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Contextual Learning Prolongs the Duration of Locomotor Sensitization to Ethanol in DBA/2J Mice

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Abstract

Rationale—Repeated exposure to many abused drugs produces a progressive increase in locomotor sensitivity, referred to as locomotor sensitization. Locomotor sensitization may persist for some time following termination of repeated drug administration, and contextual learning appears to facilitate expression of the behavioral phenomenon. However, relatively little is known about the persistence of and the degree to which contextual learning influences locomotor sensitization to alcohol (ethanol).

Objectives— The goal of the present work was determine the duration of locomotor sensitization to ethanol and the degree to which contextual learning positively influences the induction, expression, and persistence of the behavioral phenomenon in DBA/2J mice.

Methods— Sensitized (with or without ethanol-paired exposure to the testing chamber) and non - sensitized saline control mice were left undisturbed in their home cages until subsequent ethanol challenge and testing in the locomotor activity testing chambers 7, 14, 21, 28, 42, 56, and/or 70 days after cessation of the ethanol sensitization procedure. Retro-orbital sinus bloods were sampled to determine whether the sensitization procedure had altered blood ethanol clearance rates.

Results— Locomotor sensitization persisted through post-sensitization day 14, and contextual learning prolonged the expression of this phenomenon through at least post-sensitization day 28. Blood ethanol concentrations did not differ in any experiment.

Conclusions— Locomotor sensitization to ethanol persists for some time after cessation of repeated ethanol exposure and the association of contextual cues with the ethanol experience lengthens this persistence. The present data lay the groundwork for investigations into the neuroadaptive changes that underlie locomotor sensitization to ethanol in mice.

Keywords

ethanol; locomotor activity; sensitization; contextual learning; mice

Introduction

Repeated administrations of a number of abused drugs produce a progressive enhancement in sensitivity to the locomotor stimulant actions of the compounds, a process referred to as locomotor sensitization. Locomotor sensitization has been postulated to play a role in the development and maintenance of drug seeking behavior. Indeed, maladaptive behaviors such as obsessive craving and compulsive drug-seeking have been linked to this phenomenon (Hunt and Lands 1992; Robinson and Berridge 1993; Spanagel 1995; Phillips et al. 1997). However,

strong empirical data supporting such a role are lacking, and other behavioral phenomena such as drug tolerance are also likely to have a role. Nevertheless, the continued study of sensitization to abused drugs in rodents may provide insights into the progression from casual drug use to dependence in humans.

Locomotor sensitization is believed to be a neuroadaptive process. That is, the progressive increase in sensitivity to the drug is thought to be supported by alterations in the neural mechanisms mediating the acute locomotor stimulant response. It has been suggested that these neural mechanisms become progressively more sensitive to the drug and remain so for long time periods after repeated administrations of the drug have ceased. For example, locomotor sensitization to the classic psychomotor stimulants cocaine, amphetamine, and morphine have been shown to persist for periods of up to 3 (Shuster et al. 1977), 12 (Paulson et al. 1991), and 8 (Babbini et al. 1975) months respectively, and these long-lasting behavioral changes have been associated with molecular neuroadaptations in brain circuits believed important in addiction processes (Ron and Jurd 2005). Such long-lasting neuroadaptive changes in sensitivity to abused drugs might explain how addicted individuals relapse after long periods of abstinence (Robinson and Berridge 1993; Ron and Jurd 2005; Sato 1992; Sato et al. 1983).

Although ethanol is classified as a sedative-hypnotic, the compound produces biphasic effects. Whereas low doses often produce a locomotor stimulant response, higher ethanol doses produce sedation and hypnosis. Interestingly, the low-dose locomotor stimulant actions of ethanol have been shown to sensitize in both rodents (Masur and Boerngen 1980; Masur et al. 1986) and humans (Newlin and Thomson 1991). Certain genetic populations appear to be more sensitive to developing ethanol sensitization. Newlin and Thomson (1991, 1999) demonstrated that whereas sons of alcoholics sensitize to the motor effects of chronic ethanol exposure, sons of non-alcoholics develop tolerance. Studies in mouse models of locomotor sensitization offer similar findings; different genetic mouse models develop different degrees of ethanol-induced locomotor sensitization, with some exhibiting little or no sensitization (Phillips et al. 1995a; 1995b).

Although much work has been done to characterize locomotor sensitization to the classic psychomotor stimulants, little work has been done to characterize ethanol-induced locomotor sensitization. For example, little is known about the persistence of ethanol sensitization in mice, and the identification of possible neuroadaptive processes that might underlie the behavioral phenomenon has proven difficult. Lessov and Phillips (1998) demonstrated that sensitization may last for up to 23 days in female mice from an out bred mouse population (originating from the HS/Ibg heterogeneous mouse stock). However, several slightly different variations on the experiment did not arrive at a common conclusion, a result the authors attributed to the inherent genetic variability of the out bred mouse population, and no other genetic mouse populations were examined. More recently Fish et al. (2002) examined this issue in male outbred CFW mice. They reported ethanol sensitization persisting for at least 58 days following cessation of the sensitization procedure. Several studies have attempted to identify specific neuroadaptive changes that might parallel locomotor sensitization to ethanol, including changes in D2 receptor (Sousza-Formigoni et al. 1999) NMDA receptor (Quadros et al. 2002a), D1 receptor and dopamine transporter (Quadros et al. 2002b), and D4 receptor (Quadros et al. 2005) binding. However only one of these studies found a parallel change in binding; striatal D2 receptor binding was found to be increased in ethanol-sensitized mice (Sousza-Formigoni et al. 1999). There are currently no reports demonstrating a neurophysiological change associated with locomotor sensitization to ethanol.

Another issue that hasn't been well characterized with regard to ethanol sensitization is the degree to which contextual learning influences the phenomenon. Indeed, the induction and expression of drug-induced sensitization are both influenced by contextual cues; that is, the

environmental cues surrounding repeated drug experience can acquire the ability to accentuate the behavioral phenomenon (Robinson et al. 1998). Indeed, several studies have demonstrated enhanced expression of behavioral sensitization to the classic psychomotor stimulants cocaine, amphetamine, and morphine when the behavior is assessed in the same testing chamber in which the animals experienced the repeated drug exposures (Badiani et al. 1995; 2000). Two studies posed similar questions about ethanol sensitization and have come to similar conclusions. Cunningham and Noble (1992) were the first to address this issue using an ethanol conditioned place preference paradigm in mice. They observed more robust locomotor sensitization in the environment in which the animals had experienced the repeated ethanol injections. Finally, evidence that ethanol sensitization is associated with contextual learning was also demonstrated more recently by Quadros et al. (2003) who showed that mice that developed locomotor sensitization to ethanol exhibited stronger contextual fear conditioning compared to mice that did not sensitize, or to control mice that received only repeated saline injections.

