Video Article

Murine Drinking Models in the Development of Pharmacotherapies for Alcoholism: Drinking in the Dark and Two-bottle Choice

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Abstract

Alcohol Use Disorder (AUD) is a major problem with more than an estimated 76 million people worldwide meeting the diagnostic criteria. Current treatments are limited to three FDA-approved medications that are largely ineffective even when combined with psychosocial intervention, as is evident by the high relapse rate. As such, the search for more novel treatments represents an important public health goal. To this end, the following protocol utilizes two simple rodent drinking models to assess the preclinical efficacy of lead anti-alcohol compounds: two-bottle choice (TBC) and drinking in the dark (DID). The former allows mice to voluntary drink in moderation while the latter induces mice to voluntary consume a large amount of alcohol in a short period that mimics binge drinking. The simple and high throughput nature of both of these paradigms allow for rapid screening of pharmacological agents or for identifying strains of mice that exhibit certain voluntary drinking behavior.

Video Link

The video component of this article can be found at https://www.jove.com/video/57027/

Introduction

For the past 25 plus years, significant effort has been put towards developing medications for the treatment of Alcohol Use Disorder (AUD)[1].Although many advances have been made, AUD still remains a major public health problem, affecting over 18 million Americans, and costing over $220 billion annually[2,3]. Currently there are only three FDA-approved medications, disulfiram, naltrexone, and acamprosate, all of which have yielded inconsistent results in clinical trials and limited success even when combined with psychosocial intervention in the clinic settings[4,5,6,7].

A primary reason for failures of current AUD therapy is linked to the heterogenous nature of AUD[8]. While both environmental and genetic factors contribute to the development of AUD, heritability accounts for an estimated 50 - 60% of the risk of onset[9]. Similar to the treatment of depression, it is widely accepted that patients suffering from AUD will need a variety of medications that are tailored to meet the needs of each patient[10].

Clearly, there is an urgent need for more effective treatments that would be facilitated if the already arduous and time consuming process of drug discovery were streamlined[3]. To this end, the following protocol demonstrates the preclinical applicability of two rodent drinking models widely used to examine the neurobiological basis of AUD[11]. More specifically, the method introduced herein can assess the efficacy of candidate compounds at reducing alcohol consumption in both "moderate" and "binge drinking" scenarios utilizing the two-bottle choice (TBC) and drinking in the dark (DID) paradigms, respectively. Both paradigms examine non-operant ethanol self-administration, whereby mice ingest ethanol orally and at will, and therefore illustrate high face and construct validity as a model of human alcoholism[11].

In TBC drinking, also known as free choice drinking, preference drinking, or social drinking, two bottles of solution are continuously available in the home cage. One bottle contains water, and the other contains a diluted solution of ethanol, whereby the concentration of ethanol can be varied (*e.g.*, 5 - 30% v/v)[11,12]. The mice have constant access to both bottles, and therefore, can choose how much to drink from each bottle.

This model assesses the ethanol consumption of each mouse (g/kg), as well as the ethanol preference ratio (volume of ethanol consumed ÷ total volume liquid consumed). It is routinely used to compare drinking levels across different strains of mice, or after a specific genetic manipulation (*e.g.*, gene knockout or knockdown) and results in blood ethanol concentrations (BECs) similar to what is found in humans when drinking in moderation[13,14].

In the DID procedure, 3 h after the start of the dark cycle, the home cage bottle of water is exchanged with a bottle of 20% (v/v) ethanol solution for a limited access drinking session. The drinking sessions occur as a consecutive 4-day cycle, lasting 2 h on days 1 - 3 and 4 h on day 4. Days 1-3 serve as an alcohol-habituation period before the testing on day 4. Consequently, mice will reliably consume enough ethanol to attain BECs

>100 mg/dL and as a result, exhibit the behavioral effects of intoxication found in humans that are binge drinking[13,14,15]. Water access is available at all times other than the drinking session.

