Neuroscience 131 (2005) 263–273

BEHAVIORAL SENSITIZATION TO ETHANOL IS MODULATED BY ENVIRONMENTAL CONDITIONS, BUT IS NOT ASSOCIATED WITH CROSS-SENSITIZATION TO ALLOPREGNANOLONE OR PENTOBARBITAL IN DBA/2J MICE

P. J. MEYER,[b,c] A. A. PALMER,[1b,c] C. S. MCKINNON[b,c] AND T. J. PHILLIPS[a,b,c]*

[a]*Veterans Affairs Medical Center, R&D 32, 3710 Southwest US Veterans Hospital Road, Portland, OR 97239, USA*

[b]*Portland Alcohol Research Center, Portland, OR, USA*

[c]*Department of Behavioral Neuroscience, Oregon Health & Science University, Portland, OR 97239, USA*

Abstract—Rationale: The ability of ethanol to facilitate $GABA_A$ receptor-mediated transmission may result in $GABA_A$ receptor alterations during repeated ethanol administration, and lead to dynamic behavioral changes, including sensitization to the locomotor stimulant effect of ethanol. Since alterations in $GABA_A$ receptors are likely to alter sensitivity to GABAergic drugs such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and pentobarbital, we determined whether enhanced sensitivity to ethanol was associated with enhanced sensitivity (cross-sensitization) to these drugs. Two procedures that produced differences in the magnitude of expression of ethanol-induced locomotor sensitization were used.

Methods: After habituation to testing procedures for 2 days, female DBA/2J mice were injected with ethanol or saline for 12 days. On the following day, locomotion was recorded after a challenge injection of ethanol (2 g/kg), allopregnanolone (10 or 17 mg/kg), or pentobarbital (10 or 20 mg/kg). Due to evidence that exposure to the test chambers influenced sensitization, in some experiments, mice were exposed to the test apparatus on the day prior to challenge.

Results: Exposure to the test apparatus prior to drug challenge attenuated the expression of ethanol sensitization, compared with mice without this pre-exposure. Cross-sensitization was not observed to either allopregnanolone or pentobarbital under any condition; however, some groups of repeated ethanol-treated mice displayed tolerance to the initial stimulant effects of allopregnanolone and pentobarbital.

Conclusions: These studies indicate that behavioral sensitization to ethanol is not associated with cross-sensitization to pentobarbital or allopregnanolone, and that the expression of ethanol sensitization is influenced by the relative novelty of the test chamber. In addition, these results do not support a mechanism in which alterations in the neurosteroid or barbiturate modulatory sites of the $GABA_A$ receptor are responsible for the expression of sensitization to the locomotor stimulant effects of ethanol. **© 2005 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: alcohol, neurosteroid, barbiturate, neuroactive steroids, locomotion, GABA.

Alcoholism is a disease whose development depends on multiple exposures to alcohol (ethanol). The neurobiological adaptations associated with the development of alcoholism have yet to be fully characterized. One broadly defined possibility is that there is an increase in sensitivity to the reinforcing or incentive motivational effects of ethanol upon multiple ethanol exposures (Koob and Le Moal, 1997, 2001; Rodd-Henricks et al., 2001; Schmidt et al., 2000). In some rodents, ethanol stimulates locomotor activity (Dudek et al., 1991; Lister, 1987; Rodd et al., 2004), and behavioral sensitization (an increase in this response) develops following repeated ethanol administration (Correa et al., 2003; Hoshaw and Lewis, 2001; Masur and Boerngen, 1980; Phillips et al., 1995). The locomotor stimulant and reinforcing properties of ethanol and other abused drugs appear to have some neurobiological substrates in common (Amalric and Koob, 1993; Tzschentke and Schmidt, 2000; Wise and Bozarth, 1987), although differences in their regulation are also known to exist. Similarly, studies using sensitized mice have suggested a correlation between behavioral sensitization and the intake of abused drugs (Robinson and Berridge, 1993; Cornish and Kalivas, 2001), including ethanol (Grahame et al., 2000; Lessov et al., 2001a; Phillips et al., 1995). However, the neuroadaptations underlying behavioral sensitization to ethanol have been less studied. Understanding the neurobiological mechanisms of ethanol sensitization may provide useful insights into the adaptations that are critical for the development of dependence in animal models and human alcoholics.

A limited number of studies have investigated the biological processes associated with behavioral sensitization to ethanol; these few studies have indicated that several neural systems are involved. Changes in opioid systems have been suggested to underlie the development of ethanol-induced behavioral sensitization (Camarini et al., 2000b; Miquel et al., 2003), and pharmacological antagonists of *N*-methyl-D-aspartate (NMDA) receptors, a subclass of glutamate receptors, have been reported to atten-

[1] Present address: Columbia Genome Center, Columbia University, New York, NY, USA.

*Correspondence to: T. J. Phillips, Veterans Affairs Medical Center, R & D 32, 3710 Southwest US Veterans Hospital Road, Portland, OR 97239, USA. Tel: +1-503-220-8262x56674; fax: +1-503-721-1029.
E-mail address: phillipt@ohsu.edu (T. J. Phillips).

Abbreviations: Allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; ANOVA, analysis of variance; EHS, ethanol–home cage–saline; EMS, ethanol–monitor–saline; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; SHE, saline–home cage–ethanol; SME, saline–monitor–ethanol; THIP, 4,5,6,7-tetrahydroisoxazolo-(5,4,-*C*)pyridim-3-ol.

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doi:10.1016/j.neuroscience.2004.11.005

uate the development and expression of ethanol-induced behavioral sensitization (Broadbent et al., 2003; Broadbent and Weitemier, 1999; Camarini et al., 2000a; Chester et al., 2001). Recently, brain catalase levels have been proposed as an important factor in ethanol-induced sensitization (Correa et al., 2004). At the molecular level, ethanol-induced behavioral sensitization has been associated with increases in dopamine D_2 receptor binding (Souza-Formigoni et al., 1999), although no effect of the dopamine receptor antagonist, haloperidol, on the development of ethanol-induced behavioral sensitization was found (Broadbent et al., 1995). Further, dopamine D_2 receptor gene knockout mice displayed enhanced, rather than reduced, ethanol-induced sensitization (Palmer et al., 2003). Finally, results have been mixed when baclofen, a $GABA_B$ receptor agonist, was used to examine the development of ethanol-induced locomotor sensitization (Broadbent and Harless, 1999; Chester and Cunningham, 1999), and 4,5,6,7-tetrahydroisoxazolo-(5,4,-*C*)pyridim-3-ol (THIP), a $GABA_A$ receptor agonist which acts at the GABA binding site, did not affect sensitization (Broadbent and Harless, 1999). To our knowledge, the effects of $GABA_A$ receptor antagonists, or of ligands for other $GABA_A$ modulatory sites, on ethanol-induced behavioral sensitization are unknown.

