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An alcohol withdrawal test battery measuring multiple behavioral symptoms in mice

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Abstract

Despite acceptance that risk for alcohol use disorder (AUD) has a large genetic component, the identification of genes underlying various components of risk for AUD has been hampered in humans, in part by the heterogeneity of expression of the phenotype. One aspect of AUD is physical dependence. Alcohol withdrawal is a serious consequence of alcohol dependence with multiple symptoms, many seen in multiple species, and can be experienced over a wide-ranging time course.

In the present 3 studies, we developed a battery of withdrawal tests examining behavioral symptoms from multiple domains that could be measured over time in mice. To permit eventual use of the battery in different strains of mice, we used male and female mice of a genetically heterogeneous stock developed from intercrossing 8 inbred strains.

Withdrawal symptoms were assessed using commonly used tests after administration of ethanol in vapor for 72 continuous hrs. We found significant effects of ethanol withdrawal versus air-breathing controls on nearly all symptoms, spanning 4 days following ethanol vapor inhalation. Withdrawal produced hypothermia, greater neurohyperexcitability (seizures and tremor), anxiety-like behaviors using some apparatus (such as reduced transitions between light and dark compartments), anhedonia (reduced sucrose preference), straub tail, backward walking and reductions in activity, but not changes in thermal pain sensitivity, hyperreactivity to handling, or anxiety-like emergence behaviors in other apparatus. Using these data, we constructed a refined battery of withdrawal tests. Individual differences in severity of withdrawal among different tests were weakly correlated at best. This battery should be useful for identifying genetic influences on particular withdrawal behaviors, which should be reflecting the influences of different constellations of genes.

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Keywords

alcohol withdrawal syndrome; behavioral domains; mouse; test battery

Introduction

Recent revisions have been made to the diagnostic criteria for alcoholism, including its name: alcohol use disorder (AUD; DSM-V, American Psychological Association, 2013), and a person who experiences at least two of the eleven diagnostic criteria can be diagnosed with AUD. One criterion is the experience of alcohol withdrawal symptoms when alcohol intake is stopped or when the effects of alcohol are wearing off. The human acute EtOH withdrawal syndrome includes irritability, nausea, vomiting, insomnia, tremor, hyperalgesia, hyperthermia, hyperventilation, tachycardia, anxiety, and neural hyperexcitation, manifestations of which can include hallucinations, delusions, and grand mal seizures (Lieber, 1995). It is also clear that risk for alcohol dependence has a significant genetic component in humans (e.g., Kwako et al., 2016; Rietschel & Treutlein (2013). Given the heterogeneity of diagnostically-relevant symptoms, there have been many attempts over the years to define subtypes of AUD; although current versions recognize genetic contributions, these efforts have largely been based on clustering of individual symptoms (Kwako et al., 2016; Jellinek, 1960; Cloninger et al., 1981; Babor et al., 1992; Bucholz et al., 1996; Epstein et al., 2002). One implication of this is that even if ethanol intake is roughly constant, the time course for waxing and waning severity of different withdrawal signs is likely to be somewhat different, as each sign likely represents the influence of different physiological/biochemical processes. Since the earliest descriptions, we have known different withdrawal symptoms have different time courses in human alcoholics (Heilig et al. 2010). Severe withdrawal is long lasting, and protracted signs can be seen for months or longer, particularly in cognitive sequelae (Parsons, 1998; Brandt et al., 1983) and affective dysregulation [e.g., anhedonia, craving; see (Hatzigiakoumis et al., 2011; Heilig et al., 2010)]. Anhedonia and craving are important drives for relapse drinking (Koob & Volkow 2010). However, little is understood about the specific genetic contributions underlying the individual symptoms of withdrawal. One possibility is that different constellations of withdrawal symptoms reflect different patterns of genetic influence; alternatively, there may be some underlying core dysregulations (Crabbe, 2012). There are few human genetics studies targeting specific EtOH withdrawal signs (e.g., Nelson et al. 2004; Hughes 2009; Gizer et al. 2011] as opposed to a diagnosis of alcohol dependence. However, a recent twin study found that signs of withdrawal and tolerance appeared to reflect distinct genetic contributions to risk for alcohol dependence. To the extent that parallel animal data were available, they tended to support the human findings (Kendler et al. 2012).

In three experiments described here, we sought to model in mice a broader range of behavioral deficits experienced by humans undergoing alcohol withdrawal (e.g., changes in body temperature, pain sensitivity, mood) using established, reliable mouse behavioral tests. We next sought to combine behaviors into a withdrawal battery that would enable us to examine multiple behavioral domains in the same mice. We reasoned that testing multiple behaviors would be seen as an advantage by many laboratories working with scarce

resources (such as mutant mice that are generally in short supply). We have previously developed other behavioral batteries for alcohol-related behaviors (e.g., ataxia, Crabbe et al., 2005). In an intense effort, Philip and colleagues (2010) collected phenotypic data from over 250 behavioral measures, including alcohol withdrawal HIC, in several behavioral batteries using the extended BXD recombinant inbred mouse strain panel (Philip et al., 2010). Recently, several anxiety- and depression-like behaviors were shown to be affected up to 3 weeks after removal of ethanol when serially tested in C57BL/6J mice after 6 weeks of binge ethanol intake relative to mice given water (Lee et al., 2014). For the present studies, we examined multiple withdrawal signs in the genetically heterogeneous stock developed by Dr. Robert Hitzemann in Northport, NY from an intercross of 8 inbred strains (HS/Npt; Hitzemann et al., 1994). We previously tested mice from this stock for chronic ethanol withdrawal assessed solely as HIC severity and showed that they experience a robust withdrawal seizure response (Crabbe et al., 2012). We selected informative and time-compatible behavioral tests that are affected during the first 4 days of withdrawal following chronic vapor inhalation. Several HIC scores were assessed across the time-course of two of the batteries to demonstrate that withdrawal was occurring and that its magnitude was comparable to our previously published work with individual withdrawal signs. In our final battery, we targeted two blood ethanol concentrations (BECs) to test potential dose sensitivity of these symptoms. Lastly, we assessed phenotypic correlations among withdrawal behaviors in order to determine whether tests from different behavioral domains might be reflecting similar underlying mechanisms. Although these experiments were not designed to assess this, positive phenotypic correlations would be expected among groups of measures sharing similar genetic influences.

Methods

Animals

220 HS/Npt male and female mice were used in these studies. All studies were performed in accordance with NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the VA Portland Health Care System's Institutional Animal Care and Use Committee.

Mice were born within the Veterinary Medical Unit and were weaned at 21 ± 1 days of age into same-sex groups of 2-5 littermates in colonies maintained on a 0600-1800 light/dark cycle at $22 \pm 1^\circ\text{C}$. Cages were made of polycarbonate ($28 \times 17 \times 11.5$ cm) containing about 2.5 cm of Bed-o-cobs® bedding (The Andersons, Inc., Maumee, OH, USA). Purina brand food (5LOD, PMI Nutrition International, Brentwood, MO) was suspended in a wire-top and food and water were available *ad lib*. All mice were adult at the time of testing (58-79 days of age).

Chronic Ethanol Vapor Inhalation

Details regarding our vapor inhalation system have been published many times previously (e.g., Greenberg et al., 2016). Mice of different genotypes and sexes are differentially sensitive to ethanol vapor concentration, so each sex was treated in its own vapor chamber in order to achieve subjects equated for blood ethanol concentrations (BECs). Mice were

pseudorandomly assigned to vapor or air conditions within cages, so that approximately equal numbers of mice of each sex were in each vapor condition. A slightly larger percentage of mice were assigned to the ethanol vapor condition than were assigned to the air condition in each study due to expected attrition during either the dependence induction phase or the withdrawal phase. Initial body weights, basal body temperatures, and BECs after 72 hrs of vapor inhalation are shown for female and male mice in Table 1. Vapor inhalation began at 0700. BECs were elevated by intraperitoneal (i.p.) injection of either 1.5 or 2.0 g/kg (20% v/v) ethanol solution (Decon Laboratories, King of Prussia, PA) and the alcohol dehydrogenase inhibitor, pyrazole (68.1 mg/kg; Sigma-Aldrich, St Louis, MO) mixed in 0.9% physiological saline. Pyrazole helps stabilize blood ethanol concentrations, which aids in survival (Goldstein, 1972). Ethanol plus pyrazole injections were delivered in a volume of 9.51 (for 1.5 g/kg) or 12.67 (for 2.0 g/kg) ml/kg body weight; daily pyrazole booster injections were given in a volume of 10 ml/kg body weight. Mice were then housed in wire mesh racks within a large polycarbonate chamber into which ethanol vapor was pumped. Ethanol vapor was created by liquid ethanol microinjection into a regulated air supply line (55 L/min) using a continuously operating piston pump, and ethanol vapor concentrations were adjusted by increasing or decreasing the ethanol flow rate. Small fans circulated the ethanol vapor within the chamber, and air/vapor was exhausted for each chamber at calibrated rates. Ethanol vapor concentrations were set initially to achieve the target BEC for that group of mice and vapor was analyzed continuously by an Agilent model 6850 gas chromatograph to ensure stability over time. For air-exposed mice, the same dose of pyrazole alone was injected as 12.57 ml/kg on day 1 and 10 ml/kg body weight on subsequent days. The air chambers operated identically except that ethanol was not administered through the air line.

