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1 **TITLE:**
2 Murine Drinking Models in the Development of Pharmacotherapies for Alcoholism: Drinking in
3 the Dark and Two-Bottle Choice
4
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39 **SUMMARY:**
40 Alcohol Use Disorder (AUD) is a major national health problem and the development of more
41 effective treatments are required to offset the needs of this patient population. To this end, the
42 following protocol utilizes two simple rodent drinking models to assess the preclinical efficacy
43 of lead anti-alcohol compounds.
44

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.

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[4-7].

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.

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

94

95   In the DID procedure, 3 h after the start of the dark cycle, the home cage bottle of water is
96   exchanged with a bottle of 20% (v/v) ethanol solution for a limited access drinking session. The
97   drinking sessions occur as a consecutive 4-day cycle, lasting 2 h on days 1-3 and 4 h on day 4.
98   Days 1-3 serve as an alcohol-habituation period before the testing on day 4. Consequently, mice
99   will reliably consume enough ethanol to attain BECs >100 mg/dL and as a result, exhibit the
100   behavioral effects of intoxication found in humans that are binge drinking[13-15]. Water access is
101   available at all times other than the drinking session.

102

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

113

114   **PROTOCOL:**

115

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

118

119   **1.   Experimental Setup and Assembly**

120

121   1.1.   Acquire all of the following supplies and chemicals before the start of the study: mice,
122   cages/metal cage tops, bedding, food, water, ethanol, pipets, sipper tops, shrink wrap, utility
123   knife, zip ties, tape, Bunsen burner, scale, headlamp

124

125   1.2.   Obtain C57BL/6J mice, either from a commercial source or an in-house colony, keeping
126   in mind that mice can be group housed until time of testing to reduce costs.

127

128   Note: The total number of mice procured depends on the complexity of the experimental
129   design. Plan to accommodate roughly 12-15 mice per group, with pilot studies no smaller than
130   5-7 mice per group. In the representative results shown below we utilized a simple two-group
131   set-up to assess the cause-effect relationship using a single dose (5 mg/kg) of the drug (MOX).

132

133 1.3.   Follow the steps below to assemble the bottles[18].
134
135 1.3.1.   Heat a utility knife using a Bunsen burner.
136
137 1.3.2.   Using this knife, cut roughly an inch off the top and bottom ends of a plastic 18 mL
138 serological pipet.
139
140 1.3.2.1.   Note that smaller volume pipets (*i.e.*, 10 mL) can also be used to increase the
141 precision of the measurement.
142
143 1.3.3.   Warm the pipet under a heat gun.
144
145 1.3.4.   Insert the ball bearing sipper tube into the "bottom" end of the pipet (in other words,
146 the opening nearest to the 18 mL dash-line).
147
148 1.3.5.   Seal the sipper tube in place with shrink wrap using a commercially available shrink
149 wrap gun.
150
151 1.3.6.   Cap off the other opening with a silicone stopper.
152
153 **2.   Animal Habituation**
154
155 2.1.   Beginning at least 1 week prior to the start date of the experiment, transfer the mice to
156 the room where the experimental procedures are to be carried out so that they may acclimate
157 to the husbandry conditions (including the ambient temperature (21 ± 1 °C) and 12-h reverse
158 light/dark cycle, with lights off at 12 noon). Be sure to follow institutional guidelines and notify
159 the appropriate channels before moving animals from one location to another.
160
161 2.1.1. If mice are being transferred from a standard light/dark cycle allow 2 weeks of
162 additional habituation time.
163
164 2.2.   Fill the newly made bottles to the brim with water. Make sure that the cap is closed
165 securely and devoid of any air bubbles or leaks from the spout. If solution is leaking, re-secure
166 the cap. Remove any air bubbles by simply tapping on the bottle so that the air can escape the
167 tube.
168
169 2.3.   Upon arrival, single house each mouse in standard polycarbonate/polysulfone cages
170 with bedding and a metal grid cage top; remove the cage lid as it will no longer be used.
171
172 2.3.1. Provide access to food and water bottle(s) *ad libitum*.
173
174 2.3.2. Secure each bottle to the cage top by wrapping a plastic zip tie around each bottle to
175 hold it in place. Trim any excess plastic from the zip tie to ensure that it does not protrude into
176 the cage.

177

178 Note: For the drinking in the dark (DID) procedure, only a single bottle of water is required.
179 Habituation to the two bottle choice (TBC) paradigm, however, requires that the cage set-up
180 include two bottles of water. If the metal grid hopper provided is designed to hold only a single
181 bottle of solution, gently bend apart its bars to create space for an additional plate to
182 accommodate the second bottle for TBC.

183

184 2.4.   Set up at least 3 mouse free control cages. This will allow for the monitoring of any fluid
185 loss caused by evaporation or spillage from the bottles, which is simply a natural occurrence
186 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
187 equations).

188

189 2.5.   Beginning on day 4 of the 1-week single housing acclimation, measure and record the
190 daily body and food weights of each mouse, using a scale, (in grams) as well as the water intake,
191 using the etchings alongside the inverted bottle to record the highest point of the meniscus (in
192 mL).