The goal of the present study was two fold. First, we wished to further explore the persistence of locomotor sensitization to ethanol using DBA/2J inbred mice. Many generations of brother-sister breeding within this genetic mouse population has yielded mice that are genetically identical, aside from differences on the sex chromosomes. Importantly, DBA/2J mice develop robust locomotor sensitization to ethanol (Phillips et al. 1994; 1995a; 1995b; 1996), and the fact that they are inbred should eliminate some of the individual variability (due to genetic sources) in sensitivity to the development of ethanol-induced locomotor sensitization seen in out bred mouse strains. Second, we wanted to examine the influence of contextual learning on locomotor sensitization to ethanol using our paradigm, as well as whether contextual learning might influence the persistence of this behavioral phenomenon. We hypothesized that ethanol, like other drugs of abuse, produces long-lasting sensitization that is enhanced by contextual learning.

Methods

Animals

The subjects for the present work were naïve female DBA/2J mice (aged about 60 days at the beginning of behavioral testing) purchased from the Jackson Laboratory (Bar Harbor, ME). Female mice were chosen based on literature suggesting that they develop locomotor sensitization to ethanol to a greater extent than male mice. Mice were shipped to Laboratory Animal Resources at Binghamton University, housed four to a cage, and allowed to acclimate to our vivarium for at least one week prior to the initiation of behavioral testing. Mice were maintained on a 12hr light-dark cycle with lights on at 0700 and temperature and humidity maintained at $21\pm1^{\circ}\text{C}$ and $50\pm1\%$, respectively. Mice were allowed free access to food and water except during behavioral testing. Ethanol and saline injections were given intraperitoneally (ip). All procedures were in accordance with the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2003), and were approved by the Binghamton University Animal Care and Use Committee.

Locomotor Activity Testing Chambers

Locomotor activity testing was conducted using the VersaMax Animal Activity Monitoring System (Accuscan Instruments Inc., Columbus, OH). Locomotion was detected by interruption of eight pairs of intersecting photocell beams (2 cm above the chamber floor) evenly spaced along the walls of the 40×40 cm test chamber. This equipment was situated in sound-attenuating box chambers (inside dimensions, 53 cm across × 58 cm deep × 43 cm high) equipped with a house light and fan for ventilation and background noise. The locomotor activity testing equipment was interfaced with a Dell computer. Testing continued for 15 min during which

time consecutive photocell beam interruptions were translated to distance traveled in cm by the VersaMax computer program. Data were collected in 5-min time intervals.

Sensitization Procedure

The general sensitization procedure was similar to that used by Phillips et al. (1995a), and is shown in Table 1. The mice in each cage were randomly assigned to one of two groups, a repeated-saline group (RS), or a repeated-ethanol group (RE). Days 1 and 2 of the procedure served to habituate mice to intraperitoneal (ip) injections and subsequent testing in the locomotor activity chambers. On these days mice were moved to the experimental room, allowed at least 30 min to habituate to the testing room environment, were weighed, injected with sterile 0.9% saline, and immediately placed in the center of the activity testing chambers for 15 min (or 10 min in experiment 3). On day 3 mice were again moved to the experimental room and allowed 30 min to habituate, were weighed, injected, and tested in the locomotor activity chambers for 15 min (or 10 min in experiment 3). However, mice assigned to the RE group received 2.0 g/kg ethanol (20% v/v in saline), whereas those assigned to the RS group received an equivalent amount of saline immediately prior to testing. On days 4–13 (or 14, see experiment 2), mice continued to be moved to the experimental room for the 30 min habituation period each day. However, mice assigned to the RE group received 2.5 g/kg ethanol (mice in the RS group continued to receive an equivalent volume of saline). None of the mice were tested in the locomotor activity testing chambers. On day 14 (or 15, see experiment 2), mice were moved to the activity testing room, and allowed to habituate to the room for at least 30 min. However, all mice received a challenge injection of 2.0 g/kg ethanol and were immediately tested in the locomotor activity testing chambers for 15 min (or 10 min in experiment 3).

Experiment 1

The goal of experiment 1 was to determine the duration of locomotor sensitization to ethanol in DBA/2J mice. Mice were treated as described above, except that at 7-day intervals after completion of the 14-day sensitization procedure they were assessed for the continued presence of sensitization to the locomotor stimulant actions of ethanol. Mice were moved to the experimental room, allowed at least 30 min to habituate, were weighed, injected with 2.0 g/kg ethanol, and immediately tested in the activity chambers on post-sensitization days 7, 14, and 21 (experimental days 21, 28, and 35).

Experiment 2

In experiment 1, each mouse received an ethanol injection on post-sensitization days 7, 14, and 21. To the extent that such injections might have influenced the persistence of locomotor sensitization to ethanol, we wished to assess the duration of sensitization in separate groups of mice that were left undisturbed in their home cages for the duration of their respective post-sensitization phases. For this, mice were subjected to a similar sensitization procedure except that 1) an error resulted in the additional day of 2.5 g/kg ethanol or equivalent volume saline injections prior to the ethanol test day, making it a 15-day sensitization procedure, and that 2) immediately following the ethanol challenge day (day 15) mice in the RE and RS groups were further subdivided into eight separate post-sensitization groups (RE-7, RE-14, RE-21, RE-28) and (RS-7, RS-14, RS-21, RS-28). Mice in the RE-7 and RS-7 groups were moved to the experiment room, allowed at least 30 min to acclimate to the testing room environment, were injected with a challenge dose of 2.0 g/kg ethanol, and immediately tested in the locomotor activity testing chambers on post-sensitization day 7 (experimental day 22). Likewise, the other groups were treated identically, except that mice in the RE-14 and RS-14 were left undisturbed in their home cages until ethanol injection and testing on post-sensitization day 14 (experimental day 29), RE-21 and RS-21 on post-sensitization day 21 (experimental day 36), and RE-28 and RS-28 on post-sensitization day 28 (experimental day 43).