There are several variations of limited access drinking. For example, in the intermittent access model, mice receive two bottles (one containing water and the other containing 20% (v/v) ethanol) only on Monday, Wednesday, and Friday with a 24 h and 48 h withdrawal period on weekdays and weekends, respectively[16]. After several weeks of intermittent access, the mice will gradually and voluntarily escalate drinking levels, eventually attaining BECS similar to what is observed in the DID model. The DID, however, appears to be the most commonly used model to assess binge-like drinking behavior. Other models of intermittent drinking exist, but they rely on restricting access to food or vapor chamber induced increases in voluntary self-administration, which makes them less representative of voluntary human alcohol consumption[16].

Protocol

All procedures described here have been approved by the Institutional Animal Care and Use Committees of the University of Southern California Health Sciences Campus.

1. Experimental Setup and Assembly

1. Acquire all of the following supplies and chemicals before the start of the study: mice, cages/metal cage tops, bedding, food, water, ethanol, pipets, sipper tops, shrink wrap, utility knife, zip ties, tape, Bunsen burner, scale, headlamp.
2. Obtain C57BL/6J mice, either from a commercial source or an in-house colony, keeping in mind that mice can be group housed until time of testing.
   NOTE: The total number of mice procured depends on the complexity of the experimental design. Plan to accommodate roughly 12 - 15 mice per group, with pilot studies no smaller than 5 - 7 mice per group. In the representative results shown below we utilized a simple two-group set-up to assess the cause-effect relationship using a single dose (5 mg/kg) of the drug (MOX).
3. Follow the steps below to assemble the bottles[18].
   1. Heat a utility knife using a Bunsen burner.
   2. Using this knife, cut roughly an inch off the top and bottom ends of a plastic 18 mL serological pipet.
      1. Note that smaller volume pipets (*i.e.*, 10 mL) can also be used to increase the precision of the measurement.
   3. Warm the pipet under a heat gun.
   4. Insert the ball bearing sipper tube into the "bottom" end of the pipet (in other words, the opening nearest to the 18 mL dash-line).
   5. Seal the sipper tube in place with shrink wrap using a commercially available shrink wrap gun.
   6. Cap off the other opening with a silicone stopper.

2. Animal Habituation

1. Beginning at least 1 week prior to the start date of the experiment, transfer the mice to the room where the experimental procedures are to be carried out so that they may acclimate to the husbandry conditions (including the ambient temperature (21 ± 1 °C) and 12-h reverse light/dark cycle, with lights off at 12 noon). Be sure to follow institutional guidelines and notify the appropriate channels before moving animals from one location to another.
   1. If mice are being transferred from a standard light/dark cycle allow 2 weeks of additional habituation time.
2. Fill the newly made bottles to the brim with water. Make sure that the cap is closed securely and devoid of any air bubbles or leaks from the spout. If solution is leaking, re-secure the cap. Remove any air bubbles by simply tapping on the bottle so that the air can escape the tube.
3. Upon arrival, single house each mouse in standard polycarbonate/polysulfone cages with bedding and a metal grid cage top; remove the cage lid as it will no longer be used.
   1. Provide access to food and water bottle(s) *ad libitum*.
   2. Secure each bottle to the cage top by wrapping a plastic zip tie around each bottle to hold it in place. Trim any excess plastic from the zip tie to ensure that it does not protrude into the cage.
      NOTE: For the drinking in the dark (DID) procedure, only a single bottle of water is required. Habituation to the two bottle choice (TBC) paradigm, however, requires that the cage set-up include two bottles of water. If the metal grid hopper provided is designed to hold only a single bottle of solution, gently bend apart its bars to create space for an additional plate to accommodate the second bottle for TBC.
4. Set up at least 3 mouse free control cages. This will allow for the monitoring of any fluid loss caused by evaporation or spillage from the bottles, which is simply a natural occurrence that happens as cages are placed on and off the cage rack (see step 2.6.3, 3.6.1, and 4.6.1 for equations).
5. Beginning on day 4 of the 1-week single housing acclimation, measure and record the daily body and food weights of each mouse, using a scale, (in grams) as well as the water intake, using the etchings alongside the inverted bottle to record the highest point of the meniscus (in mL).
   1. While it is standard scientific practice to read the lowest point of the concave meniscus, because the bottles remain in an inverted position during the measurement, record the highest point of the meniscus.
6. Assess the parameters of 2.5 using the equations below:
   1. Measure the Body Weight Change (g): weight of current day (g) - weight of previous day (g).
   2. Measure the Food Intake (g): weight of food on previous day (g) - weight of food on current day (g).
   3. Measure the Water Intake (mL): [volume of water on current day (mL) - volume of water on previous day (mL)] - average water loss from all control cages (mL).