193

194 2.5.1. While it is standard scientific practice to read the lowest point of the concave meniscus,
195 because the bottles remain in an inverted position during the measurement, record the highest
196 point of the meniscus.

197

198 2.6.   Assess the parameters of 2.5 using the equations below:

199

200 2.6.1. Measure the Body Weight Change (g): weight of current day (g) - weight of previous day
201 (g).

202

203 2.6.2. Measure the Food Intake (g): weight of food on previous day (g) - weight of food on
204 current day (g).

205

206 2.6.3. Measure the Water Intake (mL): [volume of water on current day (mL) - volume of water
207 on previous day (mL)] - average water loss from all control cages (mL).

208

209 2.7.   Repeat step 2.5 consecutively, on days 5-7, to allow for the determination of a baseline
210 for the three days immediately prior to the introduction of ethanol. If a consecutive recording
211 cannot be collected, extend the acclimation period to allow for the evaluation of baseline
212 measurements.

213

214 2.8.   Once water intake has stabilized to ± 10% variability from the mean of the last 3 days,
215 begin ethanol access with either TBC (unlimited access) or DID (limited access).

216

217 Note: On rare occasion, one to two additional days may be needed for subjects to attain this
218 stability.

219

220 **3.   24-Hour Two Bottle Choice (TBC)**

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

3.1.   Prepare a 10% (v/v) ethanol solution at a 1,000 mL volume by adding 105.3 mL of 190 proof grain ethanol (~95% ethanol) to 894.7 mL of H_2O; be sure to shake thoroughly. Given that ethanol evaporates quickly, replace the solution twice a week.

Note: Other concentrations of ethanol can be used as well, but the authors recommend a 10% concentration for this model.

3.2.   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.

3.3.   Add more solution to the water bottle, as needed.

3.4.   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.

3.5.   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).

3.6.   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:

3.6.1.   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).

3.6.2.   Measure the 10% Ethanol Intake (g/kg): [10% ethanol Intake (mL) x 0.07893 g/mL] / body weight (kg).

3.6.3.   Measure the Preference %: [10% Ethanol Intake (mL) / Water (mL)] x 100.

3.7.   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.

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.

265

3.8.   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.

3.8.1.   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**.

4.1.   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.

4.1.1.   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.

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.

4.2.   Prepare a 20% (v/v) ethanol solution (20E) at a 1,000 mL volume by adding 210.5 mL of 190 proof grain ethanol (~95% ethanol) to 789.5 mL of H_2O; be sure to shake thoroughly.

4.3.   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.

308   4.4.   During the entire DID session (steps 4.5 – 4.8), use a red-light headlamp as to not
309   disturb the animals.
310
311   4.5.   At the start of the DID drinking session, scheduled to begin 3 hours into the dark cycle,
312   record the volume of water for each mouse. Then, replace each water bottle with a bottle of
313   the 20E solution and record the starting ethanol volume.
314
315   4.6.   Read and record the final ethanol volume 2 hours later, at the end of the drinking
316   session on Days 1-3 and 4 hours later on Day 4. Analyze the 20% ethanol intake using the
317   following equations.
318
319   4.6.1. Measure the 20% Ethanol Intake (mL): [volume of ethanol at end of drinking session
320   (mL) - volume of ethanol at start of drinking session (mL)] - average ethanol loss from all control
321   cages (mL).
322
323   4.6.2. Measure the 20% Ethanol Intake (g/kg): [20% Ethanol Intake (mL) x   0.15786 g/mL]
324   / body weight (kg).
325
326   4.7.   On Day 4 only, immediately after recording ethanol volumes, and before re-introducing
327   access to water, collect blood to assess the blood ethanol concentration of each mouse
328   (optional).
329
330   Note: Any non-terminal blood collection method may be used, such as retro-orbital sinus blood
331   collection, or saphenous vein blood collection. For useful protocols see references for
332   Parasuraman *et al.*[21] and Yardley *et al.*[20].
333
334   4.7.1. Perform analysis using various methods including LCMS, or commercially available
335   machinery (see **Table of Materials**).
336
337   4.8.   Replace all ethanol bottles with water bottles and record water volume.
338
339   **REPRESENTATIVE RESULTS:**
340   In the following representative investigations, social drinking was modeled using the two-bottle
341   choice (TBC) paradigm. Briefly, mice had access to two bottles of solution, one of which
342   contained water, and the other a 10% (v/v) ethanol solution. Subjects were subsequently split
343   and evenly assigned to drug treatment groups, moxidectin (MOX) vs saline control, so that each
344   group would have averaged ethanol intake levels with the least amount of variation.
345
346   Initial baseline 10E intake during the 24-h period stabilized at 14.46 ± 1.85 g/kg (n = 8) before
347   injections began, and subsequently re-stabilized at 14.14 g/kg post saline injections. To assess
348   the effects of an acute dose (5 mg/kg) of MOX on ethanol intake and preference, drinking
349   activity was assessed pre MOX, MOX, and post MOX . We found that a single dose of MOX
350   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
351

