Published in final edited form as:

Physiol Behav. 2016 October 15; 165: 257–266. doi:10.1016/j.physbeh.2016.08.006.

Effects of acute alcohol withdrawal on nest building in mice selectively bred for alcohol withdrawal severity

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Abstract

Nest building has been used to assess thermoregulatory behavior and positive motivational states in mice. There are known genetic influences on ethanol withdrawal severity as well as individual/thermoregulatory nest building. Withdrawal Seizure-Prone (WSP-1, WSP-2) and Withdrawal Seizure-Resistant (WSR-1, WSR-2) mice were selectively bred for high vs low handling-induced convulsion (HIC) severity, respectively, during withdrawal from chronic ethanol vapor inhalation. They also differ in HIC severity during withdrawal from an acute, 4 g/kg ethanol injection. In our initial study, withdrawal from an acute dose of ethanol dose-dependently impaired nest building over the initial 24 h of withdrawal in genetically segregating Withdrawal Seizure Control (WSC) mice. In two further studies, acute ethanol withdrawal suppressed nest building for up to two days in WSP-1 females. Deficits in nest building from ethanol were limited to the initial 10 h of withdrawal in WSR-1 females and to the initial 24 h of withdrawal in WSP-1 and WSR-1 males. Effects of ethanol on nest building for up to two days were found in WSP-2 and WSR-2 mice of both sexes. Nest building deficits in female mice from the first replicate could not be explained by a general decrease in locomotor behavior. These results suggest that nest building is a novel behavioral phenotype for indexing the severity of acute ethanol withdrawal, and that genes contributing to this trait differ from those affecting acute withdrawal HIC severity.

Keywords

Genetics; Nest building; Acute ethanol; Withdrawal; Selective breeding

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Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.physbeh.2016.08.006>

The authors declare that there is no conflict of interest.

1. Introduction

Alcohol (ethanol) withdrawal is a core component of alcohol use disorders with many genetic contributions [41]. This genetic complexity may explain why cessation of chronic ethanol exposure is associated with a wide variety of behavioral states in rodent models, including decreased locomotor activity [29], behavioral despair [30], and disrupted thermoregulation [50]. The handling-induced convulsion (HIC) index was developed over 40 years ago as a quantitative index of severity of withdrawal from chronic exposure to ethanol vapor [21], and enhanced sensitivity to convulsive treatments have been reported following a single high dose of ethanol in mice [43]. Many pharmacological [7] and genetic [44,45] studies have increased our understanding of exacerbated HICs during withdrawal following a single ethanol exposure. Although less is known about behavioral responses other than HICs during this period, there is recent evidence from mouse models suggesting acute withdrawal-associated modulations of pain sensitivity, affective state, and learning [27,58]. The genetic contributions to many behavioral responses during acute ethanol withdrawal have also not been explored. Thus, behavioral assays that can detect acute ethanol withdrawal states in mice and can provide genetic information could be of great use.

Nest building is an activity that can be observed in the home cage of mice. When a mouse of either sex is given nesting material, it will retrieve it, deposit it in a central site, and begin to build walls. Nests differ in quality, but a complete, high-quality nest has enclosing walls that can serve to provide shelter and minimize heat loss [11,17]. The latency for a mouse in a laboratory setting to retrieve nesting material and begin manipulating it for several seconds is approximately 30 min to 1 h [26], and other variations of nest-building tests indicate that nesting material is incorporated into an established nest site in under 7 min [51]. Other studies suggest that mice will work to obtain nesting material or employ it for thermoregulation. Mice will key press to dispense paper nesting material [52,53], and they will expend time and energy moving it from one cage to another held at a preferred temperature [16]. Whether nest building is an intrinsically motivated behavior or an instrumental response to the availability of a substrate for thermoregulation, these studies suggest the sequence is a highly motivated process. Decreased nest building in small mammals has been suggested to reflect anhedonic state [9,12]. Although ethanol withdrawal and nest building are complex traits, there is evidence for genetic contributions for each. Thus, nest building could be assessed during acute ethanol withdrawal to further understand the genetics that underlie withdrawal-induced impairments in affective states and thermoregulation.

Evidence for genetic influences on acute ethanol withdrawal has come from selectively-bred and inbred strains of mice [4]. With bidirectional selective breeding, individuals from a population are chosen to mate based on their high or low scores on a trait of interest (i.e., their phenotypic value). As this process is repeated across generations, a selected line eventually approaches homozygosity for all trait-relevant genes; this progression is known as allele fixation. The oppositely-selected line will fix different alleles for the same genes, and/or alleles for other influential genes. These genes can influence other traits, revealed when the divergently-selected lines differ in those genetically correlated responses to selection. Pairs of selectively-bred lines are often generated in two replicates from

independent sets of families, and evidence for a genetic correlation is stronger when both replicate pairs display a difference in a correlated response in the same direction [8].

To study ethanol withdrawal severity, two replicate pairs of selected lines of mice were bred from a genetically heterogeneous founder population for increased [i.e., the Withdrawal Seizure-Prone (WSP-1 and WSP-2)] and decreased [i.e., the Withdrawal Seizure-Resistant (WSR-1 and WSR-2)] HIC scores during withdrawal after a period of chronic ethanol vapor inhalation [5]. Thus, correlated traits that are seen to differ between WSP vs WSR mice, particularly when differences are seen in both replicates in the same direction, are assumed to be influenced by genes underlying ethanol withdrawal severity [8]. WSP mice from both replicates also display greater HIC responses after withdrawal from acute ethanol than their corresponding WSR lines, which provides strong evidence for a genetic correlation between withdrawal severity from acute and chronic ethanol exposure [6,31].

Evidence for genetic influences on nest building has similarly stemmed from mice bidirectionally selected for the quantity of nesting material used at room temperature [38]. By generation 15, mice from both replicates of a high-nesting selected line had achieved an eightfold difference in nesting compared to the low selected mice, with future generations achieving a 40-fold difference between high and low lines [39]. Tests for genetic influences on nest building have been performed for over a half decade, beginning with comparisons of inbred strains and F1 crosses [2,36]. Differences in nesting behavior across inbred strains have suggested heritable differences in the behavior [40] that also interact with environmental variables, such as changes in room temperature [36]. Although there is evidence for gene-environment interactions producing differences in nest building, we are not aware of any studies that have investigated interactions with alcohol or other drugs of abuse. Here, we investigate the interaction between genes and alcohol withdrawal for effects on qualitative nest building in mice.

