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A Genetic Comparison of Behavioral Actions of Ethanol and Nicotine in the Mirrored Chamber

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CAO, W., T. BURKHOLDER, L. WILKINS AND A. C. COLLINS. *A genetic comparison of behavioral actions of ethanol and nicotine in the mirrored chamber.* PHARMACOL BIOCHEM BEHAV **45**(4) 803–809, 1993.—Human alcoholics are almost invariably heavy users of tobacco, perhaps because both ethanol and nicotine may have anxiolytic activity. However, studies in humans have not uniformly detected anxiolytic effects because significant individual differences in anxiolytic actions of these agents seem to exist. One factor that seems to contribute to these individual differences is tolerance to ethanol. Individuals who are more sensitive to depressant actions of alcohol seem to show anxiolytic actions more readily. Consequently, we examined the relative sensitivities of the ethanol-sensitive (to the anesthetic actions of ethanol) long-sleep (LS) and ethanol-resistant short-sleep (SS) mouse lines to diazepam, ethanol, nicotine, and ethanol-nicotine combinations in the mirrored chamber test. This test measures approach-conflict behavior. Ethanol and nicotine evoked changes in mirrored chamber activities that resembled those elicited by diazepam. These effects were seen at doses that did not markedly affect locomotor activity, thereby suggesting that these changes in behavior represent anxiolytic actions. The LS-SS mice did not differ in sensitivity to diazepam, but the SS were more uniformly responsive to the other drugs. Only the SS showed clear evidence for interactions between ethanol and nicotine. If the changes in mirrored chamber behavior elicited by ethanol, nicotine, and combinations of the two drugs occur because of anxiety reduction, it seems that the SS mouse line is more responsive to anxiolytic actions of these drugs.

Ethanol Nicotine Diazepam Anxiety Genetics

ALTHOUGH it is widely believed that alcohol reduces anxiety, not all individuals who use alcohol seem to experience anxiety reduction. Men who exhibit Type A behavior (25) and men who have high levels of impulsivity and aggressiveness (29) show some reduction in anxiety following alcohol ingestion, but Type B personalities and less impulsive or aggressive men may not. Sensitivity (tolerance) to ethanol may also influence the level of anxiety reduction that ethanol ingestion produces. Men who are least sensitive (most tolerant) to ethanol's effects on standing stability are also least sensitive to ethanol's anxiolytic effects (19). As a consequence of these and other observations, Wilson (35) has argued that the questions that should be addressed are at what dose, in whom, under what circumstances, and on what measures does alcohol reduce anxiety?

Several studies suggest that animals (rats and mice) also exhibit reduced anxiety following alcohol treatment, but it is not always clear whether the effects evoked by ethanol are actually anxiolytic actions. For example, rats trained to press a lever for food reward exhibit reduced lever pressing when this act is accompanied by low intensity electrical shock. Ethanol injection partially restores lever-pressing activity which could arise because of anxiolytic activity (1,32). Unfortunately, it is not clear whether these results represent anxiolytic effects of ethanol or effects of ethanol on learning and memory processes or even analgesic actions of alcohol. However, ethanol injection produces what may be anxiolytic activity in two other tests (elevated plus maze and escape digging) that purportedly measure anxiety (11,31), and genetic factors seem to influence this action of alcohol, at least for the elevated plus maze test (31). In contrast, low doses of ethanol (0.25, 0.5 g/kg) may be anxiogenic as measured by activity in a light-dark box (24).

Tobacco also seems to have anxiety-reducing properties, but tobacco use may also serve as a stressor (13). Direct attempts to measure anxiolytic actions of smoking suggest that tobacco has one or more anxiolytic components (26,27). Because anxiolytic actions of tobacco are not seen in nicotine-free cigarettes (14), it seems likely that nicotine is the most effective anxiolytic agent in tobacco. Animal studies support

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the argument that nicotine has anxiolytic actions. Rats show what may be anxiolytic actions of nicotine in the elevated plus maze test (2,34) and mice show anxiety reduction following nicotine injection in a two-compartment white/black test box (3,4).