Experiment 3

In experiments 1 and 2 we determined that ethanol locomotor sensitization in DBA/2J mice persisted for at least 14 days after cessation of the repeated ethanol injections. However, our paradigm deliberately minimized the possibility that contextual learning could have influenced the duration of ethanol sensitization. Indeed, for the 11 days following the initial exposure to ethanol and preceding the day 14 challenge, repeated ethanol-treated mice did not experience the ethanol stimulant effect in the locomotor activity testing chamber. We therefore wished to modify our sensitization procedure to determine whether allowing the repeated ethanol-treated mice to condition to the locomotor activity testing chamber might influence the duration of ethanol sensitization (see Table 2). Mice were subjected to the same 2-day saline habituation procedure detailed above for experiments 1 and 2 above, but on day 3 were divided into three separate groups. The first of these groups received an injection of ethanol (2 g/kg on day 3; 2.5 g/kg on days 4–13), was immediately placed in the locomotor activity testing chamber for 10 min, received a saline injection, and was then returned to the home cage (RE-RS-E). The second group received an injection of saline, was immediately placed in the locomotor activity testing chamber for 10 min, received an injection of ethanol (2 g/kg on day 3; 2.5 g/kg on days 4–13), and was returned to the home cage (RS-RE-E). Both groups received an ethanol injection (2.0 g/kg) on ethanol challenge days 14, 28, 48, 56, 70, and 84. A third group (RS-RS-E) served as a non-sensitized (acute ethanol) control group as it received saline injections both before and after the locomotor activity test up until ethanol challenge on days 14, 28, 42, 56, 70, and 84 at which point it too received ethanol (2.0 g/kg). Thus, whereas the first group always received the ethanol injection immediately prior to testing in the chambers, the second group always received the ethanol immediately before being returned to the home cage. Our goal was to determine whether pairings of the ethanol stimulant experience with the context of the locomotor activity testing chamber would come to elicit learning-enhanced locomotor sensitization to ethanol.

Blood ethanol clearance

To determine whether the repeated ethanol injection (sensitization) procedure altered the rate of blood ethanol clearance, we sampled retro-orbital sinus bloods (50 µl) immediately after the ethanol challenge injections and subsequent locomotor activity testing on days 14, 21, 28, and 35 in experiment 1, on either days 15 and 22 (RE-7 and RS-7), 15 and 29 (RE-14 and RS-14), 15 and 36 (RE-21 and RS-21), or 15 and 43 (RE-28 and RS-28) in experiment 2, and on days 14, 28, 42, 56, 70, and 84 in experiment 3 for determination of blood ethanol content. Determination of blood ethanol content was achieved using an Analox Alcohol Analyzer (Analox Instruments, Lunenburg, MA).

Statistical analysis

Initial inspection of the data revealed a more robust acute locomotor stimulant response to ethanol (compared to saline-treated controls) when only the first 10 min of the 15-min locomotor activity test were examined. Thus, the last 5 min of the 15-min locomotor activity test was ignored, and only data for the first 10 min of the locomotor activity test are shown for experiments 1 and 2. Data were analyzed by mixed two-way within subjects analyses of variance (ANOVA) with day as the within subjects factor and treatment as the between subjects factor using the Statistica Version 7 statistical package (Tulsa, OK). For experiment 2, the repeated ethanol and saline control data for the individual post-sensitization periods (7 days, 14 days, 21 days, and 28 days) were analyzed separately. Follow-up post-hoc tests (Newman-Keuls tests) were carried out where appropriate for experiments 1 and 2. However, because this approach would unnecessarily restrict alpha to an ever greater extent with the inclusion of each additional locomotor activity testing day in experiment 3 (in an effort to control family-wise error), an priori decision was made to separately compare each of the daily activity scores

of the RE-RS-E group to its own activity scores on days 2 or 3, each of the daily activity scores of the RS-RE-E group to its own activity scores on day 3, and the activity scores of each of the experimental groups (RE-RS-E and RS-RE-E) to the control group (RS-RS-E) on days 3–14, 28, 42, 56, 70, and 84. Subsequent simple contrasts were carried out to satisfy this decision. Results were considered significant at $p < 0.05$.

Results

Experiment 1

The duration of ethanol-induced locomotor sensitization in DBA/2J mice was first assessed in a single group of animals that were first exposed to a 14-day sensitization procedure and then challenged with ethanol every 7 days until evidence for locomotor sensitization disappeared. These data are shown in Figure 1A. A mixed factor 2-way ANOVA indicated significant main effects of both treatment [$F(1,22)=64.8, p < 0.0001$] and day [$F(6,132)=113.7, p < 0.001$], and a significant interaction of these factors [$F(6,132)=13.5, p < 0.0001$]. Newman-Keuls post-hoc tests indicated that whereas repeated ethanol- and saline-treated animals did not differ in sensitivity to the saline habituation injections on days 1 and 2, repeated ethanol-treated animals exhibited a robust acute locomotor stimulant response on day 3 of the procedure ($p < 0.001$). Moreover, repeated ethanol injections in RE mice enhanced this sensitivity on challenge day 14 compared to the RS controls who received their first ethanol exposure on that day ($p < 0.001$), and compared to the acute locomotor stimulant response of the same animals measured on day 3 ($p < 0.0001$). Thus, repeated ethanol injections produced robust locomotor sensitization in the RE group.

Following cessation of the daily ethanol or saline injections, mice from both the RE and RS groups were allowed to sit undisturbed in their home cages for 7 days. On post-sensitization day 7, the mice from both groups received a challenge injection of ethanol and were immediately tested in the locomotor activity chambers. Newman-Keuls post-hoc tests indicated that RE mice were still sensitized relative to their RS control counterparts on post-sensitization day 7 ($p < 0.01$). Moreover, RE mice were also still sensitized relative to their acute stimulant response on day 3 ($p < 0.0001$). The above procedure was repeated on post-sensitization days 14 and 21. On post-sensitization day 14 RE mice continued to significantly differ from their RS controls ($p < 0.02$) as well as from their acute stimulant response on day 3 ($p < 0.0001$). However, only the within group comparison was significantly different on post-sensitization day 21 (compared to their relative activity score on day 3, $p < 0.02$). Thus, the present data suggest that locomotor sensitization to ethanol persists for at least 14 days after cessation of the sensitization procedure in DBA/2J mice.