We developed a rubric for scoring nest building in a genetically heterogeneous stock (HS/Npt/Pdx) of mice. HS/Npt/Pdx mice had increased HIC scores during withdrawal from 3 days of chronic ethanol vapor exposure, and chronic ethanol dose-dependently suppressed nest building for up to 32 h into withdrawal [22]. This suppression was also observed in mice after a single, acute injection of ethanol. Here, we further assessed the effects of acute ethanol withdrawal on nest building and tested for genetic influences and sex differences, with the null hypothesis that nest building would decrease after acute ethanol withdrawal. We first demonstrated dose-dependent deficits following acute ethanol withdrawal in another genetically heterogeneous mouse stock, the Withdrawal Seizure Control (WSC/Pdx) mice, which serve as the non-selected control line for the WSP and WSR mice and share their genetic background. We then tested whether there were systematic genetic differences in the nest-building deficit by studying the four WSP and WSR genotypes.

In a separate line of studies using HS/Npt/Pdx mice, we found that reduced nest building was observed up to 24 h into withdrawal from an acute 4 g/kg injection of ethanol before ethanol-withdrawn mice began to recover [22]. In earlier selected generations of WSP mice, signs of acute ethanol withdrawal as measured by HICs began to subside 12 h after injection of 4 g/kg ethanol before returning to baseline levels at 24 h [31]. HICs also returned to

baseline in Swiss-Webster and inbred strains of mice (BALB/cJ and DBA/2J) 12 h after injection of 4 g/kg ethanol [44,45]. We tracked nest building during the first day of acute ethanol withdrawal when we hypothesized nest-building deficits would occur, and during the second day of withdrawal when we hypothesized nest-building deficits would subside.

Finally, we investigated whether nest-building deficits reflected overall decreases in locomotor behavior using activity monitoring chambers in combination with acute ethanol withdrawal in female WSP-1 and WSR-1 mice. We tested three hypotheses: 1) that decreased activity 10–24 h after acute ethanol withdrawal might underlie nest building deficits specific to female WSP-1 mice, 2) that decreased activity could explain the delayed recovery from withdrawal-induced nest building deficits 24–32 h after injection in female WSP-1 mice, and 3) that the decreased nest building, either due to acute ethanol withdrawal or genetic factors, in all female groups compared to female WSR-1 mice given saline evident at 10 h after injection simply reflected lower activity in those groups during hrs 6–10.

2. Materials and methods

2.1. Animals and husbandry

Naïve male and female WSP-1/Pdx and WSP-2/Pdx and WSR-1/Pdx and WSR-2/Pdx mice and their non-selected controls (WSC/Pdx) were used. The founder population for these mice were from a heterogeneous stock (HS/Ibg) derived from an 8-way cross of inbred strains (A/J, AKR, BALB/c, C3H, C57BL/6, DBA/2, RIII and AKR). The inbred strains are a mix of *M. m. domesticus*, *M. m. musculus*, *M. m. castaneus*, and possibly *M. m. bactrianus*, but the most prominent strain is *M. m. domesticus* [55]. WSP and WSR mice were selectively bred for 25 generations [5], and have since been maintained under relaxed selection with quasi-random mating (within line). The tested mice were from generations S₂₆G₁₃₄ and S₂₆G₁₃₅, where S_{xx} refers to the number of selection generations, and G_{yy} refers to the total number of breeding generations that have elapsed since the beginning of selection. After weaning and prior to experiments, same-sex mice were housed 1–5 animals per plastic cage (28 × 17 × 11.5 cm; Thoren Caging Systems, Hazelton, PA, USA) lined with ECOFresh bedding (Absorption Corporation, Ferndale, WA, USA) with stainless-steel wire tops; rodent chow 5001 (PMI Nutrition International, Brentwood, MO, USA) and tap water were available ad libitum. All colony and procedure rooms were on a 12 h:12 h light: dark cycle (lights on at 0600 PDT) at a temperature of 21 ± 1 °C. We aimed to have 10 mice per sex and ethanol treatment group for each experiment. We previously reported that this number of animals produced significant ethanol effects on nest building [22]. Mice were euthanized via CO₂ asphyxiation followed by cervical dislocation after the completion of behavioral testing. All procedures were in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and approved by the VA Portland Health Care System Institutional Animal Care and Use Committee.

2.2. Drugs and administration

2.2.1. Acute ethanol injection—Ethanol (Decon Laboratories, Inc., King of Prussia, PA, USA) was diluted in 0.9% saline (20% v/v) and injected intraperitoneally (i.p.) at a dose of 2

or 4 g/kg. Animals were randomly assigned to treatments within sex and line using a random-number generator. Animals were further randomized for injection order and behaviorally tested in the same order. Injection volumes were based on body weight; volumes of saline equivalent to those for the maximal ethanol dose were administered to control mice. Ethanol doses were chosen from other acute withdrawal experiments where 4 g/kg was an effective dose for eliciting increased HICs that begin to peak at about 6 h after injection [31,44,45, 57].

2.2.2. Experiment 1: dose and time response to acute ethanol withdrawal in WSC mice

A total of 38 male and female WSC mice (5–9 per treatment group and sex; Table 1) were 102–109 days old (28.22 ± 0.52 g body weight) at the start of Experiment 1. Mice began a nest-building test 6 h after being injected with 2 g/kg ethanol, 4 g/kg ethanol or saline.

2.2.3. Experiment 2: nest building in acutely-withdrawing WSP-1 and WSR-1 mice

A total of 86 male and female WSP-1 and WSR-1 mice (10–11 per treatment group, genotype and sex; Table 2) were 72–80 days old (26.40 ± 0.27 g body weight) at the start of Experiment 2. Experimental procedures were identical to those described for Experiment 1, except all mice were either injected with the higher dose of ethanol (4 g/kg) or saline before beginning the nest-building test.

