 2 and 3). Therefore, data were converted to percent of vehicle to adjust for baseline differences. In general, FNZ was a poor anxiolytic in this test. The primary measures of anxiety, latency to enter the chamber, and time spent in the chamber were not affected by FNZ treatment in either mutant or wild-type mice. A two-way ANOVA indicated that the genotypes differed in their number of entrances into the mirrored chamber and entrances into the mirrored passage, $F(1,70)=7.46$, $P<.01$; $F(1,70)=13.2$, $P<.001$, respectively. This appeared to be due to an overall greater number of entrances at all doses by mutant mice. Entrances into the chamber and passage were also dose dependent, $F(2,70)=4.87$, $P<.01$; $F(2,70)=7.33$, $P<.001$, respectively; however, this did not represent an anxiolytic response, but rather a sedative response to the 0.06 mg/kg dose.

4. Discussion

The purpose of this investigation was to characterize the anxiolytic effects of ethanol in PKCγ null mutant mice, which have previously demonstrated decreased ethanol sensitivity in behavioral and biochemical assays, as well as exhibiting reduced baseline anxiety (Bowers et al., 1999, 2000; Harris et al., 1995). The decreased ethanol sensitivity in the null mutants observed in earlier studies was specific to ethanol and did not generalize to two other GABAergic drugs, FNZ and pentobarbital; therefore, the present study included a comparison of ethanol to the anxiolytic, FNZ, to determine whether ethanol's specificity could also be observed in anxiety-related behaviors. The major findings from this study are: (1) in the elevated plus-maze PKCγ null mutants exhibited decreased sensitivity to the anxiolytic effects of ethanol, manifested as a shift to the right in the dose–response curve and this change in sensitivity was specific to ethanol because the anxiolytic effects of FNZ were not different between genotypes; (2) in the black/white box PKCγ appeared to regulate the anxiolytic effects of FNZ, but not ethanol; and (3) the overall efficacy of ethanol and FNZ as anxiolytics appeared to depend on the type of anxiety test used and the presence or absence of PKCγ.

The differences in the results from the three mazes suggests a three-way interaction of PKCγ, ethanol or FNZ, and the aspects of anxiety that are specifically measured in each of these mazes. This complicated interaction can be partially explained by examining the simpler interactions of (1) PKCγ and behavior in each of the mazes, and (2) the genetic regulation of ethanol and benzodiazepines in reducing anxiety in different anxiety mazes. The third component, ethanol and FNZ effects on PKCγ's modulation of GABAergic function has been addressed biochemically by Harris et al. (1995). Firstly, although naïve mutant mice displayed significant reduced baseline anxiety in all three tests (Bowers et al., 2000) that suggested a significant role of PKCγ in the regulation of anxiety, differential effects of the PKCγ null mutation by itself in each of the mazes was observed. The degree of reduced anxiety in the mutant mice versus wild-type mice did vary qualitatively in each maze. For example, anxiety-related behaviors were markedly reduced in the mirrored chamber, e.g., latency to enter the chamber was decreased threefold in mutants compared to wild-type mice. On the other hand, in the black/white box and elevated plus-maze, anxiety-related behaviors were reduced on average by half. These qualitative differences may reflect unique anxiety-related aspects of each maze. In fact, results of recent work have shown that the distinctions in aversive stimuli between anxiety tests such as height, open spaces, bright light, novelty, or mirrored spaces likely tap into different aspects of state anxiety that could reflect the heterogeneity of anxiety disorders in humans. Factor analyses and principal component analyses have shown that within a particular test different behavioral measures represent independent factors and reaction to novelty and exploration measures in these tests are associated with different psychological variables (Beuzen and Belzung, 1995; Bowers et al., 2000). It has been suggested that the heterogeneity of anxiety disorders in humans is due to dysregulation of different neurochemical pathways, evidenced by the efficacy of different classes of anxiolytic drugs for treatment of the different types of anxiety. In support of this hypothesis, File et al. (1993) have shown that serotonin and GABA functions are differentially modulated in rat cortex and hippocampus after exposure to the elevated plus-maze and social interaction tests of anxiety and are dependent on the type of test and/or test condition. It is of interest that PKC phosphorylation regulates 5-HT_2 receptor function as well as $GABA_A$ receptor function (Rahimian and Hrdina, 1995).