352 excess of 30%. 10E intake and preference both remained significantly lower than saline on the
353 day immediately following MOX treatment (by more than 25%, and 15% respectively) shown as
354 post MOX injections in **Figure 2**).
355
356 In an unpublished pilot study, binge drinking was modeled using the drinking in the dark (DID)
357 procedure whereby mice had daily limited access (2 h) to one bottle containing 20E beginning
358 3-h into the circadian dark phase, for 3 consecutive days, with a longer access period (4 h) on
359 day 4 (**Figure 4**). Female mice (n = 12, 6 mice/group) were administered saline injections (i.p.)
360 on days 1-4 with baseline 20E established on day 4 (Pre-Drug). On Days 1-3 of the 2nd weekly
361 cycle, all mice received yet another daily saline injection. The ethanol intake values from day 3
362 were then used to split the mice into two groups (n = 6/ group) that subsequently received
363 either one injection (i.p.) of MOX (5 mg/kg) or saline on Day 4 (Drug). The following week all
364 mice received daily saline injections on days 1-4 and ethanol intake was measured yet again on
365 Day 4 (post Drug). Acute administration of 5 mg/kg MOX was analyzed and found to
366 significantly reduced alcohol intake in excess of 54%, compared with pre-Drug injections
367 (t = 7.635, p < 0.0001)
368
369 **FIGURE LEGENDS:**
370 **Figure 1. Two-Bottle Choice Schematic**
371
372 **Figure 2. Two Bottle Choice (TBC).** MOX (5 mg/kg) reduces 10% v/v ethanol (10E) intake **(A)** and
373 preference **(B)** in female C57BL/6J mice using a 24-h access two-bottle choice paradigm. After
374 attaining stable drinking levels for 3 consecutive days, MOX was administered. Bars represent
375 average 10E intake levels from the day prior to the MOX injection (white; Pre MOX), the day of
376 the MOX injection (black; MOX) and the day after the MOX injection (gray; Post MOX). Values
377 represent the mean $\pm$ SEM for 12 mice. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001
378 versus Pre MOX (leftmost horizontal line) or Post MOX (rightmost horizontal line), Tukey's
379 multiple comparison post-hoc test. Modified from Huynh N. *et al*.[19]
380
381 **Figure 3. Drinking in the Dark Schematic**
382
383 **Figure 4. Drinking in the Dark (DID).** MOX (5 mg/kg) reduces 20% v/v ethanol (20E) intake in
384 female C57BL/6J mice using a drinking in the dark paradigm. Bars represent average 20E intake
385 levels on the week prior to the MOX injection (Pre-Drug), the week of the MOX injection (Drug)
386 and the week after the MOX injection (Post-Drug). Values represent the mean $\pm$ SEM for 12
387 mice (6/group) between saline and MOX groups. ****P < 0.0001 versus Pre-Drug (leftmost
388 horizontal line) or Post-Drug (rightmost horizontal line), Tukey's multiple comparison post-hoc
389 test.
390
391 **DISCUSSION:**
392 Worldwide estimates indicate that as many as 76 million people meet the criteria to warrant a
393 diagnosis for Alcohol Use Disorder (AUD). Unfortunately, pharmaceutical treatments currently
394 available are largely ineffective and further development is necessary to offset the needs of this
395 clinical population[20]. To this end, the following protocol aims to facilitate this endeavor by

396 exemplifying two of the most basic rodent drinking paradigms: two-bottle-choice (TBC) and
397 drinking in the dark (DID). Both models measure non-operant self-administration of ethanol,
398 whereby mice ingest ethanol orally. In the TBC paradigm, ethanol (10% v/v) and water are both
399 available continuously, resulting in comparably low blood ethanol levels that are a consequence
400 of an episodic and gradual ethanol consumption pattern. This model is best for assessing lower
401 levels of ethanol intake as a preference tastant and therefore, is said to be similar to human
402 "moderate" drinking. **Figure 2** above shows representative results for the TBC paradigm.

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

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

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

435 While the field of alcoholism has various animal models to investigate the different
436 physiological and behavioral aspects of AUD[8], the following protocol describes two commonly
437 used paradigms, which allows for comparisons of results across laboratories. A second benefit is
438 that these methods are straightforward, making their use convenient during acute and chronic
439 studies similar to the examples provided above. Additionally, unlike other commonly used