2.2.4. Experiment 3: nest building in acutely-withdrawing WSP-2 and WSR-2 mice

A total of 80 male and female WSP-2 and WSR-2 mice (8–11 per treatment group, genotype and sex; Table 3) were 54–71 days old (25.11 ± 0.38 g body weight) at the start of experiment 3. Experimental procedures were identical to those described for Experiment 2.

2.2.5. Experiment 4: effects of acute ethanol withdrawal on locomotor activity in WSP-1 and WSR-1 female mice

We tested a total of 48 female WSP-1 and WSR-1 mice (75–90 days old; 23.34 ± 0.29 g body weight) for locomotor activity before and after injection of 4 g/kg ethanol or saline (24 per treatment; Table 4). Three passes of experimentally naïve mice were used, with 16 mice in each pass (8 per treatment). One week before testing, mice were moved to a procedure room to acclimate to a 12:12 h L/D cycle (lights on at 0900 h), tail marked, and weighed. Because standard water bottles could not be adapted to the activity monitors, a HydroGel™ pack (ClearH₂O, Portland, Maine USA) was placed in a small petri dish and introduced into the home cage in order to familiarize the mice to the novel substance on the day prior to testing.

Starting at lights on during the test day, mice were moved to the activity monitoring procedure room where they were allowed to acclimate for 1 h while the monitors were prepared. A thin layer of Bed-o-Cob bedding was placed directly into the locomotor activity box such that no photocell beams were blocked. A small petri dish with HydroGel was placed in a corner of each box as a liquid source for animals during testing, and 4 blocks of normal chow were placed in each corner of the activity box. At 1000 h, one mouse per activity monitor began an “acclimation test.” One hr after the start of testing, each mouse was individually removed in order of placement, injected i.p. with 4 g/kg ethanol or saline,

and placed back into the center of its respective monitor to begin the “treatment test” for the 32 h remaining in the monitoring session. Internal lights for the activity monitor chambers were kept on the same 12 h:12 h light/dark L/D cycle to which the mice had been acclimated for the duration of testing.

2.3. Behavior

2.3.1. Nest-building test—Mice were acclimated to a procedure room for 1 week on a 0800–2000 h L/D cycle. Starting at 0800 h on the first day of testing, mice were weighed and singly housed in cages lined with ECOfresh bedding. One hr after single housing, mice were injected with 4 g/kg ethanol or an equivalent volume of saline and returned to single housing. Six hrs after injection (1500 h), mice started the nest-building test. Eight g of Enviro-Dri® (FiberCore, Cleveland, OH, USA) shredded paper nesting material was introduced and distributed evenly on the bottom of the cage opposite the water bottle spout and a picture was taken from an overhead angle. Nests were scored on three consecutive days of testing at the following times after injection (by an observer blind to group): 10 h (day 1: 1900 h), 24 h, 28 h, 32 h (day 2: 0900 h, 1300 h, 1700 h) and 48 h (day 3: 0900 h). The schedule of nest scoring allowed for the observation of nest building deficits and tracking of recovery over time. The nest scoring methods are described in more detail elsewhere [22] and were derived from published descriptions [11,18]. Briefly, if the nesting material was untouched, or if some of it had been moved, a score of 0 or 1 was given, respectively. If a centralized nest site was present, the height of the nest wall for each quadrant was separately scored (range = 2 to 5) and the average was recorded. If the centralized nest site was flat against the cage bedding, the quadrant was given a score of 2. Once the nest walls became cupped, scores of 3, 4, or 5 were given based on whether the nest walls were less than half, approximately half, or greater than half the height of a full nest dome, respectively. The full dome height had been established to average 9.38 ± 0.60 cm [22].

2.4. Activity monitoring

In Experiment 4, activity was assessed using 16 automated monitors (AccuScan Instruments, Columbus, OH) with one mouse per chamber. The boxes ($40 \times 40 \times 30$ cm high) were constructed of clear plastic and confined within ventilated light- and sound-attenuated cabinets [48]. An internal light source (3.3 W incandescent bulb) was used to maintain the light cycle to which mice had been acclimated. During test sessions, activity was monitored from grids of 8×8 infrared beams affixed 6 cm above the test chamber floor (breaks for vertical movements) or 2 cm above the floor (breaks for horizontal movements). The primary sample duration for the activity monitoring software was defined as a 15-min epoch, across 132 epochs, for a total session length of 33 h. Each 15-min epoch was further summed into 1 h bins for analysis, because initial examination showed that the data were similarly represented by these shorter and longer time phases. Data were summed across time periods of interest before being analyzed, as described in the Results section.

2.5. Statistical analyses

SPSS (IBM) version 20 was used for statistical analyses. Data were analyzed using analysis of variance (ANOVA), unless noted. While nest building scores are ordinal, non-parametric analyses of the current data and other data collected similarly [22] yielded outcomes nearly identical with the more familiar parametric ANOVA results we report. Data normality for activity data was assessed with histograms, Q-Q plots and Shapiro-Wilk tests. Homogeneity of variance was assessed with Levene's test. For nest building experiments, three- or four-way ANOVA was initially performed to assess effects of ethanol treatment, genetic line, and sex with time point as a repeated measure. Experiment 2 and Experiment 3 were conducted as separate parallel experiments at different times, so they were analyzed as independent 4-way ANOVAs.

We separately assessed whether nest building deficits appeared during two time periods of acute ethanol withdrawal: 1) during the initial day of withdrawal when signs of acute ethanol withdrawal have been previously observed (10 and 24 h after injection of ethanol), and 2) during the second day of withdrawal when these signs typically subside (28, 32 and 48 h after injection). The details of this time course are described in the Introduction. When significant interactions were found, lower order ANOVAs were conducted and significant interactions pursued to the level of individual comparisons conducted with Bonferroni corrections. Distributions of the horizontal and vertical activity data during the post-treatment period from Experiment 4 were non-normal, with positive skews. These data were normalized using a square-root transformation prior to statistical analysis, and data are presented graphically as transformed. All significant results ($p < 0.05$) are reported.