The literature varies as to whether ethanol injection procedures producing locomotor sensitization alter the rate of blood ethanol clearance. For example, sensitized mice might be more sensitive to the locomotor stimulant actions of ethanol, in part, due to the development of a slower rate of blood ethanol clearance. Therefore, we sampled retro-orbital sinus bloods immediately following the 15-min locomotor activity test for determination of blood ethanol concentration. These data are shown in Figure 1B. A mixed factor ANOVA with day as the within subjects factor and treatment as the between subjects factor detected a significant main effect of day [$F(3,54)=269.3, p < 0.0001$], but not of treatment or an interaction of these factors. Thus, although blood ethanol levels varied over days, RE and RS groups did not differ. These results suggest that changes in sensitivity to ethanol's locomotor stimulant actions among the RE mice were not due to changes in the rate of blood ethanol clearance.

Experiment 2

The goal of experiment 2 was to eliminate the potential confound of repeated ethanol challenge injections in mice, particularly 14 and 21 days post-sensitization. Thus, following a 15-day sensitization procedure, RE and RS mice were further divided into eight separate groups, each differing in the length of the post-sensitization period. Whereas RE-7 and RS-7 mice were again challenged with ethanol on post-sensitization day 7, RE-14 and RS-14 were again challenged with ethanol on post-sensitization day 14, RE-21 and RS-21 on post-sensitization day 21, and RE-28 and RS-28 on post-sensitization day 28. Separate mixed factor ANOVAs were conducted to compare the data for each separate post-sensitization period.

Figures 2A–D show the data for each separate post-sensitization period. Separate mixed factor ANOVAs with day as the within subjects factor and treatment as the between subjects factor were conducted for the RE-7 and RS-7 groups, the RE-14 and RS-14 groups, the RE-21 and RS-21 groups, and the RE-28 and RS-28 groups. The analysis of the RE-7 and RS-7 data supported the results from experiment 1. Analysis revealed significant main effects of treatment [$F(1,32) = 35.9, p < 0.0001$] and day [$F(4,128) = 141.5, p < 0.0001$], and a significant interaction of these factors [$F(4, 128) = 15.0, p < 0.0001$]. Newman-Keuls post-hoc tests showed that compared to RS-7 mice, RE-7 mice exhibited a robust locomotor stimulant response on day 3 ($p < 0.001$). Sensitivity to this response was enhanced after the 15-day sensitization procedure; RE-7 and RS-7 responses significantly differed when compared on day 15 ($p < 0.001$), and the RE-7 response on day 15 significantly differed from that on day 3 ($p < 0.0001$). Furthermore, both between group and within group comparisons showed that RE-7 mice remained sensitized 7 days following cessation of the sensitization procedure. Ethanol's locomotor stimulant properties significantly differed both between RE-7 and RS-7 mice on day 22 ($p < 0.01$), and when the locomotor stimulant responses of RE-7 mice were compared on days 3 and 22 ($p < 0.0001$).

Similar results were obtained for RE-14 and RS-14 mice. Mixed factor ANOVA detected significant main effects of treatment [$F(1,32) = 34.2, p < 0.0001$] and day [$F(4,128) = 169.0, p < 0.0001$], and a significant interaction of these factors [$F(4,128) = 18.4, p < 0.0001$]. Newman-Keuls post-hoc tests revealed that compared to RS-14 mice that had received saline on day 3, RE-14 mice exhibited a significant acute locomotor stimulant response on the same day ($p < 0.001$). RE-14 mice demonstrated a sensitized locomotor stimulant response on day 15 when compared to the ethanol response of RS-14 control mice ($p < 0.001$) and when compared to their own stimulant response on day 3 ($p < 0.0001$). Consistent with the results of experiment 1, this sensitized response persisted for 14 days with RE-14 mice continuing to demonstrate significant locomotor sensitization to ethanol when compared to the RS-14 mice on the same day ($p < 0.02$) and when compared with their own acute stimulant response on day 3 ($p < 0.0001$).

Figures 2C and 2D show the data for RE-21 and RS-21, and RE-28 and RS-28 animals, respectively. Similar to the results of the mixed factor ANOVAs above, these analyses revealed significant main effects of treatment (RE-21 and RS-21, [$F(1, 23) = 23.6, p < 0.0001$]; RE-28 and RS-28, [$F(1,22) = 72.6, p < 0.0001$] and day (RE-21 and RS-21, [$F(4,92) = 122.6, p < 0.0001$]; RE-28 and RS-28, [$F(4,88) = 171.7, p < 0.0001$], and a significant interaction of these factors (RE-21 and RS-21, [$F(4,92) = 18.1, p < 0.0001$]; RE-28 and RS-28, [$F(4,88) = 23.9, p < 0.0001$]). Subsequent Newman-Keuls post-hoc tests revealed that whereas RE-21 and RE-28 mice exhibited a significant locomotor stimulant response to ethanol (compared to their respective saline controls; p 's < 0.001), and developed robust locomotor sensitization by day 15 (compared to both their respective saline controls, p 's < 0.001 , and their own acute stimulant responses on day 3, p 's < 0.001), only within group sensitization was present by post-sensitization days 21 and 28 (compared to their relative activity scores on day 3, p 's < 0.01).

Blood ethanol concentrations were also determined in each of the above post-sensitization experiments (Figure 3A–D). Inspection of the data revealed that blood ethanol concentrations generally decreased from the final day of the 15-day sensitization procedure to the post-sensitization challenge day. This observation was supported when separate mixed factor ANOVAs with treatment as the between subjects factor and day as the within subjects factor were performed for each post-sensitization period. Although not seen in the analysis for RE-7 and RS-7 mice, main effects of day were detected in each of the analyses for the other post-sensitization groups (RE-14 versus RS-14, $[F(1,32) = 9.9, p < 0.01]$; RE-21 versus RS-21, $[F(1,10) = 67.3, p < 0.001]$; RE-28 versus RS-28, $[F(1,10) = 7.7, p < 0.02]$). However, there were no significant main effects of treatment, or interactions of treatment and day in any of the analyses. Thus, although blood ethanol concentrations varied by day, repeated ethanol exposures producing locomotor sensitization did not appear to differentially alter blood ethanol concentration. These results, as did those in experiment 1 above, do not support a role for changes in ethanol metabolism as an underlying factor supporting locomotor sensitization to ethanol.

Experiment 3

We next determined whether contextual learning might influence the duration of ethanol sensitization in DBA/2J mice. To determine whether the contextual learning might enhance ethanol sensitization or the duration of the behavioral phenomenon, three groups of mice were given an ethanol or saline injection each day for 11 consecutive days and immediately tested in the locomotor activity chambers. Ethanol treatment was either paired with the activity chamber (paired, RE-RS-E), paired with the home cage (unpaired, RS-RE-E), or never experienced (saline control, RS-RS-E) during the induction phase of the experiment. Each of these groups received ethanol on challenge days 14, 28, 42, 56, 70, and 84.