One problem with pharmacological blockade studies like those just reviewed, is that many drugs reported to block behavioral sensitization also have effects on the acute locomotor response to ethanol. We recently showed that the effect of higher doses of the NMDA receptor antagonist, MK-801, on ethanol-induced sensitization is related to the effect of MK-801 on the acute ethanol response. A lower dose of MK-801, which had no effect on the acute locomotor response to ethanol, actually potentiated the development of sensitization (Meyer and Phillips, 2003). This suggests that the ability of MK-801 and some other drugs to block or attenuate the development of sensitization may be a result of their ability to alter the acute effects of ethanol. Similar arguments have been made for psychostimulant sensitization, pointing out the importance of state-dependency in behavioral sensitization (e.g. Gronig et al., 2004; Stephens et al., 2000). Another method for studying neurochemical determinants of sensitization, cross-sensitization, removes this interpretational confound.

In cross-sensitization studies, behavioral sensitization is induced by exposure to one drug, and then sensitized and non-sensitized (non-drug treated) animals are compared for their behavioral response to a novel drug. Cross-sensitization is evidenced by an enhanced behavioral response to the novel drug in sensitized compared with non-sensitized animals, and infers that the neurobiological mechanisms involved in determining the response to the novel drug have been altered in the sensitized animals. Evidence for cross-sensitization has been obtained between ethanol and cocaine, ethanol and morphine, and ethanol and restraint stress (Itzhak and Martin, 1999; Lessov and Phillips, 2003; Nestby et al., 1997; Roberts et al., 1995), suggesting specific changes in dopamine, opioid and stress-related pathways. In the current studies, due to a large literature supporting a role for $GABA_A$ receptor mediated processes in neuroadaptation to ethanol, such as that associated with tolerance and dependence (reviewed by Chandler et al., 1998; Kumar et al., 2004), we examined cross-sensitization to two $GABA_A$ receptor acting compounds.

Acutely, ethanol has been found to enhance $GABA_A$ receptor function, and its effects are altered by prior ethanol exposure (Allan and Harris, 1987; Buck and Harris, 1990a,b). These effects are dependent on the dose of ethanol used, as well as the subunit composition of the $GABA_A$ receptor (Wallner et al., 2003). In addition, there are several modulatory sites on the $GABA_A$ receptor that can alter its function. One is a proposed binding site for neuroactive steroids, such as 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), an endogenous metabolite of progesterone (Im et al., 1990; Purdy and Paul, 1999; Ueno et al., 2004). Acute ethanol administration has been found to rapidly increase the concentrations of neuroactive steroids that act as positive allosteric modulators of the $GABA_A$ receptor in the brains of certain strains of rats and mice (Barbaccia et al., 1999; Finn et al., 2004; Gabriel et al., 2004; O'Dell et al., 2004). Thus, one proposed mechanism for the effects of ethanol on GABAergic signaling is the induction of allopregnanolone in the brain (VanDoren et al., 2000). Previous studies have found a genetic association between sensitivity to the acute locomotor effect of ethanol and allopregnanolone (Korpi et al., 2001; Palmer et al., 2002a,b). The role that neuroactive steroids may play in ethanol-induced locomotor sensitization has not been studied.

Another modulatory site on the $GABA_A$ receptor is a barbiturate binding site. Similar to allopregnanolone, there appears to be a genetic association between sensitivity to the acute locomotor stimulant effects of ethanol and pentobarbital (Phillips et al., 1992; Palmer et al., 2002a). Cross-tolerance has been found between ethanol and barbiturates (Bitran and Kalant, 1993; Le et al., 1992), although the role of $GABA_A$ receptor changes has been questioned (Allan et al., 1992; Mihic et al., 1992). This barbiturate binding site has not been investigated for a role in ethanol-induced sensitization. In the present experiments, we hypothesized that ethanol-sensitized mice would display enhanced locomotor stimulant responses to allopregnanolone and pentobarbital.

Behavioral sensitization is a complex phenomenon that is known to depend not only on pharmacology, but also on environmental factors (Badiani et al., 2000; Fraioli et al., 1999; Ohmori et al., 1995; Quadros et al., 2003; Wise et al., 1996), which may or may not involve common neuroadaptive processes. Initial studies presented in this report led us to suspect that the relative novelty of the test chamber substantially influenced the expression of ethanol sensitization. Thus, by manipulating the novelty of the chamber, we examined cross-sensitization using two procedures that produced differences in the expression of behavioral sensitization to ethanol. We hypothesized that cross-sensitization to allopregnanolone and pentobarbital

would be more pronounced using a paradigm that produced higher levels of behavioral sensitization, compared with a paradigm that produced relatively lower levels of behaviorally expressed sensitization to ethanol.

EXPERIMENTAL PROCEDURES

Subjects and housing

Female DBA/2J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA), and allowed to adapt to the Veterans Affairs Medical Center animal research facility for at least 1 week prior to the initiation of testing. This strain of mice was chosen for its susceptibility to ethanol-induced behavioral sensitization (Phillips et al., 1995). Females were chosen for consistency with our previous work (Phillips et al., 1992, 1995), and because others have found that female rodents displayed greater susceptibility to drug-induced behavioral sensitization (Becker et al., 2001; Camp and Robinson, 1988; Hu and Becker, 2003; Robinson, 1984). Mice were housed in groups of three to four in clear, air-filtered polyacrylamide cages (28×18×13 cm), lined with corn-cob bedding. Food (Purina Laboratory Rodent Chow 5001; Purina Mills, St. Louis, MO, USA) and tap water, suspended from wire-mesh lids, were available *ad libitum* except during activity testing. At the time of testing, all mice were between 57 and 99 days old and weighed between 14 and 35 g. Activity testing occurred between 08:00 and 16:00 h; the lights were on from 06:00 to 18:00 h in the colony room in which the mice were housed. Room temperature was maintained between 20 and 22 °C. All procedures were performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals. Experiments were designed in such a way as to minimize suffering and utilize the smallest number of animals possible.