3. Results

3.1. Experiment 1: acute ethanol withdrawal dose-dependently reduced nest building in WSC mice

Experiment 1 was designed to test for effects of ethanol dose on nest building during withdrawal in unselected WSC mice. Results are shown in Fig. 1. Three-way ANOVA for scores at 10 and 24 h after injection revealed a significant main effect of ethanol dose ($F_{2,32} = 4.143$, $p = 0.025$) and a significant main effect of time ($F_{1,32} = 78.899$, $p < 0.001$), but not sex ($F_{1,32} = 0.514$, $p = 0.541$). No interactions were significant (All F values < 1). Nest scores were reduced in ethanol-withdrawing mice, but their improvement over time paralleled saline-administered controls (Fig. 1-left panel). Post-hoc tests for dose revealed that 4 g/kg ethanol-treated mice had lower nest scores than mice administered saline ($p = 0.005$). Mice treated with 2 g/kg ethanol did not have nest scores that were significantly different from those of saline-treated mice ($p = 0.153$), or 4 g/kg ethanol-treated mice ($p = 0.460$).

Three-way ANOVA for the final three nest assessments (28, 32 and 48 h after injection) revealed the effects of ethanol dose were no longer present during the second day of withdrawal (Fig. 1-right panel). There was a significant main effect of time ($F_{2,64} = 5.057$, $p = 0.009$), as nest scores improved over time over the second day of testing. There were no other significant main effects or interactions (All F values < 1.165 , p values > 0.289).

3.2. Experiment 2: acute ethanol withdrawal suppressed nest building in WSP-1 vs WSR-1 mice: genotype-specific and sex-specific effects

Nest scores were first assessed for the first day of withdrawal. As expected from Experiment 1, ethanol reduced nest scores and the quality of nest scores increased over time. Four-way ANOVA revealed significant main effects of treatment ($F_{1,78} = 19.204$, $p < 0.001$) and time ($F_{1,78} = 296.844$, $p < 0.001$), and there was a significant treatment $\times$ line $\times$ sex $\times$ time ($F_{1,78} = 9.606$, $p = 0.003$) interaction. We next performed three-way ANOVAs, analyzing the sexes separately (Fig. 2).

In females, (Fig. 2A-left panel), there were significant main effects of treatment ($F_{1,40} = 11.428$, $p = 0.002$), line ($F_{1,40} = 8.276$, $p = 0.006$) and time ($F_{1,40} = 120.595$, $p < 0.001$), and a significant treatment $\times$ line $\times$ time interaction ($F_{1,40} = 10.874$, $p = 0.002$). Follow up analyses showed significant lower-order interactions (not shown). Individual comparisons revealed that WSR-1 females given saline showed the highest nest scores at 10 h after injection. They had higher nest scores than WSR-1 mice treated with ethanol ($t_{1,21} = 4.785$, $p < 0.001$) and WSP-1 mice treated with ethanol ($t_{1,20} = 3.507$, $p = 0.006$), but not WSP-1 mice treated with saline ($t_{1,19} = 1.894$, $p = 0.222$). At 24 h after injection, ethanol-treated WSP-1 had the lowest nest scores, lower than WSP-1 given saline ($t_{1,19} = 2.651$, $p = 0.048$), WSR-1 mice given saline ($t_{1,20} = 3.207$, $p = 0.012$) and ethanol-treated WSR-1 mice ($t_{1,21} = 3.713$, $p = 0.003$).

Analysis of nest scores for the second day of withdrawal in females (Fig. 2A-right panel) revealed significant main effects of treatment ($F_{1,40} = 4.566$, $p = 0.039$), line ($F_{1,40} = 11.332$, $p = 0.002$) and time ($F_{2,80} = 18.034$, $p < 0.001$), as well as significant treatment $\times$ time ($F_{2,80} = 4.311$, $p < 0.017$) and line $\times$ time ($F_{2,80} = 6.882$, $p < 0.002$) interactions. There was a non-significant trend toward a treatment $\times$ line interaction ($F_{1,40} = 3.945$, $p = 0.054$). Ethanol-treated WSP-1 mice tended to have lower nest scores than WSP-1 mice given saline ($t_{1,19} = 2.386$, $p = 0.084$), whereas there was no significant difference between WSR-1 mice given saline vs ethanol.

In males (Fig. 2B), analysis of nest scores for the first day of withdrawal revealed a significant main effect of treatment ($F_{1,38} = 7.977$, $p = 0.008$) and a significant main effect of time ($F_{1,38} = 183.231$, $p < 0.001$), reflecting lower scores in ethanol-withdrawn mice and an increase in scores between 10 and 24 h. However, there was no significant main effect of line or significant interactions (Fig. 2B-left panel). Analysis of nest scores in males during the second day of withdrawal revealed a significant main effect of time ($F_{2,76} = 19.479$, $p < 0.001$) and a significant line $\times$ time interaction ($F_{2,76} = 3.189$, $p = 0.047$). WSP mice tended to have lower nest scores than WSR mice at 28 h after injection ($t_{1,40} = 1.728$, $p = 0.092$), regardless of treatment, but not at any other time point (Fig. 2B-right panel).

3.3. Experiment 3: effects of acute ethanol withdrawal on nest building in WSP-2 and WSR-2 mice

Results for experiment 3 are shown by sex, treatment and line in Fig. 3 so that data can more easily be compared with Fig. 2. Initial analysis of nest scores by four-way ANOVA for the first day of withdrawal (Fig. 3A and B, left panels) revealed significant main effects of

treatment ($F_{1,72} = 16.602$, $p < 0.001$) and time ($F_{1,72} = 86.999$, $p < 0.001$), and a significant line $\times$ time interaction ($F_{1,72} = 19.842$, $p < 0.001$). To better illustrate the line $\times$ time interaction, data are shown collapsed on sex in Fig. 3C. WSR-2 mice had higher nest scores than WSP-2 mice ($t_{1,78} = 2.987$, $p = 0.004$) at 10 h after injection, but lower scores than WSP-2 mice at 24 h after injection ($t_{1,78} = 2.512$, $p = 0.014$) (Fig. 3C-left panel). The main effect of treatment was due to a suppression of nest scores by ethanol treatment, regardless of genetic lines and sex (Fig. 3D, left panel).

