Effects of binge-like ethanol drinking on nest building behavior in mice

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Abstract
Nest building is a natural behavior that can readily be analyzed in mice in the home cage environment. Nest building is involved in thermoregulation, positive motivational states, and motor function, and has been proposed as an index for ethanol withdrawal severity in mice. However, nest building outcomes after voluntary ethanol consumption have not been examined. Here, we tested male and female C57BL/6J mice on a 4-day drinking in the dark (DID) paradigm of binge-like drinking with either ethanol or a water control and analyzed nest scores at two time points (48 hours and 7 days) after the last DID session. At 48 hours after the last DID session, there were no differences between the two groups in nest quality. At 7 days after DID, ethanol-drinking animals showed significantly lower nest scores than the water group. No differences were found between the ethanol- and water-drinking groups in locomotor activity or anxiety-like behavior at this timepoint, indicating that nest building deficits in the ethanol group were likely not due to underlying differences in these behaviors. Together, these results validate the use of nest building as a naturalistic assessment of withdrawal-associated behavioral changes following voluntary binge-like ethanol consumption.

Key words: nest building, alcohol, binge drinking, withdrawal, mice

Introduction

Binge drinking is defined as a pattern of alcohol (ethanol) drinking that results in blood ethanol concentrations (BEC) of 0.08% or higher, which typically equates to five or more drinks on one occasion for men and four or more for women. In the United States, roughly 28.7% of young adults report weekly binge drinking, and 90% of adults who drink excessively report binge drinking[1]. This drinking pattern can lead to significant individual and societal costs and represents a serious public health concern. Additionally, binge drinking is associated with a higher risk of developing an alcohol use disorder and related problems such as withdrawal symptoms[2]. Ethanol withdrawal symptoms can include negative affective changes such as anxiety, depression, and agitation[3].

In mice, previous studies have shown that ethanol withdrawal can cause lasting changes in affective and goal-directed behaviors, including nest building behavior[4]. Nest building is a highly conserved behavior in mice that is seen both in the wild and in laboratory animals[5]. Nest building is relevant for thermoregulation, predator defense, and parental care, and has been proposed to represent a potential model of an "activity of daily living" in mice[6,7]. Nest building is a sensitive index of general well-being in mice and has been shown to be impacted by physiological states such as stress and sickness, as well as specific brain lesions and genetic mutations[5,8]. Several studies have also reported nest building deficits during ethanol withdrawal, following either acute ethanol injection or ethanol vapor inhalation exposure[3,9]. Nest building may therefore represent a simple, non-invasive means of assessing ethanol withdrawal severity in mice.

In the present study, we sought to determine whether voluntary binge-like ethanol drinking is sufficient to produce lasting deficits in nest building. Mice were tested on a 4-day drinking in the dark (DID) paradigm with either 20% ethanol or water, and nest quality was evaluated at 48 hours (acute withdrawal) and 7 days (early abstinence) after the final binge-like drinking day. We hypothesized that the withdrawal following this paradigm would promote the same deficits previously seen in nest building behavior after other types of ethanol exposure, therefore validating the use of this test for the evaluation of withdrawal-associated behavioral changes after voluntary ethanol consumption.

Material and methods

Animals and husbandry

Forty adult male and female C57BL/6J mice (n=10/sex/drinking group) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were allowed to acclimate to the animal facility and a 12hr/12hr reverse light-dark cycle with lights off at 08:00 for two weeks prior to the start of testing. One week before the start of testing, mice were singly-housed in standard shoebox cages on Sani-chip bedding with a sipper tube water bottle, and food and water were provided *ad libitum.* Mice were also provided with a single 2" square cotton nestlet as enrichment to allow familiarization with the nest building material. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of New

Mexico Health Sciences Center and were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals

Drinking in the Dark (DID)

Prior to the start of the experiment, mice were pseudorandomly assigned to either the ethanol or water drinking group. On each day of the DID procedure, mice were weighed approximately 30 min before the start of the drinking session. Three hours after lights-off, water bottles were removed and replaced by 10 ml tubes fitted with a ball bearing metal sipper and containing either 20% ethanol (v/v in water) or water depending on group assignment. On Days 1-3, fluid levels were recorded, and tubes were left in place for 2 hours, after which fluid levels were recorded again. Tubes were then removed and water bottles were returned to the cages. On Day 4, the procedure was the same as on the previous days, except that drinking tubes were left in place for 4 hours, and fluid levels were recorded after 2 and 4 hours.

