Repeated Cycles of Binge-Like Ethanol Drinking in C57BL/6J Mice Augments Subsequent Voluntary Ethanol Intake But Not Other Dependence-Like Phenotypes

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Arts in the Department of Psychology (Behavioral Neuroscience)

Chapel Hill
2013

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ABSTRACT

Benjamin R. Cox: Repeated Cycles of Binge-Like Ethanol Drinking
in C57BL/6J Mice Augments Subsequent Voluntary
Ethanol Intake But Not Other Dependence-Like Phenotypes
(Under the direction of Dr. Todd E. Thiele)

Background: Recently, procedures have been developed to model specific facets of human alcohol abuse disorders, including those that model excessive binge-like drinking (i.e., "drinking in the dark", or DID) and excessive dependence-like drinking (i.e., resulting from intermittent ethanol vapor exposure). Similar neuropeptide systems modulate excessive ethanol drinking stemming from both procedures, raising the possibility that both paradigms are actually modeling the same phenotypes and triggering the same central neuroplasticity. Therefore, the goal of the present project was to study the effects of a history of binge-like ethanol drinking, using DID procedures, on phenotypes that have previously been described with procedures to model dependence-like drinking.

Methods: Male C57BL/6J mice first experienced 0 to 10 4-day binge-like drinking episodes (3 days of rest between episodes). Beginning 24-h after the final binge-like drinking session, mice were tested for anxiety-like behaviors (with elevated plus maze (EPM) and open-field locomotor activity tests), ataxia with the rotarod test, and sensitivity to handling-induced convulsions (HICs). One week later, mice began a 40-day 2-bottle (water versus ethanol) voluntary consumption test with concentration ranging from 10 to 20% (v/v) ethanol.

Results: A prior history of binge-like ethanol drinking significantly increased subsequent voluntary ethanol consumption and preference, effects most robust in groups that

initially experienced 6 or 10 binge-like drinking episodes and completely absent in mice that experienced 1 binge-like episode. Conversely, a history of binge-like ethanol drinking did not influence anxiety-like behaviors, ataxia, or HICs.

Conclusions: Excessive ethanol drinking stemming from DID procedures does not initially induce phenotypes consistent with a dependence-like state. However, the subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the early stages of ethanol dependence, suggesting that DID procedures may be ideal for studying the transition to ethanol dependence.

ACKNOWLEDGEMENTS

I would like to thank Dr. Todd E. Thiele for his guidance and mentorship throughout this project. I would also like to thank Dr. Jennifer A. Rinker, Dr. Emily G. Lowery-Gionta, Dr. Montserrat Navarro, Dr. Gretchen M. Sprow and Dr. Thomas L. Kash for their technical expertise and guidance, as well as Jeff J. Olney and Rhiannon D. Thomas for their technical assistance.

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ANOVA	Analysis of variance
BEC	Blood ethanol concentration
CeA	Central amygdala
CRF	Corticotropin releasing factor
CRF1R	Corticotropin releasing factor 1 receptor
DID	Drinking-in-the-dark
EPM	Elevated plus maze
EtOH	Ethanol
g/kg	Grams per kilogram
HIC	Handling-induced convulsions
NPY	Neuropeptide Y
RPM	Revolutions per minute
v/v	Volume to volume

CHAPTER 1

INTRODUCTION

Early pre-clinical alcoholism research primarily relied on animal models that involved voluntary consumption of ethanol, in which rats or mice were given 24-h/day access to ethanol and water simultaneously in separate bottles. However, voluntary ethanol consumption may not be the most appropriate model for human alcohol abuse disorders, as rodents typically consume low amounts of ethanol which do not generate blood ethanol levels thought to be pharmacologically meaningful. Recently, procedures have been developed to model specific facets of human alcohol abuse disorders. Such procedures include those that model excessive binge-like drinking prior to the development of dependence (Boehm et al., 2008; Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012; Sprow and Thiele, 2012), excessive ethanol intake stemming from ethanol dependence (Funk et al., 2007; Gilpin et al., 2011; Roberts et al., 1996), excessive relapse-like ethanol drinking (Spanagel and Holter, 2000; Spanagel et al., 1996; Sparta et al., 2009) and ethanol seeking behaviors (Le et al., 1999; Le et al., 1998; Weiss and Liu, 2002). These models have been useful for discovering the neurochemical pathways and the neurocircuitry involved in alcohol-related behaviors.