At 24 hrs, and again at 48 hrs, all mice were removed from vapor (or air) inhalation and weighed. Immediately after being weighed, a subset of ethanol-treated mice had a 20 μ l blood sample drawn from the tip of the tail for analysis by gas chromatography. All mice then were injected i.p. with 68.1 mg/kg pyrazole in saline in a volume of 10 ml/kg body weight. After 72 hrs of vapor or air inhalation (0700 = time 0), all mice were removed from the chambers and weighed, blood sampled or sham sampled. Mice were then returned to clean cages with their original cage mates to await withdrawal testing and remained group housed except when placed on an apparatus for testing (except the sucrose preference test, where mice were individually housed). If the experiment included HIC testing at removal from vapor, this was performed before the animals were weighed.

Blood Ethanol Concentration Assay

BECs collected from mice during and immediately following vapor inhalation from each WD Battery experiment were analyzed on an Agilent model 6890N gas chromatograph on a DB-ALC2 column. Those from WD Batteries 1 and 2 were assayed using a method described previously (Rustay & Crabbe, 2004). BECs from WD Battery 3 were analyzed using a headspace method (Finn et al., 2007). The two methods produce nearly identical results (unpublished data). Each day, samples collected from the subset of sentinel subjects were analyzed to determine whether or not to change the vapor concentration for the next 24 hr period in an effort to hold BECs as constant as possible across the 3 days. All BECs

collected after the final day of exposure were then analyzed to determine final concentration, which best represents the dose of ethanol chronically experienced by the animal (Crabbe et al., 1983).

Experiments

Figure 1 diagrams behavioral testing over time for each of three withdrawal (WD) batteries. Each experiment is represented within a background blue rectangle, and each named box and the number to its upper left in the grey bar indicate the time during WD that mice were tested. Individual behavioral test durations are as described in the respective sections describing apparatus and other testing conditions in the paragraphs below. Briefly, using WD Battery 1, we sought to replicate in HS/Npt mice multiple withdrawal-sensitive behaviors shown previously at approximately these hours of WD to reflect treatment effects (Kosobud & Crabbe, 1986; Crabbe et al., 2012; Philibin et al., 2008). We also included a test of anxiety-like behavior (Gorin et al., 2005) and assessed body temperatures (Crawshaw et al., 1994). Based on the results of WD Battery 1 (see Results, Anxiety-Like Behavior), we designed WD Battery 2 to test anxiety-like behavior without previously testing other withdrawal signs, followed by body temperature to verify the previous findings, and added a sucrose preference test to assess possible anhedonia (Muscat and Willner, 1992). Lastly, WD Battery 3 was designed as a dose-response test of sensitive behaviors: we included a later test of anxiety-like behavior and added a hot plate test that had been shown to be sensitive to genetic differences in chronic withdrawal (Greenberg, unpublished data). We targeted average BECs expected from our experience to produce Medium (Med) or High levels of dependence and withdrawal in separate groups of mice.

Withdrawal Tests

All tests were conducted during the light cycle. When a test overlapped with the time of lights off (1800), the lights remained on until the test was complete. Before and between withdrawal tests, mice were housed with their original cage mates, except during the sucrose preference test.

Neurohyperexcitability

Handling-Induced Convulsions—Originally developed by Goldstein and Pal (1971) to quantify withdrawal seizures, the HIC involves lifting the mouse by the tail and observing for one or several convulsion signs. Our laboratory refined the scale to include 7 points (Crabbe et al., 1991). If no signs are observed upon lifting, the mouse is spun by its tail in an arc (180 - 270°) and again observed. If no signs are observed, the mouse is given a score of zero. If a tonic (less severe seizure involving stiffening of the forelimbs) or tonic-clonic (more severe seizure with tonic components but also involving involuntary spasmodic clonic episodes) seizure is observed upon lifting, its severity is graded as a score of 4-6, while if spinning the animal by its tail is required, severity is graded as 2-3. A score of 1 indicates a myoclonic facial grimace after spinning, while a very rare score of 7 is given if the mouse convulses without lifting (e.g., in response to cage disturbance). This convulsion may or may not be fatal; if the mouse recovers quickly and is ambulating normally within the first minute after the convulsion, it remains in the study; otherwise, cervical dislocation is performed to

insure death. The usual range of scores is 2-6 during chronic withdrawal using our conditions (Metten & Crabbe, 2005). The HIC can be scored for ethanol withdrawal repeatedly for 24 hrs or longer, typically at intervals of 1 hr, and the area under the withdrawal HIC curve over time is a reliable, quantitative measure of withdrawal severity following either acute high-dose administration (Metten & Crabbe, 1994) or chronic vapor inhalation (Metten et al 2005). HIC scoring during WD Battery 1 was performed at WD Hrs 0, 8, 11, 25, and during WD Battery 3, was performed at WD Hrs 0, 10, 26, and 48.

Tremor—As with seizures, tremor is observed in all mammalian species tested (Friedman, 1980). Tremor is typically assessed either by presence/absence or frequency during a defined interval; however, some attempts have been made to quantify severity in animals (e.g., Derr & Derr, 1986). In the present studies, we scored tremor and three other behaviors on an open platform during a two-minute test (Kosobud & Crabbe, 1986). The platform test we use comprises measures of neurohyperexcitability (tremor), activity (line crossings) and two other alcohol withdrawal-sensitive behaviors (backward walking [Freund, 1969] and Straub tail [Bilbey et al., 1960]; Kosobud & Crabbe, 1986). The apparatus is a stainless steel rectangular pan (35.5 × 48 cm) with 1.9 cm walls. The pan was marked with straight lines to create 5 cm² squares. Mice were placed on the center square facing away from the observer for two minutes and were simultaneously filmed for later scoring of some behaviors. Two behaviors were scored live by observers blind to treatment condition (air vs ethanol): tremor occurrences and score (0- no tremor; 1- brief tremor lasting 1 sec; 2- sustained tremor lasting longer than 1 sec), Straub tail occurrences and worst score (0- tail completely limp or normal; 1- tail stiff and suspended off ground, parallel to the ground; 2- tail stiff and held at 45° relative to the ground). We also calculated the proportion of animals showing tremor and Straub tail (see Results).

Behavior on the platform was video-recorded from directly above and the following behaviors were scored from the video by observers blind to treatment condition: total line crossings (all 4 feet across a line in a forward motion); number of instances of backwards walking as measured by the hind limbs crossing a line, and number of squares traversed backwards, either consecutively or in separate instances (Kosobud & Crabbe, 1986). Because Kosobud & Crabbe (1986) observed differential effects of WD Hr depending on the behavior observed, we conducted this 2-minute long test at two time points, WD Hrs 5 & 9 in WD Battery 1 and one time at WD Hr 7 in WD Battery 3.

Ataxia

Accelerating Rotarod (ARR)—We previously established that this commonly used test of motor incoordination is also sensitive to genetic differences in ethanol withdrawal severity (Philibin et al., 2008). The apparatus is a modified Accurotor Rota Rod (Accuscan Instruments, Columbus, OH) with a 63 cm fall height and 6.5 cm diameter rotating dowel covered in 320 grit wet/dry sandpaper to provide a uniform surface and reduce slipping. Four mice were placed on and perpendicular to the stationary rod (0 rpm) facing the wall opposite the experimenter, each in a separate lane divided by Plexiglas rounds. The motor was then turned on, set to continuously accelerate at 20 rpm/min. Maximum speed was set at 90.0 rpm (no mouse ever achieves this speed; Rustay et al., 2003). When a mouse fell into a

deep well containing ~15 cm fluffed sawdust, it broke a photocell beam marking the latency to fall. After all mice had fallen, a 30 sec intertrial interval (ITI) began. 8 trials were given, each separated by a minimum 30 sec ITI. ARR performance was calculated as the average latency to fall across all 8 trials, while ARR improvement was calculated as the average of the last 2 trials minus the average of the first 2 trials (Philibin et al., 2008). This test was conducted at WD Hr 7 during WD Battery 1 and at WD Hr 9 during WD Battery 3.

Vertical Screen—Within a few minutes after completing the Open Platform test at WD Hr 9 during WD Battery 1, mice were placed in the center of a vertical screen (1.22 m × 2.44 m) made of 3.2 mm² hardware cloth (Hutchins et al., 1981). The bottom of the screen was placed inside a tray of wood shavings in case a mouse fell. We added dark colored yarn to the back side of the screen to demark 10 cm² squares. Squares traversed were counted during a 3-minute test (Hutchins et al., 1981). After the test, mice were housed with their original cage mates and allowed to rest before the next test.

Anxiety-Like Behavior

Elevated Zero Maze testing was conducted in WD Battery 1 at WD Hr 24. Our elevated zero maze (EZM) is made of dark grey PVC (Flair Plastics, Inc., Portland, OR). It has a single, circular track 5.5 cm wide surrounding an open space of diameter 33.6 cm, enclosed by clear Plexiglas walls in 4 sections. Each section is ¼ the length of the track. Two sections on opposite quadrants have walls 1 cm high (“open” arms). The other two sections have walls 10 cm high (“closed” arms). The legs raised the maze 52 cm off of the floor. Each of 4 mazes was placed in a bin of wood shavings in case a mouse fell. Light levels in the room were set at ~15 lux, measured in the center of the maze at track level, one hour before the test. The maze's walls were removed before the first mouse and between each mouse, and tracks and walls were cleaned with 10% isopropyl alcohol. Four mazes were used simultaneously with dividers set so the mice on the apparatus could not see each other during testing. Testing was performed similarly to procedures used previously in our laboratory (Milner & Crabbe, 2008). A mouse was lifted by the tail and immediately supported under its feet with gloved hands and placed in an open arm facing toward a closed arm such that the mouse's head was in the closed arm, and the technician moved out of sight. Behavior was video recorded from above for scoring of the following variables during the 5 minute test: (1) the number of entries into each arm (defined as when the mouse places all 4 feet inside the arm); (2) time spent in each arm; (3) line crosses in each arm and total line crosses (using a template over the video screen divided into 12 equal segments); (4) number and locations of stretched-attend postures; number of head dips (defined as when the mouse's head crosses the open arm wall and dips down to below track level); (5) numbers of boli and urine puddles were counted immediately after the test.