Analysis of nest score data for the second day of withdrawal (Fig. 3, right panels) identified significant main effects of treatment ($F_{1,72} = 5.781$, $p = 0.019$) and time ($F_{1,72} = 34.667$, $p < 0.001$), and a significant treatment $\times$ time interaction ($F_{1,72} = 12.798$, $p < 0.001$). Nest scores were lower in ethanol-treated than in saline-treated mice at 28 ($t_{1,78} = 4.114$, $p < 0.001$), and 32 h ($t_{1,78} = 2.139$, $p = 0.036$), but not 48 h ($t_{1,78} < 1$) after injection.

3.4. Experiment 4: withdrawal-associated locomotor reductions did not parallel withdrawal-associated deficits in nest building in female WSP-1 or WSR-1 mice

Horizontal and vertical activity were monitored for 33 continuous hrs, and all untransformed data are shown in supplementary Fig. S1. During the initial 1-h acclimation period, female WSP-1 mice were more active than female WSR-1 mice (see Fig. S1 Panel A; $F_{1,44} = 10.764$, $p = 0.002$), but there was no significant effect of treatment and there were no significant interaction effects. For vertical activity, female WSR-1 mice were significantly more active than female WSP-1 mice (Fig. S1, Panel B; $F_{1,44} = 7.619$, $p = 0.008$), regardless of treatment.

Prior to analyzing the activity data collected after treatment with saline or ethanol, data from the first 15 min epoch following injection were removed because the experimenter's hand entered the test chamber in order to administer the injection. The bin from hr 2 of 33 was reduced to 45 min because we removed the first 15-minute epoch of that bin from the analysis (see Results). We first examined the time periods that corresponded with nest building deficits observed in female WSP-1 mice but not female WSR-1 mice (i.e., 10–24 h after injection; see Fig. 2A-left panel). Results for this experiment are shown in Fig. 4 by treatment and line so that data can be compared with Fig. 2. The three-way ANOVA for horizontal activity data revealed significant main effects of treatment ($F_{1,44} = 12.429$, $p = 0.001$) and time ($F_{13,572} = 43.546$, $p < 0.001$). There were also significant treatment $\times$ time ($F_{13,572} = 3.841$, $p < 0.001$) and line $\times$ time ($F_{13,572} = 8.376$, $p < 0.001$) interactions. A two-way ANOVA for summed horizontal activity data revealed significant main effects of treatment (Fig. 4A, $F_{1,44} = 9.747$, $p = 0.003$) and line ($F_{1,44} = 6.926$, $p = 0.012$), but the treatment $\times$ line interaction was not significant.

For vertical activity data collected 10–24 h after injection, the three-way ANOVA revealed significant main effects of treatment ($F_{1,44} = 12.883$, $p = 0.001$), line ($F_{1,44} = 6.883$, $p = 0.012$) and time ($F_{13,572} = 49.394$, $p < 0.001$), as well as significant treatment $\times$ time ($F_{13,572} = 10.690$, $p < 0.001$) and line $\times$ time ($F_{13,572} = 3.415$, $p < 0.001$) interactions. For vertical activity data summed across 10–24 h after injection (Fig. 4B) there was a significant main effect of treatment ($F_{1,44} = 15.290$, $p < 0.001$) but not line ($F_{1,44} = 3.229$, $p = 0.079$), and the treatment $\times$ line interaction was not significant.

ANOVA for horizontal activity data across 24–32 h after injection revealed significant main effects of line ($F_{1,44} = 10.500$, $p = 0.002$) and time ($F_{7,308} = 2.888$, $p = 0.006$), but not treatment. There were no significant interactions. For horizontal activity data summed across 24–32 h after injection, the only significant effect was the main effect of line (Fig. 4C, $F_{1,44} = 8.389$, $p = 0.006$). ANOVA for vertical activity data 24–32 h after injection revealed significant main effects of line ($F_{1,44} = 4.778$, $p = 0.034$) and time ($F_{7,308} = 4.762$, $p < 0.001$), but not treatment ($F_{1,44} = 0.048$, $p = 0.828$). There were no significant interactions. When data were summed across 24–32 h after injection, there were no significant statistical results (Fig. 4D).

ANOVA for horizontal activity data during hrs 6–10 revealed significant main effects of treatment ($F_{1,44} = 18.391$, $p < 0.001$), line ($F_{1,44} = 6.712$, $p = 0.013$) and time ($F_{3,132} = 46.423$, $p < 0.001$), as well as a significant treatment $\times$ time ($F_{3,132} = 4.180$, $p = 0.007$) interaction. Two-way ANOVA for the horizontal activity summed across hrs 6–10 revealed a significant main effect of treatment (Fig. 4E, $F_{1,44} = 16.770$, $p < 0.001$), but no main effect of line ($F_{1,44} = 2.211$, $p = 0.144$) or interaction between the two. ANOVA for vertical activity data during this time period revealed significant main effects of treatment ($F_{1,44} = 26.309$, $p < 0.001$), line ($F_{1,44} = 34.389$, $p < 0.001$) and time ($F_{3,132} = 45.396$, $p < 0.001$), as well as significant treatment $\times$ time ($F_{3,132} = 12.465$, $p = 0.001$) and line $\times$ time ($F_{3,132} = 27.251$, $p < 0.001$) interactions. Two-way ANOVA for summed vertical activity data revealed significant main effects of treatment (Fig. 4F, $F_{1,44} = 20.002$, $p < 0.001$) and line ($F_{1,44} = 34.017$, $p < 0.001$), but the treatment $\times$ line interaction was not significant.