Recent evidence has been presented suggesting that neuropeptide systems, specifically neuropeptide Y (NPY) and corticotropin releasing factor (CRF), modulate excessive binge-like ethanol drinking in C57BL/6J mice that have been described as non-dependent. Using the procedure called “drinking in the dark” (DID; (Rhodes et al., 2005;

Rhodes et al., 2007)), central administration of CRF-1 receptor (CRF1R) antagonists and NPY were found to prevent binge-like ethanol drinking in mice. Interestingly, CRF1R antagonists and NPY failed to alter low level non-binge-like ethanol drinking, suggesting that central CRF and NPY systems are recruited only after sufficient blood/brain ethanol concentrations are achieved, which may motivate continued binge-like drinking (Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012). Interestingly, central administration of CRF1R antagonists or NPY have also been shown to protect against excessive dependence-like ethanol drinking in rodents that have had a prior history of repeated intermittent ethanol vapor exposure, but these compounds failed to alter low level ethanol intake in non-dependent animals (Gilpin et al., 2011; Roberto et al., 2010). The striking similarity between results obtained with models of binge-like ethanol drinking in "non-dependent" animals and in "dependent" animals has led our group to propose that overlapping systems may be involved (Lowery-Gionta et al., 2012; Sparrow et al., 2012; Thiele, 2012). Theoretically, CRF signaling is increased and NPY signaling is blunted when sufficient brain ethanol levels are achieved during binge-like drinking, stimulating continued excessive ethanol intake. Alterations in neuropeptide signaling are thought to initially be transient, but with repeated episodes of binge-like drinking these neuroplastic changes may become rigid, contributing to excessive dependence-like drinking.

One possibility is that procedures to promote binge-like ethanol drinking and procedures to induce dependence-like ethanol drinking are actually modeling the same phenotypes and trigger the same neuroplastic changes in the brain. Thus, it might be argued that the amount of ethanol exposure achieved with DID procedures is sufficient to induce a dependence-like state, and in fact that exposure to either DID procedures or ethanol vapor

accomplish the same end-point. If this is true, one would predict that DID procedures should promote phenotypes consistent with ethanol dependence.

Therefore, the goal of the present project was to study the effects of a history of binge-like ethanol drinking on phenotypes that have previously been described following intermittent ethanol vapor exposure (Becker and Lopez, 2004; Crabbe et al., 1991; Kliethermes et al., 2004; Lopez et al., 2011; Philibin et al., 2012). A dependence-like state is typically associated with subsequent increases of voluntary ethanol consumption and self-administration (Becker and Lopez, 2004), and mice have been shown to exhibit elevated anxiety-like behaviors (Kliethermes et al., 2004), increased ataxia (Philibin et al., 2012), and increased sensitivity to handling-induced convulsion (HICs) following 24-h or more of withdrawal after ethanol vapor exposure (Beadles-Bohling and Wiren, 2006; Homanics et al., 1998). In fact, it could be argued that these withdrawal-induced phenotypes promote increased ethanol consumption via negative reinforcement (Koob, 2003; Koob and Le Moal, 2001). Here, we assessed anxiety-like behaviors, ataxia, HIC, and finally voluntary ethanol consumption in mice with varying amounts of experience with binge-like ethanol drinking using DID procedures.

CHAPTER 2

METHODS

Animals

Male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were 6-8 weeks of age and weighed 20-30 g upon arrival. Mice were housed individually in standard plastic cages and allowed to habituate to the environment for at least 1 week before experimental procedures were initiated. The animal colony room was maintained at ~22°**C** with a 12h/12h light/dark cycle (lights on at 0700 h). Mice had *ad libitum* access to food throughout all experiments and *ad libitum* access to water except during ethanol access, as noted below. All procedures used were in accordance with the National Institute of Health guidelines and were approved by the University of North Carolina Institutional Animal Care and Use Committee.

Drinking-in-the-dark Procedure

All experiments utilized a 4 day drinking-in-the-dark (DID) procedure that our group and others have used previously to generate high levels of ethanol intake that are associated with blood ethanol concentrations (BECs) in excess of 80 mg/dl, typically greater than 100 mg/dl (Lowery-Gionta et al., 2012; Lowery et al., 2010; Rhodes et al., 2005; Rhodes et al., 2007; Sparrow et al., 2012). On days 1-3, beginning 3 h into the dark cycle, water bottles were removed from all cages and replaced with a bottle containing 20% (v/v) ethanol solution. Mice had 2 h of access to ethanol, after which the ethanol bottles were removed from cages and water bottles were replaced. The same procedure was followed on day 4 except that ethanol access was extended to 4 h. Each 4 day DID procedure is referred to as a