7. Repeat step 2.5 consecutively, on days 5 - 7, to allow for the determination of a baseline for the three days immediately prior to the introduction of ethanol. If a consecutive recording cannot be collected, extend the acclimation period to allow for the evaluation of baseline measurements.
8. Once water intake has stabilized to ± 10% variability from the mean of the last 3 days, begin ethanol access with either TBC (unlimited access) or DID (limited access).
   NOTE: On rare occasion, one to two additional days may be needed for subjects to attain this stability; don't be alarmed if additional time is needed for the values to display ±10% variability from the mean of the last 3 days.

3. 24-Hour Two Bottle Choice (TBC)

Note: A schematic is prepared in **Figure 1**.

1. Prepare a 10% (v/v) ethanol solution at a 500 mL volume by adding 52.65 mL of 190 proof grain ethanol (~95% ethanol) to 447.35 mL of H_2O; be sure to shake thoroughly. Given that ethanol evaporates quickly, replace the solution in every 3-4 day's interval.
   NOTE: Other concentrations of ethanol can be used as well, but the authors recommend a 10% concentration for this model.
2. On the first day of TBC, (day 8 at the earliest) empty 1 of the 2 water bottles, in each cage, and fill it to the brim with the freshly prepared ethanol solution. Given that ethanol and water are difficult to be distinguished visually, clearly label the bottles with their corresponding contents. Simply apply a piece of masking tape to the bottle and labeling it with a marker or by writing directly on the bottle.
3. Add more solution to the water bottle, as needed.
4. Place bottles back on the cage, making sure that all caps are closed securely and devoid of any air bubbles or leaks from the spout. If the solution is leaking, re-secure the cap. Remove any air bubbles by simply tapping on the bottle so that the air can escape the bottle.
5. Alternate the position of the bottles every other day as to correct for conditioned place preference related influences in drinking activity (see **Discussion** for more).
6. In addition to the daily measurements from 2.5 and 2.6, which have been ongoing, begin to read and record ethanol intake levels as well. Analyze the 10% ethanol intake and preference ratio using the following equations:
   1. Measure the 10% Ethanol Intake (mL): [volume of ethanol on current day (mL) - volume of ethanol on previous day (mL))] - average ethanol loss from all control cages (mL).
   2. Measure the 10% Ethanol Intake (g/kg): [10% ethanol Intake (mL) x 0.07893 g/mL] / body weight (kg).
   3. Measure the Preference %: [10% Ethanol Intake (mL) / Water (mL)] x 100.

7. As soon as ethanol intake has become stable, administer a single control (saline) intraperitoneal (i.p.) injection (0.01 mL/g of body weight) to each mouse during the daily measurement routine. In this way, subjects become accustomed to the injection itself.
   1. Stability is defined as ± 10% variability from the mean of the last 3 days (same as in section 2.8).
      NOTE: It can take up to 1 week for ethanol levels to stabilize. This is especially true if mice are being re-used from a previous experiment and have had previous exposure to ethanol. The control is simply the solvent used to dissolve the drug.

