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Nest building is a novel method for indexing severity of alcohol withdrawal in mice

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Abstract

Withdrawal after chronic ethanol (EtOH) affects body temperature, goal-directed behavior and motor function in mice and increases general central nervous system excitability. Nest-building tests have been used to assay these states but to this point have not been employed as measures of EtOH withdrawal severity. We first refined nest-scoring methods using a genetically heterogeneous stock of mice (HS/Npt). Mice were then made physically dependent following three days of chronic EtOH vapor inhalation to produce average blood EtOH concentrations (BECs) of 1.89 mg/mL. EtOH withdrawal affected the progression of nest building over time when mice were tested 2–4 days after removal from three days of chronic exposure to EtOH. In a separate group of mice, chronic EtOH vapor inhalation (BECs 1.84 mg/mL) suppressed nest building over days 1–2 but not days 2–3 of withdrawal. In a following experiment, EtOH withdrawal dose-dependently slowed recovery of nest building for up to 32 h. Finally, we determined that long-lasting nest-building deficits extend to mice undergoing withdrawal from a high dose (4g/kg) of acute EtOH. Sex differences for nest building were absent following EtOH exposure. In mice naïve to EtOH treatments, male mice had lower pre-test body temperatures and increased nest scores across a two-day testing period compared to females. These results suggest that nest building can be used to assess chronic and acute EtOH withdrawal severity in mice.

Keywords

Ethanol; Withdrawal; Chronic; Acute; Nest; Mice

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1. Introduction

Although alcohol withdrawal is a complex syndrome, many studies using rodent models have focused on a single physical response; the enhanced sensitivity to and/or severity of seizures. When quantified as a convulsion score in mice, this response can be used as a measure that is sensitive to the duration of exposure and dose of alcohol [1]. In mice, handling-induced convulsions (HICs) have been used for four decades to assess the severity of withdrawal from chronic EtOH vapor exposure, and they are also observed during withdrawal from a single, high-dose injection of EtOH [1, 2]. Although seizures following chronic alcohol administration occur in a number of species [3, 4], HICs in particular have only been described in mice. Additionally, convulsions represent only one effect that appears during a limited time window of withdrawal. In humans, the subjective effects of alcohol withdrawal may be present for up to weeks or months after abstinence [5]. Therefore, behavioral tests that can identify alcohol withdrawal states for extended time periods and can be potentially applied to many model organisms would be valuable.

Nest building is a behavior performed by mice and many other species. A mouse provided with nesting material will move it to a centralized location and build up walls. Nests in mice have been quantified based on the quantity of material used [6] or the quality of the nest, with high-quality nests having built-up walls that produce cupped or dome shapes [7, 8]. Nest measures have been used to assess thermoregulatory behavior [9], positive motivational states [10, 11] and sensorimotor function in mice [12]. These states are disrupted in mice over various time periods during withdrawal from chronic EtOH. During the initial 24 hours of withdrawal from three days of chronic EtOH vapor inhalation, dysregulated body temperature [13] and motor abnormalities (e.g., increased tremor) [14] were observed in mice from a genetically heterogeneous stock and in mice selectively bred for severe EtOH withdrawal HICs, respectively. Longer-lasting motor impairments on the balance beam and accelerating rotarod were dose-dependently induced by chronic EtOH vapor inhalation and observed up to 3 days after withdrawal in mice from a genetically segregating background [15]. Effects of alcohol withdrawal on affective states may also be longer-lasting [5], and C57BL/6J mice display increased depression-like behavior (i.e., increased immobility during a forced swim test) for up to 14 days after withdrawal from two-bottle choice voluntary alcohol drinking [16].

Here, we refined nest-scoring methods to test for effects of EtOH withdrawal in a genetically heterogeneous stock of mice (HS/Npt). Nest building allows for a reduction of heat loss and has been studied extensively for thermoregulatory purposes in singly-housed mice. Mice selectively bred for greater weight of cotton used for nesting at room temperature had higher body temperatures than the low-nesting line (positive correlated response) [17]. In our initial experiments, we assessed body temperature and nest building before and after withdrawal from chronic EtOH vapor inhalation. In separate groups of mice, we tested for time- and dose-dependent effects of chronic EtOH. Finally, we tested whether effects of EtOH withdrawal on nest building extended to an acute, high-dose EtOH injection. With these experiments, we present nest building as a novel marker of EtOH withdrawal severity that can be detected for extended periods of time during withdrawal, and potentially be applied to many species.