single binge-like ethanol drinking "cycle" or "episode" below. Between each binge-like drinking cycle, mice were given 3 days of rest with no access to ethanol. In Experiment 1 described below, mice were separated into groups based on body weights. Groups of mice experienced 1 (n = 13) or 6 (n = 13) binge-like drinking cycles and a third control group drank water throughout the binge-like drinking portion of the experiment (n = 13). In Experiment 2 described below, animals were divided into 5 groups based on body weights: The groups experienced 1, 3, 6, or 10 binge-like drinking cycles, and a fifth control group drank water only throughout the binge-like drinking portion of the experiment (n =10/group). For both experiments, initiation of binge-like drinking was staggered between groups such that all mice experienced their last binge-like drinking cycle at the same age.

Elevated Plus Maze (EPM): Assessment of Anxiety-like Behavior

The EPM test is a pharmacologically validated model for the assessment of anxiety in rodents (Pellow et al., 1985). For both Experiments 1 and 2, the EPM test was initiated 24-h after the completion of the final binge-like drinking test. We have described the apparatus and the 5-min procedure previously (Fee et al., 2004). The behavior in the EPM was recorded using a digital video camera with night vision capability, and measures were scored by condition-blinded individuals trained to identify the various dependent measures. With the EPM test, anxiety-like behavior is reflected as reduced number of entries into the open arms and in the time spent in the open arms, along with an increase in the number of entries and amount of time spent in the closed arms of the EPM.

Open Field Locomotor Activity Test: Assessment of Anxiety-like Behavior

The open-field test is commonly used to assess anxiety-like behavior in rodents, in which avoidance of the center portion of the open arena is thought to reflect heightened

anxiety (Choleris et al., 2001; Fee et al., 2004). After the completion of the EPM test in Experiments 1 and 2, mice were transported in their home cages to an adjacent room and allowed to rest for 10 min. We have described the apparatus and procedure elsewhere (Fee et al., 2004). Testing sessions were 10 minutes in duration, and marginal time and distance traveled (cm), and central time and distance traveled (cm) in the open-field arena were measured over the course of the session.

Accelerating Rotarod Test: Assessment of Ataxia

Recent observations show that mice experiencing ethanol withdrawal exhibit elevated ataxia, and have difficulty maintaining balance on a rotarod apparatus (Philibin et al., 2012). After open-field testing in Experiment 2, mice were allowed to rest for 15 min in their home cage and were then placed on a rotarod apparatus (Ugo Basile, Italy) with an initial rotation speed of 4 rpm. The speed was gradually increased to 40 rpm over a period of 5 min and latency-to-fall and rpm at the time of fall were recorded. The test was repeated three more times, with 5 min between each test. Average latency-to-fall and rpm at time of fall were averaged over the 4 test trials.

Handling-Induced Convulsions (HIC): Assessment of Seizure Susceptibility

Thirty min after administration of the rotarod tests in Experiment 2, HIC tests were conducted. For HIC testing, each mouse was picked up (from its home cage) by the tail, and if this failed to elicit a convulsion, the mouse was spun gently through a 180^0 arc. The behavior of each mouse during the HIC test was video recorded and later scored independently by 2 condition-blinded raters (scores were averaged for analyses). The HIC rating scale ranged from 0-7, with a score of 0 given to mice showing no convulsions and a

score of 7 given to mice exhibiting severe tonic-clonic convulsions. A detailed description of HIC procedures and the scoring scale is described elsewhere (Crabbe et al., 1991).

Continuous 2-Bottle Voluntary Ethanol Consumption

Approximately one week after the completion of binge-like ethanol drinking in Experiments 1 and 2, the mice were tested for voluntary ethanol consumption using a home cage 2-bottle choice procedure. Over 8 days, mice were given 24 h access to 2-bottles in their homecage, one containing tap water and the other containing a 10% (v/v) ethanol solution. The concentration of ethanol was changed every 8 days as follows: 10, 15, 20, 15, and 10% ethanol. The positions of the bottle were alternated every day to control for position preferences. Each drinking bottle was weighed every day to calculate fluid intake and body weights were recorded every 4 days.