440 alcohol paradigms, such as operant conditioning and vapor chamber paradigms, the
441 methodology we have described here can be carried out without the need for specialty
442 equipment. Anecdotally, it is likely that nearly all components of the drinking apparatus can be
443 found in a prototypical institutional vivarium. There are also various ways to alter the protocols
444 described here so that they can best fit the goals of each experiment. For instance, our DID
445 procedure is best for testing the acute effects of a drug on a single drinking session (during day
446 Day 4). However, we have shown that multi-day drug dosing can be assessed by augmenting
447 the drinking sessions to 4 consecutive days of 2 hour access, as opposed to the current 2 hours
448 of access on days 1-3 and 4 hours on day 4[19]. Mice can also be transferred from one paradigm
449 to another, or re-tested for additional doses / different compounds, with a simple 1-2 week
450 washout period in between.
451
452 It should be mentioned that any drug-induced change in drinking observed through these
453 methods must be thoroughly investigated to determine whether the effects are selective for
454 alcohol or if they might be the result of toxicity. For more information on controls the reader
455 should refer to Yardley *et al*.[20] No single paradigm can model all aspects of this condition.
456 Instead, each paradigm typically examines a few of the key attributes associated with AUD. The
457 TBC and DID models described here, have been linked to the binge/intoxication stage of the
458 addiction cycle. For a more thorough understanding of the utility of the compound, multiple
459 preclinical drinking models should be utilized.
460