Light/Dark Box—The apparatus used for testing in WD Batteries 2 and 3 comprise two connected black acrylic and clear compartments with walls 30 cm high (Flair Plastics, Inc., Portland, OR). The light compartment is 28 cm² and made of clear acrylic. It is lit from above by a 60W white light bulb such that light level in the center of the compartment is ~250 lux. The dark compartment is 28 × 17 cm, made of opaque black acrylic, and has a lid to ensure that little light enters the compartment. The apparatus and testing procedures have

been described previously (Milner & Crabbe, 2008). Cleaning procedures were as described for the EZM. Four Light/Dark boxes were used, with dividers to prevent each mouse from observing another. The mouse was lifted by the tail, supported under its feet, and placed in the light compartment next to the wall furthest from the dark compartment. The numbers of boli and urine puddles were counted immediately after the test. Behavior was video recorded from above for scoring of the following variables during the 5 minute test: (1) total time spent on the light side; (2) initial latency to enter the dark side; (3) initial latency to exit the dark side; (4) number of nose pokes from the dark side to the light side; (5) the number of transitions between the light and dark sides; (6) voluntary time on the light side (total time spent on the light side minus initial latency to enter the dark side; (7) rearings; (8) line crosses in the light side (the area was divided into 25 5 cm² segments).

In WD Battery 2, the Light/Dark Box test was the first behavior assessed, without prior HIC scoring. The test was conducted at WD Hr 12 (1900). Overhead room lights were turned off at WD Hr 11, and dim red lighting was employed for the technicians to see for testing. Lights remained off until all mice completed testing, when red lights and apparatus lights were turned off. Animals were thus returned to normal lights off during the dark phase. Lights were turned on again at 0600.

In WD Battery 3, the Light/Dark Box test was conducted at WD Hr 24 using the same testing conditions. Note that animals were tested for other behaviors the previous day (Figure 1).

Reactivity to handling—We also tested reactivity to handling using the Wildness Capture Scale developed by Wahlsten et al. (2003). During WD Battery 1, each time a mouse was captured (e.g., from the home cage or a test apparatus) a score was given on a scale from 0 – 6. A score of 1 indicates evasion by running around the cage at least once; a score of 2 indicates jumping onto the side of the cage; 3 indicates jumping out of the cage and onto the table. Scores of 4 – 6 refer to how far they run, whether they jump from the table, and how quickly they are caught. In total, 22 capture scores per mouse were obtained over the 27 hrs of the testing period.

Anhedonia/Depression

Sucrose preference—Reduced sucrose preference is an established model of depression-like behavior (Katz, 1982). Our procedure assessed sucrose preference by offering mice two 25 ml tubes (graduated cylinders fitted with stoppers and 2” stainless steel drinking spouts with a 3 mm opening) containing fluid for 24hrs/day for 2 days. One tube always contained tap water, and the other contained 58.35 mM sucrose. Mice were weighed before the 48 hr test began. Volumes were read initially and at 24 hr intervals, and tube positions were switched after reading volume at 24 hrs. Sucrose preference was calculated as (mls of sucrose divided by mls of total fluid) times 100. Total fluid consumed was also measured and analyzed as mls/20g body weight (mls × 20g/actual body weight in g).

Activity

General locomotor activity was measured as line crossings during tests of some other behaviors. Line crossing data were collected at WD Hrs 5, 7 and 9 on the Open Platform (WD Batteries 1 & 3), at WD Hrs 12 and 24 during the Light/Dark Box test (WD Batteries 2 & 3) and WD Hr 24 on the Elevated Zero Maze (WD Battery 1).

Body Temperature

Hyperthermia is often observed in humans during ethanol withdrawal and it is a risk factor for later development of *delirium tremens* (Hall & Zador, 1997; Monte et al., 2009). Studies in animal models have shown that either hyperthermia or hypothermia can be shown, depending on the circadian phase (Damaggio et al., 2014a,b). We have also shown disruption of thermoregulation during withdrawal from vapor inhalation (Crawshaw et al., 1994). Our temperature measurement methods have been described previously (e.g., Crabbe et al., 2006). Before each measurement of temperature, mice were isolated in ventilated polycarbonate boxes (8 × 9 × 9 cm) for 60 minutes to prevent differences in temperature among mice due to behavioral thermoregulation (e.g., huddling, or not, with cage mates). After the acclimation period, each mouse was removed from its compartment and placed in a cylindrical restrainer. We used a Sontek Thermalert TH8 digital thermometer (Sontek Inc., Clifton, NJ) to measure rectal temperatures with a 1.8 cm long probe, lubricated with glycerol and inserted into the rectum. In WD Battery 1, we included body temperature as the last behavior measured (WD Hr 26). We estimated basal body temperature in this experiment one week after the experiment ended, at the same time of day as the temperature assessment during withdrawal, in order to avoid potential circadian confounds (Tankersley et al., 2002; Damaggio et al., 2014a). In WD Batteries 2 and 3, we assessed basal body temperatures 1 to 4 days before placing mice into the vapor inhalation chambers. We compared baseline body temperatures among the three WD Battery studies, and found that they were comparable within sex (see Table 1). Therefore, for all three WD Batteries, withdrawal-induced changes in temperature were assessed by measuring temperatures 24-27 hrs after removal from vapor inhalation and subtracting the withdrawal temperature from the baseline temperature. Note that an HIC score was obtained in WD Batteries 1 and 3 immediately prior to acclimation to the individual compartments of the ventilated box for 60 min.

Pain Sensitivity

Increased pain sensitivity during alcohol withdrawal has been measured using thermal, mechanical (e.g., von Frey), chemical (e.g., formalin injection), and electric current assays (e.g., Jochum et al., 2010; Smith et al., 2016). In these studies, we used the hot plate test, a supraspinal thermal assay, at 27 hrs (Mogil et al., 1999). Using procedures and timing examined in our laboratory by Dr. Gian Greenberg (personal communication), we placed the mouse on the hot plate (preheated to 52°C) and recorded latency in seconds to first front paw lift or lick, and/or hind paw lift, lick or flutter. Mice were removed immediately after hind paw response, or if there was no response, at 90 sec to avoid possible tissue injury. Additionally, the side of the mouse opposite to the experimenter was video recorded for rescoring later. Video scoring occasionally showed that mice made a sign on its other side

sooner than the sign recorded by the observer. When this occurred, latency to that sign was adjusted downward. Not all mice reacted with a front paw movement, so we used hind paw latency as the index. Although mice sometimes jump off of the hot plate without making one of the front or hindpaw movements (Mogil et al., 1999), only one mouse did so in this experiment (time of jumping was used as the response latency).

Statistical Analyses

The data for each behavioral test within each battery were analyzed separately by factorial ANOVA comparing ethanol- and air-exposed groups and males vs females. When the initial ANOVA showed a non-significant interaction of Sex $\times$ Treatment, either with or without a significant main effect of Sex, analyses were performed again excluding Sex as a factor. Where significant interactions of Sex $\times$ Treatment were observed, data for each sex were analyzed separately by ANOVA. *Post hoc* analyses were conducted using Tukey's HSD test (Keppel, 1991). Non-parametric data were analyzed using chi-square or Fisher's Exact Test (Siegel, 1956). Correlations were performed using Pearson's *r*. Thresholds for significance were set at $\alpha = 0.05$, two-tailed. Data analyses were performed using SYSTAT, version 13. Graphs were made using SigmaPlot, version 12.

Results

Results are presented by broad behavioral domain rather than by WD Battery experiment. For tests that were included in more than one WD Battery, only one representative data set is presented and findings for the other(s) are summarized here. To avoid redundancy, their analyses are in Supplementary Materials.

Table 1 presents, for each experiment, the final sample sizes, body weights, basal body temperatures, and BECs after vapor inhalation by sex and treatment group (air vs ethanol vapor exposure). In order to equate ethanol treatment across subjects (e.g., to compare the sexes), it is sometimes necessary to match subjects for BEC at the end of vapor inhalation (Terdal & Crabbe, 1994; Metten & Crabbe, 2005). Usually, that means excluding an occasional mouse with either too low a BEC or one too high for the target BEC. We first performed ANOVA on BECs across the relevant factor (e.g., sex, vapor exposure concentration) and then determined whether there were any significant differences in BECs. If there were, we then removed animals accordingly until BECs were not significantly different across groups. These matching procedures were always performed before analyzing the behavioral measures. The data in Table 1 show results after BEC matching.

WD Battery 1 started with 62 mice (male: 20 WD, 10 Control; female: 22 WD, 10 Control). For male mice, 4 died during ethanol vapor inhalation, 4 were removed for $>20\%$ body weight loss, and 2 were removed to match BECs between sexes. One female mouse was also removed to match BECs between sexes, leaving 51 mice total in the experiment (see Table 1).

Sixty-two mice began WD Battery 2 (male: 20 WD, 10 Control; female: 22 WD, 10 Control). One female mouse was excluded for abnormally high baseline temperature (39.6°C). Two males and 2 females died during vapor inhalation, and 5 mice (1 female and 2

male Controls, and 2 males in the WD group) were excluded for >20% body weight loss. Two mice (1 per sex) were removed to match BEC between sexes. Three male mice died during withdrawal, and all of their data were excluded. This left 47 mice (Table 1).