Drugs

Ethanol (20% vol/vol, diluted in 0.9% saline) was obtained from Pharmco Products (Brookfield, CT, USA). Allopregnanolone was synthesized by and purchased from Dr. Robert Purdy (San Diego, CA, USA), and then solubilized in a 20% (wt/vol) solution of 2-hydroxypropyl-β-cyclodextrin (Research Biochemicals International, Natick, MA, USA). Sodium pentobarbital was obtained from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 0.9% saline.

General activity testing procedure

On days on which an activity test was scheduled, mice were moved in their home cages into the testing room 45–60 min before testing began. This time allowed acclimation to the testing environment. Mice were placed into the activity monitors immediately after being weighed and receiving an i.p. injection. Locomotor activity data were collected in 5-min units for 20 or 30 min. On days on which an activity test was not scheduled, mice were not transferred to the testing room; instead they received injections in the colony room and were immediately returned to their home cages.

Mice were tested in automated locomotor activity monitors (40 cm×40 cm×30 cm; w×l×h; Accuscan Instruments, Columbus, OH, USA) that were individually housed in opaque, sound-insulated cabinets. An internal fan provided ventilation and background noise. A 15 W fluorescent bulb lit the inside of each activity monitor during testing. Eight intersecting infrared beams located 2 cm above the test floor of the monitor determined the location of the mouse within the chamber. As mice moved within the activity monitors, beam interruptions were recorded and later translated by software to yield horizontal distance traveled (in cm), which was the dependent measure used for all analyses.

Experiments 1–2

These initial experiments utilized procedures we had previously established for measuring cross-sensitization to cocaine and morphine (Lessov and Phillips, 2003). The experimental protocols are outlined in Table 1. On days 1 and 2, all mice received injections of saline before being placed into the activity monitors. The first day habituated the mice to the testing procedures, and the second provided a measure of baseline activity level. Ethanol treatment group mice received 2 g/kg of ethanol on day 3, before being placed in the activity monitors. On days 4–13, they received 2.5 g/kg ethanol injections in their home cages. This higher dose of ethanol was chosen from previous experiments in our laboratory showing that it produces robust sensitization to a test dose of 2 g/kg ethanol (Lessov and Phillips, 2003; Lessov et al., 2001b). Saline treatment group mice were treated and tested similarly, except that only saline injections were given on days 1–13.

On day 14, ethanol- and saline-treated mice received 2 g/kg ethanol or saline injections, respectively, before being placed into the activity monitors. The purpose of this test was to demonstrate behavioral sensitization in the group that had been repeatedly exposed to ethanol. In experiment 1, on day 15, each of these groups was further subdivided into two subgroups that received 10 or 17 mg/kg allopregnanolone to assess cross-sensitization. These doses of allopregnanolone were carefully selected from previous data in our laboratory as doses known to produce modest-higher levels of locomotor stimulation in DBA/2J mice (Palmer et al., 2002b). We avoided doses that acutely induced locomotor depression because we desired to study increased sensitivity to stimulation, rather than tolerance to initial depressant effects. However, from the levels of stimulation induced by these doses, we believed that either an increase or a decrease in stimulant response would be detectable. In experiment 2, the ethanol and saline groups were subdivided into three subgroups that received 2 g/kg of ethanol or one of two doses of pentobarbital (10 or 20 mg/kg). Again, these doses of pentobarbital were chosen to produce modest-higher levels of stimulation in DBA/2J mice (Crabbe et al., 2002). The ethanol challenge group was added to experiment 2 to verify sensitization to ethanol on the cross-sensitization challenge day. The test duration was 30 min for experiment 1 and 20 min for experiment 2 on all activity test days. The 20 and 30 min test sessions were used to capture the peak stimulant responses to pentobarbital and allopregnanolone, which had been previously established in DBA/2J mice (Dudek et al., 1994; Palmer et al., 2002a). There were 12 mice in each treatment group.

Experiment 3

Experiment 2 suggested that exposure to the monitor on day 14 resulted in attenuated levels of behavioral sensitization on day 15. In this experiment, mice from four groups were treated identically on all days except day 14 (see Table 1). Treatments and testing on days 1–13 were identical to those for the ethanol-treated groups of experiments 1 and 2. On day 14, the mice were differentiated into four groups, whose treatment and testing were designed to determine whether exposure to the test environment, 24 h prior to a final ethanol test, would affect the magnitude of behaviorally expressed sensitization (Table 1). Two groups were not exposed to the test environment on day 14, and two groups were. Groups SHE (saline–home cage–ethanol) and EHS (ethanol–home cage–saline) remained in the colony room for their treatments; mice in group SHE were injected with saline, returned to their home cages for 60 min, and then injected with 2 g/kg of ethanol, whereas mice in group EHS were injected with 2 g/kg of ethanol, followed 60 min later by a saline injection. Group EMS (ethanol–monitor–saline) mice were injected with 2 g/kg of ethanol before being placed in the activity monitor for 30 min, returned to their home cages for 30 min, and then injected with saline. Group

Table 1. Summary of the experimental design for experiments 1–5[a]

Experiment	Group	Day 1–2 (Test)	Day 3 (Test)	Day 4–13 (Home)	Day 14	Day 15 (Test)
1	Ethanol-treated	S	E2	E2.5	E2-Test	A10 A17
	Saline-treated	S	S	S	S-Test	A10 A17
2	Ethanol-treated	S	E2	E2.5	2E-Test	P10 P20 E2
	Saline-treated	S	S	S	S-Test	P10 P20 E2
3	EHS	S	E2	E2.5	E2-Home-S	E2
	SHE	S	E2	E2.5	S-Home-2E	E2
	EMS	S	E2	E2.5	E2-Test-S	E2
	SME	S	E2	E2.5	S-Test-2E	E2
4	Ethanol-treated	S	E2	E2.5	2.5E-Home	A10 A17 E2
	Saline-treated	S	S	S	S-Home	A10 A17 E2
5	Ethanol-treated	S	E2	E2.5	E2.5-Home	P10 P20 E2
	Saline-treated	S	S	S	S-Home	P10 P20 E2

[a]S, saline injections; E2, E2.5, 2 or 2.5 g/kg ethanol injections, respectively; A10, A17, 10 or 17 mg/kg allopregnanolone injections, respectively; P10, P20, 10 or 20 mg/kg pentobarbital injections, respectively; Home, injections occurred in the colony room and mice were returned to their home cages; Test, injections occurred in the testing room and mice were placed into the activity monitors.