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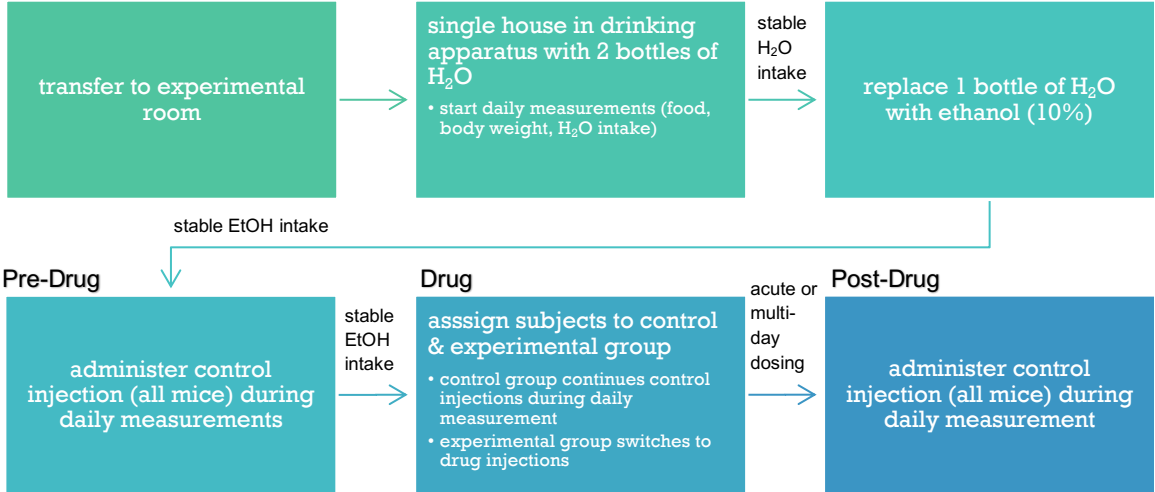471 1. Litten, Falk, Ryan, Fertig. Discovery, Development, and Adoption of Medications to Treat
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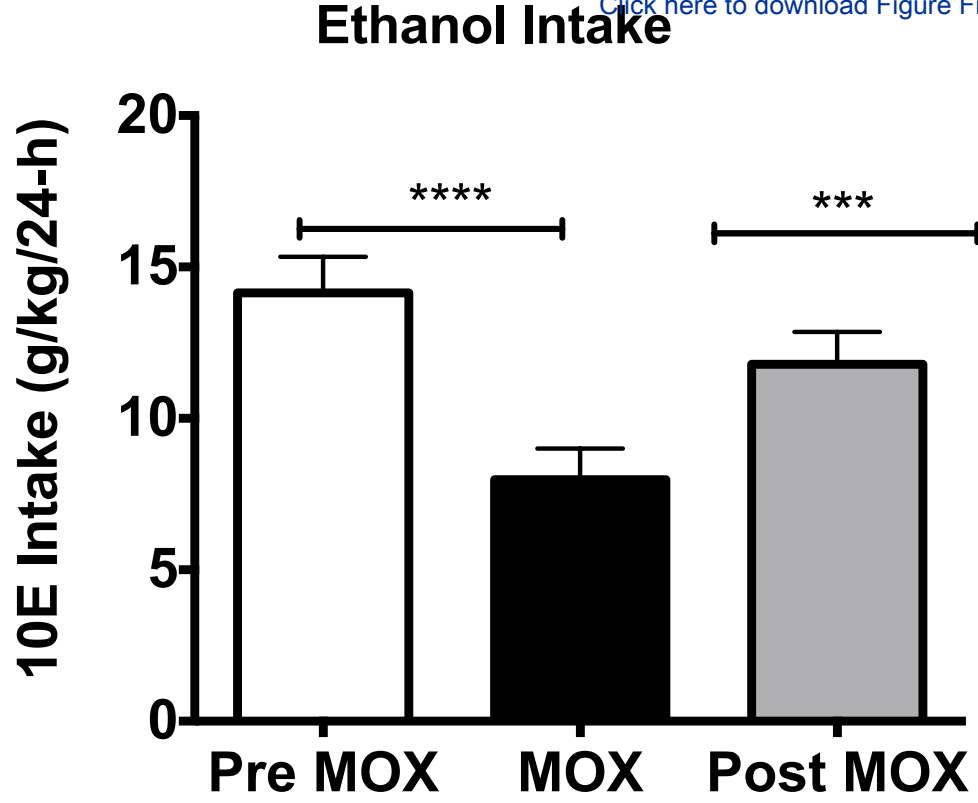481 doi:10.1016/j.amepre.2015.05.031 (2015).