WD Battery 3 used 96 mice (18/sex/BEC group and 12/sex Control). The two BEC groups were housed in separate vapor chambers and each sex was housed in a separate rack but together in the BEC group-appropriate vapor chamber. Two males and 1 female in the High BEC group and 4 males and 1 female in the Medium BEC group died during ethanol vapor inhalation. Five female mice were excluded for >20% body weight loss (4 High BEC, 1 Medium BEC). One female mouse in the High BEC group was excluded due to extreme hypothermia during withdrawal (8.5°C). Lastly, 2 female mice from the Medium BEC group were removed to match BEC between sexes, leaving 80 mice total in the experiment (see Table 1).

Results are organized by broad WD behavioral domain, and representative results are presented and shown in figures. Selected additional analyses and figures for behaviors assessed in more than one battery at similar time points are shown in Supplementary Materials. For WD Battery 3, the matching procedures achieved roughly equal BECs at each level for the two sexes. BECs showed both a significant main effect of vapor concentration (resulting in High>Medium BEC) and sex $\times$ concentration interaction ($F_{(1,52)} > 4.31$, $p < 0.05$) but not a main effect of sex ($F < 1$). Female mice showed concentration-dependent BECs (Tukey's HSD, $p < 0.001$), but male mice did not ($p > 0.46$). We summarize the results of the dose-response study, WD Battery 3, at the end of the Results.

Table 2 lists experimental effect sizes for each behavioral measure for the depicted WD behaviors. Effect sizes for measures included in the Supplemental Materials are in Supplemental Table S1.

Neurohyperexcitability

Handling-Induced Convulsions

WD Battery 1: HICs at WD Hrs 0, 8, 11, and 25: As observed previously (Crabbe et al., 2012), HS/Npt mice treated with ethanol vapor displayed robust withdrawal indicated by HICs (Figure 2). The magnitudes of the 4 HIC scores were very similar to those observed previously (Figure 2 A). (WD>Air; M>F; Withdrawing mice showed greater average HICs than air controls, and male mice showed greater average HICs than females $F_{(1,47)} > 10.60$, $p < 0.01$), but the interaction was not significant ($F_{(1,47)} = 1.37$, $p > 0.24$). Scores varied significantly over time ($F_{(3,141)} = 43.10$, $p < 0.0001$) and Time $\times$ Treatment ($F_{(3,141)} = 48.29$, $p < 0.0001$), but Sex did not interact with either ($F_{(3,141)} < 1.12$, $p > 0.34$). At WD Hr 0, there was no effect of Treatment ($F < 1$); however, the Treatment effect was observed at all other Time points (WD>Air; $F_{(1,49)} > 10.97$, $p < 0.01$). Next, we estimated the area under the curve of HIC scores over time for each individual mouse by extrapolation (AREA25; Figure 2 B); see supplemental materials for calculation). AREA25 was significantly greater in ethanol treated mice than in air controls ($F_{(1,47)} = 69.3$, $p < 0.0001$), and again, male mice showed greater AREA25 than did female mice ($F_{(1,47)} = 10.95$, $p < 0.01$), but these factors did not interact ($F_{(1,47)} = 1.04$, $p > 0.31$).

These results were similar to those in WD Battery 3, where withdrawing animals had greater AREA scores over both 26 and 48 hrs. However, AREA scores were not significantly different between BEC groups given different doses of ethanol vapor in WD Battery 3 (see Supplemental Results; Figure S1).

Tremor and Other Measures

WD Battery 1: Open Platform at WD Hrs 5 & 9: Data from the Open Platform test are shown in Figure 3. Tremor (Panel A) was observed in 6/26 withdrawing animals at WD Hr 5, but in none of the air control animals. This difference was nonetheless statistically significant by the chi-square test ($\chi^2=4.04$, 1df, $p<0.05$). A greater proportion of withdrawing animals (11/32) showed tremor at WD Hr 9, while again, none of the air controls did ($\chi^2=8.33$, 1df, $p<0.01$). Straub tail (Panel B) was similarly shown more frequently by withdrawing mice than air controls. At WD Hr 5, the proportions of animals were 17/32 and 1/19, respectively ($\chi^2=11.96$, 1df, $p<0.001$), while at WD Hr 9, the proportions were 12/32 and 1/19, respectively ($\chi^2=6.52$, 1df, $p<0.01$). Backwards walking (Panel C) was not shown by air controls at either WD Hr. The proportions of withdrawing animals walking backwards was 14/32 at WD Hr 5 ($\chi^2=11.46$, 1df, $p<0.001$) and 12/32 at WD Hr 9 ($\chi^2=9.32$, 1df, $p<0.01$). Line crossing data are shown in Panel D. Mice undergoing withdrawal were significantly less active at either time point than air controls (Treatment $F_{(1,47)}=44.0$, $p<0.0001$). A significant Sex $\times$ Treatment interaction at both time points ($F_{(1,47)}=4.70$, $p<0.05$) resulted from each sex showing withdrawal effects ($ps<0.05$), with the differences between air and withdrawal scores in females being larger than in males (e.g., a difference of 29 vs 13 line crossings at WD Hr 5, respectively). Additionally, air treated female mice made more line crossings than air treated male mice ($ps<0.05$).

Ataxia

Accelerating Rotarod

WD Battery 3: ARR at WD Hr 9: The effect of ethanol withdrawal on motor coordination was assessed using the accelerating rotarod as we have previously described (Philibin et al., 2008; 2012). In this experiment, we tested whether the received dose of ethanol (as assessed by BECs) affected the degree of impairment on the ARR at WD Hr 9. Main effects of Treatment (Air>WD; $F_{(2,74)}=32.67$, $p<0.0001$), Sex (F>M; $F_{(1,74)}=7.48$, $p<0.01$) and Trial ($F_{(7,518)}=15.99$, $p<0.0001$) were significant, in addition to a significant interaction of Trial $\times$ Treatment ($F_{(14,518)}=3.99$, $p<0.0001$). Figure 4 A shows the average latencies over trial for each BEC group, collapsed over Sex, since Sex interacted with neither the between groups factor, BEC group ($F_{(2,74)}<1.95$, $ps>0.15$) nor the within subjects factor, Trial ($F_{(7-14,518)}<1.12$, $ps>0.35$). The analysis of average performance showed both significant, dose-related impairment in ethanol groups ($F_{(2,74)}=32.67$, $p<0.0001$) and better performance in females ($F_{(1,74)}=7.48$, $p<0.01$), but no interaction ($F_{(2,74)}=1.95$, $p=0.15$; Figure 4 B, left). For improvement, shown in Figure 4 C, the main effect of Treatment was significant ($F_{(2,74)}=16.54$, $p<0.0001$), but the two BEC dose groups did not differ in improvement from each other (Tukey's HSD, $p=0.24$). Results were similar to those observed in WD Battery 1 at WD Hr 7 (see Supplementary Results; Figure S2).

Anxiety-Like Behavior

WD Battery 3: Light-Dark Box at WD Hr 24: Results of this test at WD Hr 24 (see Figure 5) were similar to the LD Box test results of WD Battery 2 at WD Hr 12 (see Supplemental Results; Figure S3). However, anxiety-like behavior on the elevated zero maze at the same time (WD Hr 24) did not show significant effects of withdrawal (see Supplemental Results, Figure S3).

No effects of Treatment, Sex, or their interaction were observed for total time in the Light (mean: 95 ± 11 sec; all $F_{(2,69)} < 2.31$, $p > 0.10$; Figure 5 A). Eleven of 75 mice never left the Light (air: 1, Med BEC: 8, High BEC: 2), and therefore were assigned latencies to enter the dark of 300 sec (the length of the test). ANOVA on latency to enter the dark revealed no effect of Sex or interaction of Sex with Treatment ($F_{(1-2,69)} < 1.70$, $p > 0.19$). Collapsing on Sex, we then performed ANOVA using just the Treatment factor ($F_{(2,72)} = 3.68$, $p < 0.05$); the Med BEC group remained in the light side significantly longer than the air group ($p < 0.05$; Figure 5 B).

Initial latency to exit the Dark compartment also was longer in the ethanol groups ($F_{(2,58)} = 3.72$, $p < 0.05$; Figure 5 C), but neither Sex nor the interaction were significant (both $F_{(1-2,58)} < 1.94$, $p > 0.15$). Analysis of just the Treatment condition showed a significant effect ($F_{(2,61)} = 3.17$, $p < 0.05$); however, only the High BEC dose group tended to show longer latency to exit the dark than the air group ($p = 0.053$). Nose pokes from the dark side to the light side were observed. This behavior was reduced in ethanol groups ($F_{(2,58)} = 5.19$, $p < 0.01$; Figure 5 D) and females displayed more than males ($F_{(1,58)} = 5.76$, $p < 0.05$), but there was no interaction ($F_{(2,58)} = 2.0$, $p < 0.14$). Each of the two BEC groups made fewer nose pokes than the air control group ($p < 0.05$), but did not differ from each other.

More transitions between compartments were made by the air group than by either BEC group ($p < 0.001$; Figure 5 E). There was neither an effect of Sex nor an interaction ($F < 1.14$, $p > 0.32$). Ranges for each group were 0-26 (air), 0-17 (Med BEC), and 0-8 (High BEC).