This leads to the second component of the interaction. It would seem likely that the anxiolytic efficacy of alcohol and FNZ would also be variable depending on which aspect of anxiety is expressed, which pathway is involved, and perhaps whether PKCγ is available to phosphorylate receptors in these pathways. The results of the present study suggest that in these mice (1) PKCγ interacts with ethanol, but not FNZ, to regulate the fear of open spaces in the plus-maze; (2) PKCγ appears to interact with FNZ, but not ethanol, to regulate anxiety related to brightly lit, open environments in the black/white box; and (3) PKCγ does not mediate the actions of ethanol or FNZ in reducing possible anxiety related to the perceived social stimuli generated by the mirrored chamber. Indeed, studies of alcohol use among individuals diagnosed with anxiety disorders and studies of alcohol's effects on anxiety in human populations do suggest that some types of anxiety

disorders are more responsive to the anxiolytic effects of alcohol than others (Eddy, 1979; Kushner et al., 1996; Pohorecky, 1991). The clinical efficacy of benzodiazepines used in the treatment of anxiety disorders also appears to depend on the type of anxiety being treated. Generalized anxiety disorder and panic disorder but not phobias or obsessive–compulsive disorder are usually successfully treated with benzodiazepines (Friedel, 1988; Sheehan and Raj, 1990). Genetic influences on personality also play a role in the anxiolytic profiles of both ethanol and benzodiazepines. Sher and Levenson (1982) suggest that aggressive, impulsive, and antisocial individuals, as well as Type I alcoholics who display anxious behavior (Cloninger, 1987) receive greater anxiolytic benefit from alcohol than less aggressive personality types. Similar results have been found for responses to diazepam measured in sons of alcoholics (SOAs). When compared to controls, SOAs report significantly greater euphoric responses to DZ, and exhibit decreased responses to DZ in two eye-movement tasks (Cowley et al., 1992, 1994).

Evidence for genetic variation in drug responses is even better exemplified in rodent studies. For example, mice selected for differences in initial sensitivity to ethanol, the long-sleep (LS) and short-sleep (SS) mice, display different responses to ethanol and diazepam in the elevated plus-maze. The less ethanol sensitive SS mice show decreased anxiety after ethanol, but not diazepam, whereas, the more ethanol sensitive LS mice exhibit the opposite response; i.e., anxiolysis due to DZ, but not ethanol (Stinchcomb et al., 1989). It should be noted, however, that these mice do not exhibit different responses to these two drugs in the mirrored chamber (Cao et al., 1993). Similar results were observed in rats selected for ethanol consumption, the ethanol preferring (P) and nonpreferring (NP) rats. When tested in an anticonflict test and the plus-maze after ethanol or chlordiazepoxide (CDZ) injections, P rats responded to ethanol's anxiolytic effects, but NP rats did not (Stewart et al., 1993). However, both lines exhibited decreased anxiety-related behaviors after CDZ. Grieble et al. (2000) have shown that in the light/dark test and elevated plus-maze there are significant strain differences in response to diazepam treatment among nine strains of mice, some of which were unaffected by the drug even if they demonstrated high levels of baseline anxiety in the tests. In addition, DZ was differentially effective in reducing anxiety in each of the two mazes dependent on genotype. The interaction of drug treatment and anxiolytic responses in each of the mazes observed in the present study is most likely regulated by the null mutation; however, the possibility remains that sequential testing of the mazes may have contributed to these differences.