Tobacco and alcohol are frequently used together (8), but the reasons behind this are largely unknown. One possible explanation is that both of these agents have modest anxiolytic activity. The studies reported here measured the effects of diazepam (a standard anxiolytic drug), ethanol, nicotine, and ethanol-nicotine given together on several activities in the mirrored chamber test (33). This test may be a model for assessing approach-avoidance conflict behavior. The long-sleep (LS) and short-sleep (SS) mouse lines that were selectively bred for differences in duration of ethanol-induced hypnosis (sleep time) (22) were used because of the observation that in human males sensitivity to alcohol's ataxia-producing effects may predict sensitivity to anxiolytic actions (19). Because the LS and SS mice also differ in sensitivity to an acute dose of nicotine (5-7,9) the use of these animals may also provide some information concerning potential genetic regulation of anxiolytic actions of nicotine.

METHOD

Animals

Experimentally drug-naive male LS/IBG and SS/IBG mice were used in this study. The mice were raised at the Institute for Behavioral Genetics, and weaned at 25 days of age. The mice were separated by line, and were housed with five like-sex littermates in a climatically controlled environment (temperature 19-21°C). The animals were acclimated to a 12L : 12D cycle and allowed free access to food (Wayne Lab Blox) and water. All animals were 60-100 days old when tested and kept for a minimum of 30 min in the testing room before performing the experiment.

Drug Source and Administration

Diazepam (a generous gift of Dr. Peter Sorter, Hoffman-La Roche Inc.) was dissolved in 10% cyclodextrin and then diluted with physiological saline solution. (−)Nicotine was obtained from Sigma Chemical Co. (St. Louis, MO). Ethanol was obtained from Midwest Grain Products Co. (Pekin, IL). The drugs were injected intraperitoneally in a volume of 0.01 ml/g body weight. Control groups received a vehicle injection. Diazepam was given 10 min prior to the initiation of testing, ethanol was administered 5 min prior to the initiation of testing, and mice injected with nicotine were tested immediately after injection. These times were chosen on the basis of information concerning rate of LS-SS drug absorption (9,30).

Mirrored Chamber Activity

The chamber of mirrors described by Toubas et al. (33) was constructed and used in these experiments. The chamber consists of a mirrored cube open on one side that is placed inside a square Plexiglas box. The mirrored cube measuring 30 cm on a side was constructed of five pieces of mirrored glass with one mirrored side and an opposite side painted dark brown. The three mirrored side panes, a top pane, and the floor pane faced the interior of the cube. The container box was 40 × 40 × 30.5 cm. The mirrored cube was placed in the center of the container to form a 5 cm corridor that completely surrounded the mirrored chamber. A mirror was also placed on the container wall so that it faced the single open side of the mirrored chamber. Except for this one mirrored portion on the container wall, all portions of the container walls were dark brown.

Group-housed mice were brought into the testing room and allowed to acclimate to the new environment for at least 30 min. Mice were placed in the chamber of mirrors and scored only once. Mice were injected with vehicle or drug and were placed at a single, fixed starting point in a corner of the chamber. The mouse was allowed to explore the chamber for 5 min. The time of the first entry into the chamber of mirrors was determined along with the number of entries, time per entry, and total time/5-min test period. In addition, the number of times that the animal entered into the pathway between the mirrored chamber and the mirror that was placed on the container wall (pathway entries) was also scored. The criterion for entry into the chamber or pathway was all four feet being placed on the floor panel of the mirrored chamber or in the pathway. The apparatus was washed thoroughly with 10% ethanol between tests to minimize environmental effects related to testing multiple animals.

Open Field Activity

One of the major concerns regarding measurement of potential anxiolytic actions of drugs is whether changes in activity represent anxiolytic actions or effects on locomotor activity. Therefore, dose-response curves were constructed for the effects of ethanol and nicotine on activity in a circular open field arena. The arena consists of a white Plexiglas square (58.5 × 58.5 cm, l × w) that had a Plexiglas cylinder placed inside (58.5 cm diameter). Mice were injected with saline, ethanol, or nicotine and were then immediately placed in the middle of the arena. The arena had photocells spaced every 15 cm and activity was recorded when the animals broke a light beam. In order to assure that locomotion rather than grooming, sniffing, or stereotypy was measured, the Commodore computer was programmed to ignore any readings spaced with intervals of less than 0.2 s. The test was run under white light and activity was measured for 15 min after injection.

Data Analysis

All mirrored chamber and open field data were analyzed by two-way analysis of variance (ANOVA). Subsequently, the data obtained from each mouse line (LS,SS) were also analyzed using a one-way ANOVA. In addition, the correlations between drug effects on each measure in the mirrored chamber were determined for both mouse lines.