The data for the first 13 days of the experiment are shown in Figure 4A. Because locomotor activity was assessed each day throughout the 14-day procedure we were able observe induction of ethanol sensitization. Repeated measures ANOVA detected significant main effects of treatment ($[F(2,30) = 38.8, p < 0.0001]$), day ($[F(12,360) = 4.3, p < 0.0001]$), and a significant interaction of these factors ($[F(24,360) = 6.3, p < 0.0001]$). Although we were interested in only a limited number of group comparisons, we recognized that Statistica would perform Newman-Keuls post-hoc tests for every possible comparison following the significant interaction of treatment and day. Because this approach would unnecessarily restrict alpha to an ever greater extent with the inclusion of each additional induction day (in an effort to control family-wise error), an priori decision was made to separately compare each of the daily activity scores of the RE-RS-E group to its own activity scores on days 2 or 3, each of the daily activity scores of the RS-RE-E group to its own activity scores on day 3, and each of the experimental groups (RE-RS-E and RS-RE-E) to the control group (RS-RS-E) on each induction day (3–13). When the locomotor activity of the RE-RS-E mice was compared using this approach they exhibited a significant acute locomotor stimulant effect on day 3 (compared to day 2, $p < 0.01$; compared to the RS-RS-E control group on the same day, $p < 0.05$), as well as an enhancement of this effect when compared to the activity scores of the RS-RS-E controls on days 3 and 5–13 (p 's < 0.05). Interestingly, when a similar series of contrasts were performed for the RS-RE-E group, their activity scores on days 4–13 also significantly differed from their activity scores on day 3 (p 's < 0.05), as well as from those of the RS-RS-E control group on days 4–13 (p 's < 0.05). Thus, whereas repeated ethanol treatment immediately prior to testing in the locomotor activity chambers resulted in elevated locomotor activity scores (and presumably the induction of locomotor sensitization) in the RE-RS-E group, the repeated saline treatment prior to activity testing (and repeated ethanol treatment immediately after) over the same time period appeared to reduce locomotor activity in the RS-RE-S group.

The influence of contextual learning on the magnitude and duration of locomotor sensitization to ethanol is shown in Figure 4B. Two-way repeated measures ANOVA revealed a marginally significant main effect of treatment ($[F(2,27) = 3.1, p = 0.06]$) and significant main effect of day ($[F(8,216) = 67.6, p < 0.0001]$), as well as a significant interaction of these factors ($[F(16,216) = 2.9, p < 0.0001]$). An a priori decision was made to compare the locomotor stimulant responses of the RE-RS-E paired to the RS-RS-E control, the RS-RE-E unpaired to the RS-RS-E control, and the RE-RS-E paired to the RS-RE-E unpaired groups on days 14, 28, 42, 56, 70, and 84, and to compare the activity scores of the RE-RS-E and RS-RE-E groups on these days to their own scores on either day 3 (RE-RS-E) or day 14 (RS-RE-E). Such contrasts revealed that mice that always received ethanol immediately prior to testing in the locomotor activity testing chamber (paired group; RE-RS-E) demonstrated a significant sensitized response on day 14 when compared to the acute ethanol RS-RS-E control group ($p < 0.01$), and when compared to their own acute locomotor stimulant response on day 3 ($p < 0.02$). We had hypothesized that mice that had received saline prior to spending time in the locomotor activity chamber and ethanol immediately before returning to the home cage (unpaired; RS-RE-E) would exhibit lesser locomotor sensitization because they had never before experienced ethanol in the chamber. However, not only did they develop significant locomotor sensitization to ethanol when compared to the RS-RS-E acute ethanol control mice on day 14 ($p < 0.001$), but their sensitized response did not significantly differ from that of the RE-RS-E paired mice that same day ($p = 0.67$), suggesting the two groups sensitized to similar degrees. Thus, the pairing of contextual cues associated with the locomotor activity testing chamber with the ethanol experience did not appear to enhance the magnitude of sensitization.

Contextual learning did not enhance locomotor sensitization. However, when the groups were again challenged with ethanol 14 (experimental day 28), 28 (experimental day 42), 42 (experimental day 56), 56 (experimental day 70), and 70 (experimental day 84) days after the repeated ethanol injection procedure ended contextual learning did appear to prolong the duration of ethanol sensitization. RE-RS-E paired mice displayed a marginally significant sensitized response on experimental day 28 (compared to their relative acute stimulant response on day 3, $p = 0.07$; the between groups comparison was not significant) and 42 (compared to RS-RS-E control group on day 42, $p = 0.05$; compared to relative acute stimulant response on day 3, $p < 0.02$). Between groups contextual learning-enhanced locomotor sensitization appeared to disappear by day 56. However, the RE-RS-E paired mice continued to demonstrate within group sensitization out to day 84 (p 's < 0.03). In contrast, mice in the RS-RE-E unpaired group failed to demonstrate significant locomotor sensitization on any of the post-sensitization challenge days (compared to the RS-RS-E acute ethanol controls on experimental days 28, 42, 56, 70, and 84), and exhibited significantly lower ethanol-stimulated locomotion when their day 28 and 42 scores were compared to their own day 14 scores ($p < 0.03$). Furthermore, the ethanol-stimulated locomotion of the RE-RS-E group was also marginally greater than that of the RS-RE-E group on experimental day 42 ($p = 0.06$). Thus, modification of our ethanol sensitization procedure to allow mice the opportunity to associate the contextual cues of the locomotor activity chamber with ethanol experience prolonged the duration of locomotor sensitization to ethanol. The pairing of the ethanol injection with the locomotor activity chamber resulted in sensitization that persisted for at least four weeks, twice as long as was seen when such learning was avoided.

Similar to experiments 1 and 2 above, blood ethanol concentrations were determined immediately following locomotor activity testing on days 14, 28, 42, 56, 70, and 84. These data are shown in Figure 4C. Similar to the results of experiments 1 and 2 above, the overall two-way repeated measures ANOVA only detected a main effect of day ($[F(5,130) = 13.6, p < 0.001]$) with no significant main effect of treatment or interaction of these factors. Thus, blood ethanol concentrations again varied by ethanol challenge day, but did not differ between groups on any day.