All together, the results from the elevated plus maze and the black/white box indicate that the mechanism by which PKCγ regulates the anxiolytic effects of ethanol and FNZ is not the same. However, the pathway for each drug most likely involves $GABA_A$ receptors, a neurotransmitter system that has been significantly implicated in the etiology of several anxiety disorders (Kaspar, 1994). Protein kinase C appears to be involved in ethanol's actions at several ion channel systems including $GABA_A$ receptors. However, the mechanism by which this interaction occurs is not clear; several independent in vitro studies have reported controversial results (Stubbs and Slater, 1999). For example, Wafford and Whiting (1992) stated that phosphorylation of the γ2L subunit of the $GABA_A$ receptor was essential for ethanol potentiation of the receptor. However, Zhai et al. (1998) did not find a requirement of PKC phosphorylation of this receptor to elicit ethanol's effects. Some of the discrepancies in these results may be due to the lack of specificity in studying phosphorylation of selective $GABA_A$ subunits by individual PKC isozymes. Recently, Kumar et al. (2000) reported a direct association of PKCγ with the α1 subunit of the $GABA_A$ receptor and receptor phosphorylation in the presence of PKCγ, but not PKCδ or PKCε. The acute effects of ethanol on PKCγ's action at the $GABA_A$ receptor is unknown; however, this same study reported a decrease in the association of PKCγ with the $GABA_A$ receptor after chronic ethanol consumption. This evidence indicates that ethanol does influence PKCγ's phosphorylation of this receptor suggesting that alterations in receptor function may occur even after acute ethanol administration. The increased sensitivity to FNZ *in the absence* of PKCγ may be related to an inhibitory role of the enzyme in FNZ's actions at the $GABA_A$ receptor at low doses. The mechanism of action may not involve direct phosphorylation of the receptor after FNZ treatment because recombinant receptors with a deletion in the PKC phosphorylation site of the γ2L subunit show normal functional responses to benzodiazepines (Wafford and Whiting, 1992). The γ2 subunit has been shown to be necessary for benzodiazepine actions at the $GABA_A$ receptor (Kucken et al., 2000). However, this does not rule out an interaction of PKCγ and some of the $GABA_A$ alpha subunits, particularly α1, which have been shown to be necessary to form the binding site for benzodiazepines (Sigel and Buhr, 1997).

To summarize, we have shown that the enzyme, PKCγ, is one factor in the regulation of individual differences in the sensitivity to the anxiolytic actions of both ethanol and FNZ; however, anxiolytic responses to these drugs are dependent on aspects of anxiety unique to the elevated plus-maze or the black/white box. The results of this study suggest that phosphorylation of receptor proteins by PKCγ in neurochemical pathways that mediate anxiety partially explains why alcohol and benzodiazepines alleviates anxiety in some individuals in some contexts, but not others.

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References

Abeliovich A, Chen C, Goda Y, Silva AJ, Stevens CF, Tonegawa S. Modified hippocampal long-term potentiation in PKCγ-mutant mice. Cell 1993;75:1253–62.

Bale TL, Contarino A, Smith GW, Chan R, Gold LH, Sawchenko PE, Koob GF, Vale WW, Lee K-F. Mice deficient for corticotropin-releasing hormone receptor-2 display anxiety-like behavior and are hypersensitive to stress. Nat Genet 2000;24:410–4.

Belzung C, Le Pape G. Comparison of different behavioral test situations used in psychopharmacology for measurement of anxiety. Physiol Behav 1994;56:623–8.

Beuzen A, Belzung C. Link between emotional memory and anxiety states: a study by principal component analysis. Physiol Behav 1995;58:111–8.

Bowers BJ, Owen EH, Collins AC, Abeliovich A, Tonegawa S, Wehner JM. Decreased ethanol sensitivity and tolerance development in γ-protein kinase C null mutant mice is dependent on genetic background. Alcohol: Clin Exp Res 1999;23:387–97.

Bowers BJ, Collins AC, Tritto T, Wehner JM. Mice lacking PKCγ exhibit decreased anxiety. Behav Genet 2000;30:111–21.

Cao W, Burkholder T, Wilkins L, Collins AC. A genetic comparison of behavioral actions of ethanol and nicotine in the mirrored chamber. Pharmacol, Biochem Behav 1993;45:803–9.

Chalouff F, Durand M, Mormede P. Anxiety- and activity-related effects of diazepam and chlordiazepoxide in the rat light/dark and dark/light tests. Behav Brain Res 1997;85:27–35.

Chen C, Rainnie DG, Greene RW, Tonegawa S. Abnormal fear response and aggressive behavior in mutant mice deficient for alpha-calcium-calmodulin kinase II. Science 1994;266:291–4.