RESULTS

A correlational analysis of the five measures in both of the mouse lines following diazepam treatment detected significant correlations among the time to first entry, time per entry, number of entries, and total time in the chamber measures. The number of entries into the pathway was not significantly correlated with any of the other measures. Consequently, the pathway data will be presented for all drugs separately.

The effects of diazepam on the four correlated measures are presented in Fig. 1. Overall LS-SS differences in latency to first entry into the chamber were obtained, but diazepam

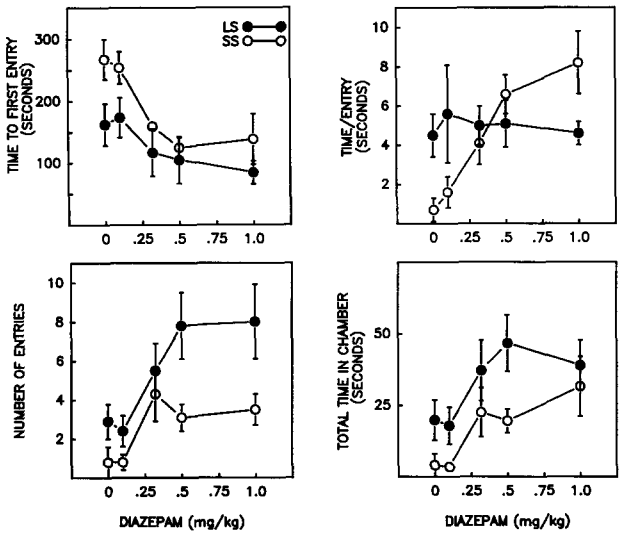

FIG. 1. Effects of varying doses of diazepam on several mirrored chamber activities. LS and SS mice were injected with vehicle or one of several doses of diazepam and mirrored chamber activities measured for a 5-min time period starting 10 min after injection. Each point represents the mean ± standard error of 8-12 animals for the LS mice and 8-10 animals for the SS.

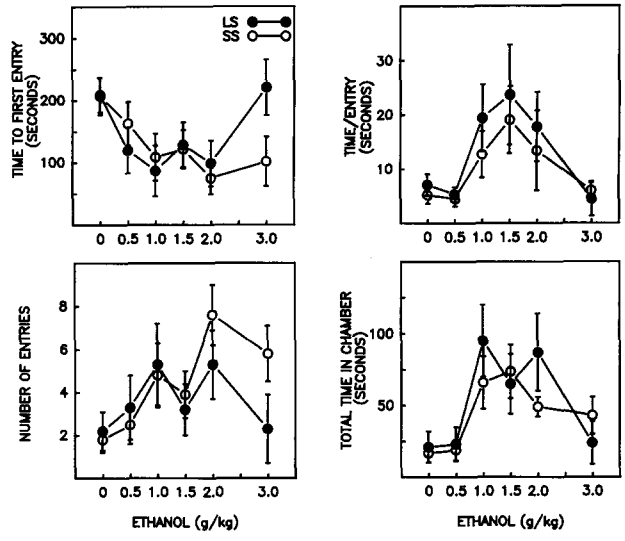

FIG. 2. Effects of ethanol injection on several mirrored chamber activities. LS and SS mice were injected with saline or one of several doses of ethanol. Mirrored chamber behaviors were measured for a 5-min test period starting 5 min after injection. Each point represents the mean ± standard error of 9-18 animals for the LS and 6-12 animals for the SS.

reduced the latency to first entry similarly in both mouse lines. The two-way ANOVA did not detect an overall LS-SS difference in time per entry (Fig. 1, upper right panel) nor did it detect an overall effect of diazepam dose, but a significant line-by-dose interaction was obtained for this measure. The significant interaction term arises because diazepam injection increased the time per entry in the SS, but was without effect in the LS [SS, $F(4, 37) = 7.93$, $p < 0.001$; LS, $F(4, 44) = 0.11$]. Diazepam treatment tended to increase the number of entries into the chamber, but no overall LS-SS differences and no significant interactions were seen. In addition, diazepam tended to increase the total time spent in the chamber, similarly in the two mouse lines. These results suggest that anxiolytic activity results in a decreased latency to first entry, an increase in the number of entries, time per entry, and total time spent in the chamber.