Data Analysis

To obtain a measure that corrected for individual differences in body weight, grams of ethanol consumed per kilogram of body weight were calculated. Ethanol preference ratios were also calculated by dividing the volume of ethanol consumed by the total fluid (ethanol + water) consumption. For all experiments, differences between groups were analyzed using analysis of variance (ANOVA). With significant interaction effects, or main effects in the absence of significant interactions, post hoc comparisons were performed using Bonferroni tests to parse out group differences. In all cases, $p<0.05$ (two tailed) was used to indicate statistical significance.

CHAPTER 3

RESULTS

Drinking-in-the-dark

In Experiment 1, there were no significant differences between the groups that experienced 1 (5.42 $\pm$ 0.28 g/kg/4h) or 6 (6.05 $\pm$ 0.29 g/kg/4h) binge-like drinking cycles in terms of the amount of ethanol consumed during the final binge-like drinking session [$F_{(1,25)} = 2.486$, $p = 0.127$]. Similarly, in Experiment 2 there were no significant differences between the groups that experienced 1 (4.43 $\pm$ 0.29 g/kg/4h), 3 (5.09 $\pm$ 0.41 g/kg/4h), 6 (6.16 $\pm$ 0.36 g/kg/4h), or 10 (5.36 $\pm$ 0.31 g/kg/4h) binge-like drinking cycles in the amount of ethanol consumed during the final binge-like drinking session [$F_{(3,36)} = 1.374$, $p = 0.226$]. These data suggest that up to 10 binge-like drinking cycles did not significantly increase the level of binge-like ethanol drinking. Although we did not assess blood ethanol concentrations (BECs) in an attempt to avoid the potential confounding effects of stress on subsequent measures, the amount of ethanol consumed by mice on the final day of binge-like ethanol drinking was consistent with amounts that we have previously reported and which produced BECs of 80 mg/dL or greater (Lowery-Gionta et al., 2012; Lowery et al., 2010; Sparrow et al., 2012; Sparta et al., 2008).

Elevated Plus Maze (EPM): Assessment of Anxiety-Like Behavior

Data from the EPM tests for Experiments 1 and 2 are presented in Table 1. Data sets from both experiments failed to reveal any significant effects of binge-like ethanol drinking on subsequent anxiety-like behavior. In Experiment 1, relative to the water control group,

mice that experienced 1 or 6 cycles of binge-like ethanol drinking exhibited no significant differences in open arm time [$F_{(2,36)} = 0.146$, $p = 0.865$] or percentage total time spent in open arms [$F_{(2,36)} = 0.145$, $p = 0.856$]. Furthermore, there were no group differences in closed arm time [$F_{(2,37)} = 1.056$, $p = 0.358$] or percentage of total time spent in closed arms [$F_{(2,37)} = 1.058$, $p = 0.357$]. A lack of group differences in total arm entries [$F_{(2,37)} = 1.05$, $p = 0.360$] suggests that the history of binge-like ethanol drinking did not impact overall activity (H_2O = 18.38 ± 1.20 entries; 1-cycle = 20.46 ± 1.07 entries; 6-cycle = 18.92 ± 0.84 entries).

In Experiment 2, relative to the water control group there was no evidence of alterations of anxiety-like behavior in mice that experienced 1 to 10 cycles of binge-like ethanol drinking. Thus, there were no group differences in open arm time [$F_{(4,44)} = 2.012$, $p = 0.109$] or percentage total time spent in open arms [$F_{(4,44)} = 2.007$, $p = 0.110$]. Furthermore, there were no group differences in closed arm time [$F_{(4,45)} = 2.016$, $p = 0.108$] or percentage of total time spent in closed arms [$F_{(4,45)} = 2.013$, $p = 0.109$]. Finally, there were no significant differences between groups on the number of total arm entries [$F_{(4,45)} = 0.448$, $p = 0.774$], suggesting that the history of binge-like ethanol drinking did not impact overall activity (H_2O = 22.2 ± 1.10 entries; 1-cycle = 21.3 ± 1.59 entries; 3-cycle = 21.5 ± 1.68 entries; 6-cycle = 22.7 ± 1.32 entries; 10-cycle = 20.3 ± 1.03 entries).