2. Materials and methods

2.1 Animals and husbandry

Naïve male and female mice from a genetically heterogeneous stock (HS/Npt) were bred in the VA Portland Health Care System's Veterinary Medical Unit and were 64–82 days old at the start of experiments. With the exception of one singly-housed animal, mice were housed 2–5 animals per plastic cage (28 x 17 x 11.5 cm) lined with Bed-O-Cob bedding (The Andersons, Maumee, OH, USA) and stainless steel wire bar tops with rodent chow 5001 (PMI Nutrition International, Brentwood, MO, USA) and tap water available *ad libitum*. These mice were derived from an 8-way cross of inbred mouse strains for which details have been previously described [18]. The colony room was held on a 12 h:12 h light:dark cycle [lights on at 2130 Pacific Daylight Time (PDT)] at a temperature of 21+/-1°C, and procedural rooms were kept within the same temperature range. Mice were not exposed to nesting material prior to being included in experiments. Animal numbers for each experiment are detailed in Table 1. All procedures were in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and approved by the Portland VA Health Care System's Institutional Animal Care and Use Committee.

2.2 Behavior

2.2.1 Nest-building test—Mice were singly housed and 8 g of Enviro-Dri® (FiberCore, Cleveland, OH, USA) shredded paper nesting material was flattened on the half of the cage opposite the water bottle spout and a picture was taken from an overhead, high angle. The start time of the nest test depended on the design for experiments described below, and nests were scored at various time points during the light phase. Images were captured at each time point for scoring confirmation and comparison with the initial placement of nesting material. The nest-scoring rubric was adapted from methods previously described in mice [7, 8]. If the nesting material was untouched, a nest was scored a 0. If the majority of nesting material was moved but scattered throughout the cage, the nest was scored a 1. Scores 2–5 were based on a centralized site built towards a maximum score of 5. We determined from Experiment 1 (Figure S1) that complete dome heights (measured from the floor of the cage to the apex of the dome) ranged from 7.60 to 10.10 cm (mean 9.38 ± 0.60 cm). Therefore, once a centralized site was present, the nest was conceptually split into quadrants, and the height of the nest wall for each quadrant determined the score. A score of 5 was given when the wall height was greater than half the height of a dome (>5cm), a score of 4 was given when a wall was approximately half the height of a dome (4–5cm), a score of 3 was given when the nest wall was less than half the height of a dome, but still cupped (<4cm), and a score of 2 was given when the wall was flat against the cage bedding. The experimenter scored all four quadrants of the nest, took measurements as needed, and a picture was taken from an overhead, high angle. The four scores were averaged for a total nest score. Mice had *ad libitum* access to food and water throughout the experiments.

2.2.2 Handling-induced convulsions—To confirm withdrawal severity during Experiment 2, we tested mice for HICs following exit from the EtOH vapor chambers. Mice were first observed for HICs after being picked up gently by the tail. If mice did not display

a convulsion, they were spun 180 degrees and scored using a 0–7 rubric that has previously been described [19].

2.2.3 Body temperature—Body temperatures were assessed at baseline and during withdrawal in Experiment 2. Mice were first singly housed without nesting material for at least 60 min prior to temperature assessments. Mice were then weighed and placed briefly in restrainers before taking rectal temperatures with a glycerol-lubricated probe for 5s (TH-8; Sontotek, Clifton, NJ). We tested for EtOH withdrawal induced changes in body temperature by subtracting the baseline body temperature from the body temperature recorded during withdrawal.

2.3 Drugs and administration

2.3.1 EtOH injections—Mice were injected intraperitoneally (i.p.) with EtOH (Decon Laboratories, Inc., King of Prussia, PA) diluted in 0.9% saline (20% v/v). Injection volumes were based on body weight with a dose that depended on the individual experiments described below, and a volume of saline equivalent to the maximal EtOH dose in the experiment was administered to control mice.

2.3.2 Vapor inhalation—Mice were exposed to EtOH vapor by inhalation (to induce physical dependence) or breathed air for 72 h continuously. These methods have been previously described [20]. Mice were first weighed and injected i.p. with a priming dose of EtOH depending on target blood EtOH concentration (BEC). Priming doses were 1.50 g/kg (0.010 mL 20% EtOH/g body weight), 2.00 g/kg (0.013 mL 20% EtOH/g body weight) and 2.50 g/kg (0.016 mL 20% EtOH/g body weight) for target BECs of 1.50 mg/ml, 2.00 mg/ml and 2.50 mg/ml, respectively. Mice were then placed in wire mesh cages within Plexiglas inhalation chambers at 0700h on day 1. Air control mice received injections of saline in volumes equivalent to the maximal dose in the experiment. All mice (air and EtOH) received a dose of pyrazole hydrochloride (68.1 mg/kg, Sigma, St. Louis, MO, USA, mixed with the EtOH or saline) during this initial injection in order to inhibit EtOH metabolism and maintain stable BECs during the 24 h vapor exposure. A subset of mice from EtOH groups was randomly chosen for tail-blood samples to estimate BECs and adjust chamber vapor concentrations as necessary using previously described procedures [21, 22]. All mice were briefly removed from the inhalation chambers 24 and 48 h after chamber entrance, weighed, and given booster injections of pyrazole. 72 h after chamber entrance, all mice were removed from chambers in order of placement, weighed, and had a tail-blood sample taken. Air control mice underwent sham blood collection with a tail snip. The tail was dipped briefly in hot wax followed by cold water to seal the blood collection site.