Discussion

Locomotor sensitization to ethanol is believed to result from molecular neuroadaptations associated with the repeated ethanol administrations that produce the behavioral phenomenon. If true, the expectation is that these neuroadaptations will persist for as long as the locomotor sensitization itself. The goal of the present work was to determine the duration of ethanol sensitization in DBA/2J mice, and the extent to which the contextual cues associated with the repeated ethanol experience would influence this duration. In experiment 1, mice were given a series of repeated ethanol or saline injections and were then periodically challenged with ethanol to determine the persistence of ethanol-induced locomotor sensitization. Ethanol-treated mice developed robust locomotor sensitization to ethanol, and this sensitization lasted through post-sensitization day 14. In experiment 2, we determined whether the repeated post-sensitization challenge injections in experiment 1 artificially prolonged the duration of sensitization. The duration of sensitization observed in experiment 2 was similar to that seen in experiment 1; sensitization persisted through post-sensitization day 14, but was gone by post-sensitization day 21. We therefore concluded that repeated injections during the ethanol challenge phase of the experiment do not alter the duration of ethanol sensitization. In experiment 3, we re-examined the duration of sensitization, this time including a contextual learning group in which the repeated ethanol injections during the induction phase were always paired with the locomotor activity chamber, presumably allowing the mice to associate the contextual cues of the chamber with the ethanol experience. Based on the results of experiment 2, we chose to use a similar repeated ethanol challenge approach. In order to conserve the number of mice necessary to adequately assess the effects of contextual learning on the duration of sensitization. Although contextual learning did not increase magnitude of sensitization to ethanol's locomotor stimulant actions, it prolonged the persistence of the behavioral phenomenon. Locomotor sensitization to ethanol persisted for at least 28 days after cessation of repeated ethanol administrations; twice as long as that seen in experiments 1 and 2.

Several different labs have examined the duration of ethanol-induced locomotor sensitization in mice. Lessov and Phillips (1998) undertook similar experiments and concluded that ethanol sensitization in mice persists for up to 23 days although there was some disagreement among individual experiments within the larger study; whereas one experiment suggested that ethanol-induced locomotor sensitization lasted for at least 23 days, another suggested that it may dissipate by day 17. In a more recent study Fish et al. (2002) demonstrated continued ethanol sensitization persisting out to post-sensitization day 58.

Why is there so much disagreement between the studies? One possible explanation for these seemingly disparate findings might relate to the magnitude of sensitization achieved in each study. Procedural differences, for example the number of repeated daily ethanol administrations, may have influenced the initial magnitude of the behavioral phenomenon, therefore influencing its persistence after the repeated ethanol administrations had ceased. Our sensitization paradigm in experiments 1 and 2 was similar to that of Lessov and Phillips (1998), so it is not immediately clear why we were only able to observe durations out to 14 days post-sensitization while they observed the behavioral phenomenon out to 23 days. However, it is notable that in the first of the experiments detailed by these authors they employed a 10-day repeated ethanol administration procedure and both the magnitude and duration of sensitization was not as great as that seen in the later experiments (11 days following the 10-day procedure versus 23 days following the 11-day procedure). This explanation, however, doesn't explain why Fish et al. (2002) observed such long-lasting sensitization; they also used a 10-day procedure but observed sensitization persisting to 58 days. Moreover, Lessov and Phillips (1998) observed sensitization persisting to 23 days post-sensitization using a nearly identical procedure as we used in experiments 1 and 2. Thus, the number of daily ethanol administrations does not appear to fully explain our differing results.

The doses of ethanol administered also differed between the studies. We used an identical ethanol dosing regimen as Lessov and Phillips (1998), giving ethanol doses of 2.5 g/kg (20% v/v, ip) on experimental days 4–13, and 2 g/kg (20% v/v, ip) on experimental days 3, 14 and the ethanol challenge days. However, Fish et al. (2002) gave 10 daily doses of 2.4 g/kg (15% w/v, ip) during the induction phase of the experiment, and 2 g/kg (15% w/v, ip) on the post-sensitization challenge days. Thus, while it is unlikely that dose influenced the differing duration of sensitization between our study and the Lessov and Phillips (1998) study, it could explain the longer duration of sensitization observed in the Fish et al. (2002) study.

Yet another procedural difference between the works involved the genotypes of the mice studied. Whereas Lessov and Phillips (1998) and Fish et al. (2002) each used a different out bred mouse population, we chose DBA/2J inbred mice for the present work. Because these mice are inbred and therefore genetically identical, their use reduces the inherent genetic variability associated with the use of out bred mice. Furthermore, we wanted to assess duration of ethanol-induced locomotor sensitization in mice that display a particularly high propensity for developing the phenomenon. DBA/2J inbred mice are particularly vulnerable to development of locomotor sensitization to ethanol (Phillips et al. 1994, 1995; 1996). Nevertheless, the duration of ethanol sensitization was not as robust as that seen in the out bred mouse stocks, at least not until we introduced the contextual learning component in experiment 3. We therefore speculate that even longer-lasting sensitization might have been possible if context-enhanced sensitization were examined in an out bred mouse population, such as the CFW out bred mouse stock tested by Fish et al. (2002). In that study, although the CFW out bred mice were not tested in the locomotor activity chambers after every ethanol injection during the induction phase, the ethanol group was allowed to experience ethanol in the locomotor activity testing chamber on four occasions over the course of the 10-day induction phase of the procedure, presumably allowing for some contextual learning to occur in these animals. If the CFW out bred mouse stock is indeed more susceptible to development of locomotor sensitization to ethanol, and contextual learning positively influences that susceptibility, than one might expect a longer duration of sensitization in these animals.