4. Discussion

4.1. Evidence for nest building as a novel ethanol withdrawal phenotype and overlapping genetic influence with HIC severity

WSC mice in Experiment 1 displayed dose-dependent, acute ethanol withdrawal-induced nest building deficits. These findings suggest that nest building is a sensitive measure of ethanol withdrawal, with nest building deficits increasing relative to the degree of acute ethanol exposure. In Experiment 2, we observed partial evidence that nest building depression during withdrawal reflects genetic influences similar to those influencing ethanol withdrawal HICs. Acute ethanol withdrawal reduced nest scores in female WSP-1 mice for up to two days, but it suppressed nest building in female WSR-1 mice only at 10 h into withdrawal. Nest building was also reduced in male WSP-1 and WSR-1 mice during the first day of withdrawal. The effects of acute ethanol withdrawal on nest building appear to be longer-lasting than effects on HICs at the higher ethanol dose we used. Elevated HIC scores were previously shown 6–12 h following administration of an acute, high dose (4 g/kg) of ethanol in male and female WSP but not WSR mice of both genetic replicates, but they returned to baseline levels 24 h after injection [31]. It is likely that the effects of acute ethanol withdrawal on HICs and nest building do not wax and wane in complete parallel, because there is only partial overlap in the genes and neural circuitry which mediate the two behavioral responses.

Sex-dependent effects of acute ethanol withdrawal on physiology may be related to the sex differences we observed for nest building in Experiment 2. Adrenalectomy/gonadectomy

increased HICs in female WSP-1 and WSR-1 mice exposed to a single 4 g/kg ethanol injection compared to sham surgery mice [57]. This effect occurred earlier and was more prolonged compared to males, and it was associated with decreased progesterone levels, as animals having the highest acute withdrawal severity had the lowest progesterone levels (i.e., a negative correlation). Female WSR-1 mice that had undergone sham surgeries displayed the greatest increase in progesterone following acute withdrawal that was not observed in WSR-1 male mice. This work suggests that female WSP-1 and WSR-1 mice, particularly WSR-1 mice, produce a neuroadaptive response that increases progesterone as a protective mechanism. This response may explain why female WSR-1 mice had a faster recovery than female WSP-1 mice from acute withdrawal-associated nest-building deficits in Experiment 2. Progesterone implants, similar to cold temperatures, dose-dependently stimulated nest building in virgin female mice from a genetically heterogeneous outbred stock (HS/Ibg) [54].

Results from Experiment 3 with the second replicate of selectively-bred mice suggest that the effects of ethanol withdrawal may supersede any effects of genetic background on nest-building behavior. In addition to suppressing WSP-2 nest building, the effect of ethanol treatment extended to WSR-2 mice. WSR-2 mice are resistant to the effects of ethanol withdrawal on HICs. However, there were overall genetic differences in nest score, some of which resemble their differences in HIC magnitude. In Experiment 2, female WSP-1 mice had lower nest scores than female WSR-1 mice 10 h after injection of saline. Earlier generations of WSP-1 administered saline or air had greater HIC scores than WSR-1 mice acutely administered saline or chronically exposed to air [6,31]. We also identified effects of line during different time points from Experiment 3. WSP-2 mice had lower nest scores than WSR-2 mice 10 h after ethanol and saline injection but increased nest scores 24 h after ethanol or saline injection.

One likely explanation for why the genetic differences we observed in nest building did not strictly parallel HIC-selected differences is that there are partially separate genetic influences on nest building behavior and HICs during ethanol withdrawal. However, future experiments could provide support for similar genes influencing HICs and nest building during ethanol withdrawal. Although we did not assess correlations between HIC severity and nest-building scores within the same mice, this direct correlational approach could support the use of either measure interchangeably during ethanol withdrawal. We chose not to test for HICs because nest building is negatively influenced by conditions with a stressful aspect, such as housing male mice with unknown male cagemates [49].

4.2. Nest building deficits during withdrawal do not reflect withdrawal-associated impairments of overall activity

Our final experiment was conducted as a control to determine whether reduced nest building may be confounded by differences in activity, specifically due to low motivation, disordered motor performance, malaise, or other factors. To our knowledge, our Experiment 4 is the first time that activity has been monitored continuously over acute ethanol withdrawal in a home cage-like setting. One specific hypothesis was that female WSP-1 mice that displayed long-lasting ethanol withdrawal-associated deficits in nest building might also have

decreased overall activity, but that this decrease would not occur in female WSR-1 mice that were resistant to the nest building deficits. Experiment 4 revealed reduced horizontal activity 10–24 h after ethanol injection, but, contrary to the hypothesis, this effect was carried mostly by suppressed horizontal activity in ethanol-withdrawn WSR-1 mice that did not display decreased nest building during this time period. Female WSP-1 mice in Experiment 2 continued to display nest building suppression up to 32 h after injection of ethanol, but there was no effect of ethanol treatment or selected line on horizontal activity from 24 to 32 h after injection. Thus, generally lower activity was not the source of the nest building deficits. Addressing our second specific hypothesis, the reduced nest building in withdrawing female WSR-1 mice during earlier time points was not paralleled by reduced activity. In general, our results provide evidence that nest building deficits during ethanol withdrawal are not due to general malaise, consistent with activity levels we have reported following chronic ethanol withdrawal in these lines [29].

In Experiment 4, we also compared activity in female WSP-1 and WSR-1 mice administered saline. Saline-injected female WSP-1 mice had lower nest scores than saline-injected female WSR-1 mice during the period 6–10 h after injection. However, WSP-1 mice tended to have greater activity than WSR-1 mice 6–10 h after either ethanol or saline injection. Examinations of circadian nest building activity in female C57BL/6J mice revealed that mice began building nests soon after presentation of nesting material with short breaks during the light phase; but during the start of the dark phase, they had periods of locomotor activity during which nests were destructed [26]. Therefore, increased activity in WSP-1 mice near the end of the light phase and start of the dark phase may explain the decreased nest scores observed during these early time points.