In addition to the 11 mice that stayed in the light compartment, 28 mice did not return to the light after their initial exit (air: 5, Med BEC: 10, High BEC: 13), leaving 36 mice that returned voluntarily. Voluntary time in the light for the 36 mice making 2 or more transitions showed a significant reduction in ethanol groups ($F_{(2,30)} = 3.43$, $p < 0.05$; Figure 5 F) and a trend for male mice to spend more time in the light than females ($F_{(1,30)} = 3.50$, $p = 0.07$), but no interaction ($F_{(2,30)} < 1.33$, $p > 0.28$). Collapsing on Sex left a non-significant effect of Treatment ($F_{(2,33)} = 2.39$, $p > 0.10$).

Total numbers of line crossings in the light and instances of rearing were collected. Both behaviors were reduced in mice undergoing withdrawal. (See the next section for analyses on line crossings). For rearing, the effect of Treatment ($F_{(2,69)} = 4.21$, $p < 0.05$) but not Sex or their interaction ($F < 1$) were significant. The High BEC group significantly differed from the air controls ($p < 0.05$; Figure 5 G), while neither of the other two comparisons was significant.

Activity

Summary of Activity Measures—In the course of testing on the Open Platform, EZM, and Light/Dark Box, we obtained activity data during each of the WD Batteries, in all cases quantified as the number of lines crossed in a forward motion. Line crossings were obtained on the open platform in a 2-minute test at three WD Hrs (5, 7, 9) in WD Batteries 1 and 3. Results of each platform test showed that withdrawal reduced line crossings relative to the control conditions ($p < 0.0001$; Figure 3 D).

Line crossing data on the Elevated Zero Maze were also scored during WD Battery 1 at WD Hr 24. In this test, no significant effect of withdrawal was detected ($F_{(1,49)} = 2.42$, $p > 0.12$). Data also were available from the Light-Dark Box in WD Batteries 2 and 3. Line crossings at WD Hr 12 were reduced significantly in the withdrawal group ($F_{(1,45)} = 107.9$, $p < 0.0001$; see Figure S3 H) and there were no significant main or interaction effects with Sex ($F_s = 1$). Line crossings were reduced significantly in both BEC groups compared with air controls ($F_{(2,69)} = 7.03$, $p < 0.01$) at WD Hr 24. Females made fewer line crossings than males ($F_{(1,69)} = 4.01$, $p < 0.05$), but Sex did not interact with Treatment ($F < 1$). Subsequent analyses showed that each BEC group made significantly fewer line crossings compared with air controls (High vs. air: $p < 0.01$; Med vs air: $p < 0.05$; Figure 5 H), but the withdrawal groups did not differ from each other.

Body Temperature

WD Battery 2: WD Hr 24: Figure 6 A shows that main effects of Treatment ($F_{(1,43)} = 13.50$, $p < 0.001$) and Time ($F_{(1,43)} = 4.93$, $p < 0.05$), as well as Time $\times$ Treatment ($F_{(1,43)} = 12.03$, $p < 0.01$) were significant. A significant main effect of Sex was also observed ($F > M$; $F_{(1,43)} = 27.2$, $p < 0.0001$) as well as a trend toward a Time by Sex interaction ($F_{(1,43)} = 3.19$, $p = 0.08$), but the three-way interaction was not significant ($F < 1$). Thus, we next analyzed baseline and withdrawal temperatures separately. Baseline temperatures were higher in females ($F_{(1,43)} = 20.35$, $p < 0.0001$) but were not affected by future treatment group. WD temperatures were significantly lower in withdrawing mice ($F_{(1,43)} = 15.38$, $p < 0.001$). Summary hypothermia scores were calculated as the difference between WD temperature and baseline (greater numbers indicating greater WD hypothermia). Unlike air controls, withdrawing animals showed average reductions in body temperature of about 0.7 degrees C (Figure 6 B). In WD Batteries 1 and 3, withdrawing mice were also hypothermic at WD Hr 26 or WD Hr 27 (see Supplementary Results, Figures S4 & S5).

Hot plate test

WD Battery 3: WD Hr 28: The most common response on the hot plate was to flutter the hindpaw (57/80 mice) and a forepaw lick prior to either hindpaw lick or hindpaw flutter was also common (60/80). All 80 mice showed either a hindpaw lick or a hindpaw flutter (latency means $\pm$ SE: Med BEC group, 17.8 ± 2.5 sec, High BEC group, 17.3 ± 1.6 sec, air group, 15.2 ± 1.9 sec, respectively; data not shown). Unexpectedly, ANOVA revealed that the hindpaw response was not sensitive to ethanol WD, Sex, or their interaction (all $F_s < 1$).

Sucrose Preference

WD Battery 3: WD Hr 50: Withdrawing mice had lower sucrose preference ($F_{(2,73)}=4.47$, $p<0.05$); preference increased across days ($F_{(1,73)}=25.18$, $p<0.0001$) and there was a significant interaction of Treatment $\times$ Sex ($F_{(2,73)}=4.31$, $p<0.05$). No other effects or interactions were significant ($F_{(1-2,73)}<1.67$, $ps>0.19$). We next analyzed the sexes separately. Analyses using female mice showed no significant effects of Treatment ($F_s<1$) on either Day (Figure 7 A). In male mice, Treatment and Day were both significant ($F_{(1-2,38)}>10.36$, $ps<0.001$; Figure 7 B), but did not interact ($F<1$). On Day 1, both BEC groups showed lower sucrose preference than the air group ($ps<0.05$), while on Day 2, only the Medium BEC group differed ($p<0.05$). On either day, male mice in the two BEC groups did not differ from each other. Average sucrose preference is shown in Figure 7 C. Male, but not female, mice showed reduced sucrose preference in both BEC groups (Med BEC $p<0.001$; High BEC $p<0.05$; Figure 7 C).

In contrast to results on sucrose preference, total fluid intake was not reduced significantly by either ethanol withdrawal ($F_{(2,74)}=2.99$, $p<0.06$) or Sex ($F_{(1,74)}=3.62$, $p=0.06$), or their interaction ($F<1$; Figure 7 D). Means and standard errors for Treatment were 4.72 ± 0.22 (air), 5.26 ± 0.23 (Med BEC), and 5.44 ± 0.23 ml/20g (High BEC), respectively. Mean overall intakes for Sex were 5.41 ± 0.20 (females) and 4.94 ± 0.17 ml/20g (males).

Other Tests

Vertical Screen test

WD Battery 1: WD Hr 9.25: Withdrawing mice showed significantly fewer number of squares traversed on the vertical screen than the control group ($F_{(1,47)}=28.56$, $p<0.0001$; data not shown). Female mice crossed more squares than males ($F_{(1,47)}=7.23$, $p<0.01$), but Sex did not interact with Treatment ($F_{(1,47)}=2.26$, $p>0.13$).

Reactivity to Handling

WD Battery 1: Capture scores throughout all behavioral testing: One return score for 1 mouse from the EZM to the cage was inadvertently not recorded; 1121 scores were collected. There were no differences between air control and withdrawing animals at any time or averaged over time in capture scores. Capture scores greater than zero were infrequent (35/51 mice showed 0 every time they were handled and only 29/1121 scores were non-zero).

Dose-response summary—With a single exception, WD behaviors were not differentially sensitive to the two BEC levels achieved in WD Battery 3. The single exception was average performance on the accelerating rotarod, which showed decreasing performance with increasing BECs (Figure 4 B).

Phenotypic Correlations among WD Behaviors—We next examined correlational structure among these withdrawal behaviors using WD Battery 3 data. Prior to analyses, we calculated withdrawal scores for each behavior by subtracting the sex-specific air group's mean score from each individual ethanol treated animal's raw score, with sign adjusted to

indicate that greater positive score meant greater withdrawal severity. The variables are preceded by “WD” to differentiate these scores from those used for examination of treatment effects by ANOVA.

Correlations among behaviors are shown in Tables 3 & 4. Table 3 shows the phenotypic correlations of all withdrawing animals, regardless of ethanol dose ($n = 56$). The two WD_AREA scores (WD_AREA26 and WD_AREA48) also were significantly correlated with each other ($r = 0.93$, $p < 0.0001$) and the two WD scores for ARR performance and improvement were significantly correlated ($r = 0.49$, $p < 0.0001$). There were three trends for significance (WD_ARR performance with both WD_AREA scores, $r_s = 0.23 - 0.25$, $p_s < 0.09$; and WD_hypothermia with WD latency on the hotplate, $r = 0.25$, $p = 0.06$). This latter correlation was unexpected given the lack of treatment effect on hot plate latency to respond. Scatterplots (examined for every correlation) supported the conclusions that these correlations are generally weak.

Table 4 shows the same correlations broken down by High vs Medium BEC groups. The two WD_AREA scores were still correlated significantly ($r_s > 0.91$, $p_s < 0.0001$), and with both rotarod measures when only the High BEC group data were analyzed ($r_s = 0.44 - 0.53$, $p_s < 0.05$). Interestingly, WD_AREAs and WD_Performance were clearly not significant ($0.02 < r < 0.09$) in the Med BEC group. In contrast, weak *negative* correlations ($-0.30 < r < -0.27$) were found between WD_AREA scores and WD_Improvement. The scatterplots of these variables within the Med BEC group appeared to be fairly linear, indicating that the status of this relationship may have been clarified with additional subjects. Also significant within the High BEC group was the negative correlation between WD_hypothermia and WD_sucrose avoidance (i.e., loss of sucrose preference), with $r = -0.41$, $p < 0.05$, which was not significant (and positive) in the Med BEC group. Within the Med BEC group, only the correlation of WD_Performance and WD_Improvement on the ARR was significant, ($r = 0.61$, $p < 0.001$).