SME (saline–monitor–ethanol) mice were injected with saline before being placed into the monitor for 30 min, returned to their home cages for 30 min, and then injected with 2 g/kg of ethanol. The purpose of the second injection in all groups was to equate the amount of ethanol exposure. On day 15, all groups received injections of 2 g/kg of ethanol before being placed into the activity monitors for a 30-min test. Because all mice received comparable exposures to ethanol and differed only in the context of the exposure on day 14, differences in activity levels of the groups on day 15 would reflect the influence of context on the expression of ethanol-induced behavioral sensitization. However, if the context of exposure was unimportant, then activity levels of all groups on day 15 should be similar. There were 11–12 mice per treatment group.

Experiments 4 and 5

Results from experiment 3 demonstrated that the behavioral expression of ethanol-induced sensitization was of greater magnitude when mice were not tested in the activity monitors on the day preceding the ethanol challenge. Experiments 4 and 5 determined whether ethanol-sensitized mice would display cross-sensitization to allopregnanolone or pentobarbital when this procedure was used. Mice were treated and tested identically to those from experiments 1 and 2 on days 1–13, except that the test length for the pentobarbital study was extended to 30 min, to enable comparison to other experiments in this series. A description of experimental procedures is summarized in Table 1. On day 14, the mice were not transferred to the testing room and were not tested in the activity monitors. Instead, ethanol- and saline-treated mice remained in the colony room, where they received 2.5 g/kg ethanol or saline injections, respectively, and were immediately returned to their home cages. On day 15, mice from each treatment group were further subdivided into three groups that received 2 g/kg of ethanol or allopregnanolone (10 or 17 mg/kg; experiment 4), or three groups that received 2 g/kg of ethanol or pentobarbital (10 or 20 mg/kg; experiment 5). There were 11–13 mice per treatment group.

Statistical analysis

The main dependent variable in all of these experiments was distance traveled (in cm) on challenge day 15. For experiments 1, 2, 4, and 5, day 15 data were analyzed by two-way analysis of variance (ANOVA) with Repeated Treatment (Ethanol or Saline) and drug Dose as the between groups factors. When ethanol-challenged groups were included on day 15, Student's *t*-tests were conducted to determine whether saline- and ethanol-treated groups differed in their responses to ethanol. In addition, for experiments 1 and 2, repeated measures ANOVA was conducted with Day (1, 2, 3, and 14) as the repeated measure and Repeated Treatment (ethanol or saline) and drug Dose as the between groups factors. Simple main effects analyses were followed by planned group comparisons when appropriate to determine whether mice treated repeatedly with ethanol were behaviorally sensitized by comparing their initial response to ethanol on day 3 to their response on day 14.

For experiment 3, data were analyzed by ANOVA with Treatment Order (ethanol then saline, saline then ethanol) and Treatment Location (monitor, home cage) as the between groups factors, and Day (1, 2, 3, and 15) as a repeated measure. Simple main effects analyses and planned group com-

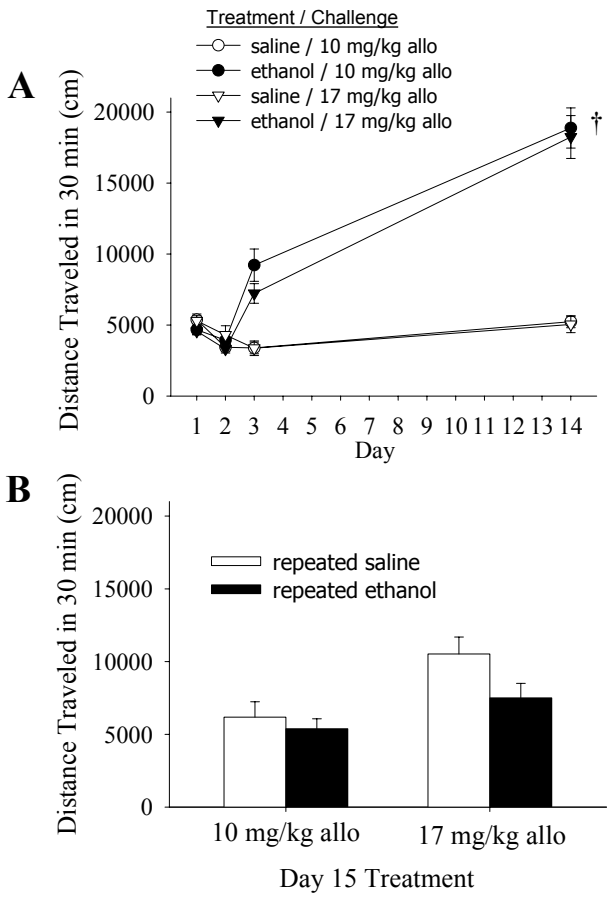

Fig. 1. Effects of repeated ethanol treatment on the response to allo. The responses to the repeated saline and ethanol treatments from days 1–14 are shown in A. Dagger indicates significant increases in activity ($P<0.01$) on day 14 compared with day 3 in the ethanol treated groups. Responses of repeated saline- and ethanol-treated mice to allo on day 15 are shown in B. Values shown are means±S.E.M.

parisons were used to detect behavioral sensitization on day 15 compared with day 3, and also whether there were group differences in response to ethanol on day 15. Data obtained on day 14 were not included in these analyses because not all groups were tested on this day. Instead, a paired *t*-test was used to determine whether the response to ethanol in mice from group EMS was significantly enhanced on this day, compared with their own response on day 3.

RESULTS

Experiment 1

There were differences in locomotor activity across days 1 through 14 for the repeated ethanol- and saline-treated mice [Fig. 1A; $F(3,132)=98.4$; $P<0.01$, for the Repeated Treatment×Day interaction]. Simple main effects analyses followed by planned comparisons revealed that the ethanol-treated mice expressed significant behavioral sensitization on day 14 ($P<0.01$), compared with day 3. There was no change in the response to saline on day 14 compared with day 3 in the saline-treated mice. As can be seen in Fig. 1A, groups to be subsequently tested for cross-sensitization to different doses of allopregnanolone were well matched for magnitude of sensitization or activity after saline. On allopregnanolone challenge day 15, a significant effect of allopregnanolone Dose [$F(1,44)=10.6$, $P<0.01$] indicated that allopregnanolone had a dose-dependent effect on activity (Fig. 1B). Paired *t*-tests indicated that both the 10 and 17 mg/kg doses increased activity levels in the repeated saline-treated groups, compared with their day 3 activity levels [$t(11)=3.3$; $P<0.01$ and $t(11)=7.7$; $P<0.01$, respectively]. However, there was no indication that repeated ethanol treatment enhanced the response to allopregnanolone. Instead, there was a statistical trend ($F(1,44)=3.7098$, $P=0.06$, for the effect of Repeated Treatment) toward a *diminished* response (i.e. tolerance to the stimulant effect of allopregnanolone) in the ethanol-pretreated mice. These results indicate a lack of cross-sensitization to allopregnanolone in mice behaviorally sensitized to ethanol.