2.4 Statistical analyses

SPSS (IBM) version 20 was used for statistical analyses by analysis of variance (ANOVA). To assess nest scores across time, repeated measures 3-way ANOVA was performed to test for effects of treatment and sex as between-subjects factors and time points as the within-subjects factor. Significant results were followed up with lower-order ANOVAs. In experiment 4, we followed up significant effects of EtOH dose with Tukey's HSD post-hoc tests to compare different doses. Correlational analyses were performed with Spearman

correlations to test for relationships between body temperatures and nest scores and between BECs and nest scores.

2.5 Experimental design

Schematics of the design of individual experiments are incorporated into the figures depicting the results.

2.5.1 Experiment 1: Nest building progression in HS/Npt mice—8 male and female HS/Npt mice (4 per sex) were moved to a procedure room and singly housed at 0830h. Starting at 0900h, mice were given nesting material and observed every two hours (1100h, 1300h, 1500h) during the light phase for continuous nest building. A final observation took place during the following day at 1300h. Pictures were captured from an overhead, high angle and from the cage sides.

2.5.2 Experiment 2: Nest building and body temperature before and after chronic EtOH withdrawal—We began this experiment with 24 HS/Npt mice in the EtOH group (12 per sex) and 16 in the air group (8 per sex). 2 male mice from the EtOH group died in the chambers, and the data presented are from the 38 mice remaining. Mice were acclimated to a procedure room held on a 12 h:12 h light:dark cycle (lights on 0600 PDT) for seven days. On day 1 of testing, baseline body temperatures and body weight were recorded at 0800h. Mice began the nest-building test at 1000h. Nests were scored 4 h, 28 h, and 52 h after the start of the test. All nesting material was removed after the end of the test, and mice remained singly housed from days 4–7.

On day 8 starting at 0800h, mice were placed into EtOH vapor chambers or a chamber with only air for three days. We targeted a BEC of 2.00 mg/mL and confirmed mice were dependent following vapor exposure by testing for HICs immediately after and 8 h after removal from the chambers. We hypothesized that HICs would be elevated in EtOH-treated mice 8 h after removal from the chambers (a time we previously observed increased HICs [20]) compared to scores immediately after chamber exit when EtOH is present at measurable concentrations in the blood and exerts a well-known anticonvulsant effect. 24 h after exit from the chambers, mice were assessed for body temperature. Mice then began a nest-building test at 1000h, 26 h after exit from the chambers. Nests were scored 30, 54, and 78 h after exit from chambers (4 h, 28 h, and 52 h after the start of the test).

2.5.3 Experiment 3: Nest building during early time points of chronic EtOH withdrawal—We next tested whether nest building was impaired during the height of EtOH withdrawal severity measured by HICs. HS/Npt mice reach peak HIC scores between 4–7 h after removal from three days of continuous EtOH vapor inhalation with an average BEC of 1.84 mg/mL [20]. We began this experiment with 71 mice (21 male EtOH mice, 19 female EtOH mice, 16 male air mice, 15 air female mice). 8 male mice and 4 female mice from the EtOH group died. Data are from the remaining 59 mice in the study. First mice were acclimated to a procedural room held on a 12 h:12 h light:dark cycle (lights on 0600 PDT) for twelve days. Starting at 0700h on the first day of experimentation, mice underwent an EtOH vapor inhalation paradigm with a targeted BEC of 2.00 mg/ml. Mice began a first

nest-building test 5 h after removal from chambers, and nests were scored 7, 9, and 24 h after removal from chambers (when HIC scores are elevated during withdrawal [20]). Mice began a second nest-building test 27 h after removal from chambers, and nests were scored 29, 31, 33, and 48 h after chamber exit (when HICs have subsided).