To be certain that possible distribution issues did not impair our ability to detect significant correlations, we transformed each WD index to a z-score and performed additional analyses; all conclusions were identical to those using actual scores (not shown).

Discussion

Several commonly used mouse behavioral tests reflected the state of ethanol withdrawal in these studies. Importantly, in addition to the neurohyperexcitability shown by increasing HIC severity and tremor, multiple other behavioral domains showed ethanol withdrawal after vapor inhalation, including temperature regulation, anhedonia/depression, motor incoordination/ataxia, and anxiety-like behavior. Several behaviors showed significant ethanol withdrawal effects in the same direction in more than one experiment. For example, thermoregulatory effects of withdrawal were consistently observed as hypothermia at approximately 24-27 hrs into withdrawal (Figures 6 B and (Supplemental) Figures S4 & S5). Motor impairment was consistently observed at approximately 7-10 hrs of withdrawal and was observed on two apparatus (rotarod and vertical screen) confirming previously published findings (Philibin et al., 2008; 2012; Kosobud & Crabbe, 1986).

When using a battery of behavioral tests, it is necessary to consider the possibility that prior handling may influence the results of tests performed later during the battery, as has been reported in some cases (e.g., Crawley & Paylor, 1997; Crawley et al., 1997; Crawley, 2008; McIlwain et al, 2001). While there are remarkably few data on this point, it is generally accepted that widely spaced tests are less likely to be compromised, and it is usually suggested by investigators using multiple tests that they were performed in the order of least to most stressful. We designed the test order in our batteries to assess different behavioral domains at times appropriate to the behavior, rather than in an assumed “least-to-most-invasive” pattern. We found that the magnitudes of withdrawal severity of individual behaviors within a battery were consistent with published findings that had measured single behaviors in mice using similar ethanol vapor exposure, resultant blood ethanol concentrations, and times during withdrawal (HIC: Crabbe et al., 2012; rotarod: Philibin et al., 2012; platform behaviors: Kosobud & Crabbe, 1986). Therefore, the combination of behavioral tests into several versions of withdrawal test batteries appears not to have compromised the conclusions reached regarding individual behavioral outcomes. In addition, that tests performed later during withdrawal still showed significant effects of withdrawal argues against the concern that measurements collected earlier interfere with subsequent tests. Together, these three experiments show that mice may be serially tested during withdrawal for these specific different behaviors at times appropriate for the domain. Of course, other behaviors, domains and/or specific tests might be more susceptible to such order effects. For an excellent discussion of these issues, see Wahlsten (2011). We exposed mice continuously to ethanol vapor for 72 hr. Many recent studies with mice have adopted variants of the chronic intermittent exposure paradigm pioneered by Lopez & Becker (2005) as it leads to escalations of limited access drinking during withdrawal in C57BL/6J and other murine genotypes (Crabbe et al., 2012). Other behaviors during withdrawal from chronic intermittent vapor exposure have rarely been reported in mice. However, anxiety-like behavior in rats (e.g., Zhao et al., 2007) and hyperthermia in mice (Damaggio & Gorman, 2014a,b) have been seen and these findings differ from ours here (see discussion below). While we know that HIC severity in mice responds to many of the same genetic influences after both exposure regimens (Metten & Crabbe, 2005; Metten et al., 2010), it would be interesting to explore a broad behavioral withdrawal battery after chronic intermittent exposure.

The repeated finding of withdrawal hypothermia (range of means: 0.65 – 1.30 °C) in all three experiments shows that it is reliable in HS/Npt mice at approximately 24 hours into withdrawal during the early part of the light cycle (lights on at 0600; withdrawal beginning at 0700). The thermoregulatory system in mammals is complex and has marked circadian rhythmicity. Hyperthermia is sometimes observed in the first 24 hrs of withdrawal in humans and can be a risk factor for more severe symptoms later during withdrawal (e.g., Monte et al., 2009). We chose the ~24 hr time point because body temperatures in our mice would likely have been at the low point in the circadian cycle at the time we assessed it (Tankersley et al., 2002); thus, the withdrawal response might have been expected to be hyperthermia. At the time of day that we assessed temperatures (1 hr after lights on), otherwise naïve male mice (C57BL/6J, C3H/HeJ, and their F1) with implanted abdominal transmitters show core body temperatures of approximately 0.75 °C below their 24-hr average core temperatures

and about 1.75 °C below their peak core temperature (at just after lights out; Tankersley et al., 2002). Both of these inbred strains were included in the development of the HS/Npt mice tested in the present studies (Hitzemann et al., 1998). Damaggio & Gorman (2014b), using a series of elegantly designed studies, found that C57BL/6J male mice subjected to multiple exposures of chronic intermittent ethanol vapor inhalation and withdrawal show hyperthermia of differing degrees depending on whether withdrawal started at night or during the day (when it was more severe). However, using our continuous vapor inhalation procedures, we showed in another heterogeneous stock, HS/Ibg, that withdrawing male mice exhibited similar core body temperatures during the first 4-10 hrs of withdrawal compared with air controls, but preferred a lower ambient temperature. We interpreted this to indicate that regulated body temperature was not altered during ethanol withdrawal, but that mice sought to dissipate extra heat due to the hypermetabolic state accompanying withdrawal (Crawshaw et al., 1994). The procedures used for our current battery cannot explain the mechanisms of the core temperature differences, nor why our results differ from those of Damaggio & Gorman (2014a,b), who used very different procedures and a single inbred strain. One simple difference is that we included both sexes in our studies here; however, there were no sex differences in change in body temperature, just in absolute temperatures during withdrawal (Figure 6 A vs B). Another clear difference between the studies was our use of 72 hr continuous vapor exposure vs their chronic intermittent exposure.

The domain of anxiety-like behavior showed apparatus specificity. At the same time during withdrawal (WD Hr 24) and at the same approximate BEC, increased anxiety-like behaviors were shown in the light-dark box, but not on the elevated zero maze (Figures 5 C-F, Supplemental Results). We have previously reported the absence of an effect of 48 hrs of withdrawal on behaviors such as % open arm time and open arm entries on the EZM when the apparatus was novel (Kliethermes et al., 2004). Furthermore, there were no differences between C57BL/6J and DBA/2J inbred mouse strains experiencing withdrawal compared with their respective controls on the elevated plus maze (EPM; a predecessor apparatus) at 6-10 hrs after 72 hrs of chronic continuous ethanol vapor inhalation (Finn et al., 2000). The EZM and EPM are often used to demonstrate withdrawal anxiety-like behaviors in rats at multiple time points of withdrawal (e.g., Zhao et al., 2007), suggesting species differences in behavior. These authors used a 4-week-long chronic intermittent vapor inhalation procedure (targeting BECs of 1.50-2.20 mg/ml) to induce dependence in male Wistar rats and measured withdrawal on the EPM during acute (~8 hrs), early protracted (2 weeks) and late protracted (6,12 weeks) withdrawal phases. They found that anxiety-like behavior increased at ~8 hrs, decreased to control levels at 2 weeks, but rebounded during late protracted withdrawal (Zhao et al., 2007). Closed arm entries (generalized activity) were not significantly reduced during the acute or early protracted phases of withdrawal (Zhao et al., 2007). They did not analyze total (closed + open) entries. In contrast, our experiment (WD Battery 2), withdrawal assessed in the light-dark box at WD Hr 12 also showed increased anxiety-like behaviors (Figure S3 and Supplemental Results), but this occurred in combination with nearly complete reductions of line crossings and transitions into/out of the dark compartment (Figures S3 E, H). Interpretation of our finding at WD hr 12 as an anxiety-like behavior may therefore have been compromised by hypoactivity at this early time point during withdrawal [mice making few to no entries into open arms cannot

reasonably be inferred to be avoiding an experienced anxiety, since it is presumably not elicited by remaining in the closed arms - see (Kliethermes, 2005)]. Assessment of anxiety-like behavior in mice, in contrast to rats, may require tests that do not place as much emphasis on locomotor behavior (e.g., marble burying [e.g., Lee et al., 2014]). Another possible explanation for the difference in findings is that anxiety-like behavioral testing in rats is most often performed after liquid diet or chronic intermittent vapor exposure. We could find no anxiety-like behavioral study that used chronic continuous vapor inhalation in rats. However, the results of Zhao et al. (2007) suggest that examination of anxiety-like behavior during late protracted withdrawal in mice could be useful, although it was beyond the scope of the present work.

The domain of anhedonia/depression-like behavior was assessed by the sucrose preference test; whether withdrawal was seen depended on sex in WD Battery 3. The sucrose preference test in males, but not females, showed results consistent with an interpretation of increased depression-like behavior or decreased hedonic effects of a pleasurable solution (Muscat and Willner, 1992; Katz, 1982). We have conducted this test in several other studies, and have reproduced the finding of decreased sucrose preference by withdrawing animals, but we have not always observed the differential responses of the sexes in this test (unpublished data). We have never observed a sex-dependent drop in sucrose preference in other genotypes; thus, the result with HS/Npt may be genotype-specific. However, there was also a tendency for withdrawing HS/Npt males, but not females, to show reduced sucrose preference in WD Battery 2 (Supplemental Results, Figure S5). Such a result argues not only that both sexes should be tested (Crabbe, 2016), but also that sufficient sample sizes to detect interactions of sex with treatment should be employed (Wahlsten, 2007; 2011). The withdrawing mice did not show reductions in total fluid intake (see Figure 7 D), which would be expected if this were a generalized effect on drinking *per se*.