A potential problem with the design of experiment 1 was that the periodic ethanol challenge injections that were given every seven days might have been sufficient to “boost” ethanol sensitization, maintaining it for longer than it otherwise might have been. Such a boost in ethanol sensitization could have resulted from conditioned locomotion as might be expected with repeated pairings of the locomotor activity testing chambers. Therefore, we executed experiment 2 in which separate groups of mice received 12 days of repeated ethanol or saline injections followed by ethanol challenge and testing in the locomotor activity chambers either 7, 14, 21, or 28 days later. Importantly, these mice were left undisturbed in their home cages until their pre-assigned ethanol challenge day. The data from experiment 2 confirmed those of experiment 1; ethanol-induced locomotor sensitization was seen in the ethanol-treated groups on post-sensitization days 7 and 14, but not on post-sensitization days 21 or 28. We therefore concluded that the periodic ethanol challenge injections did not alter the duration of sensitization, and decided to employ a similar strategy in experiment 3 to assess the importance of contextual learning. We even introduced a 14-day interval between ethanol challenge injections, just to be sure. Nevertheless, the ethanol-stimulated locomotion of the RS-RE-E unpaired group appeared to gradually increase on experimental days 56 and 70, nearly matching that of the RE-RS-E paired group by experimental day 84. Furthermore, the ethanol-stimulated activity of the RS-RS-E acute ethanol control group also appeared to increase on experimental day 84. We speculate that although three post-sensitization challenge injections spaced 14 days apart were not sufficient to produce cue-enhanced sensitization, further challenge injections actually began to produce this type of sensitization in both the RS-RE-E unpaired and RS-RS-E acute ethanol control groups. Future work will explore this possibility by assessing the duration of context-enhanced sensitization in separate groups of mice using a similar approach

as that used in experiment 2. It is entirely possible that such a design would demonstrate even longer-lasting cue-enhanced sensitization among the RE-RS-E paired group. Indeed, it is notable that in the present work the RE-RS-E group continued to demonstrate within group sensitization out to experimental day 84 and only lost between group sensitization when the ethanol-stimulated locomotion of the unpaired RS-RE-E group began to creep up.

In addition to assessing the duration of locomotor sensitization to ethanol in DBA/2J mice we were also interested in determining whether the repeated ethanol injection procedure also produced changes in blood ethanol clearance rates, and whether any such changes persisted for as long as the locomotor sensitization. Indeed, evidence has been mixed on this issue. For example, Lessov and Phillips (1998) reported elevated blood ethanol concentrations in several groups that had undergone the repeated ethanol injection (sensitization) procedure, perhaps suggesting that the enhanced sensitivity to ethanol-induced locomotion may have been the result of lower ethanol clearance rates. Neither the animals in experiment 1, 2, or 3 displayed evidence of altered blood ethanol clearance rates. Blood ethanol concentrations were similar in both repeated ethanol-, and repeated saline-treated animals, regardless of whether the ethanol-treated animals experienced ethanol exclusively in the locomotor activity testing chambers. Lessov and Phillips (1998) concluded that alterations in the rate of blood ethanol clearance rates, if present in their studies, were not significantly associated with the magnitude of ethanol-induced locomotor sensitization. More recently, Quadros et al. (2005) demonstrated that blood ethanol levels were not associated with the expression of ethanol sensitization following a 21-day ethanol treatment procedure in out bred mice. Our studies support this conclusion as there was no indication that sensitization was associated with reduced rates of blood ethanol metabolism (higher blood ethanol concentrations).

A number of research groups have postulated that long-lasting locomotor sensitization may predispose individuals to relapse after long intervals of drug abstinence (Robinson 1993; Ron and Jurd 2005; Sato et al. 1983). Indeed, locomotor sensitization to cocaine and amphetamine has been shown to persist for up to 3 (Shuster et al. 1977) and 12 (Paulson et al. 1991) months respectively, perhaps explaining why individuals addicted to these substances relapse after many months of abstinence. Our work, along with that of Lessov and Phillips (1998) and Fish et al. (2002), suggests that ethanol locomotor sensitization is not as long lasting, persisting for up to 2 months, depending on genotype. If the collective data are relevant to humans and locomotor sensitization does indeed predispose addicted individuals to relapse, the data may imply that alcoholics (compared to cocaine or amphetamine addicts) are less likely to relapse after longer periods of relative abstinence. The human literature isn't clear on whether such a relationship exists between the drugs. Additional work will be necessary to adequately address this issue.

Few studies have directly examined the potential influence of context on the strength of ethanol locomotor sensitization, and none have attempted to identify a role for contextual learning on the long-lasting nature of the behavioral phenomenon. Indeed, contextual learning has been shown to enhance the expression of sensitization to the classic psychomotor stimulants cocaine, amphetamine, and morphine (Badiani et al. 1995; 2000). Cunningham and Nobel (1992) reported that the magnitude of locomotor sensitization to ethanol was indeed stronger in the environment in which animals had experienced the repeated ethanol administrations. In the present work we did not observe a statistically significant increase in magnitude of ethanol sensitization in mice that had been allowed to associate the ethanol experience with the locomotor activity testing chambers. The reason for these differing results is not immediately clear, but may be due to the different behavioral testing paradigms used in the studies. For example, Cunningham and Nobel (1992) addressed this issue using an ethanol place conditioning paradigm. Nevertheless, it is notable that repeated pairing of the locomotor activity testing chamber and the repeated ethanol injections in the present work appeared to

prolong the expression of the behavioral phenomenon after termination of the repeated ethanol injections. Such prolonged cue-enhanced duration of sensitization may, as has been suggested in studies showing cue-enhanced strength of sensitization, influence the propensity for relapse upon re-exposure to the drug associated context.

Locomotor sensitization is believed to be associated with molecular neuroadaptations in brain regions associated with drug addiction (Ron and Jurd 2005). The persistence of such molecular neuroadaptations may support the continued expression of the phenomenon. A number of neurotransmitter systems have been implicated in the induction and expression of ethanol-induced locomotor sensitization (Phillips and Shen 1996), and any one of these may be the focus of such molecular neuroadaptations. However, few studies have actually reported any such associated neuroadaptive changes. For example, despite several studies examining dopamine receptor and/or transporter binding in mouse brain before and after exposure to an ethanol locomotor sensitization paradigm (Quadros et al., 2005; Quadros et al., 2002b; Souza-Romigoni et al., 1999), only one actually reported an associated change in binding: striatal D2 receptor binding was increased in sensitized compared to non-sensitized and saline-control mice (Souza-Formigoni et al., 1999). Nevertheless, that cue-enhanced locomotor sensitization persists for at least 28 days after cessation of the repeated daily ethanol administrations in DBA/2J mice suggests that any such molecular neuroadaptations should also persist for this period.