Nest Building Tests

24 hours after the end of the final DID session, the existing nesting material was removed from the cage and replaced with a fresh cotton nestlet. Mice were left undisturbed in the home cage for 24 hours after nesting material replacement, and then the nest quality was scored. Six days after the final DID session, the nesting material was again removed and replaced with a fresh nestlet, and nests were again scored 24 hours after replacement. Nest scoring was based on the scale previously described by Deacon[6] and used the following criteria: untouched nestlets received a score of 1, shredded nestlets that had been moved and scattered without a centralized nest site received a score of 2, centralized nest sites without any walls received a score of 3, centralized nest sites with walls received a score of 4, and centralized nest sites with fully domed walls received a score of 5. Nests with qualities fitting in multiple scoring categories were awarded half points between the identified categories.

Open Field Test

The open field test was performed 7 days after the final DID session, immediately following the second nest score assessment. The test apparatus consisted of an arena made of acrylic transparent walls and white floor (27 x 27 x 20cm; Med Associates, St. Albans, VT) and placed inside of an illuminated sound-attenuating cubicle. Infrared light sensors separated by 1.5 cm intervals were used to automatically detect horizontal and vertical activity at 1.5 and 6 cm above the floor level, respectively. The center space was defined as 8x8 beams (12.72 cm x 12.72 cm). On the experiment day, the animals were moved to the experiment room and allowed to acclimate for 1 hour. Mice were then placed individually into the center of the arena and allowed to explore freely for 15 minutes. Locomotor activity (distance traveled in cm) and the time spent in the center of the arena were automatically recorded. Between animals, the chambers were cleaned with 10% isopropyl alcohol to eliminate odor cues.

Statistical analyses

Statistical analyses were carried out using SPSS (IBM; version 29.0.1.0(171)). To determine whether there was an effect of drinking group on nest scores either during acute withdrawal or early abstinence, nest scores at 48 hours and at 7 days were analyzed by two-way analysis of variance (ANOVA) with factors of treatment (water or ethanol) and sex. Total locomotor activity (distance traveled in cm) and percent of total time spent in center of the chamber were also analyzed using two-way ANOVA with factors of treatment group and sex. Data are shown collapsed on sex when no significant treatment group x sex interaction was found. Spearman's correlation was used to determine if there was a significant relationship between cumulative DID ethanol intake and nest score at either timepoint. Significance was set at $\alpha=0.05$ for all tests.

Results

Drinking in the Dark and Nest Building

Figure 1 shows experimental timeline (Fig. 1A) and average g/kg intake of ethanol on each day of the DID test (Fig. 1B). Ethanol intake in this experiment was consistent with previously reported levels for C57BL/6J mice and would be expected to results in intoxicating BECs[10,11,12]. Figure 2 shows nest scores on each of the post-drinking assessments. At 48 hours after the last drinking session, there was no significant effect of treatment group [$F(1,35)=0.10$, $p=0.750$], no main effect of sex [$F(1,35)=2.04$, $p=0.162$], and no significant treatment x sex interaction [$F(1,35)=3.17$, $p=0.084$] (Fig. 1A). At 7 days following drinking, however, there was a main effect of treatment group [$F(1,36)=7.79$, $p=0.008$], with the ethanol-drinking animals having poorer nest quality scores than the water-drinking control animals (Fig. 1B). There was also a main effect of sex [$F(1,36)=6.24$, $p=0.017$], with female mice having poorer quality nests than male mice, but there was no significant treatment x sex interaction [$F(1,36)=0.02$, $p=0.884$]. There was no significant correlation between cumulative ethanol intake across the DID procedure and nest score at either 48 hours or 7 days after drinking ($p\geq0.275$).

Open Field Test

To assess whether deficits in nest building were potentially due to withdrawal-associated changes in locomotor activity and anxiety-like behavior, an open field test was performed 7 days after the end of DID (immediately following scoring of nest quality). Figure 2 shows total distance traveled in 15 min (Fig. 2A) and the percent of total time spent in the center of the arena (Fig. 2B). For both variables analyzed, there were no main effects of treatment group and no significant treatment x sex interactions [$F(1,36) \leq 2.64$, $p\geq0.113$ for all], though there was a significant main effect of sex on center time [males>females; $F(1,36) =8.58$, $p=0.006$].