Similar experiments were done using ethanol, but unlike the results obtained with diazepam, the number of entries into the pathway correlated significantly with all four of the other measures (minimum $r^2 = -0.56$, $p < 0.05$ for the comparison of pathway entries with time to first entry). Nonetheless, Fig. 2 presents the data obtained for the same four measures reported in Fig. 1. The two-way ANOVA suggested that ethanol changed each of the measures similarly in the LS and SS mouse lines. However, when one-way ANOVA were done within each mouse line, a different story emerged. SS mice treated with ethanol showed significant dose-dependent decreases in latency to enter the mirrored chamber, $F(4, 35) = 5.34$, $p < 0.01$, along with significant increases in the number of entries, $F(4, 35) = 6.43$, $p < 0.01$, and total time in the chamber, $F(4, 35) = 6.44$, $p < 0.01$. The apparent increase in time per entry was not statistically significant, $F(4, 35) = 1.64$. In each case where significant effects were seen, the post hoc analyses indicated that the groups treated with the 1.5 and 2.0 g/kg ethanol doses were different from the other treatment groups. The only significant effect of ethanol seen in the LS was for the total time measure, $F(4, 57) = 3.39$, $p < 0.05$, with the 1.5 and 2.0 g/kg doses being different from saline control.

Figure 3 presents the effects of nicotine injection on the same four mirrored chamber measures. Correlational analyses of the combined LS-SS data showed, once again, that time to first entry, time per entry, number of entries into the chamber,

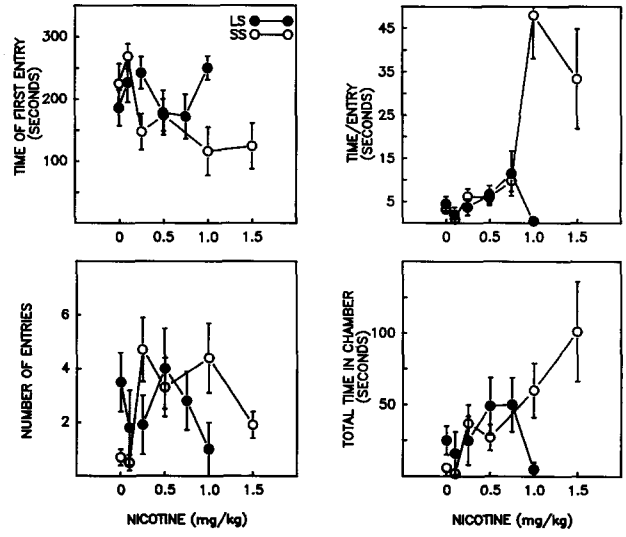

FIG. 3. Effects of nicotine injections on several mirrored chamber activities. LS and SS mice were injected with saline or one of several doses of nicotine. Immediately after injection the animal was placed in the mirrored chamber and activity was scored for a 5-min time period. Each point represents the mean ± standard error of 10-15 animals for the LS and 10-15 animals for the SS.

and total time in chamber were highly correlated. Thus, nicotine affects these measures similarly. The number of entries into the pathway was significantly correlated with time to first entry ($r^2 = -0.30$, $p < 0.01$) and number of entries ($r^2 = 0.31$, $p < 0.01$). The two-way ANOVA failed to detect any significant overall effects following injection with 0, 0.1, 0.25, 0.5, and 1.0 mg/kg doses. The 1.5 mg/kg dose was not used in the LS because they were virtually immobile at this dose (see Fig. 6). The one-way ANOVA indicated that SS mice show a dose dependent decrease in time to first entry, $F(5, 59) = 3.23$, $p < 0.01$; post hoc comparisons detected significant decreases in latency, compared to control, following injection with the 1.0 and 1.5 mg/kg nicotine dose. Nicotine injection elicited dose dependent increases in the SS mice in time per entry [$F(5, 59) = 6.13$, $p < 0.001$; the 1.0 and 1.5 mg/kg doses differed from control], number of entries [$F(5, 59) = 3.53$, $p < 0.01$; the 0.25, 0.5, and 1.0 mg/kg doses differed from control], and total time in the chamber [$F(5, 59) = 5.82$, $p < 0.001$; the 1.0 and 1.5 mg/kg differed from control]. The LS mice, in contrast, did not show any significant changes in behavior following nicotine treatment.