8. Once an ethanol baseline with low variability is re-established, split the mice into dosing groups using the ethanol intake values so that all groups have roughly similar average ethanol intake values.
   1. Designate one group as the control (continuing to receive saline) and the other as the experimental (i.p. injection of the investigated drug at 0.01 mL/g of body weight). Begin daily drug dosing, either for an acute or multi-day duration. Subsequently, the control can be re-introduced to test the post-drug effects (optional).
      NOTE: Because drinking is monitored across a 24-h long period, the time of dosage administration is not dependent on the dark cycle.

4. Drinking in the Dark (DID)

NOTE: A schematic is prepared in **Figure 3**.

1. On each day of scheduled ethanol access (Days 1 - 4) record the measurements, for water volume, food intake, and body weight, and perform drug dosing. Do this during a preselected time during the light cycle that is chosen in accordance with the pharmacokinetics of the drug so that the compound is at/or approaching the maximum brain concentration during the drinking period.
   NOTE: Remember days 1 - 3 are meant to simply acclimate the mice to drinking copious levels of ethanol in a short period of time. While mice do not reach BEC levels comparable to the human 0.08; these "training" days ensure that on day 4, during the slightly longer drinking session, they will in fact drink to that level.
   1. Give all mice the control (saline) on days 1 - 3, and either the control or drug on day 4. This DID procedure occurs over the span of 3 weeks to include a pre-drug (week 1), drug (week 2), and post-drug (week 4) drinking session.
   2. NOTE: Note that during the drug-dosing week, day 3 ethanol intake levels are used to assign mice to either the control or drug group in a way where the ethanol intake levels of both groups have the least variability. This is unlike TBC, which assigns groups based on a 3 day average.

2. Prepare a 20% (v/v) ethanol solution (20E) at a 500 mL volume by adding 105.25 mL of 190 proof grain ethanol (~95% ethanol) to 394.75 mL of H_2O; be sure to shake thoroughly.
3. Fill the ethanol bottles before the start of the drinking session so that as soon as the DID begins the water bottles can simply be replaced with the alcohol bottles.
4. During the entire DID session (steps 4.5 - 4.8), use a red-light headlamp as to not disturb the animals.
5. At the start of the DID drinking session, scheduled to begin 3 hours into the dark cycle, record the volume of water for each mouse. Then, replace each water bottle with a bottle of the 20E solution and record the starting ethanol volume.
6. Read and record the final ethanol volume 2 hours later, at the end of the drinking session on Days 1 - 3 and 4 hours later on Day 4. Analyze the 20% ethanol intake using the following equations.

1. Measure the 20% Ethanol Intake (mL): [volume of ethanol at end of drinking session (mL) - volume of ethanol at start of drinking session (mL)] - average ethanol loss from all control cages (mL).
2. Measure the 20% Ethanol Intake (g/kg): [20% Ethanol Intake (mL) x 0.15786 g/mL] / body weight (kg).

7. On Day 4 only, immediately after recording ethanol volumes, and before re-introducing access to water, collect blood to assess the blood ethanol concentration of each mouse (optional).
   NOTE: Any non-terminal blood collection method may be used, such as retro-orbital sinus blood collection, or saphenous vein blood collection. For useful protocols see references for Parasuraman *et al.*[21] and Yardley *et al.*[20].
   1. Perform analysis using various methods including LCMS, or commercially available machinery (see **Table of Materials**).

8. Replace all ethanol bottles with water bottles and record water volume.

Representative Results

In the following representative investigations, social drinking was modeled using the two-bottle choice (TBC) paradigm. Briefly, mice had access to two bottles of solution, one of which contained water, and the other a 10% (v/v) ethanol solution. Subjects were subsequently split and evenly assigned to drug treatment groups, moxidectin (MOX) vs. saline control, so that each group would have averaged ethanol intake levels with the least amount of variation.