2.5.4 Experiment 4: Dose-dependent response to chronic EtOH withdrawal—

We tested whether nest-building deficits during early time points of chronic EtOH withdrawal were dose dependent and tracked their recovery. We targeted BECs of 1.50 mg/ml, 2.00 mg/ml and 2.50 mg/ml. We began this experiment with 80 mice (12 mice per sex per group for the 2.50 mg/mL dose, 10 mice per sex per group for 1.5 and 2.0 mg/mL dose, and 8 mice per sex per group for the air group). 1 female from the 1.5 mg/mL and 1 male from each EtOH group died in the chambers. Data presented are from the remaining 76 mice. Mice were acclimated to a procedure room held on a 12 h:12 h light:dark cycle (lights on 0600 PDT) for six days. Mice entered EtOH vapor chambers at 0700h to begin a 3-day paradigm. The nest-building test commenced 5 hours after exit from the chambers, and nests were scored at 9, 24, 28, 32, 48, 52, and 56 h after exit from the chambers.

2.5.5 Experiment 5: Effects of acute EtOH withdrawal on nest building—

We tested whether deficits in nest building extended to early time points of acute EtOH withdrawal. 43 male and female mice (10–11 per sex per group) were used. Mice were acclimated to a procedure room for 11 days on a 12h:12h light/dark cycle (lights on 0800 PDT). On day 1 of testing, mice were singly housed at 0800h for 1 h. Mice were injected with 4g/kg EtOH (0.025 mL 20% EtOH/g body weight) or an equivalent volume of saline starting at 0900h. The 4g/kg dose was chosen based on other experiments in which 4g/kg was an effective dose for eliciting increased handling-induced convulsions (HICs) that peak 6–9 h after an injection [20]. We began testing mice during withdrawal from acute EtOH at 6 h after injection. Nests were scored over 2 consecutive days of testing at 10 h 24 h, 28 h, and 32 h after injection.

3. Results

3.1 Experiment 1: HS/Npt mice build complete, enclosed dome nests within 32 hour

Mice had moved nesting material into a centralized site and had begun to construct walls by 4 hours after material presentation. Cupped nests were present by 6 hours after presentation, and mice had built domes with enclosing walls by 32 hours after presentation (Figure S1). By the end of testing, any complete, enclosed dome heights were measured (from the floor of the cage to the apex of the dome); heights ranged from 7.60 to 10.10 cm (mean 9.38 $\pm$ 0.60 cm).

3.2 Experiment 2: Body temperature predicts nest-building scores before vapor chamber exposure but not after

When mice exited the vapor chambers, BEC values for EtOH-treated mice were 1.89 ± 0.08 , and there was no sex difference ($F_{1,20} < 1$). HIC scores taken immediately and 8 h after exit from the vapor chambers revealed a significant main effect of EtOH treatment ($F_{1,34} = 79.879$, $p < 0.001$) and time ($F_{1,34} = 113.004$, $p < 0.001$) and a significant treatment X time

interaction ($F_{1,34} = 163.386$, $p < 0.001$). There was no significant main effect of sex or any other interactions (All F values < 1). HICs increased over time after exit from the chambers in EtOH-withdrawing mice but not air controls (Fig. 1A), establishing that EtOH-treated mice were physically dependent.

Baseline nest scores were lower in female mice ($F_{1,34} = 5.781$, $p = 0.022$), and they improved over time in parallel with male mice (Fig. 1B; $F_{2,68} = 23.661$, $p < 0.001$). There were no other main effects or interactions (All F values < 1). A two-way ANOVA to assess body temperature before the start of the nest-building test revealed a significant main effect of sex ($F_{1,34} = 9.389$, $p < 0.01$) but neither effects of subsequent vapor assignment nor an interaction were significant ($F_{1,34} < 1$). Females had greater body temperatures than males (Table 2). Body temperature before the baseline nest-building test predicted nest score. Collapsed across sexes, there was a significant, negative correlation between body temperature and baseline nest score 4 h after nest material presentation (Fig. 1C, $\rho = -0.555$, $p < 0.001$). When data were split by sex, the correlations remained significant in both males ($\rho = -0.541$, $p = 0.021$) and females ($\rho = -0.474$, $p = 0.035$). Correlations with nest scores at 28 h and 52 h were negative but not significant (all $\rho > -0.208$, data not shown).

Three-way ANOVA for the three nest score time points during withdrawal (30, 54, and 78 h after withdrawal) revealed significant main effects of time ($F_{2,68} = 16.988$, $p < 0.001$) and a trend toward a treatment X time interaction ($F_{2,68} = 2.586$, $p = 0.083$). There were no other significant main effects or interactions (All F values < 1). The quality of nest scores increased over time, and the pattern of this increase tended to differ depending on treatment (Fig. 1D).