Experiment 2

Repeated ethanol and saline treatments resulted in different patterns of activity across days [Fig. 2A; $F(3,198)=131.3$; $P<0.01$, for the Repeated Treatment×Day interaction]. Simple main effects analyses followed by planned comparisons revealed that ethanol-treated mice exhibited significant behavioral sensitization to ethanol on day 14, compared with their acute response to ethanol on day 3 ($P<0.01$). Again, treatment groups were well matched for magnitude of sensitization (Fig. 2A). The saline-treated mice exhibited a modest but significant increase in their response to saline on day 14, compared with day 3 ($P<0.05$). On pentobarbital challenge day 15, a significant effect of pentobarbital Dose [$F(1,44)=50.6$; $P<0.01$] indicated that pentobarbital had a dose-dependent effect on activity (Fig. 2B). Paired *t*-tests indicated that both the 10 and 20 mg/kg doses increased activity levels in the repeated saline-treated groups, compared with their day 3 activity levels [$t(11)=10.1$; $P<0.01$ and $t(11)=15.3$; $P<0.01$, respectively]. However, there was no evidence for cross-sensitization to pentobarbital in repeated ethanol-treated mice. In fact, an effect of Repeated Treatment [$F(1,44)=4.9$; $P<0.05$] reflected reduced sensitivity to pentobarbital in the repeated ethanol-treated mice, compared with repeated saline-treated mice. These results suggest the presence of tolerance to the locomotor stimulant effects of pentobarbital, rather than cross-sensitization, in mice that

Abbreviations used in the figures

allo allopregnanolone
EHS ethanol–home cage–saline
EMS ethanol–monitor–saline
pento pentobarbital
SHE saline–home cage–ethanol
SME saline–monitor–ethanol

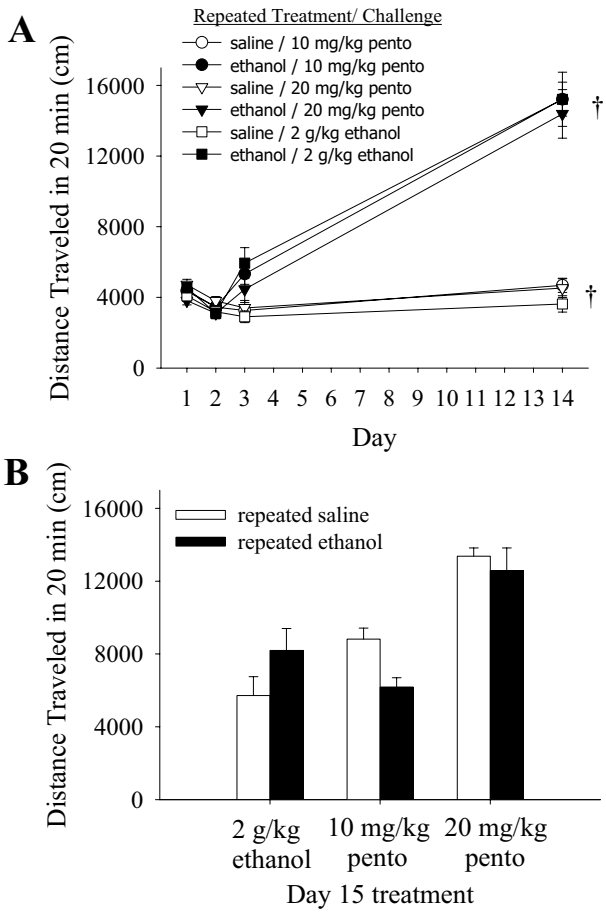

Fig. 2. Effects of repeated ethanol treatment on the response to pento. The responses to the repeated saline and ethanol treatments from days 1–14 are shown in A. Daggers indicate significant increases in activity (Ps<0.05) on day 14 compared with day 3. The responses of these repeated saline- and ethanol-treated mice to ethanol and pento on day 15 are shown in B. A significant effect of Repeated Treatment reflected decreased sensitivity to pento in ethanol-treated mice ($P<0.05$). Values shown are means±S.E.M.

exhibited robust behavioral sensitization to ethanol on the previous day.

Despite evidence for significant ethanol-induced behavioral sensitization on day 14, there was no significant difference in the response to ethanol between the repeated ethanol- and repeated saline-treated mice on day 15 (Fig. 2B, $t(22)=1.6$; $P=0.13$). As is evident in Fig. 2, the response of the repeatedly ethanol-treated mice to ethanol was markedly attenuated on day 15, compared with day 14 (approximately 8000 vs approximately 15000, respectively), whereas the acute response of the repeated saline group to ethanol on day 15 was similar to the acute response of the repeated ethanol group on day 3 (approximately 6000 vs approximately 5000, respectively). This suggests that sensitization to ethanol was minimally expressed on day 15, 1 day after robust behavioral sensitization had been observed in the repeated ethanol group.

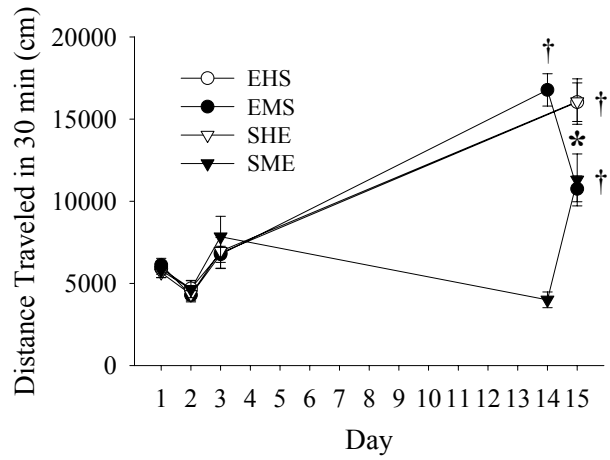

Fig. 3. Effects of exposure to the activity chambers on day 14 on the expression of ethanol sensitization. Treatment was identical for all groups except on day 14. Daggers depict significant increases in activity (Ps<0.01) compared with day 3 for the indicated groups. The asterisk indicates the effect of Treatment Location ($P<0.01$), in which groups exposed to the monitor on day 14 had significantly lower activity levels than groups that were not exposed. Values shown are means±S.E.M.