Discussion

Ethanol withdrawal is a complex state that includes different phenotypes that occur at different timepoints and durations of abstinence. Acute withdrawal typically occurs during the

first 48-72 hours after cessation of ethanol in humans (24-48 hours in rodents) and is usually defined by symptoms related to nervous system hyperactivity. In contrast, early abstinence (3-6 weeks in humans, 1-2 weeks in rodents) symptoms may include anxiety-like behaviors and disturbances in sleep with the absence of physical symptoms. Finally, protracted abstinence can last for more than a month and is characterized by the prevalence of negative affective changes[3,13,14]. For all phases of withdrawal, specific symptoms may vary depending on the duration, pattern, and intensity of prior ethanol exposure.

In this study, we found that a single 4-day DID procedure was sufficient to cause a significant impairment in nest quality of ethanol-drinking C57BL/6J mice. This impairment was not seen 48 hours after the last drinking session (acute withdrawal), but was significantly different from water-drinking control animals at 7 days after the end of DID (early abstinence). This is some of the first evidence for ethanol withdrawal-associated impairment of nest building during early abstinence following voluntary binge-like drinking. The absence of a drinking group difference during acute withdrawal is consistent with a previous study which found no differences in nest scores at 24 hours after the last day of ethanol exposure in mice that received a two-bottle choice drinking paradigm[15]. Interestingly, several other studies have found deficits in nest building during acute ethanol withdrawal following acute injections or vapor inhalation, though these studies also used different mouse strains[3,9]. This suggests that both route of ethanol administration and genetic background may impact the timing of withdrawal effects on nest building deficits.

We found no significant effect of drinking group (ethanol vs. water) on locomotor activity and anxiety-like behavior when tested 7 days after DID. This indicates that the group difference in nest building performance at this timepoint was likely not due to underlying locomotor deficits or alterations in anxiety level. While locomotor sedation has been commonly reported during acute ethanol withdrawal in mice and could potentially explain reduced nest building activity[3,16], these locomotor effects are typically gone by the early abstinence period during which we observed impaired nest building. This suggests that the time course of locomotor deficits and nest building deficits during withdrawal in our experiment shared minimal overlap. Alternatively, locomotor impairment could potentially drive the acute withdrawal differences in nest building deficits that have been previously reported, but our drinking paradigm may have been insufficient to produce these locomotor effects. Future experiments that test locomotor activity during acute withdrawal following DID may help to resolve this distinction. Similarly, examining nest building behavior at later timepoints following acute ethanol injection of vapor inhalation would be useful to determine whether the early abstinence nest building deficit we observed here is conserved across different ethanol exposure paradigms. It may be that nest building deficits during withdrawal are due to several different underlying causes, with acute withdrawal deficits reflecting locomotor or thermoregulatory changes during this time period, whereas early abstinence deficits might be more closely related to cognitive impairments or negative affective states that predominate during this phase of withdrawal.

The specific neurobiology underlying nest building behavior is still not fully characterized, but there is some evidence that impairment in nest building can arise from disrupted oxytocinergic transmission. A previous study found that genetic manipulations that result in a decrease in oxytocin neurons in the hypothalamus were associated with an increase in the latency to construct nests in female mice[15]. Some studies have found oxytocin treatment can improve ethanol withdrawal symptoms in both humans and rodent models[17,18], and different polymorphisms of the oxytocin receptor gene are associated with altered susceptibility to affective changes during withdrawal[19]. Oxytocin signaling has also been shown to be crucial for hippocampal function by regulating neuronal excitability, network oscillatory activity, and modulation of fast-spiking interneurons[20,21]. Nest building performance is sensitive to hippocampal damage[5], and impairments in hippocampal function or reduced hippocampal volume result in deficits in nest building behavior[22,23,24]. There may therefore be a relationship between ethanol withdrawal, oxytocin signaling, and nest-building behavior that is related to the hippocampal role in spatial memory and orientation, as well as memory-based recognition of "nest-like" materials by mice[25,26]. Withdrawal-associated alterations in GABA and glutamate signaling could potentially also play a role in the observed nest building deficits. There is evidence that GABAergic and glutamatergic neurons in the lateral preoptic area are activated in response to nesting material in male mice[27]. Ethanol exposure and withdrawal can both alter GABAergic and glutamatergic signaling and GABA/glutamate balance in brain[28,29,30,31], and these changes could potentially lead to reductions in nest building behavior. Further mechanistic studies will be needed to determine the underlying neurotransmitter systems and brain regions that are responsible for withdrawal-associated impairment of nest building.