Figure 4 presents the effects of cotreatment with ethanol and nicotine. The doses used (0.5 g/kg ethanol; 0.1 and 0.25 mg/kg nicotine) elicited minimal effects, when given alone, in both mouse lines. The two-way ANOVA detected no overall line differences for any of the four measures, but significant overall effects of drug dose were detected for the time per entry and total time measures. Significant line-by-dose interactions were obtained for all of the measures. The one-way ANOVA showed that LS mice did not exhibit any changes in behavior following ethanol-nicotine injection, whereas significant effects were seen in the SS for all of the measures [time to first entry, $F(2, 19) = 6.64$, $p < 0.01$, time per entry, $F(2, 19) = 7.91$, $p < 0.01$, number of entries, $F(2, 19) = 7.37$, $p < 0.01$, and total time [$F(2, 19) = 11.40$, $p < 0.001$]. Thus, drug interactions were seen in the SS mice, but not in the LS.

As is evident from the data presented in Fig. 5, diazepam was without effect on pathway activity although overall LS-SS differences were observed. Ethanol, in contrast, had marked effects on this behavior, but this effect was seen only in the SS mice following ethanol, [$F(4, 35) = 6.82$, $p < 0.001$; doses of 1.5 g/kg and larger produced significant increases]. LS mice did not show any change in their pathway activity following ethanol, $F(4, 57) = 1.38$. Nicotine treatment also had pronounced effects on pathway activity. The two-way ANOVA detected significant effects of mouse line, nicotine dose, and line-by dose interactions. Nicotine injection elicited a dose-dependent decrease in activity in the pathway in the LS mice which was also seen in the SS, but not until much larger doses were used. The one-way ANOVA also detected significant decreases from control in pathway entries in the LS mice and biphasic actions on pathway entrances in the SS mice. The 0.2, 0.5, and 1.0 mg/kg nicotine dose increased pathway entries in this mouse line; significant decreases were attained after injection with the 1.5 mg/kg nicotine dose. Ethanol-nicotine cotreatment seemed to affect the two mouse lines differently as suggested by a significant line-by-dose interaction. The significant interaction term presumably arose because cotreatment tended to decrease pathway activity in the LS and increase it in the SS.

Changes in mirrored chamber activities could simply reflect changes in locomotor activity. Therefore, drug effects on locomotor activity were measured in another apparatus, a circular open field arena. These results are depicted in Fig. 6. Nicotine produced dose-dependent decreases in open field activity in the LS mice, whereas an increase at low doses followed by a decrease at high doses was seen in the SS. The two-way

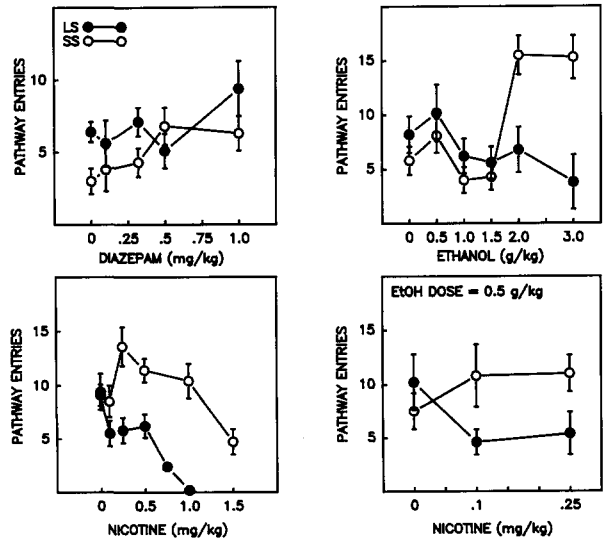

FIG. 4. Effects of drug injection on the number of pathway entries. LS and SS mice were injected with diazepam, ethanol, nicotine, or ethanol-nicotine, and number of entrances into the pathway between the outer box and the mirrored chamber scored for a 5-min test period. Each point represents the mean ± standard error. The *n* values for each group are reported in the legends to Figs. 1, 2, 3, and 5.