Experiment 3

Due to the apparent effect of monitor exposure on the expression of ethanol sensitization seen in experiment 2, we hypothesized that group EMS (see Table 1), which was treated with ethanol in the activity monitors on day 14, would display less sensitization on day 15 compared with group EHS, which was treated with ethanol on day 14, but not pre-exposed to the monitors. If monitor exposure, rather than specifically exposure in the presence of ethanol, is the critical variable, then group SME, which was treated with saline in the activity monitors on day 14, should also display blunted sensitization compared with group SHE, which were not exposed to the monitors on day 14.

Results for experiment 3 are summarized in Fig. 3. There were no group differences in locomotor behavior on days 1, 2 or 3, indicating that the four groups were well matched for their basal activity levels and acute ethanol responses. On day 14, mice from group EMS received ethanol and mice from group SME received saline before exposure to the activity monitors, which is reflected in the large difference in the activity levels of these two groups. The mean distance traveled by group EMS following ethanol treatment on day 14 was significantly greater than on day 3 [$t(11)=7.6$; $P<0.01$], a demonstration of behavioral sensitization to ethanol. Repeated measures ANOVA of data obtained on days 1–3 and 15 revealed a significant Treatment Location×Day interaction [$F(3,129)=11.1$; $P<0.01$], indicating that the groups' patterns of responses were different across days. There was no main effect or interaction with Treatment Order. Simple main effects analyses followed by planned comparisons revealed that both the sets of mice that received their injections in the monitors (groups EMS and SME) and those treated in their home cages (groups EHS and SHE) had larger responses to ethanol on day 15 compared with day 3 (Ps<0.01),

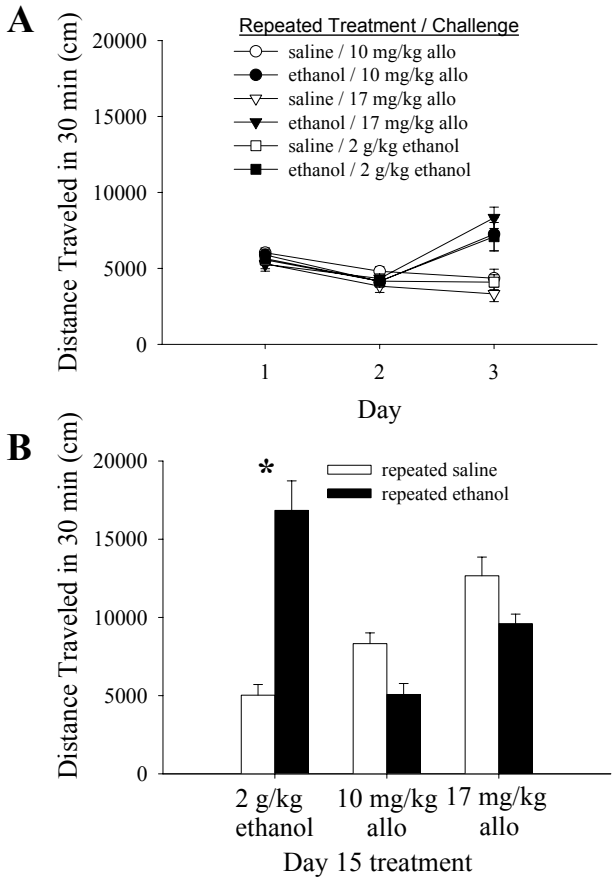

Fig. 4. Effects of repeated ethanol treatment on the response to allo in a paradigm that produces robust ethanol sensitization. Locomotor responses after saline injections on days 1–2 and the acute response to ethanol on day 3 are shown in A. Responses of repeated saline- and ethanol-treated mice to ethanol and allo on day 15 are shown in B. The asterisk indicates a significantly larger response to ethanol ($P<0.01$) in repeated ethanol-treated mice. A significant effect of Repeated Treatment reflected decreased sensitivity to allo in ethanol-treated mice. Values shown are means±S.E.M.

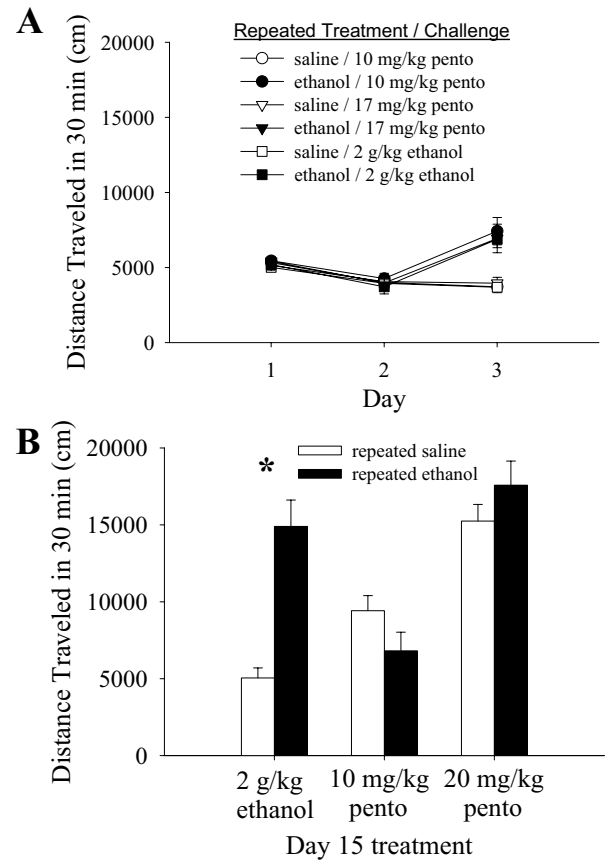

Fig. 5. Effects of repeated ethanol treatment on the response to pento in a paradigm that produces robust ethanol sensitization. Locomotor responses after saline injections on days 1–2 and the acute response to ethanol on day 3 are shown in A. The responses of these repeated saline- and ethanol-treated mice to ethanol and pento on day 15 are shown in B. The asterisk indicates a significantly larger response to ethanol ($P<0.01$) in repeated ethanol-treated mice. Values shown are means±S.E.M.

which indicates that all groups developed significant behavioral sensitization to ethanol. However, it is clear from Fig. 3 that the magnitude of sensitization varied among these groups; mice not exposed to the monitors on day 14 expressed greater sensitization [$F(1,43)=16.1$; $P<0.01$, for the effect of Treatment Location]. However, there was no interaction with Treatment Order. Thus, exposure to the monitor on day 14 attenuated the expression of ethanol sensitization on the next day, regardless of whether this exposure occurred in the presence of ethanol treatment.