There are some potential limitations of the present study that should be noted. First, nest building behavior was only evaluated at two time points that were relatively far apart (48 hours and 7 days after the last drinking session). We therefore are not able to determine when the impaired nest building first occurs, or for how long this deficit may persist. Testing a more complete timecourse of nest building behavior throughout acute withdrawal and further into early abstinence will be necessary to identify more subtle changes in this trait throughout the initial phases of ethanol withdrawal. Additionally, without BECs we cannot say definitively that the mice reached binge levels of intake. Although ethanol consumption was high and was consistent with levels that have been shown to produce intoxication in previous studies[10,11,12], future experiments including a blood sample for BEC determination after the final DID session would be beneficial. We could then also determine whether BEC is correlated with nest building scores, even in the absence of a correlation between total consumption and nest quality.

In summary, our findings provide some of the first evidence for lasting effects of voluntary binge-like ethanol drinking on nest building behavior in mice. These results add to the previous literature showing nest building deficits can serve as an index of acute ethanol withdrawal severity and support the use of this test to evaluate behavioral changes during early abstinence. Because of its ease of use, minimal invasiveness, and relevance for naturalistic

mouse behavior, nest building should be considered as a potential phenotype of interest in future studies of ethanol and other drug withdrawal.

Data availability

The data collected and analyzed in this study are available on reasonable request from the corresponding author.

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Author contributions

ALS-B was responsible for data acquisition, data analysis, and writing the original manuscript draft. AMB-L was responsible for experimental conception and design, data analysis, review and editing of the manuscript, and funding acquisition.

Competing interests

The authors declare no conflicts of interest.

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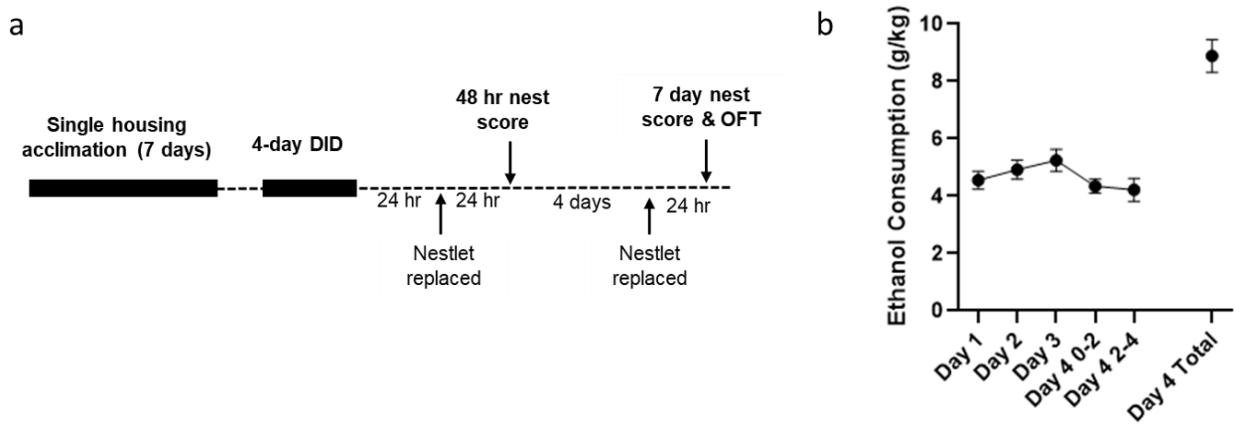

Figure 1. Experiment timeline (A) and average daily g/kg ethanol consumption during the 4-day DID procedure (B).