Body temperature 24 h after chamber exit tended to be higher in females than males (Table 2, $F_{1,34} = 4.101$, $p = 0.051$), but no main effect of treatment or interaction was seen (All F values < 1 , p values > 0.168). When testing for effects of EtOH withdrawal on the difference in body temperature from baseline, there were no main effects of treatment or sex or an interaction (Table 2, All F values < 1 , p values > 0.314).

3.3 Experiment 3: Nest building is suppressed during chronic EtOH withdrawal

Data are shown in Figure 2. The BEC values at the time of exit from chambers were 1.67 ± 0.11 , and there was no significant sex difference ($F_{1,27} = 2.675$, $p = 0.114$). Data from the first nest-building test (7, 9, and 24 h after chamber exit) and the second test (29, 31, 33 and 48 h after chamber exit) were analyzed separately. Early during withdrawal (Figure 2A), there were significant main effects of treatment ($F_{1,55} = 36.234$, $p < 0.001$) and time ($F_{2,110} = 35.187$, $p < 0.001$), and there was a trend for females to have higher nest scores than males ($F_{1,55} = 3.285$, $p = 0.075$). There were no significant interactions (All F values < 1). Collapsed across sexes, two-way ANOVA revealed that EtOH suppressed nest building ($F_{1,57} = 34.431$, $p < 0.001$), and withdrawing mice had nest scores that increased in parallel with air controls (Fig. 2A, $F_{2,114} = 36.687$, $p < 0.001$). There was no significant interaction ($F_{2,114} < 1$). BECs at the time of chamber exit predicted nest scores. There was a significant, negative correlation between the 72 h BEC and the nest scores 24 h after withdrawal (Figure 2B, Spearman's $\rho = -0.544$, $p = 0.003$).

Nest-building scores during the second test revealed a significant main effect of time ($F_{3,165} = 54.601$, $p < 0.001$) but no main effect of treatment ($F_{1,55} = 2.549$, $p = 0.116$). There were neither significant sex effects nor any interactions (All F values > 2.069 , $p > 0.156$).

3.4 Experiment 4: Nest building is dose-dependently suppressed during chronic EtOH withdrawal

To further examine the effect of alcohol withdrawal on nest building and to track recovery, we tested mice continuously from early to later time points for dose-response effects of alcohol withdrawal on nest building (see Fig. 3). BECs at time of exit from chamber for EtOH-treated mice were 1.76 ± 0.12 mg/ml, 2.12 ± 0.12 mg/ml, and 2.51 ± 0.13 mg/ml, respectively, in the low, medium and high dose groups. BECs broken down by sex and dose are displayed in Table 3. Two-way ANOVA revealed significant main effects of exposure group ($F_{2,54} = 10.378$, $p < 0.001$) and sex ($F_{1,54} = 9.577$, $p = 0.003$) but no interaction ($F_{2,54} < 1$). Males had greater BECs than females. One-way ANOVA collapsed across sexes revealed a significant main effect of dose ($F_{2,54} = 9.031$, $p < 0.001$). The low dose group had lower BECs than the high dose group ($p < 0.001$). The medium dose group tended to have different BECs from both.

Nest scores differed across doses ($F_{3,68} = 9.953$, $p < 0.001$) and times ($F_{6,408} = 162.49$, $p < 0.001$), and there was a significant dose X time interaction ($F_{18,408} = 7.042$, $p < 0.001$). Pictures of nests in this experiment from a representative mouse from each group are shown in Figure S2. There was no significant main effect of sex or any other significant interactions (all F values > 1.299 , $p > 0.256$). Alcohol withdrawal suppressed nest scores, and the pattern of improvement differed by dose (Fig. 3A). One-way ANOVAs revealed significant effects of dose at the first withdrawal test, at 9 h ($F_{3,72} = 92.263$, $p < 0.001$) and again at 24 h ($F_{3,72} = 329.196$, $p < 0.001$). Mice from all three EtOH groups had reduced nest scores compared to air controls (all p values < 0.001). By 32 h after chamber exit, there was still a significant effect of dose ($F_{3,72} = 3.162$, $p = 0.030$), and the air group had significantly greater nest scores than the high dose group ($p = 0.017$) but did not differ from the other alcohol withdrawing groups (p values > 0.237). There were no significant effects of alcohol dose on nest scores 48 h, 52 h or 56 h after exit from the chambers ($F_{3,72} < 1.431$, $p > 0.241$). BECs at the time of chamber exit and nest scores 24 h after exit revealed a significant, negative correlation between the two variables (Figure 3B, Spearman's $\rho = -0.268$, $p = 0.039$).