Open-Field Locomotor Activity Test: Assessment of Anxiety-Like Behavior

Data from the open-field locomotor activity tests from Experiments 1 and 2 are presented in Table 2. As with the EPM test, data sets from both experiments failed to provide evidence of elevated anxiety-like behavior stemming from a history of binge-like ethanol drinking. For data from Experiment 1, there were no group differences in average distance traveled in the margins [$F_{(2,37)} = 0.545$, $p = 0.584$], average time spent in margins [$F_{(2,37)} =$

2.505, p = 0.095], average distance traveled in the center [$F_{(2,36)}$ = 0.867, p = 0.429], or average time spent in the center [$F_{(2,37)}$ = 0.507, p = 0.095]. Additionally, analysis of total distance traveled data failed to achieve statistical significance [$F_{(2,37)}$ = 0.688, p = 0.509], further support that a history of binge-like ethanol drinking did not alter overall locomotor behavior (H_2O = 2982.38 ± 226.83 cm^2/10min; 1-cycle = 3278 ± 195.19 cm^2/10min; 6-cycle = 2956.57 ± 218.52 cm^2/10min; 10-cycle = 3146 ± 134.36 cm^2/10min).

The same pattern of results were observed from the open-field test of Experiment 2. There were no group differences in average distance traveled in the margins [$F_{(4,45)}$= 0.244, p = 0.912], average time spent in margins [$F_{(4,45)}$ = 0.112, p = 0.978], average distance traveled in the center [$F_{(4,45)}$ = 0.283, p = 0.887], and average time spent in the center [$F_{(4,45)}$ = 0.112, p = 0.978], and analysis of total distance traveled failed to achieve statistical significance [$F_{(4,45)}$ = 0.133, p = 0.969] (H_2O = 2942 ± 206.4 cm^2/10min; 1-cycle = 3094.5 ± 212.46 cm^2/10min; 3-cycle = 3037.7 ± 256.41 cm^2/10min; 6-cycle = 2989.7 ± 275.36 cm^2/10min; 10-cycle = 3146 ± 134.36 cm^2/10min).

Accelerating Rotarod Test: Assessment of Ataxia

Data averaged over the 4 rotarod tests performed in Experiment 2 are presented in Table 3. Separate one-way ANOVAs performed on latency to fall data [$F_{(4,45)}$ = 0.691, p = 0.602] and average RPM at fall data [$F_{(4,45)}$ = 0.724, p = 0.58] both failed to achieve statistical significance, indicating that a history of binge-like ethanol drinking did not promote increased ataxia.

Handling-Induced Convulsions (HIC): Assessment of Seizure Sensitivity

Data from the HIC test performed in Experiment 2 are presented in Table 3. An ANOVA performed on these data failed to achieve statistical significance [$F_{(4,45)}$ = 0.651, p =

0.629], suggesting that a history of binge-like ethanol drinking did not alter seizure sensitivity.

Continuous 2-Bottle Voluntary Ethanol Consumption

Data representing the average 8-day consumption of ethanol (g/kg) and average 8-day ethanol preference at each ethanol concentration tested in Experiment 1 (Fig. 1A and B, respectively) and Experiment 2 (Fig. 1C and D, respectively) are presented in Fig. 1. Separate two-way (ethanol concentration x number of binge-like drinking cycles) ANOVAs were performed to analyze each data set. Analysis of ethanol consumption data from Experiment 1 revealed significant main effects of ethanol concentration [$F_{(4,148)} = 49.724$, $p < 0.001$] and binge-like drinking cycles [$F_{(1,37)} = 10.279$, $p < 0.001$], and a significant interaction effect [$F_{(8,148)} = 2.97$, $p = 0.004$]. Post hoc Bonferroni tests revealed that the group with a prior history of 6 binge-like ethanol drinking cycles voluntarily drank significantly more ethanol relative to the control group during access to 4 of the 5 ethanol solutions, and more than the 1-cycle group at 2 of the 5 concentrations (Fig. 1A). Analysis of ethanol preference ratio data from Experiment 1 showed significant main effects of ethanol concentration [$F_{(4,148)} = 58.905$, $p < 0.001$] and binge-like drinking cycles [$F_{(1,37)} = 8.357$, $p = 0.001$], but the interaction effect was not significant [$F_{(8,148)} = 1.923$, $p = 0.061$]. A post hoc Bonferroni test of the binge-like drinking cycles main effect revealed that only the 6-cycle binge-like ethanol drinking group preferred ethanol significantly more than the control group (Fig. 1B).