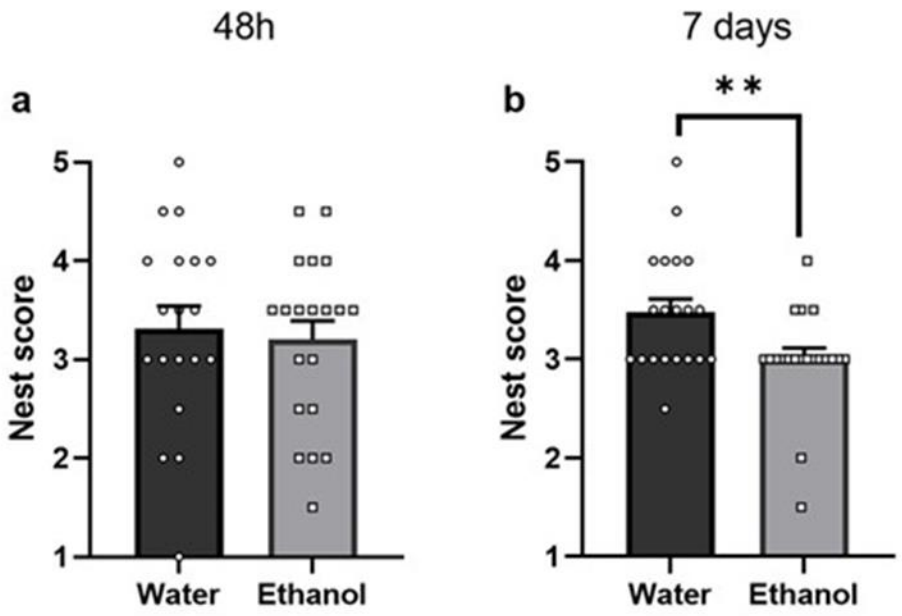

Figure 2. Nest scores at 48h (A) and 7 days (B) after the final DID session. Nests were scored on a scale from 1 to 5, with 5 being the highest in quality. Data are shown as means ± SEM and are collapsed on sex. ** indicates $p < 0.01$. N= 10/sex/treatment group.

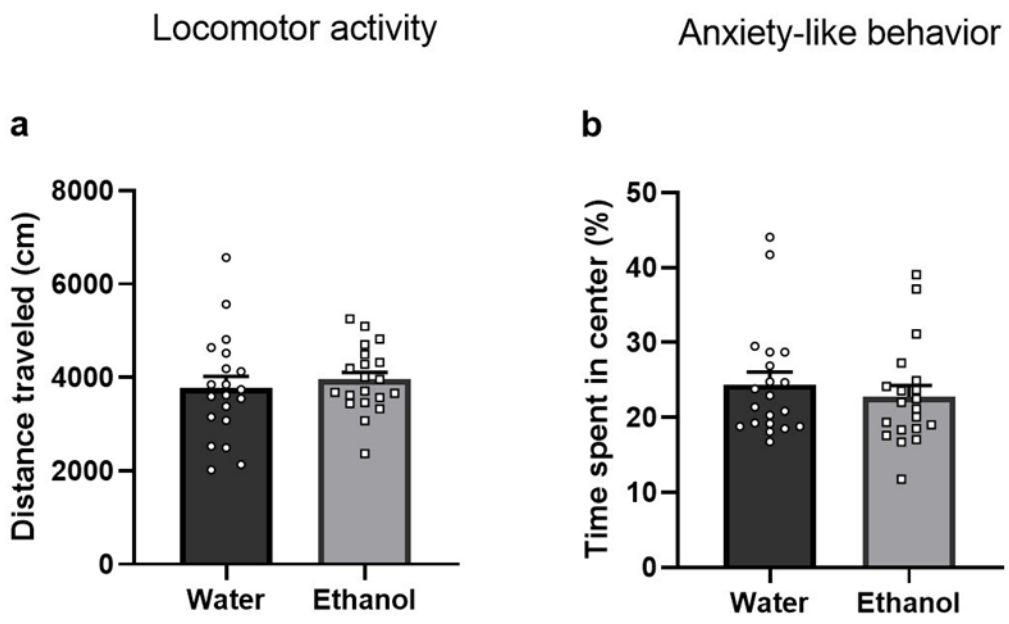

Figure 3. Open field test of locomotor activity and anxiety-like behavior 7 days after the final DID session. Total distance traveled in 15 min (A) and the percentage of total time spent in center (B) are shown. Data are shown as mean ± SEM and are collapsed on sex. N=10/sex/treatment group.