The progressive approach to design of these experiments was adopted in order to eliminate possibly redundant tests (e.g., the vertical screen), identify insensitive apparatus (e.g., the elevated zero maze), and clarify timing during withdrawal when particular behaviors were most affected (e.g., light-dark box at WD Hr 24, or at least later than WD Hr 12). Thus, we recommend the design of WD Battery 3 as a starting point, with possible substitutions of apparatus to reflect local conditions (e.g., if no accelerating rotarod is available, a vertical screen can be made inexpensively), or addition of other tests to reflect other hypotheses.

An important and surprising finding of these experiments was that severities of withdrawal across different behavioral domains were not well correlated (Tables 3 & 4). In fact, using the data from WD Battery 3, the only significant correlations were between the two WD_AREA scores (which are computationally related) and between the two rotarod ataxia measures (WD_Performance and WD_Improvement). There are few prior data addressing the co-occurrence of multiple withdrawal-related behaviors (but see Lee et al., 2014 for multiple anxiety/depression measures after drinking). Freund (1969) reported withdrawal tests based on multiple responses in mice. However, this effort did not identify whether different assays were assessing the same underlying behaviors (i.e., showed phenotypic correlation). In another early study, mice of the genetically heterogeneous (HS/lbg) stock were allowed to drink ethanol solutions in a liquid diet for multiple days to induce

dependence and were then scored for multiple behaviors, including HICs, the hole-in-the-wall test, body temperature, and the vertical screen test (McClearn et al., 1982). The behaviors taken to indicate dependence were assessed by factor analysis, which extracted a single factor. The variables generally were found to be very weakly correlated – each loaded approximately 0.2 on the withdrawal factor. It is hard to know exactly how to interpret these data, because one of the variables was ethanol dose ingested.

The positive correlation between WD_hypothermia and WD_loss of sucrose preference was only significant among High BEC group animals (Table 4), indicating the greater the hypothermia at ~24 hrs into WD, the less sucrose was preferred between 2-4 days into WD. As we did not assess body temperatures near the time of the sucrose preference test, we cannot exclude the possibility that temperatures of withdrawing mice were still affected later during withdrawal, which may, in turn, have affected their sucrose preference. However, fluid intake was not different between withdrawing and air control animals.

Our study only allowed us to examine phenotypic relationships. Results seeking to examine genetic correlations of withdrawal HIC severity and other behaviors have yielded mixed results. The majority of withdrawal behavioral assays have not yet been examined in WSP and WSR mice (Metten & Crabbe, 1996). However, we previously found that WSP and WSR mice, selectively bred for severe or mild withdrawal HICs, respectively, did not differ in withdrawal-induced rotarod performance after exposure to ~1.5 mg/ml BEC (Philibin et al., 2008). Subsequent studies showed little relationship between rotarod deficits and HICs across inbred mouse strains (Philibin et al., 2012). Similarly, results of the comparison of WSP and WSR mice withdrawing from ethanol vapor showed patterns suggesting a positive correlation of HICs with tremor and a negative genetic correlation with backward walking, where WSR mice showed greater response (Kosobud & Crabbe, 1986). Anxiety-like behavior, measured as a reduction in percent open arm time on the elevated plus maze, tended to be increased in WSP, but not WSR, mice, indicating that withdrawal seizure susceptibility and anxiety-like behavior may share some common genetic determinants (Gorin et al., 2005). WSP and WSR mice do not differ during withdrawal in Straub tail, Hole-in-Wall measures, and vertical screen activity (Kosobud & Crabbe, 1986). However, all of these studies were focused on genetic commonality and did not address phenotypic correlations.

To our surprise, most behaviors tested in WD Battery 3 were not significantly less affected by a lower ethanol dose under our conditions (BECs 1.62 versus 2.07 mg/ml; Table 1, collapsed across sexes). While there were statistically significant differences in BECs in females, but not males, in WD Battery 3, the behavioral data suggest that the difference is not behaviorally relevant. As mentioned, an exception was the accelerating rotarod test, which showed dose sensitivity for two measures, trials over time and performance, but not for the third, improvement. In another study, rotarod performance differences among groups exposed to 0, 1.0, 1.5, or 2.5 mg/ml BECs were seen except between the 1.5 and 2.5 mg/ml groups (Philibin et al., 2012). It is possible that a larger difference between BEC targets is necessary for other behaviors, but equally possible that a threshold BEC is necessary and sufficient. More simply stated, the shape and near-linear range of the presumed vapor-induced dose-effect curves for different withdrawal signs are not well known and may vary

well differ from one another. Although we did not detect differences due to BEC groups in HIC AREA scores in this study, it is possible that this was because we assessed HICs only 4 times. Dose and/or exposure duration jointly affect duration of withdrawal HIC effects (Goldstein, 1972; Crabbe, 1998). More detailed parametric explorations of the withdrawal battery would be needed to provide a full picture of the behavioral withdrawal landscape.

Results on the hot plate were also surprising in that no effect of withdrawal was seen at WD Hr 27. Hyperalgesia during alcohol withdrawal is experienced by humans (Jochum et al., 2010), and others have demonstrated that this occurs in animals (Gatch, 2009; Smith et al., 2016). Multiple pain modalities, including thermal, mechanical, and chemical sensitivity, have been shown to be sensitive to withdrawal in mice (Smith et al., 2016). Male C57BL/6J mice were given ascending concentrations of ethanol in a 24 hr/day 2 bottle choice test (2 days each at 3%, 6%, and 1 day at 10%). After experiencing withdrawal for 24 hrs, they were again given 10% ethanol for 6 days. Testing during a second withdrawal at WD Hr 24 produced hyperalgesia using either the mechanical von Frey test or the thermal tail withdrawal test (Smith et al., 2016). Thermal hyperalgesia is also observed in rats 3, 6, and 12, but not 36, hrs after removal of liquid diet (6.5% w/v ethanol for 10 days; Gatch & Lal, 1999). Using the same vapor inhalation procedures as those here, we have seen withdrawal hypoalgesia sensitivity of the hot plate test in other genotypes (Greenberg, unpublished data). Thus, the present finding may be due to our use of HS/Npt mice.

The question of whether to use a battery of tests targeting multiple behavioral domains or multiple tests within a domain merits discussion. We believe that both approaches are useful. We would argue that researchers with a novel genetic animal model or with established models that have never investigated the withdrawal state might want to use the multiple domain battery to identify whether there are particular domains that require further investigation. On the other hand, a researcher interested in a particular behavioral domain may want to investigate withdrawal severity using a battery of tests within that domain. For example, we know that the domain of ataxia is complex and by and large, the different tests assessing murine sensitivity to acute ethanol seem to reflect different genetic constructs (Crabbe et al., 2005). By demonstrating diverse withdrawal effects in heterogeneous stock animals, we infer that these experimental designs are highly likely to be generalizable to multiple strains of mice, and perhaps specific murine genotypes (e.g., transgenics). Our data demonstrate that, when conducted under controlled conditions and at the proper WD Hr, testing multiple behaviors in the same mice serially results in reliable displays of withdrawal from alcohol. Importantly, even though pronounced withdrawal was seen for the multiple behaviors tested, severities were phenotypically uncorrelated, in the main, indicating distinct underlying mechanisms. Thus, alcohol withdrawal reflects a complex collection of behavioral dysregulations, but the distinctiveness of individual sensitivities to different withdrawal responses suggests that individualized therapies may not be unobtainable.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Commonly used mouse behavioral tests from multiple behavioral domains reflected the state of withdrawal.

Domains tested included neurohyperexcitability, ataxia, anxiety-like and anhedonia-/depression-like behaviors, body temperatures, and thermal pain sensitivity.

Severity of withdrawal using different measures from different domains were not phenotypically correlated.

Use of a heterogeneous stock in development of these behavioral batteries should lead to generalizability to multiple strains of mice.

Withdrawal behaviors from different domains occurred at specific times across a 4-day time course.

Figure 1.

Withdrawal Battery Experimental Designs. Each blue box contains the design of one of the three withdrawal batteries. Across the top of each box, a grey rectangle shows the start times of each test. Medium blue rectangles show the dependence induction phase, which was 72 hrs of chronic ethanol vapor inhalation (or the air control procedure). Immediately after this phase begins the withdrawal time course at WD Hr 0. Test durations (not shown) are described in the methods section. Rest periods (light grey bars; Rest or R) separated each test where shown and mice were co-housed with their original cage mates during these periods. Handling-Induced Convulsion (HIC) scores were assessed at the red arrows in WD Batteries 1 and 3. Tan bars indicate the Open Platform test; light purple bars indicate ARR – Accelerating Rotarod; orange bars indicate an anxiety-like test – Elevated Zero Maze (WD Battery 1) and Light/Dark Box (WD Batteries 2 and 3); light teal bars indicate Body Temperature assessment, either Baseline or during Withdrawal; purple bars indicate the Hot Plate test; and green bars indicate the Sucrose Preference test.

Figure 2.

Withdrawal assessed as handling-induced convulsions, from WD Battery 1. Left panel: average HIC scores at WD Hrs 8, 11, and 25, by Treatment and Sex. Withdrawing animals of both sexes showed significantly greater scores over time compared to air controls, while males had greater scores than females overall. Right panel: withdrawing animals also showed significantly greater Area Under the Curve scores (AREA25; ** $p < 0.01$, *** $p < 0.0001$). Significant main effects of sex were also found (^a $p < 0.01$), but sex did not interact with treatment. Mean $\pm$ SE shown.

Figure 3.