We did not assess activity in ethanol-naïve WSP-2 and WSR-2 mice in this study. However, nest building results from Experiment 3 produced intriguing parallels to previous studies that monitored activity in naïve male WSP-2 and WSR-2 mice over a 24 h period, WSR-2 mice displayed scattered bouts of activity near the end of their light phase that were not observed in WSP-2 mice, but WSP-2 mice were more active than WSR-2 mice from the middle of the dark period to the early time points of the light phase [42]. These activity patterns may explain the differences in genetic line we observed on nest building in Experiment 3. WSP-2 mice had lower nest scores than WSR-2 mice at 10 h after injection (i.e., at the end of the light phase), but they then displayed sharper increases in nest building during the dark period, resulting in nest scores that surpassed those of WSR-2 mice at 24 h after injection (i.e., the beginning of the second light phase). Therefore, while our studies provide little evidence that ethanol withdrawal-induced suppression of nest building simply reflects decreased activity, our nest building experiments in combination with previous activity monitoring studies suggest that nest building behavior may parallel periods of activity in non-withdrawing mice.

4.3. Nest building deficits during ethanol withdrawal may reflect withdrawal-associated disruptions of task completion

Single-gene manipulation studies further separate deficits in nest building from decreased locomotor activity, and they parallel neuropathologies observed in models of ethanol

withdrawal. Hippocampal impairments and excitotoxicity are implicated in genetic disorders that produce deficits “in activities of daily living” (ADLs). Nesting is a spontaneously-performed, daily activity that has been used as a surrogate for ADLs in transgenic mouse models for disorders such as Huntington’s Disease and Alzheimer’s disease in the absence of locomotor activity deficits [10,13,46]. Intriguingly, deficits in nest building in transgenic mouse models for Alzheimer’s disease are rescued following treatment with memantine, a non-competitive NMDA receptor antagonist that blocks glutamate neurotoxicity [14]. Memantine also reduces chronic ethanol withdrawal severity as measured by HICs in Swiss Webster mice [56] and audiogenic seizures in rats [3,32], and it reduces hippocampal toxicity when applied to explants from mice during withdrawal from chronic ethanol exposure [56]. In humans, there is clinical evidence that memantine attenuates alcohol withdrawal severity and alcohol craving during withdrawal [1,33,34].

We performed an additional experiment in female WSP-1 and WSR-1 mice in which 10 mg/kg memantine was administered 5 h after injections of ethanol (4 g/kg) and 1 h prior to a nest-building test. This dose of memantine was previously found to attenuate ethanol withdrawal-associated seizures in mice and rats when administered 30 min prior to testing. We replicated the original genetic finding and the long-lasting effect of ethanol withdrawal on nest building in female WSP-1 mice (Data not shown). However, withdrawal-associated deficits in nest building were not reversed by noncompetitive NMDA receptor blockade in female WSP-1 or WSR-1 mice. We cannot rule out the importance of glutamatergic signaling, particularly at NMDA receptors, for ethanol withdrawal-associated nest building deficits from this single experiment.

4.4. Nest building deficits during ethanol withdrawal may reflect withdrawal-associated disruptions of thermoregulation

Although we did not test thermoregulation here, we found in a separate line of experiments that deficits in nest building are not associated with body temperature during chronic ethanol withdrawal in mice from a genetically heterogeneous background [22]. We have discussed extensively in that paper hypotheses for how disrupted thermoregulation during ethanol withdrawal may influence nest building.

4.5. Nest building deficits during ethanol withdrawal as a sign of affective state

Tests of nest building have been proposed as assays for affective behavior, particularly positive motivational states [9,12]. Nest building was delayed in C57BL/6J mice for up to 7 h following a subchronic social defeat stress paradigm, which also induced depressive-like social aversion behavior [47]. Depression-like behaviors, including learned helplessness and anhedonia, have also been detected following chronic ethanol withdrawal in rodent models [24]. More recently, depression like behavior was detected for up to 14 h following acute ethanol withdrawal in Swiss mice, as withdrawing mice displayed decreased latency to immobility and increased percent time being immobile during the tail-suspension and forced-swim tests [27]. Further testing is necessary to determine the relationship between depression-like behavior and delayed nest building during ethanol withdrawal.

Future studies should investigate brain mechanisms that link delayed nest building to negative affective states following ethanol withdrawal. Serotonergic signaling has been targeted for anxiety-like behavior during ethanol withdrawal [15] and for changes in nest building that may reflect anxiety-like behavior [37]. SSRI administration decreased nest shredding without affecting locomotor activity in Swiss mice, suggesting nest building may be an assay sensitive to anxiolytic-like effects of SSRIS[37]. Mice transgenic for modeling Down Syndrome had increased 5-HT_{2A} receptor expression in frontal cortex and displayed nest building deficits that were rescued by 5-HT_{2A} receptor blockade [25]. 5-HT_{2A} receptors have also been targeted for anxiety like behavior during ethanol withdrawal, and the 5-HT₂ antagonist mianserin blocked the anxiogenic effects of chronic ethanol withdrawal on the elevated plus maze [35]. Therefore, serotonergic signaling is a potential future direction for targeting nest building deficits during ethanol withdrawal that may reflect a change in affective state.

4.6. Conclusions

Our data indicate that nest building can be used to index severity of acute ethanol withdrawal. Effects of ethanol withdrawal on nest building were dose-dependent. Genetic background also influenced the progression of nest building over time. The long-lasting effects of the high dose of ethanol on nest building extended to mice that were bidirectionally selectively bred for chronic ethanol withdrawal severity, with the exception of female WSR-1 mice which appear to be particularly resistant to long-lasting nest building deficits. Finally, ethanol withdrawal-induced deficits in nest building did not simply reflect decreases in locomotor activity.

The observed effects of ethanol withdrawal on nest building could have been altered by a lack of previous nesting exposure prior to the start of experiments, either due to experience or because our mice were housed at temperatures that produce cold stress. Experiments in HS/Npt/Pdx mice suggest that withdrawal-associated deficits in nest building can still be detected with prior exposure to nesting material, but the cumulative relationship between cold stress and thermoregulatory effects of ethanol withdrawal on nest building was inconclusive [22]. HS/Npt/Pdx mice tended to have decreased nest scores 28 h after chronic ethanol withdrawal, and these mice had previous exposure to nesting material during a baseline nest-building test. Body temperature was related to nest scores before but not after ethanol withdrawal, so it is unclear exactly how nest-building deficits after ethanol withdrawal related to disrupted thermoregulation; however, we discussed this potential interaction more extensively in that manuscript. The exposure of mice to injections may have also influenced nest building. Mice that received injections of either lipopolysaccharide to induce sickness or saline alone by a novel handler had lower nest scores from baseline, with saline-injected mice having nests reduced by a score of 1 on average [19]. Future studies should further examine effects of stress induced by cold temperature and injections on ethanol withdrawal-related nest-building deficits.