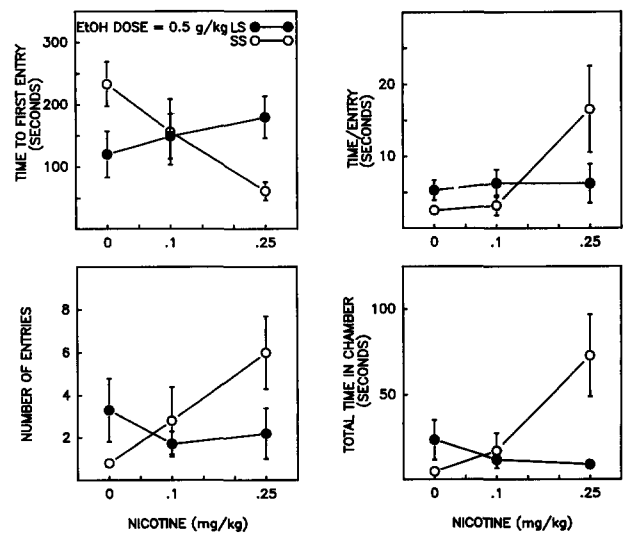

FIG. 5. Effects of ethanol-nicotine coinjection on mirrored chamber activities. LS and SS mice were injected with ethanol (0.5 g/kg) and one of three doses of nicotine (0, 0.1, 0.25 mg/kg) and mirrored chamber activities were measured for the next 5 min. Each point represents the mean ± standard error 6–10 animals for both the LS and SS.

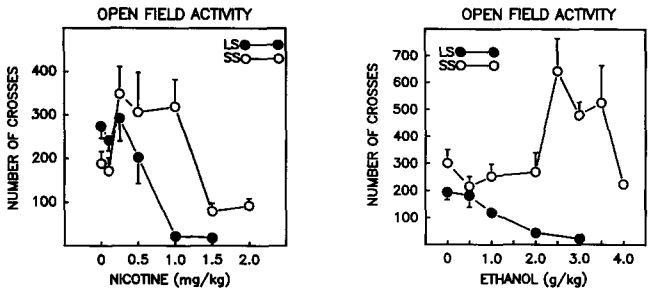

FIG. 6. Effects of nicotine and ethanol on open field activity. LS and SS mice were injected with saline or one of several doses of nicotine or ethanol. Open field activity was measured for a 15-min time period as described in the text. Each point represents the mean ± standard error. n = 6-7 for LS and 7-8 for the SS for ethanol and 9-11 for the LS and SS for nicotine.

ANOVA detected overall LS-SS differences, $F(1, 97) = 4.86$, $p < 0.05$, significant effects of dose, $F(5, 97) = 7.65$, $p < 0.01$, and a significant line-by-dose interaction term, $F(5, 97) = 4.50$, $p < 0.01$. Somewhat similar results were found with ethanol; only the SS showed activation. The two-way ANOVA detected overall LS-SS differences, $F(1, 76) = 49.31$, $p < 0.01$, and a significant line-by-dose interaction, $F(5, 76) = 6.12$, $p < 0.01$. The one-way ANOVA detected significant effects of dose in both the LS [$F(5, 35) = 7.48$, $p < 0.01$; significant reductions in activity were seen with the 1.0 g/kg, and higher, ethanol doses] and in the SS [$F(5, 41) = 3.51$, $p < 0.01$; significant increases in activity were seen following the 2.5, 3.0 and 3.5 g/kg doses]. Thus the LS mice were more sensitive to the locomotor activity depressant effects of both drugs while the SS were more sensitive to activity enhancing effects.

DISCUSSION

Although several reports have suggested that both alcohol and nicotine exert anxiolytic actions in rats and mice (1-4,11,31,32,34), virtually no attention has been paid to the possibility that genetic factors may regulate these activities. The results of the experiments reported here indicate that genetic factors are potentially important in regulating not only the anxiolytic actions of both of these drugs, but also interactions between these agents.

Toubas et al. (33) argued that the reflection of an animal in a mirror might serve as a source of anxiety. In our experiment, control animals entered the pathway between the chambers cautiously and, once in the pathway, frequently planted their rear legs, extended the front legs into the chamber, and then hastily retreated. Only after several such advance/retreats did the animal ultimately enter the chamber. Further evidence that this behavior represents anxiety comes from the observation that the classic anxiolytic, diazepam, reduced latency to enter the chamber, increased the number of entries, and time spent in the chamber. These effects were seen, in both the LS and SS mouse lines, at doses that did not alter the number of times the animal entered the pathway. Thus diazepam-induced increases in the number of entries into the chamber do not arise because the animal is in a position to enter the mirrored chamber more often. Rather, the animals entered the chamber a greater fraction of the time when treated with diazepam. These effects were seen at diazepam doses of 0.5 mg/kg or less which agrees very well with the results reported by Toubas et al. (33) who studied diazepam actions in BALB/cByJ mice using the mirrored chamber apparatus.