Initial baseline 10E intake during the 24-h period stabilized at 14.46 ± 1.85 g/kg (n = 8) before injections began, and subsequently re-stabilized at 14.14 g/kg post saline injections. To assess the effects of an acute dose (5 mg/kg) of MOX on ethanol intake and preference, drinking activity was assessed pre MOX, MOX, and post MOX. We found that a single dose of MOX significantly reduced alcohol intake in excess of 45%, compared with pre MOX injections [$F(2, 22) = 26.33$, $p < 0.0001$] (**Figure 2A**), and preference [$F(2, 14) = 17.35$, $p < 0.0001$] (**Figure 2B**) in excess of 30%. 10E intake and preference both remained significantly lower than saline on the day immediately following MOX treatment (by more than 25%, and 15% respectively) shown as post MOX injections in **Figure 2**).

In an unpublished pilot study, binge drinking was modeled using the drinking in the dark (DID) procedure whereby mice had daily limited access (2 h) to one bottle containing 20E beginning 3-h into the circadian dark phase, for 3 consecutive days, with a longer access period (4 h) on day 4 (**Figure 4**). Female mice (n = 12, 6 mice/group) were administered saline injections (i.p.) on days 1 - 4 with baseline 20E established on day 4 (Pre-Drug). On Days 1 - 3 of the 2nd weekly cycle, all mice received yet another daily saline injection. The ethanol intake values from day 3 were then used to split the mice into two groups (n = 6/ group) that subsequently received either one injection (i.p.) of MOX (5 mg/kg) or saline on Day 4 (Drug). The following week all mice received daily saline injections on days 1 - 4 and ethanol intake was measured yet again on Day 4 (post Drug). Acute administration of 5 mg/kg MOX was analyzed and found to significantly reduced alcohol intake in excess of 54%, compared with pre-Drug injections ($t = 7.635$, $p < 0.0001$)

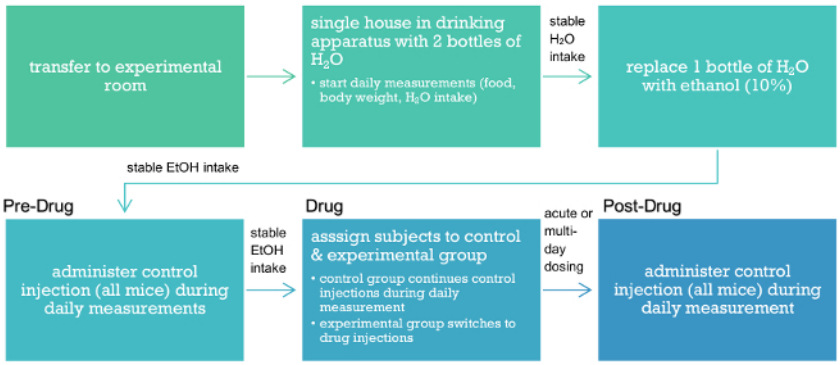

Figure 1. Two-Bottle Choice Schematic Please click here to view a larger version of this figure.

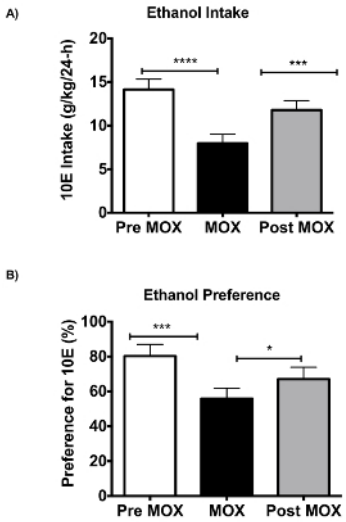

Figure 2. Two Bottle Choice (TBC). MOX (5 mg/kg) reduces 10% v/v ethanol (10E) intake **(A)** and preference **(B)** in female C57BL/6J mice using a 24-h access two-bottle choice paradigm. After attaining stable drinking levels for 3 consecutive days, MOX was administered. Bars represent average 10E intake levels from the day prior to the MOX injection (white; Pre MOX), the day of the MOX injection (black; MOX) and the day after the MOX injection (gray; Post MOX). Values represent the mean ± SEM for 12 mice. *P <0.05, **P <0.01, ***P <0.001, and ****P <0.0001 versus Pre MOX (leftmost horizontal line) or Post MOX (rightmost horizontal line), Tukey's multiple comparison post-hoc test. Modified from Huynh N. *et al.*[19] Please click here to view a larger version of this figure.