Nest-building tests have been used as behavioral assays for thermo-regulation, affective states and motor function in rodent models, all of which are modulated during ethanol withdrawal. It is possible that deficits in nest building during withdrawal may be the

manifestation of disruptions across all of these areas, and future studies are needed to understand the underlying mechanisms mediating withdrawal-induced suppression of nest building. This assay has the potential to be tested across a wide variety of species. HICs are a commonly used to assess ethanol withdrawal severity in mice, but they cannot be elicited in rats. Nest building is an innate behavior that is evolutionarily conserved across many species, including mice, gerbils [20], birds [23], and Siberian hamsters [28]. During withdrawal from an acute, high dose of ethanol, HICs usually last for 6–14 h before subsiding and returning to basal levels by 24 h [5,31]. Other behavioral phenotypes of acute ethanol withdrawal have mostly been studied for effects lasting less than a day. Nest building could be a useful phenotype/marker of withdrawal that lasts longer into the withdrawal period.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding sources

Experiments were supported by grants 101BX000313 and 101BX002106 from the U.S. Department of Veterans Affairs, National Institute on Alcohol Abuse and Alcoholism T32 AA007468, R24 AA020245, INIA U01 AA013519, the VA Portland Alcohol Research Center (PARC) P60 AA010760 and Methamphetamine Abuse Research Center (MARC) P50 DA018165.

We thank Jason Schlumbohm and Lawrence Huang for technical assistance and animal colony maintenance. Thank you to Pamela Metten and Angela Ozburn for aiding in the conceptualization and design of the nest building experiments. Thank you to Cheryl Reed, Andy Zhu and Harue Baba for assistance with design and implementation of the activity monitoring experiments.

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HIGHLIGHTS

- Nest building is dose-dependently impaired by acute ethanol withdrawal in mice.
- Nest building deficits were present in mice selectively bred for high and low withdrawal severity from chronic ethanol vapor.
- Nest building deficits could not be explained by a general decrease in locomotor activity.

Fig. 1.

Effects of acute ethanol dose on nest building in mice from a heterogeneous genetic background. Nest scores for WSC mice injected with saline, a high dose of ethanol (4 g/kg) or lower dose of ethanol (2 g/kg). Hatched bars indicate hrs during lights off. Ethanol or saline was injected at hr 0, and nesting material was added at hr 6 (arrow). Data are shown as mean $\pm$ SE, $n = 11-14$ per treatment. Data are collapsed across sex. * indicates nest scores in the 4 g/kg group were significantly lower ($p < 0.05$), compared to the saline group. EtOH = ethanol.

Fig. 2.

Effects of acute ethanol withdrawal on nest building in WSP-1 and WSR-1 mice. Graphs show nest building in (A) female and (B) male WSP-1 and WSR-1 mice administered saline or a high dose of ethanol (4 g/kg). Hatched bars indicate hrs of lights off. Ethanol or saline was injected at hr 0, and nesting material was added at hr 6 (arrow). Data are shown as mean $\pm$ SE, $n = 10-12$ per selected line, sex and treatment. * indicates a statistically significant difference ($p < 0.05$) in nest scores at individual time points from corresponding saline group. † indicates a trend for a difference ($p < 0.10$) in nest scores averaged across time points from corresponding saline group. ‡ indicates a statistically significant ($p < 0.05$) main effect of treatment on nest scores averaged across time points. EtOH = ethanol.

Fig. 3.

Effects of acute ethanol withdrawal on nest building in WSR-2 and WSP-2 mice. Although there were no statistically significant effects of sex, data are presented separately for females (A) and males (B) to facilitate comparison with Fig. 2. Data are also presented collapsed on sex (C) and selected line (D). Hatched bars indicate hrs of lights off. Ethanol or saline was injected at hr 0, and nesting material was added at hr 6 (arrow). Data are shown as mean $\pm$ SE, $n = 8-11$ per selected line, treatment and sex. * indicates a statistically significant difference ($p < 0.05$) in nest scores at individual time points from corresponding saline group. ‡ indicates a statistically significant ($p < 0.05$) main effect of treatment on nest scores

averaged across time points. § indicates a statistically significant difference ($p < 0.05$) in nest scores between lines at individual time points. EtOH = ethanol.

Fig. 4.

Effects of acute ethanol on locomotor activity. Data are presented separately by treatment and line to facilitate comparison with Fig. 2. Graphs show the square-root transformation of horizontal and vertical activity for female WSP-1 and WSR-1 mice. Data were summed across time points 10–24 h (A,B) and 24–32 h (C,D), or 6–10 h (E,F) after injection of saline or 4 g/kg ethanol. Data are shown as mean $\pm$ SE, $n = 12$ per line per treatment. * indicates a

statistically significant ($p < 0.05$) main effect of treatment. ⁺ indicates a statistically significant ($p < 0.05$) main effect of line. EtOH = ethanol.

Table 1

Sample sizes for Experiment 1.

Ethanol treatment	WSC Male	WSC Female
0 g/kg	5	6
2 g/kg	5	8
4 g/kg	5	9

Table 2

Sample sizes for Experiment 2.

Ethanol treatment	WSP-1 Male	WSP-1 Female	WSR-1 Male	WSR-1 Female
0 g/kg	10	10	11	11
4 g/kg	10	11	11	12

Table 3

Sample sizes for Experiment 3.

Ethanol treatment	WSP-2 Male	WSP-2 Female	WSR-2 Male	WSR-2 Female
0 g/kg	10	11	10	10
4 g/kg	10	11	10	8

Table 4

Sample sizes for Experiment 4.

Ethanol treatment	WSP-1 Female	WSR-1 Female
0 g/kg	12	12
4 g/kg	12	12