3.5 Experiment 5: Effects of acute EtOH on nest building

Nest scores showed significant main effects of treatment ($F_{1,39} = 9.995$, $p = 0.003$) and time ($F_{3,117} = 114.564$, $p < 0.001$). Neither effects of sex nor its interactions were significant ($F_{1,39} < 1$). There was a significant treatment X time interaction ($F_{3,117} = 3.257$, $p = 0.024$). Withdrawal from acute EtOH injection suppressed nest building and the pattern of increase of nest scores over time differed between groups (Fig. 4). EtOH withdrawal significantly reduced nest scores at 10 h ($F_{1,41} = 21.991$, $p < 0.001$) and 24 h after injection ($F_{1,41} = 5.333$, $p = 0.026$). EtOH withdrawal tended to reduce nest scores 32 hrs h ($F_{1,41} = 3.819$, $p = 0.058$) but not 28 h ($F_{1,41} = 2.527$, $p = 0.120$) after injection.

4. Discussion

4.1 Evidence for nest building as a measure of EtOH withdrawal severity

We present here a nest-scoring rubric refined from previously published nest-building methods in mice [7, 8]. Mice withdrawing from chronic EtOH vapor had reduced nest scores 5–24 h after exit from vapor chambers. BECs at the time of chamber exit in EtOH-treated mice were negatively correlated with nest scores 24 h after withdrawal, suggesting that nest-building deficits over the initial day of withdrawal are related to the dose of EtOH. This relationship was directly supported by Experiment 4, where mice displayed dose-dependent EtOH withdrawal-induced nest-building suppression. Mice receiving the highest dose continued to display nest-building deficits at 28h and 32 h after chamber exit, while mice receiving the low and medium doses recovered more rapidly. In Experiment 5, mice given an acute, high-dose injection of EtOH also displayed reduced nest building 6–32 h after injections. Our findings support the utility of nest building as a measure of chronic and acute EtOH withdrawal severity that is sensitive for up to two days after withdrawal.

4.2 Potential role of thermoregulation and other behaviors disrupted by EtOH withdrawal

Nest-building deficits in EtOH-withdrawing mice could reflect disrupted thermoregulation. In Experiment 2, body temperatures in male mice naïve to EtOH exposure were lower than female mice, and nest scores were higher in male mice than female mice. Similar sex differences in nest scores and body temperature were observed in naïve inbred (C57BL/6N and BALB/cAnN) and outbred (CD1) strains of mice that were assessed for nest building and housed in conditions similar to our study [23]. Therefore, results from our studies and others support a relationship between core body temperature and nest building. However, when the same mice from Experiment 2 were assessed for body temperature and nest building after vapor chamber exposure, there was no relationship between the two variables. Similarly, transgenic mice used for modeling Alzheimer's disease (TG2576 mice) showed nest-building deficits, but there was no relationship between nest building and body temperature in TG2576 mice [24]. Our results suggest the relationship between core body temperature and nest building is disturbed by the vapor chamber paradigm.

It could be hypothesized that animals with lower preferred temperatures (i.e., decreased thermal set point) would be less motivated to build nests, since typical colony and procedure rooms are maintained at temperatures that constitute a mild cold stress for mice. In female mice from a different but similar heterogeneous genetic background (HS/Ibg), both progesterone and exposure to cold temperatures stimulated nest-building, and it was hypothesized that the increase in thermoregulatory behavior was a response to an increase in thermal set point [25]. Results from Experiments 3 and 4 revealed that effects of chronic EtOH withdrawal on nest building were more pronounced during early stages of withdrawal (i.e., the first 24 h) when EtOH withdrawal-induced reductions in thermal set point have been observed. HS/Ibg mice were generally more motivated to seek cooler environments when tested in thermal gradients 1–26 h after chronic EtOH vapor inhalation, and this change in preference occurred in the absence of reduced core body temperature [13]. EtOH-withdrawing mice also displayed greater variability in preferred temperatures than either mice that breathed air and had been given daily injections of pyrazole in saline or injections

of saline alone during the initial 10–11 hours after exiting the vapor chambers. Mice selectively bred for high- and low-nesting behavior and naïve to any experimental treatments were tested in thermal gradient chambers for 1 h following a 3 h acclimation period [26]. Although there was no difference in selected temperature between the lines, variance in thermal preference for low-nesting mice was more than twice that of high-nesting mice. These results suggest that mice from the low selected line choose a wider range of selected temperatures than high-nesting mice, similar to the wider range in selected temperatures observed in HS/Ibg mice following chronic EtOH withdrawal.