Minimal evidence for LS-SS differences in response to diazepam were observed. The LS mice seemed to show greater dose-dependent increases in the number of entries into the chamber and in total time spent in the chamber than did the SS, but the variance of these measures was so large that LS-SS differences did not emerge. However, mouse line differences were observed for the time-per-entry measure; the SS mice showed a dose-dependent increase in this measure whereas the LS did not.

Marley et al. (20) have reported that the LS-SS mice do not differ uniformly for various responses to the benzodiazepine flurazepam. The LS mice are more sensitive to the sleep time-inducing effects of flurazepam than are the SS, but they are less sensitive to the anticonvulsant actions and do not differ in sensitivity to the hypothermic actions. Stinchcomb et al. (31) reported that the LS are more sensitive to what may be anxiolytic actions of diazepam on the elevated plus maze test. The LS-SS mice do not differ in the number or affinity of [^{3}H]flunitrazepam binding sites, but do differ in GABA-induced enhancement of flunitrazepam binding (21). In some, but not all, brain regions the SS show greater enhancement. Consequently, it is not surprising that LS-SS differences are seen for some responses to benzodiazepines, but not for others.

The data presented in Fig. 2 suggest that the LS and SS mice are equally sensitive to ethanol effects on mirrored chamber activities, but the variance was so large in the LS that significant effects of ethanol in the LS did not emerge. Some of the LS mice totally failed to enter the chamber no matter what dose of ethanol was used. Stinchcomb et al. (31) also found better evidence for what may be anxiolytic actions of ethanol in the SS mice using the plus maze test; only the SS showed ethanol-induced increases in the percent of time spent in the open arms of the plus maze. These effects were seen at the same ethanol doses that produced significant changes in mirrored chamber behaviors in the SS mouse line.

Our results agree with previous studies that showed the SS mice are sensitive to the locomotor-activating effects of ethanol (10,28). We were concerned that ethanol-induced changes in activity could influence several of the mirrored chamber measures, particularly the number of entries into the pathway and chamber, but increases in open field activity were not run in the SS until the 2.5 g/kg dose, whereas mirrored chamber activities were affected in the SS mice by ethanol doses of 1.0-2.0 g/kg. This finding increases confidence that the changes in mirrored chamber activity in the SS mice do not arise because of increases in locomotor activity.

Both mouse lines showed a trend towards diazepam-like changes in mirrored chamber activities following nicotine injection, but these changes were not significant in the LS mice. As was the case following ethanol, some of the LS mice totally failed to show changes in behaviors in the pathway no matter what nicotine dose was used. In contrast, marked LS-SS differences in nicotine effects on open field activity were seen. Several doses (0.1-1.0 mg/kg) of nicotine increased the activity of SS mice in the arena, whereas the same doses were either without effect or depressed LS activity. These findings replicate previous reports that demonstrate LS-SS differences in sensitivity to nicotine (5-7,9). Because nicotine did not uni-

formly activate the locomotor activity of the SS mice in the open field arena at doses that altered mirrored chamber activity (1.0 and 1.5 mg/kg), it seems likely that nicotine's effects on mirrored chamber activity in the SS mice reflect anxiolytic activities rather than direct locomotor actions.

Perhaps the most interesting outcome of this study is that genotype influenced ethanol–nicotine interactions; only the SS mice showed marked interactions between ethanol and nicotine. The effects of ethanol–nicotine combinations were seen at doses of ethanol and nicotine that had minimal effect when given alone. These findings suggest additive or synergistic interactions between these drugs, but these interactions may be seen only in those individuals with specific genotypes. It is tempting to speculate that genetically based regulation of ethanol–nicotine interactions explains why alcoholics show a consistent increase in smoking behavior when alcohol is made available (16,17), whereas in nonalcoholics, alcohol availability increased tobacco use in some subjects, decreased it in others, and was without effect in yet others (18,23).

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