Experiment 4

Data are summarized in Fig. 4. Ethanol sensitization and cross-sensitization groups were well matched for activity levels (Fig. 4A). To avoid the influence of monitor exposure on the behavioral expression of sensitization to ethanol, mice were not exposed to the test environment on day 14. There was clear evidence of behavioral sensitization to ethanol on day 15; the day 15 response to ethanol was significantly enhanced in the repeatedly ethanol-treated group, compared with the repeatedly saline-treated group [Fig. 4B, $t(21)=8.4$; $P<0.01$], and to their own day 3 response ($t(11)=4.0$; $P<0.01$). Allopregnanolone had significant dose-dependent effects [$F(1,43)=28.0$; $P<0.01$, for the effect of Dose], and paired t-tests indicated that both the 10 and 17 mg/kg doses increased activity levels in the repeated saline-treated groups, compared with their day 3 activity levels [$t(11)=4.8$; $P<0.01$ and $t(11)=7.9$; $P<0.01$, respectively]. However, no significant cross-sensitization was seen. In fact, a significant effect of Repeated Treatment [$F(1,43)=14.2$; $P<0.01$] reflected reduced sensitivity to allopregnanolone in repeatedly ethanol-treated mice. This suggests that tolerance (not cross-sensitization) developed to the stimulant effect of allopregnanolone, despite evidence of robust sensitization to ethanol, which is consistent with the results of experiment 1.

Experiment 5

Data are summarized in Fig. 5. Again, in this experiment, the mice were not exposed to the test environment on day 14. The response to ethanol on day 15 was significantly

enhanced in the repeated ethanol-treated group, compared with the repeated saline-treated group [Fig. 5B; $t(21)=6.4$; $P<0.01$], and to their own day 3 response ($t(11)=3.9$; $P<0.01$), providing evidence of significant behavioral sensitization. Pentobarbital had significant dose-dependent effects [$F(1,45)=44.1$, $P<0.01$, for the effect of Dose], and paired *t*-tests indicated that both the 10 and 20 mg/kg doses increased activity levels in the repeated saline-treated groups, compared with their day 3 activity levels [$t(11)=6.1$; $P<0.01$ and $t(11)=11.8$; $P<0.01$, respectively). However, there was no evidence of cross-sensitization to pentobarbital in the ethanol-sensitized mice. The Repeated Treatment×Dose interaction did not reach significance ($P=0.054$). Thus, even when test environment exposure was avoided and behavioral sensitization to ethanol was clearly demonstrated, repeated ethanol treatment did not confer an enhanced response to pentobarbital.

DISCUSSION

The present results demonstrate robust behavioral sensitization to the locomotor stimulant effects of ethanol, as has previously been reported for DBA/2J mice (Broadbent and Harless, 1999; Cunningham and Noble, 1992; Lessov et al., 2001a,b; Phillips et al., 1994; Roberts et al., 1995). Further, the results show a lack of cross-sensitization between ethanol and either allopregnanolone or pentobarbital, both of which are thought to produce their effects via interactions with specific modulatory sites on $GABA_A$ receptors. Instead, there was modest evidence of tolerance to allopregnanolone and pentobarbital in ethanol-sensitized mice in some experiments. Although an important role for the relative novelty of the testing chamber in the behavioral expression of the sensitized response to ethanol was demonstrated, the magnitude of behavioral expression did not affect the cross-sensitization results.

The test chamber was not completely novel for any of the mice by the day of the ethanol challenge; however, 10 intervening days transpired before exposure to the chamber 24 h prior to challenge. This exposure to the monitor on the day prior to challenge was associated with a large reduction in the behavioral expression of sensitization. However, this effect was not an ethanol-monitor associative effect, because ethanol-monitor and saline-monitor pairing had a comparable effect on the subsequent response. Further, the effect of monitor exposure appeared to be specific to ethanol sensitization, rather than extending to the acute response to ethanol. Repeatedly saline-treated mice that were exposed to the test environment prior to the ethanol challenge on day 15 (experiment 2) displayed a similar average acute response to ethanol compared with saline-treated mice that were not exposed to the test environment prior to the ethanol challenge (experiments 4 and 5).

The robust response to ethanol on day 14 (24 h prior to challenge) in repeatedly ethanol-treated mice of experiment 3 could be associated with stress axis activation. It is possible that the mice experienced more stress in the relatively novel environment, which may have enhanced the magnitude of sensitization. We previously found that repeated restraint stress could sensitize mice to the locomotor stimulant effects of ethanol, and that glucocorticoid antagonists could block this stress-ethanol cross-sensitization, as well as ethanol-induced sensitization itself (Roberts et al., 1995). In this manner, stress elicited by restraint and novelty may have similar effects on the development or expression of ethanol sensitization. This idea is consistent with findings suggesting that environmental novelty plays an important role in amphetamine sensitization (Fraioli et al., 1999). However, Quadros et al. (2003) found that ethanol sensitization was absent when Swiss–Webster mice were tested in an unfamiliar environment. This may be because the ethanol exposures were explicitly paired with another environment in that study, or due to a difference in stress effects on ethanol response in Swiss–Webster compared with DBA/2J mice.