Nests of different qualities retain heat differently, allowing mice some control over their selected temperature. Older studies in rats found a negative relationship between nest-building activity (i.e., the number of strips used for nests) and ambient temperature [27]. In addition to changes in the amount of nest-building behavior with changes in temperature, more recent evidence suggests that the quality of nests differ with ambient temperature. Naïve C57BL/6N, CD1 and BALB/cAnN mice that built higher-quality nests had decreased amounts of radiated heat, suggesting that high-quality nests are insulating and trap heat [23]. It was reported that the shape of nests that mice built changed with ambient temperature, with dome-like nests more common in lower temperatures and cupped shaped nests becoming more prevalent with increased temperatures [28]. Here, nests that approached dome-like structures with encapsulating walls were given a maximum score of 5. In Experiment 4, mice that breathed air reached average nest scores that were greater than 4 by 24 h after exit from the chambers (Fig. 3). In contrast, EtOH-withdrawing mice had average nest scores between 1 and 3 (i.e., nests that are open without walls that are at least half the height of a full dome) by 24 h after exit from the chambers, and they did not exceed average scores of 4 until 48–56 h after exit from the chambers. This delayed progression in EtOH-withdrawing mice towards domed nests is depicted in Figure S2, where representative mice that received medium (2.0 mg/mL) and high (2.5 mg/mL) doses of EtOH started to build nests by the second day but did not have enclosed nests until the third day of testing.

An alternative hypothesis is that disrupted locomotor activity during EtOH withdrawal underlies nest-building deficits. A separate line of experiments that examined nest-building deficits after acute ethanol withdrawal did not support this hypothesis. Mice selectively bred for severe chronic EtOH withdrawal HICs had reduced home cage activity lasting up to 24 h after withdrawal in a study using chronic EtOH vapor methods similar to those used here [29]. However, these mice showed nest-building deficits lasting up to 32 h after acute EtOH withdrawal, a time when we did not observe decreased locomotor activity [30]. In this manuscript, we discuss several other studies that dissociate nest-building deficits from changes in locomotor activity. One example comes from TG2576 mice that displayed reduced nesting in addition to other species-typical behaviors such as burrowing, but they were found to have increased activity compared to wild type mice in tests of open field and home cage activity [24]. Testing for burrowing in future studies of ethanol withdrawal could be valuable, as effects on burrowing have been detected with greater sensitivity than nest-building and occurred in the absence of disrupted locomotor activity [24, 31] Activity is generally lower during the circadian light phase, and mice are less inclined to build nests in the light than in the dark. Our tests continued throughout both light and dark phases, but

some details of our specific findings might differ if nest building and EtOH withdrawal were synchronized differently during the light-dark cycle.

In summary, the nest-building test is an assay that can be applied in the home cage to index severity of both acute and chronic EtOH withdrawal. It is a cheap, easy-to-administer test that may be applied to other models of alcohol withdrawal. Our results suggest that this assay is sensitive to the dose of EtOH but is not related to differences in core body temperatures during withdrawal. Although core body temperature does not appear to be related to nest building after vapor chamber treatment, it is possible that differences in preferred temperature may drive suppressed nest building during withdrawal. Further testing is needed to understand whether withdrawal-associated deficits in nest building are related to previously detected disturbances in thermoregulation, and to understand the mechanism(s) underlying the disruption.

Supplementary Material

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Abbreviations

EtOH	Ethanol
HIC	Handling-induced convulsion
BEC	blood ethanol concentration
i.p	intraperitoneally
h	hours
PDT	Pacific Daylight Time

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Highlights

- Chronic ethanol vapor inhalation dose-dependently suppressed nest building for up to 32 hrs after withdrawal.
- Withdrawal from an acute, high dose (4g/kg) of ethanol reduced nest building for up to 32 hrs.
- Body temperature predicted nest building in naïve HS/Npt mice but not in the same mice following ethanol vapor chamber exposure.