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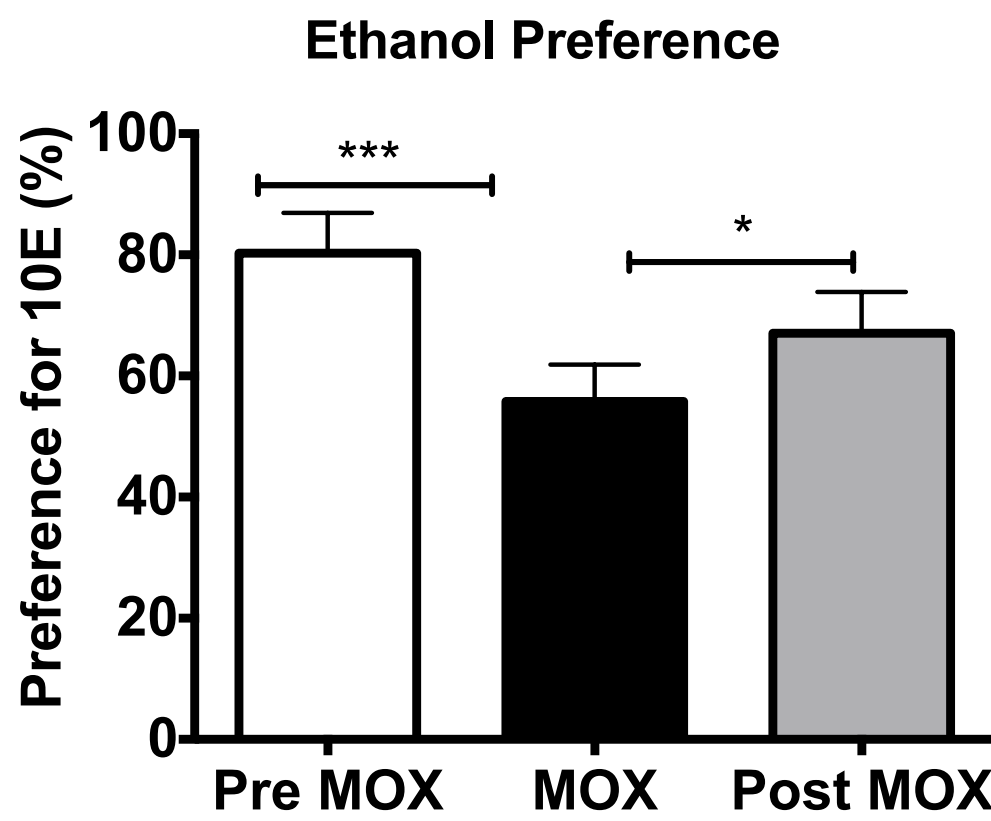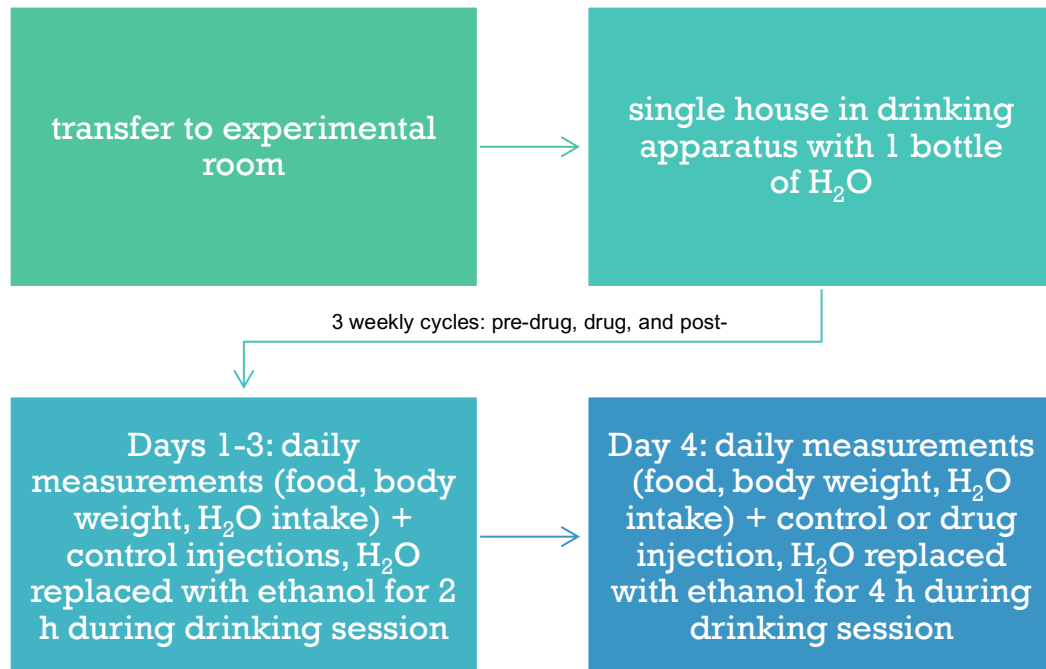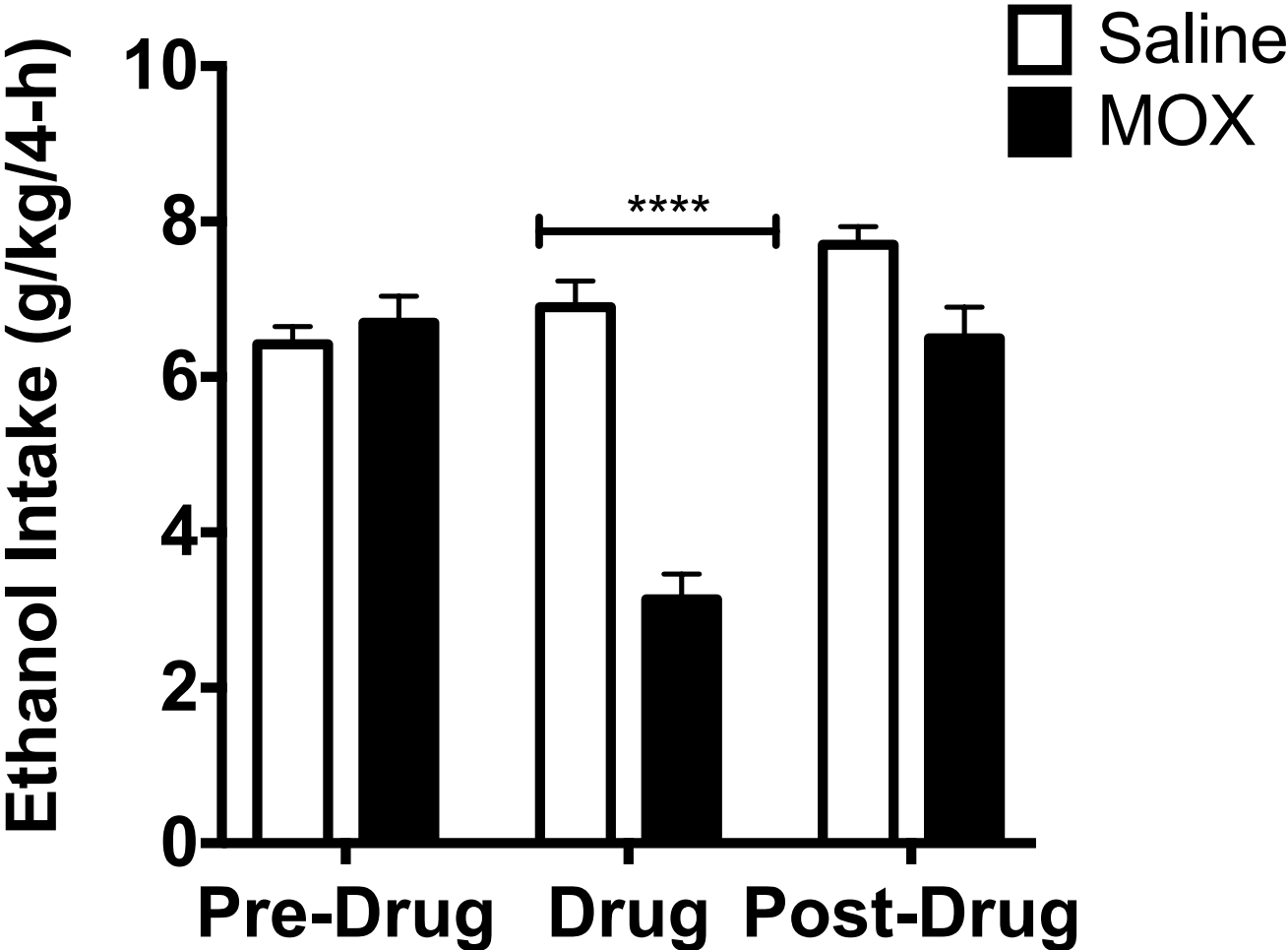555 500x.72350 (2010).
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Figure 1
Click here to download Figure Figure1.pdf