While many studies stress the similarities among drugs of abuse, and particularly those that interact with different modulatory sites on the $GABA_A$ receptor complex, these findings indicate that even drugs with similar acute effects on GABA transmission and behavior have distinguishable effects when administered repeatedly. Neuroactive steroids, such as allopregnanolone, as well as other compounds that alter $GABA_A$ receptor function, including ethanol and pentobarbital, have similar behavioral effects (Purdy and Paul, 1999). Perhaps of greatest relevance to the current work is evidence for genetic correlation between the locomotor stimulant response to ethanol and allopregnanolone in both a panel of inbred mouse strains (Palmer et al., 2002b), and selectively bred FAST and SLOW mouse lines that were bred for differential sensitivity to the locomotor stimulant effects of ethanol (Palmer et al., 2002a,b; Phillips et al., 1992). These results suggest a primary role for $GABA_A$ receptors as mediators of similarities in the *acute* effects of ethanol, pentobarbital, and allopregnanolone. However, these similarities do not appear to extend to adaptations associated with the sensitization expressed after *repeated* ethanol administration, regardless of whether behavioral sensitization to ethanol was robustly expressed on the cross-sensitization challenge day or not. Instead, these adaptations may involve actions of ethanol on other neurotransmitter systems, or may be related to changes that are more specific to actions of ethanol on the $GABA_A$ receptor. For example, the subunit composition of $GABA_A$ receptors may have been altered during repeated ethanol administration, or the number of receptors may have changed. However, these changes may have occurred within certain brain areas unrelated to pentobarbital or allopregnanolone sensitivity, or changes in subunit composition induced by ethanol may not be relevant to sensitivity to these other drugs. Thus, it is possible that changes in $GABA_A$ receptors are involved in ethanol sensitization, but due to differences in the specific effects of ethanol, allopregnanolone, and pentobarbital on $GABA_A$ receptor function and/or composition, cross-sensitization was not observed.

Few studies have examined locomotor sensitization to $GABA_A$-acting compounds. Sensitization of the locomotor effects of the benzodiazepine, diazepam, was seen in Wistar rats with repeated treatments (Matsubara and Mat-

sushita, 1982), but not in NMRI mice (Wolffgramm et al., 1994). To the best of our knowledge, there have been no reports of locomotor sensitization to allopregnanolone or of locomotor cross-sensitization between ethanol and allopregnanolone before now, although some studies have shown cross-sensitizing effects of $GABA_A$ acting compounds for traits other than locomotion. For example, chronic treatment with ethanol, followed by abrupt withdrawal, resulted in sensitization to the anticonvulsant effect of allopregnanolone in mice and rats (Devaud et al., 1996; Finn et al., 2000), and to the antidepressant effect of allopregnanolone in Porsolt's forced swim test in Swiss mice (Hirani et al., 2002). Ethanol treatment was associated with sensitization of the potentiating effect of allopregnanolone on $GABA_A$ receptor-mediated chloride flux (Grobin et al., 2001). An abstract reported sensitization of the effects of allopregnanolone on aggressive behavior by repeated ethanol treatment in CFW mice, but did not mention locomotor sensitization (Fish et al., 2001). Our data and data showing no effect of the $GABA_A$ agonist THIP on ethanol-induced sensitization (Broadbent and Harless, 1999) are consistent in suggesting that $GABA_A$ receptor changes are not involved in ethanol-induced sensitization to the locomotor stimulant effects of ethanol. However, there are certain limitations associated with these results. For example, only one mouse strain was used. Although this strain was chosen for its particular susceptibility to behavioral sensitization induced by ethanol, it may be that cross-sensitization would have been revealed in other strains of mice or other animal models. Further, while the doses of ethanol used in these experiments were chosen from prior studies to be those that induce robust behavioral sensitization, higher doses of ethanol may produce adaptations in the $GABA_A$ receptor that lead to cross-sensitization. This is unlikely, given that tolerance to allopregnanolone and pentobarbital, rather than cross-sensitization, was observed in some of the current studies.

We obtained some evidence for tolerance to the locomotor stimulant effects of allopregnanolone and pentobarbital in mice repeatedly treated with ethanol. Other studies have found cross-tolerance between the ataxic effects of pentobarbital and ethanol using some apparatus (Bitran and Kalant, 1993; el-Ghundi et al., 1989), but not others (Khanna et al., 1997; Le et al., 1992). In addition, a study of ethanol- and allopregnanolone-induced hypothermia identified tolerance to the hypothermic effects of ethanol and allopregnanolone, as well as cross-tolerance to these effects in some genotypes, but not others (Palmer et al., 2002c). One possible interpretation of the current results showing tolerance after chronic ethanol is that increased GABAergic tone caused either directly by repeated ethanol, or through ethanol-induced increases in endogenous allopregnanolone levels, resulted in down-regulation of $GABA_A$ receptors, leading to a reduced response to the locomotor effects of these drugs. However, mice were clearly not tolerant to the locomotor effects of ethanol under the same ethanol pretreatment conditions, suggesting that if this down-regulation does occur, it does not affect the locomotor stimulant response to ethanol itself. Though it is a complex issue, we have argued that tolerance to ethanol's sedative and ataxic effects is qualitatively different from sensitization to locomotor stimulation (Meyer and Phillips, 2003; Phillips et al., 1996).

Whereas some pharmacological antagonist studies are designed to examine the mechanisms involved in the *development* of sensitization (e.g. Broadbent and Harless, 1999), the current experiments assessed the mechanisms involved in the *expression* of sensitization. The neural systems that influence the development of sensitization are not necessarily the same ones that are activated during the expression of sensitization. For example, it could be postulated that the action of ethanol on GABA induces alterations in glutamate receptors, and that it is those alterations that cause enhanced sensitivity to the locomotor effects of ethanol. In this hypothetical situation, GABA receptors would be necessary for the development of ethanol sensitization, while glutamate receptors would be necessary for the expression of ethanol sensitization. Therefore, studies that implicate different roles for receptor systems in the development and expression of sensitization are not necessarily conflicting.

In summary, the present results do not support a model of enhanced $GABA_A$ receptor signaling as a neural substrate for ethanol sensitization. Cross-sensitization to the stimulant effects of the $GABA_A$ receptor agonists, allopregnanolone and pentobarbital, did not occur in mice displaying robust locomotor sensitization to ethanol. However, other modes of interaction with the $GABA_A$ receptor remain to be explored. These results are useful in defining the transmitter systems involved in sensitization to the locomotor stimulant effects of ethanol, and may contribute to our understanding of processes that influence the development of alcoholism.

Acknowledgments—This work was supported by NIAAA grants P50AA10760, T32AA07468 and F32AA05600, as well as a grant from the Department of Veterans Affairs. All of these procedures comply with the current laws of the United States of America and were performed in accordance with the Institutional Animal Care and Use Committee and National Institutes of Health guidelines for the care and use of laboratory animals.

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(Accepted 4 November 2004)
(Available online 19 January 2005)