Figure 1. Experiment 2: Effects of sex, body temperature and EtOH withdrawal on nest building

In Experiment 2 body temperature (BT) and nest building were assessed before EtOH (EtOH) exposure. Nests were initiated by giving mice materials as described (NEST) and nest quality was subsequently scored (NS) at 3 time points after the start of the test. Mice that underwent three days of chronic EtOH vapor exposure or breathed air were tested for withdrawal severity with handling-induced convulsions (HICs) before blood samples were obtained for EtOH concentrations (BEC), and they were later assessed for BT and NEST starting the second day after exit from the chambers. (A) Mice that underwent 3 days of EtOH vapor inhalation with an average BEC of 1.89 mg/mL had greater HIC scores than those that breathed air 8 hours after exit from the chambers but not immediately following exit. (B) Naïve HS/Npt male mice had greater nest scores than female mice, and nest quality increased with time. Hatched bars indicate hours of lights off. Data are shown as mean $\pm$ SE, collapsed across subsequent drug treatment groups. (C) Body temperatures in naïve male and female mice were negatively correlated with nest scores 4 h after the start of the test. Data are shown as mean $\pm$ SE, collapsed across sexes. (D) EtOH-withdrawal influenced the progression of nest building across the first two time points after chamber exit. Hatched bars indicate hours of lights off. Mice left vapor chambers at h 0, and nesting material was added at h 26 (arrow). Data are shown as mean $\pm$ SE, collapsed across sex.

Figure 2. Experiment 3: Effects of chronic EtOH withdrawal on nest building

Mice in Experiment 3 underwent a 3-day EtOH vapor paradigm before starting two NEST tests that began on the first day and second day after exit from vapor chambers (A) HS/Npt undergoing withdrawal from 3 days of chronic EtOH vapor inhalation with an average BEC of 1.84 mg/mL had decreased nest scores 7–24 h, but not 29–48 h, after exit from vapor chambers during nest-building tests that started at 5 and 27 h after exit, respectively. Hatched bars indicate hours of lights off. Mice were removed from chambers at h 0, and nesting material was added at h 5 and h 27 (arrows). (B) In EtOH-withdrawing mice, there was a significant, negative correlation between BECs and nest scores 24 h after withdrawal. Data are shown as mean $\pm$ SE, collapsed across sex.

Figure 3. Experiment 4: Effects of chronic EtOH dose on nest building

Mice in Experiment 4 were tested for dose-response to EtOH vapor inhalation in a NEST test that began during the first day of exit from the chambers and continued for three days. (A) HS/Npt undergoing withdrawal from 3 days of chronic EtOH vapor inhalation with low, medium or high doses had decreased nest scores 9, 24 and 28 h after exit from vapor chambers compared to mice that received air. The high-dose group had decreased nest scores compared to mice that received air at 32 h after chamber exit. Hatched bars indicate hours of lights off. Mice were removed from chambers at h 0, and nesting material was added at h 5 (arrow). Data are shown as mean ± *SE*, collapsed across sex. (B) Across all EtOH-withdrawing mice, there was a significant, negative correlation between BECs and nest scores 24 h after withdrawal.

Figure 4. Experiment 5: Effects of acute EtOH withdrawal on nest building

In Experiment 5 mice received an acute dose of EtOH i.p. and started a NEST test that began during the first day following injections and continued for two days. Withdrawal from acute EtOH significantly decreased nest scores 10 and 24 h after injections. Hatched bars indicate hours of lights off. EtOH or saline was injected at h 0, and nesting material was added at h 6 (arrow). Data are shown as mean $\pm$ SE, collapsed across sex.

Table 1

Sample sizes and sample losses across experiments

Experiment no.	Initial <i>N</i>	Final <i>N</i>	No. of trt groups	Final <i>N</i> /group
1	8	8	N/A	N/A
2	40	38	2	16–22
3	71	59	2	28–31
4	80	76	4	16–23
5	43	43	2	21–22

Table 2

Experiment 1 body temperatures before and after ethanol vapor chamber exposure.

Body Temperature (°C)	Male Air	Male EtOH	Female Air	Female EtOH
1 Wk Before Chamber	37.47 ± 0.27	37.28 ± 0.21	37.94 ± 0.19 ^{††}	38.07 ± 0.15 ^{††}
24 Hrs After Chamber	37.77 ± 0.21	37.41 ± 0.21	38.07 ± 0.16 [†]	37.89 ± 0.16 [†]
After - Before	0.30 ± 0.28	0.13 ± 0.25	0.13 ± 0.24	-0.18 ± 0.18

[†]Sex difference, p<0.05^{††}Sex difference, p <0.01

Table 3

Experiment 4 BECs (mg/mL) at time of exit from chamber

Exposure Group	Males	Females	Combined
Low	1.95 ± 0.12	1.58 ± 0.19 ^{††}	1.76 ± 0.12
Medium	2.47 ± 0.09	1.81 ± 0.15 ^{††}	2.12 ± 0.12
High	2.63 ± 0.20	2.40 ± 0.18 ^{††}	2.51 ± 0.13

^{††} Sex difference, p <0.01