Withdrawal severity as assessed by the behaviors on the Open Platform during WD Battery 1. Shown from left to right in Panels A - C are the % of mice that displayed the sign from the neurohyperexcitability measure, tremor (A); Straub tail (B); Backwards walking (C); and the number of Line crossings in Panel D. For the behaviors expressed as percentage of animals (A-C), shown are the results of χ^2 tests for each WD Hr separately (* p<0.05, ** p 0.01, *** p<0.001). Greater percentages of withdrawing animals showed tremor, Straub tail, and backward walking, compared to air controls. Line crossings showed effects of withdrawal at both WD Hr, with air control animals showing greater numbers of line crossings relative to withdrawing animals (both ps<0.0001).

Figure 4.

Withdrawal severity as assessed by the ataxia measure, latency to fall from the Accelerating Rotarod, from WD Battery 3. Shown in the left panel are the average latencies to fall across trials by Treatment group. Withdrawing animals showed significant dose-dependent reductions in latencies compared to air controls. Average latency to fall (Performance) was also dose-dependently reduced during withdrawal. Both the medium (Med) BEC group and the High BEC group showed reduced performance relative to the air control group *** $p < 0.0001$. The High BEC group also showed reduced performance relative to the Medium BEC group ^a $p < 0.01$. Main effects of Sex ($F > M$) were found in Latency to Fall over trials ($p < 0.01$) and Performance ($p < 0.01$), but not Improvement ($F < 1$). Improvement was significantly greater in air controls relative to either BEC group ($ps < 0.001$), but the BEC groups did not show a significant difference from each other.

Figure 5.

Withdrawal severity at WD Hr 24 as assessed by several anxiety-like behavioral measures from the Light-Dark Box test in WD Battery 3. Withdrawing animals showed no differences in total time in the light compartment. Mice of the Medium BEC group showed increased initial latency to enter the dark compartment (Panel B) and both BEC groups showed increased latency to exit the dark compartment (Panel C). Withdrawal also reduced nose pokes (D), transitions between compartments (E), and voluntary time in the light (F). Rearing behavior (G) and line crossings (H) in the light compartment were also reduced by withdrawal. *p<0.05, **p<0.01, ***p<0.001

Figure 6.

Withdrawal severity as assessed by change in body temperature from WD Battery 2. Shown in panel A are the average Basal and WD Hr 24 temperatures by Treatment and Sex.

Withdrawing animals showed significantly lower WD Hr 24 body temperatures compared with baseline (Panel A) and significantly greater hypothermia compared to air controls (Panel B). ** $p < 0.01$, *** $p < 0.0001$. Significant main effects of sex (F>M) on both basal and WD Hr 24 body temperatures were also found, but not on hypothermia. ^a $p < 0.01$. Sex did not interact with Treatment in any comparison.

Figure 7.

Withdrawal severity as assessed by the anhedonia measure, reduction in sucrose preference, from WD Battery 3. Shown in the left two panels are the daily sucrose preference percentages for females (A) and males (B) by blood ethanol concentration (BEC) group vs air controls. *Post hoc* analyses were performed for the Sexes separately due to a significant BEC group $\times$ Sex Interaction (^a $p < 0.05$). Female mice showed a significant main effect of Day (** $p < 0.01$) but no interaction of Day by BEC group. Panel C shows average sucrose preference by BEC group and Sex. Average preference for female mice also did not differ by BEC group (grey bars). Male mice (black bars) with Medium and High BECs showed reduced preference compared with the air group, depending on Day (* $p < 0.05$, ** $p < 0.01$). Males with Medium and High BECs also showed reduced average preference compared to air controls, but the two BEC groups did not differ (* $p < 0.05$, *** $p < 0.001$). Panel D shows total fluid (sucrose and water) by treatment and sex. There were no significant differences across days, among treatments or between sexes.

Table 1

Sample Sizes, Body Weights, Basal Body Temperatures, and Blood Ethanol Concentrations (BECs) for Each WD Battery.

Experiment	N		Initial Body Weight (g)		Basal Body Temperature (°C)		BECs at End of Vapor		
	F	M	F*	M	F*	M	F	M	Both
Sex->									
1: EtOH	21	11	22.75±0.51	25.88±1.31	38.38±0.10	38.01±0.24	2.25±0.07	2.38±0.12	2.30±0.06
1: Air	10	9	21.82±0.82	26.21±0.81	38.36±0.09	37.77±0.17	-	-	-
2: EtOH	19	12	23.57±0.36	29.34±0.81	37.54±0.20	37.73±0.09	2.36±0.08	2.22±0.10	2.31±0.06
2: Air	8	8	21.81±0.50	29.20±1.20	38.29±0.11	37.60±0.12	-	-	-
3: EtOH-high	12	16	22.11±0.55	26.23±0.63	38.39±0.08	37.65±0.09	2.24±0.11	1.94±0.12	2.07±0.09
3: EtOH-medium	14	14	21.61±0.43	26.66±0.54	38.37±0.07	37.51±0.11	1.53±0.10 ^d	1.71 ±0.12	1.62±0.08
3: Air	12	12	21.45±0.65	27.19±0.96	38.14±0.18	37.65±0.22	-	-	-

* significant main effect of sex on body weight in all 3 WD Batteries ($F_{(1,43-74)} > 18.7$, $p = 0.0001$), without effects of (later) treatment or sex $\times$ treatment interactions ($F_{(1,43-74)} < 1.79$, $ps > 0.18$). Asterisks also indicate significant main effect of sex on basal body temperature ($F_{(1,43-74)} > 8.58$, $p = 0.01$) that did not differ among groups assigned to later treatment ($F_{(1,2,43-74)} < 0.89$, $p > 0.34$) or sex $\times$ treatment interactions ($F_{(1,2,43-74)} < 3.42$, $p > 0.07$).

^d BECs in WD Battery 3 showed both a significant main effect of vapor concentration and sex $\times$ concentration interaction ($F_{(1,52)} > 4.31$, $ps < 0.05$) but not a main effect of sex ($F < 1$). Post hoc analyses of the interaction revealed that female mice showed concentration-dependent BECs (Tukey's HSD, $p < 0.001$), but male mice did not ($p > 0.46$).

Table 2
Effect Sizes (R^2) for WD vs Air Control or BEC group vs Air Control

WD Battery	WD Hr	Behavior	Effect Size *	
			WD vs air	BEC vs air
1	0-25	WD AREA25	0.50	
1	5	Platform: LC	0.46	
1	9	Platform: LC	0.52	
1	9	Vertical Screen	0.34	
2	24	Hypothermia	0.19	
3	9	ARR Performance		0.44
3	9	ARR Improvement		0.31
3	24	Light/Dark Box (LDB): Time in Light		0.06
3	24	Light/Dark Box (LDB): Latency to Enter Dark		0.09
3	24	Light/Dark Box (LDB): Latency to Exit Dark		0.09
3	24	Light/Dark Box (LDB): Nose Pokes from Dark to Light		0.14
3	24	Light/Dark Box (LDB): Transitions		0.23
3	24	Light/Dark Box (LDB): Voluntary Time in Light		0.13
3	24	Light/Dark Box (LDB): Rearing in the Light		0.11
3	24	Light/Dark Box (LDB): LC		0.15
3	28	Pain Sensitivity		0.02
3	50-98	Average Sucrose Preference		0.09
		Average Sucrose Preference - M		0.32
		Average Sucrose Preference - F		0.00

* Effect sizes were estimated using multiple R^2 from one-way ANOVAs on Treatment. Effect sizes for significant effects of Treatment are shown in **BOLD**.

Table 3

Phenotypic Correlations among WD Behaviors *

Pearson's r	WD_AREA26	WD_AREA48	WD_Performance	WD_Improvement	WD_Hypothermia	WD_Hot Plate
WD_AREA48		-				
WD_Performance	0.2300	0.2470				
WD_Improvement	-0.0018	-0.0498	-			
WD_Hypothermia	-0.0673	-0.0746	0.0498	0.1489	-	
WD_Hot Plate	-0.1074	-0.1516	0.2461	0.1436	0.2513	-
WD_SUC_Avoid **	-0.1656	-0.1541	-0.1625	-0.0322	-0.1265	0.0420

* All behavioral indices were transformed so that greater positive numbers described greater withdrawal severity.

** WD_SUC_Avoid: WD-induced reduction in sucrose preference

Table 4
Phenotypic Correlations among WD Behaviors by BEC Group High BEC group

Pearson's r	WD_AREA26	WD_AREA48	WD_Performance	WD_Improvement	WD_Hypothermia	WD_Hot Plate
WD_AREA48	0.9121	-				
WD_Performance	0.4938	0.5330	-			
WD_Improvement	0.4533	0.4373	0.1760	-		
WD_Hypothermia	-0.2118	-0.1776	-0.0404	0.1862	-	
WD_Hot Plate	0.0246	-0.0957	0.1871	-0.1048	0.2807	-
WD_SUC_Avoid	-0.1579	-0.0913	-0.3062	-0.2635	-0.4126	-0.1234
Med BEC group						
Pearson's r	WD_AREA26	WD_AREA48	WD_Performance	WD_Improvement	WD_Hypothermia	WD_Hot Plate
WD_AREA48	0.9507	-				
WD_Performance	0.0316	0.0880	-			
WD_Improvement	-0.2705	-0.3037	0.6145	-		
WD_Hypothermia	0.0752	0.0224	0.1325	0.1206	-	
WD_Hot Plate	-0.1859	-0.1843	0.3436	0.3006	0.2427	-
WD_SUC_Avoid	-0.1657	-0.1934	-0.0471	0.1201	0.2240	0.1565