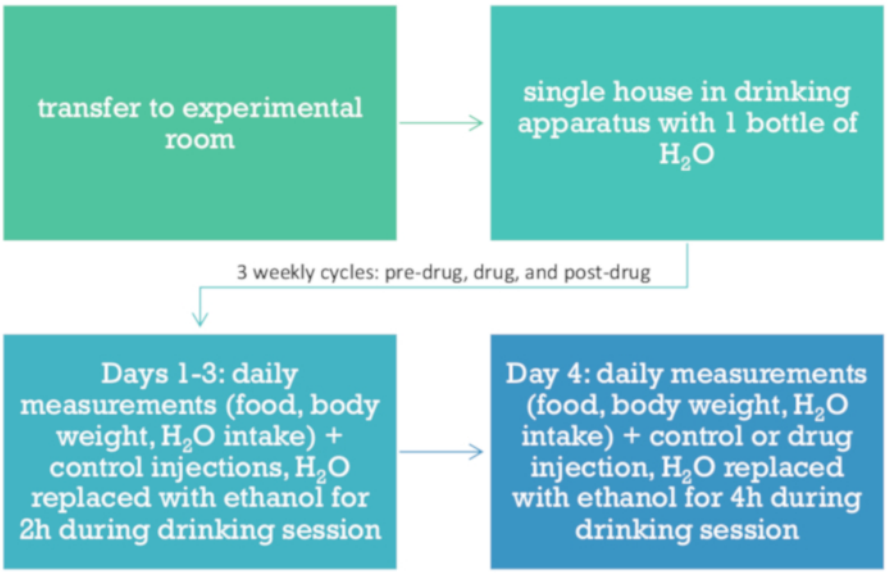

Figure 3. Drinking in the Dark Schematic Please click here to view a larger version of this figure.

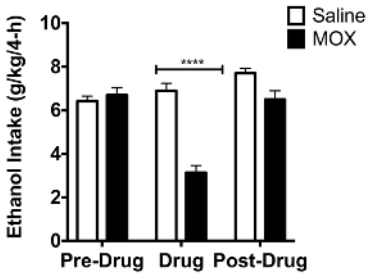

Figure 4. Drinking in the Dark (DID). MOX (5 mg/kg) reduces 20% v/v ethanol (20E) intake in female C57BL/6J mice using a drinking in the dark paradigm. Bars represent average 20E intake levels on the week prior to the MOX injection (Pre-Drug), the week of the MOX injection (Drug) and the week after the MOX injection (Post-Drug). Values represent the mean ± SEM for 12 mice (6/group) between saline and MOX groups. ****P <0.0001 versus Pre-Drug (leftmost horizontal line) or Post-Drug (rightmost horizontal line), Tukey's multiple comparison post-hoc test. Please click here to view a larger version of this figure.

Discussion

Worldwide estimates indicate that as many as 76 million people meet the criteria to warrant a diagnosis for Alcohol Use Disorder (AUD). Unfortunately, pharmaceutical treatments currently available are largely ineffective and further development is necessary to offset the needs of this clinical population[20]. To this end, the following protocol aims to facilitate this endeavor by exemplifying two of the most basic rodent drinking paradigms: two-bottle-choice (TBC) and drinking in the dark (DID). Both models measure non-operant self-administration of ethanol, whereby mice ingest ethanol orally. In the TBC paradigm, ethanol (10% v/v) and water are both available continuously, resulting in comparably low blood ethanol levels that are a consequence of an episodic and gradual ethanol consumption pattern. This model is best for assessing lower levels of ethanol intake as a preference tastant and therefore, is said to be similar to human "moderate" drinking. **Figure 2** above shows representative results for the TBC paradigm.