A number of brain sites could be the potential targets for such neuroadaptive changes. However, the mesolimbic dopamine system would appear to be a reasonable place to begin the search. Brodie and Appel (2000) demonstrated that ethanol enhances the activity of dopamine neurons in the ventral tegmental area (VTA), and ethanol has been shown to stimulate dopamine release in the nucleus accumbens (Imperato and DiChiara 1986), the terminal site of VTA dopamine neurons. Moreover, this effect was associated with its locomotor stimulant effects in vivo (Imperato and DiChiara 1986). Sensitization to ethanol's locomotor stimulant actions may also be associated with the enhanced ethanol sensitivity to VTA dopamine neurons. Indeed, chronic ethanol exposure results in heightened sensitivity of VTA dopamine neurons to ethanol (Brodie 2002). Although the dose of ethanol administered in these studies was higher than is typically administered in sensitization experiments and the C57BL/6J mice used in the work do not typically display ethanol-stimulated locomotion, Brodie's work fuels speculation that repeated ethanol exposures producing locomotor sensitization may also enhance the ethanol sensitivity of VTA dopamine neurons. Interestingly, in the same work Brodie (2002) showed that these VTA dopamine neurons were also less potently inhibited by GABA. Such findings may suggest that alterations in the GABAergic modulation of VTA dopamine neurons are important in the induction and expression of locomotor sensitization to ethanol. Future work in the lab will begin to address this issue.

The current work demonstrates that the expression of locomotor sensitization to ethanol in DBA/2J mice persists for at least 14 days following termination of repeated ethanol exposures, and up to 28 days when the repeated ethanol exposures are associated with a specific context. Such a long-lasting change in behavioral sensitivity to ethanol may suggest the presence of persistent neuroadaptive changes in brain that are made more persistent when contextual cues have been associated with the sensitization. Future work will begin to investigate these potential neuroadaptive changes focusing particular attention to GABAergic mechanisms in the VTA and nucleus accumbens.

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A. Duration

B. Blood Ethanol Concentration

Figure 1.

Duration of ethanol-induced locomotor sensitization in DBA/2J mice given periodic challenge injections of ethanol at seven day intervals. A. Duration. Ethanol-treated DBA/2J mice exhibited a significant acute stimulant response on day 3 and developed robust locomotor sensitization by day 14. Ethanol-treated mice remained sensitized on post-sensitization days 7 and 14 (experimental days 21 and 28) when compared to their saline-treated counterparts, but sensitization was lost by post-sensitization day 21 (experimental day 35). B. Blood ethanol concentrations. Blood ethanol concentrations varied by day, but repeated ethanol- or saline-treated mice did not differ in this response. Only between groups significant effects are shown. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

A. Seven Days Post-Sensitization

B. Fourteen Days Post-Sensitization

C. Twenty-One Days Post-Sensitization

D. Twenty-Eight Days Post-Sensitization

Figure 2.

Duration of ethanol-induced locomotor sensitization in separate groups of DBA/2J mice, each given an ethanol challenge injection 7, 14, 21, or 28 days post-sensitization. All RE groups exhibited a significant locomotor stimulant response on day 3, and developed between groups sensitization by day 15. RE-7 (A) and RE-14 (B) mice displayed continued ethanol sensitization on post-sensitization days 22 and 29, respectively. RE-21 (C) and RE-28 (D) mice did not exhibit sensitization on days 36 and 43, respectively. Only between groups significant effects are shown. * $p < 0.05$. ** $p < 0.01$. *** $p < 0.001$.

Figure 3.

Blood ethanol concentrations in separate groups of mice subjected to the 15-day sensitization and subsequent post-sensitization procedure depicted in Figure 2. RE-7 and RS-7 mice, as well as RE-14 and RS-14 mice, did not differ in blood ethanol concentration 5 min after conclusion of the 10 min activity test (15 min after ethanol challenge) on either the sensitization test day, or the ethanol challenge day. RE-21 and RS-21 mice, and RE-28 and RS-28 mice also did not exhibit differing blood ethanol concentrations on either day.

Figure 4.

Effect of contextual learning on the duration of locomotor sensitization to ethanol in DBA/2J mice given periodic challenge injections of ethanol at fourteen day intervals. A. Induction. Mice having always received ethanol immediately prior to testing in the locomotor activity chambers and saline immediately afterward (RE-RS-E paired group) exhibited a significant enhancement of ethanol-stimulated locomotion on days 3, and 5–13 (compared to RS-RS-E saline controls). In contrast, mice having always received saline immediately prior to testing and ethanol immediately after (RS-RE-E unpaired group) demonstrated a significant reduction in locomotor activity on days 4–13 (compared to RS-RS-E saline controls). B. Duration. Whereas RE-RS-E and RS-RE-E mice both displayed a significant sensitized response on day

14 (compared to the RS-RS-E acute ethanol controls), only RE-RS-E mice continued to display a sensitized response 4 weeks after termination of the sensitization procedure (compared to the RS-RS acute ethanol controls on experimental day 42). C. Blood ethanol concentrations. Blood ethanol concentrations varied by day, but did not differ between groups. Only between groups significant effects are shown. $*p \leq 0.05$. $**p < 0.01$. $***p < 0.001$.

Table 1

Locomotor sensitization paradigm - Experiments 1 and 2.

Treatment Groups	Habituation Days 1–2 (test)	Acute EtOH Day 3 (test)	Daily Treatments (induction) Days 4–13 (or 14) (no test)	Sensitization (expression) Day 14 (or 15) (test)	Sensitization (continued expression) Post-Sensitization Days 7, 14, 21, and/or 28 (test)
Saline Control (RS)	saline	saline	saline	EtOH (2 g/kg)	EtOH (2 g/kg)
Repeated EtOH (RE)	saline	EtOH (2 g/kg)	EtOH (2.5 g/kg)	EtOH (2 g/kg)	EtOH (2 g/kg)

EtOH = ethanol.

Table 2

Locomotor sensitization paradigm - Experiment 3.

Treatment Groups	Habituation Days 1–2 (test)	Acute EtOH Day 3 (test)	Daily Treatments (induction) Days 4–13 (test)	Sensitization (expression) Day 14 (test)	Sensitization (Duration) Days 28, 42, 56, 70, 84 (test)
EtOH chamber (RE-RS-E)					
EtOH homecage (RS-RE-E)	saline/saline	EtOH/saline	EtOH/saline	ethanol	ethanol
Acute EtOH control (RS-RS-E)	saline/saline	saline/EtOH	saline/EtOH	ethanol	ethanol
	saline/saline	saline/saline	saline/saline	ethanol	ethanol

Mice receiving ethanol or saline were injected with 2 g/kg or an equivalent volume of saline on days 3, 14, 28, 42, 56, 70, and 84, and 2.5 g/kg ethanol or an equivalent volume of saline on days 4–13.
EtOH = ethanol.