Cloninger CR. Neurogenetic adaptive mechanisms in alcoholism. Science 1987;236:410–6.

Costall B, Jones BJ, Kelly ME, Naylor RJ, Tomkins DM. Exploration of mice in a black and white test box: validation as a model of anxiety. Pharmacol, Biochem Behav 1989;32:777–85.

Cowley DS, Roy-Burne PP, Greenblatt DJ, Ries R, Walker RD, Samson HH, Hommer DW. Response to diazepam in sons of alcoholics. Alcohol: Clin Exp Res 1992;16:1057–63.

Cowley DS, Roy-Burne PP, Radant A, Hommer DW, Greenblatt DJ, Vitaliano PP, Godon C. Eye movement effects of diazepam in sons of alcoholic fathers and male control subjects. Alcohol: Clin Exp Res 1994;18:324–32.

Crabbe JC, Gallaher EJ, Cross SJ, Belknap JK. Genetic determinants of sensitivity to diazepam in inbred mice. Behav Neurosci 1998;112:668–77.

Crawley J, Goodwin FK. Preliminary report of a simple animal behavioral model for the anxiolytic effects of benzodiazepines. Pharmacol, Biochem Behav 1980;13:167–70.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy J-M, Lüscher B, Mohler H. Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. Nat Neurosci 1999;2:833–9.

Durcan MJ, Lister RG. Time course of ethanol's effects on locomotor activity, exploration and anxiety in mice. Psychopharmacology 1988;96:67–72.

Eddy C. The effects of alcohol on anxiety in problem- and nonproblem-drinking women. Alcohol: Clin Exp Res 1979;3:107–14.

File S. Recent developments in anxiety, stress, and depression. Pharmacol, Biochem Behav 1996;54:3–12.

File SE, Zangrossi H, Andrews N. Social interaction and elevated plus-maze tests: changes in release and uptake of 5-HT and GABA. Neuropharmacology 1993;32:217–21.

Friedel RO. The therapeutic value, limitations and hazards of treatment of psychiatric disorders with benzodiazepines. In: Roth M, Noyes R, Burrows GD, editors. Biological, clinical and cultural perspectives. Handbook of anxiety vol. 1. New York: Elsevier, 1988. pp. 385–97.

Gallup GG. Mirror-image stimulation. Psychol Bull 1968;70:782–93.

Grieble G, Belzung C, Perrault G, Sanger DJ. Differences in anxiety-related behaviors and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology 2000;148:164–70.

Harris RA, McQuilkin SJ, Paylor R, Abelovich A, Tonegawa S, Wehner JM. Mutant mice lacking the γ isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of γ-aminobutyrate type A receptors. Proc Natl Acad Sci USA 1995;92:3658–62.

Heisler LK, Chu H-M, Brennan TJ, Danao JA, Bajwa P, Parsons LH, Tecott LH. Elevated anxiety and antidepressant-like responses in serotonin 5-HT_{1A} receptor mutant mice. Proc Natl Acad Sci USA 1998;95:15049–54.

Hogg S. A review of the validity and variability of the elevated plus-maze as an animal model of anxiety. Pharmacol, Biochem Behav 1996;54:21–30.

Kash SF, Tecott LH, Hodge C, Baekkeskov S. Increased anxiety and altered responses to anxiolytics in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. Proc Natl Acad Sci USA 1999;96:1698–703.

Kaspar S. Neurobiology and new psychopharmacological strategies for treatment of anxiety disorders. In: Darcourt G, Mendlewicz J, Racagni G, Brunello N, editors. Current therapeutic approaches to panic and other anxiety disorders. International Academy for Biomedical and Drug Research 1994;vol. 8:15–35.

Kishimoto T, Radulovic J, Radulovic M, Lin CR, Schrick C, Hooshmand F, Hermanson O, Rosenfeld MG, Spiess J. Deletion of Crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat Genet 2000;24:415–9.

Kucken AM, Wagner DA, Ward PR, Teissere JA, Boileau AJ, Czajkowski C. Identification of benzodiazepine site residues in the γ2 subunit of the γ-aminobutyric $acid_A$ receptor. Mol Pharmacol 2000;57:932–9.