transfer to experimental room

single house in drinking apparatus with 2 bottles of H_2O
• start daily measurements (food, body weight, H_2O intake)

stable H_2O intake

replace 1 bottle of H_2O with ethanol (10%)

stable EtOH intake

Pre-Drug

administer control injection (all mice) during daily measurements

stable EtOH intake

Drug

asssign subjects to control & experimental group
• control group continues control injections during daily measurement
• experimental group switches to drug injections

acute or multi-day dosing

Post-Drug

administer control injection (all mice) during daily measurement

Figure 2

A)

Ethanol Intake

B)

Ethanol Preference

Figure 3

transfer to experimental room

single house in drinking apparatus with 1 bottle of H_2O

3 weekly cycles: pre-drug, drug, and post-

Days 1-3: daily measurements (food, body weight, H_2O intake) + control injections, H_2O replaced with ethanol for 2 h during drinking session

Day 4: daily measurements (food, body weight, H_2O intake) + control or drug injection, H_2O replaced with ethanol for 4 h during drinking session

Figure 4

Material/Equipment

Material/Equipment
1 L graduated cylinder
1 L glass bottle
100 mL graduated cylinder
Analox
ball-bearing sipper tubes
C57BL/6J Mice
disposable serological pipets
ethanol, pure, 190 proof (95%), USP, KOPTEC
heat gun
heat shrink tubing
industrial knife/blade
metal cage plate
mouse RO water
portable electronic scale
red light headlamp
silicone stoppers
thermometer
weigh boat

Company	Catalog Number
VWR	https://us.vwr.com/store/proc
Pyrex (Fisher Scientific)	https://www.fishersci.com/sho
Fisher Scientific	https://www.fishersci.com/sho
Ancare Corp.	http://www.ancare.com/produ
Jackson lab	https://www.jax.org/strain/00
VWR International (VWR)	https://us.vwr.com/store/proc
Decon Labs (VWR)	https://us.vwr.com/store/proc
Master Appliances Corp.	http://www.masterappliance.c
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---	---
---	---
---	---
Ohaus (VWR)	https://us.vwr.com/store/proc
nyteBright (Amazon)	https://www.amazon.com/LED
Fisher	---
Fisher Scientific	https://www.fishersci.com/sho
VWR International (VWR)	https://us.vwr.com/store/proc

Comments/ Description

To prepare ethanol solution.

To prepare ethanol solution.

To prepare ethanol solution.

One potential method of analyzing DID blood samples is by using the analox ma

Length: 2.5 inches, Diameter: 5/16 inches, Model: TD100

May also come from internal breeding colony

10 mL, 18 mL, or 25 mL

:om/master-heat-guns-kits/

Diameter: 3/8 inches

Should be available through the university/institutional vivarium

Should be available through the university/institutional vivarium

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The lid from a pipete tip box is an appropriate alternative

achine

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Author(s): Nhat Huynh, Natalie M Arabian, Liana Asatryan, Daryl L Davies

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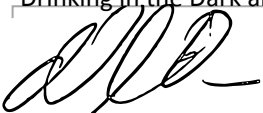
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Article Title: Murine Drinking Models in the Development of Pharmacotherapies for Alcoholism: Drinking in the Dark and Two Bottle Choice

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Editorial Comments:

1. The formatting from our prior draft has been maintained throughout our document. We continued to define all abbreviated terms upon first use and to use standard SI unit symbols (with a single space between numerical values and their limits).

2. We checked to ensure that our introduction included 1) the advantages over alternative techniques with applicable references to previous studies, 2) the context of this technique in the wider body of literature, and 3) information to help readers determine if the protocol is appropriate for their application.

3. Keywords/phrases were added to **lines 35 – 36**.

4. Non-imperative text found within the protocol was specified as a "Note" in **line 157**

5. Section 1.1 - Supplies have been specified without commercial language **line 125.**

6. Section 2.9 – Note added addressing how likely > 7 days are required prior to moving to experiments at **line 270.**

7. Section 3.2 – Edited to explain that a simple piece of tap is used to label the bottles. The label will not influence the mouse's preference for one bottle or another, as the label cannot be seen by the mouse, **line 284.**

8. Section 3.8 – Wording and terminology was edited in this section to better clarify the steps of the protocol. More specifically, the term 'vehicle' was replaced with 'control'. Additionally, we provided the volume of injectable solution used in our studies **line 309**

9. Section 3.9 – The text was clarified to better explain the dosing groups used and how subjects are assigned to their respective group.

10. Section 4.7 – Examples of possible blood collection techniques were provided, along with references for potential new methods, **line 411.**

11. Section 4 –Details were provided on when the drug was administered, **line 354**.

12. Figure titles added to each legend.

13. Figures 1 and 2 – groups and mean values were clarified, both in text and figure legends, **line 455**.

14. Figures 1 and 2- stars on horizontal lines explained in **line 475,484.**

15. The second paragraph of the representative results section was re-written to address any textual overlap with our previous publications, **line 429**.

16. References were reformatted to the appropriate style.

17. Acknowledgements section was added for all funding sources, **line 560**.

18. All commercial language was removed from the manuscript, but kept in the materials list (Including 'Analox' and 'Jackson lab').

Reviewers' comments:

Reviewer #1:

1. Please comment on the influence of sex on assay results and which sex is preferred and/or what parameters influence the choice of which sex to use.

All of the effects of a drug are not guaranteed to be observed in both genders due to the estrous cycle, genetic, and or underlying factors that can affect metabolism, etc.. As such, in drug discovery both female and male models are important for an evaluation of the true efficacy of the compound. Our studies with avermectins have shown that ivermectin and moxidectin can consistently and robustly reduce ethanol intake in both genders and across various paradigms. As such, the models discussed here are appropriate for both genders with no specific requirement pertaining to each gender.