On the other hand, in the DID model ethanol (20% v/v) is only available for a limited amount of time. Unlike TBC, this model assesses the effects of a compound on behaviorally relevant concentrations of ethanol, taking advantage of the fact that C57BL/6J mice will consume large amounts of ethanol very quickly during the most active phase of their circadian cycle. These drinking sessions occur for 4 consecutive days, beginning at the 3rd h into the dark cycle, with a duration of 2 h on days 1 - 3 and 4 h on day 4. Using this procedure, mice typically consume enough ethanol to achieve BECs >100 mg/dL and to exhibit behavioral evidence of intoxication. Our representative results for the DID paradigm are shown in **Figure 4**. Although we did not measure BECs for these mice, their drinking levels are similar to those reported in the literature that achieved BECs over 100 mg/dL[13,14,15].

Both paradigms have limitations. During instances when oral gavages are the preferred method of administration for pharmacological testing of lead compounds, esophogeal trauma caused by the procedure may hinder ethanol self-administration in both paradigms. This is especially true for the DID model, which uses a higher concentration of ethanol (20%) and which may present instances where the pharmacokinetics of the drug require the drinking session to begin before the animals have had adequate time to recover from the procedure. This would be evident in a control group drinking at levels that correspond to BECs well below the expected 0.08. In our lab, we have experienced difficulty with force feedings both with the DID and TBC. We were able to work around these issues by formulating our compounds so that they could be delivered using an orally dissolving thin strip (ODS)[19].

Moreover, in the DID model specifically, given the fact that the ethanol access period is so short in duration, drug dosing must be done during a preselected time during the light cycle that is chosen in accordance with the pharmacokinetics of the drug so that the compound is at/or approaching the maximum brain concentration during the drinking period. If a pharmacokinetic analysis cannot be performed, an alternative approach would be to conduct a time course assessment of the behavioral effects of the drug using TBC. Following this logic, by monitoring drinking activity on an hourly basis, and determining peak anti-alcohol effects, one could reasonably strategize when drugs should be administered[19].

While the field of alcoholism has various animal models to investigate the different physiological and behavioral aspects of AUD[8], the following protocol describes two commonly used paradigms, which allows for comparisons of results across laboratories. A second benefit is that these methods are straightforward, making their use convenient during acute and chronic studies similar to the examples provided above. Additionally, unlike other commonly used alcohol paradigms, such as operant conditioning and vapor chamber paradigms, the methodology we have described here can be carried out without the need for specialty equipment. Anecdotally, it is likely that nearly all components of the drinking apparatus can be found in a prototypical institutional vivarium. There are also various ways to alter the protocols described here so that they can best fit the goals of each experiment. For instance, our DID procedure is best for testing the acute effects of a drug on a single drinking session (during day Day 4). However, we have shown that multi-day drug dosing can be assessed by augmenting the drinking sessions to 4 consecutive days of 2 hour access, as opposed to the current 2 hours of access on days 1 - 3 and 4 hours on day 4[19]. Mice can also be transferred from one paradigm to another, or re-tested for additional doses / different compounds, with a simple 1 - 2 week washout period in between.

It should be mentioned that any drug-induced change in drinking observed through these methods must be thoroughly investigated to determine whether the effects are selective for alcohol or if they might be the result of toxicity. For more information on controls the reader should refer to Yardley *et al.*[20] No single paradigm can model all aspects of this condition. Instead, each paradigm typically examines a few of the key attributes associated with AUD. The TBC and DID models described here, have been linked to the binge/intoxication stage of the addiction cycle. For a more thorough understanding of the utility of the compound, multiple preclinical drinking models should be utilized.

Disclosures

DLD and LA are inventors on a patent for the repurposing of ivermectin and related avermectins for the treatment of alcohol-use disorders. The authors have no other conflicts of interest and are entirely responsible for the scientific content of the paper.

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