Kumar S, Grobin AC, Kralic JE, Morrow AL. $GABA_A$ receptor specifically binds PKCγ: effect of chronic ethanol consumption. Alcohol: Clin Exp Res 2000;24:46A.

Kushner MG, Sher KJ, Beitman BD. The relation between alcohol problems and the anxiety disorders. Am J Psychiatry 1990;147:685–95.

Kushner MG, Mackenzie TB, Fiszdon J, Valentiner DP, Foa E, Wagensteen D. The effects of alcohol consumption on laboratory induced panic and state anxiety. Arch Gen Psychiatry 1996;53:264–70.

Lister RG. The use of a plus-maze to measure anxiety in the mouse. Psychopharmacology 1987;92:180–5.

Lister RG. Ethologically-based animal models of anxiety disorders. Pharmacol Ther 1990;46:321–40.

Parks CL, Robinson PS, Sibille E, Shenk T, Toth M. Increased anxiety of mice lacking the serotonin 1A receptor. Proc Natl Acad Sci USA 1998;95:10734–9.

Pohorecky LA. Stress and alcohol interaction: an update of human research. Alcohol: Clin Exp Res 1991;15:438–59.

Rahimian R, Hrdina PD. Possible role of protein kinase C in regulation of 5-hydroxytryptamine 2A receptors in rat brain. Can J Physiol Pharmacol 1995;73:1686–91.

Ramboz S, Oosting R, Amara DA, Kung HF, Blier P, Mendelsohn M, Mann JJ, Brunner D, Hen R. Serotonin receptor 1A knockout: an animal model of anxiety-related disorder. Proc Natl Acad Sci USA 1998;95:14476–8.

Rodgers RJ, Johnson NJT. Factor analysis of spatiotemporal and ethological measures in the murine elevated plus-maze test of anxiety. Pharmacol, Biochem Behav 1995;52:297–303.

Sheehan DV, Raj A. Benzodiazepine treatment of panic disorder. In: Roth R, Noyes R, Burrows GD, editors. The treatment of anxiety. Handbook of anxiety vol. 4. New York: Elsevier, 1990. pp. 169–206.

Sher KJ, Levenson RW. Risk for alcoholism and individual differences in the stress-response-dampening effect of alcohol. J Abnorm Psychol 1982;91:350–67.

Sigel E, Buhr A. The benzodiazepine binding site of $GABA_A$ receptors. Trends Pharmacol Sci 1997;18:425–9.

Smith GW, Aubry J-M, Dellu F, Contarino A, Bilezikjian LM, Gold LH, Chen R, Marchuk Y, Hauser C, Bentley CA, Sawchenko PE, Koob GF, Vale W, Lee K-F. Corticotropin releasing factor receptor deficient mice display decreased anxiety, impaired stress response, and aberrant neuroendocrine development. Neuron 1998;20: 1093–102.

Stewart RB, Gatto GJ, Lumeng L, Li T-K, Murphy JM. Comparison of alcohol-preferring (P) and nonpreferring (NP) rats on tests of anxiety and for the anxiolytic effects of ethanol. Alcohol 1993;10:1–10.

Stinchcomb A, Bowers BJ, Wehner JM. The effects of ethanol and Ro 15-4513 on elevated plus-maze and rotarod performance in long-sleep and short-sleep mice. Alcohol 1989;6:369–76.

Stubbs CD, Slater SJ. Ethanol and protein kinase C. Alcohol: Clin Exp Res 1999;23:1552–60.

Toubas PL, Abla KA, Cao W, Logan LG, Seale TW. Latency to enter a mirrored chamber: a novel behavioral assay for anxiolytic agents. Pharmacol, Biochem Behav 1990;35:121–6.

Wafford KA, Whiting PJ. Ethanol potentiation of $GABA_A$ receptors requires phosphorylation of the alternatively spliced variant of the γ2 subunit. FEBS Lett 1992;313:113–7.

Zhai J, Stewart RR, Friedberg MW, Li C. Phosphorylation of the $GABA_A$ receptor γ2L subunit in rat sensory neurons may not be necessary for ethanol sensitivity. Brain Res 1998;805:116–22.