2. For drinking in the dark, are animals typically kept on a reverse light dark cycle? If mice are transferred from normal lighting to a reverse light cycle, how long should they be allowed to acclimate? I have seen some papers in the literature that allow two weeks. What is the timing of the light dark cycles (ie, when do lights come on and go off)?

During drinking in the dark, lights are kept on a reverse light/dark cycle with lights off at 12:00 pm and on at 12:00 am. We agree that mice transferred from a standard light/dark cycle should spend 2 weeks adjusting to the new schedule. We have incorporated this information into **line 165-169.**

3. I recommend not using the confusing term of "social" drinking for the 2BC procedure. Social, to many, implies drinking in a group, i.e., a social setting. While I understand that social can also mean moderate drinking, this term is confusing. It would be better to stick with a less confusing term like moderate or more clearly define what is meant by social.

We agree that the term "social" drinking is misleading and have instead labeled it "moderate" drinking; see **line 77**.

4. The terms bottles and sipper tubes are used interchangeably throughout the manuscript and this is confusing. Where you mean sipper tubes, that terminology should be strictly followed to avoid confusion.

We have made these changes, see **line 140, 171, 240.**

5. Section 2.6: This seems to be the only place you refer to "inverted sipper tubes". Do you really invert the sipper tubes to read them every time? If so this should be made clear and the term inverted sipper tubes should be mentioned throughout.

The tubes themselves are situated on the cage top in an inverted position; we have added the information to **line 240.**

6. Section 2.6: Standard scientific practice is to read the bottom (i.e., the lowest point) of the concave meniscus for water and ethanol solutions instead of the atypical "highest point" as written. Perhaps this is simply a semantic issue. It should either be clarified or made to conform to standard scientific practice.

It should be noted that 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, we record the highest point of the meniscus. This was noted at **line 243**.

7. Section 2.7: for clarity, change wording to "Body Weight Change".

We have made this change, **line 250.**

8. For the 2BC protocol, no details are provided about drug testing. When are drugs tested relative to (a) light dark cycle and (b) measurement of drinking? How many days is the assay performed with and without drug? Why 10% ethanol? Many use the assay at concentrations that vary from ~3-20%? Is 10% best for testing drugs? Can the assay be used at multiple concentrations of ethanol to test drug effects?

Details were added at **line 280, 341, 470.**

9. When referring to C57 or C57BL/6 it is critical to specify the substrain (e.g., C57BL/6J) because differences in substrain can make a huge difference in performance on these assays.

Changes made at **line 475, 497.**

10. Discussion, line 324: These models are NOT models of "human alcoholism". They are models of drinking behavior.

Changes made at **line 549.**

11. Discussion on important controls is missing. How can one be sure that a change in drinking due to a drug is not simply a toxic side effect, i.e., it is making the animals sick. Typically one looks at the effects of the drug on locomotor activity or some other parameter. Also, typically one would like to know if the drug effect is specific to alcohol drinking or consumption of other substances (e.g., sucrose). Also, one typically needs to know if the drug is having an effect via the intended mechanism or if it alters taste perception. Therefore, preference and consumption of saccharine and quinine is typically measured also.

We have added this information along with a reference to better illustrate these additional investigations, **line 547.**

12. A diagram illustrating the time course of these experiments would be very helpful.

We have created 2 flow charts to illustrate the time course of these experiments (**figure 3 and 4**).

13. Can animals be retested for several doses and/or different drugs? If so, what is the timing of those experiments? Is there a washout between drugs?

Yes, animals can in fact be retested, assuming they are healthy and not too old. In our experience the washout between drugs can take around 1-2 weeks, **line 540.**

Reviewer #2:

Major comments

1. *Many mouse cages have filter tops, those must be removed. The tubes must be anchored in place, otherwise the mice can rattle the tubes causing fluid to leak. We use large black paperclips. I couldn't tell, it looked like they included catalog numbers, but these should be clearly labeled for the rubber stoppers, ball bearing sipper tubes, and pipettes.*

    We have added this information at **line 223-225.**

2. *For drinking-in-the-dark it is better to use 10 ml pipettes for more accurate measurements within the shorter time period. For 2-bottle choice, 18 or 25 ml pipettes can be used.*

    We have added this information at **line 147.**

3. *Line 129: The authors should explain that it is way easier to cut the plastic pipettes with a razor blade that is heated under a Bunsen burner, using a vice grip to handle the razor blade and gloves to avoid getting burned. Both the tip and the end need to be cut off.*

    We have added this information at **line 142.**

4. *Line 132: It is way easier to insert the ball bearing sipper tube into the plastic pipette if the pipette is warmed under a heat gun. Using this method the sipper tube slips right in and gets sealed. The shrink tubing is extra precaution. The same heat gun can be used for the shrink tubing.*

    We have added this information at **line 150.**

Minor comments:

1. *In the abstract second to last sentence should be binge drinking not binges drinking.*

    We have edited this typo at **line 112.**