Analysis of the ethanol consumption data from Experiment 2 revealed significant main effects for ethanol concentration [$F_{(4,180)} = 65.420$, $p < 0.001$] and binge-like drinking cycles [$F_{(4,45)} = 8.173$, $p < 0.001$], and a significant interaction effect [$F_{(16,180)} = 2.076$, $p =$

0.011]. Post hoc Bonferroni tests revealed that relative to the control group, mice that had a prior history of 6 or 10 binge-like drinking cycles voluntarily drank significantly more ethanol during access to 4 of the 5 ethanol solutions. Mice with a history of 3 binge-like drinking cycles drank significantly more ethanol than the control group during access to 3 of the 5 ethanol solutions. Finally, mice with a history of 10 binge-like drinking cycles drank significantly more ethanol than the 1-cycle group at one concentration (Fig. 1C). Analysis of ethanol preference data from Experiment 2 showed significant main effects of ethanol concentration [$F_{(4,180)} = 59.915$, $p < 0.001$] and binge-like drinking cycles [$F_{(4,45)} = 5.82$, $p = 0.001$], but the interaction effect was not significant [$F_{(16,180)} = 0.984$, $p = 0.476$]. A post hoc Bonferroni test of the binge-like drinking main effect showed that groups with a prior history of 3 to 10 binge-like ethanol drinking cycles showed significantly elevated ethanol preference relative to the control group (Fig. 1D).

CHAPTER 4

DISCUSSION

Here we show that relative to mice with no prior history of binge-like ethanol drinking, a history of binge-like ethanol drinking using DID procedures promoted subsequent increases of voluntary ethanol consumption and preference without altering anxiety-like behaviors (assessed by EPM and open-field locomotor activity tests), ataxia (assessed by the accelerating rotarod test), or sensitivity to HICs. Furthermore, increased voluntary ethanol consumption and preference were greater in mice that experienced greater numbers of binge-like ethanol drinking cycles, with mice experiencing 6 or 10 binge-like drinking episodes showing the most robust increases of subsequent voluntary ethanol drinking and preference. Conversely, there was no instance in which mice with a prior history of just one binge-like drinking cycle exhibited a significant change from the control group in subsequent ethanol intake or preference. These observations are consistent with the idea that alterations in the neurocircuitry that modulates binge-like ethanol drinking become more robust and longer-lasting with increasing numbers of binge-like drinking cycles. Several phenotypes that have been reported with procedures that induce a dependence-like state (i.e., ethanol vapor exposure) were not evident in mice experiencing up to 10 4-day cycles of binge-like ethanol drinking. Together, these observations make a strong case that DID and ethanol vapor procedures do not lead to the same end-point, and suggest that unlike ethanol vapor exposure procedures, excessive ethanol drinking stemming from DID procedures does not initially induce a dependence-like state. However, the subsequent increases of voluntary ethanol

consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the emergence of ethanol dependence, suggesting that DID procedures may be ideal for studying the early stages of the transition to ethanol dependence. We speculate that increased anxiety-like behavior, ataxia, and sensitivity to HIC may emerge with greater experience with binge-like ethanol drinking (i.e., more than 10 cycles of DID exposure).

The most striking results from the present data set were the robust and long-lasting increases of voluntary ethanol consumption and preference stemming from a history of binge-like ethanol drinking. These observations parallel those obtained with vapor inhalation models that have been shown to induce subsequent increases of voluntary ethanol consumption in rats (Funk et al., 2007; Gilpin et al., 2011) and mice (Becker and Lopez, 2004; Finn et al., 2007; Lopez et al., 2011). Importantly, increases of subsequent voluntary ethanol consumption in the present report resulted from animals voluntarily drinking excessive amounts of ethanol in a pattern and level that closely matches human binge drinking, rather than from forced exposure to ethanol vapor. Thus, as a model to study the neurobiology underlying the transition to an ethanol dependence-like state (Thiele, 2012), repeated binge-like drinking episodes using procedures such as DID arguably have greater face validity than models employing repeated intermittent ethanol vapor exposure.

Previous reports have shown that up to 24-h of withdrawal from ethanol vapor exposure is associated with elevated anxiety-like behaviors (Kliethermes et al., 2004), increased ataxia (Philibin et al., 2012), and increased sensitivity to HICs (Beadles-Bohling and Wiren, 2006; Becker and Veatch, 2002; Homanics et al., 1998) in mice. There are numerous possibilities that may explain differences between the present report and previous

studies, including the amount, pattern, and route of ethanol exposure, the amount of ethanol withdrawal time before tests were administered, and the strain of mice used. For example, while HICs have been observed up to 24-h after ethanol withdrawal, HICs tend to be more robust in the first several hours after ethanol withdrawal (Beadles-Bohling and Wiren, 2006; Homanics et al., 1998), and strains other than C57BL/6J mice are more sensitive to withdrawal-induced HICs (Homanics et al., 1998). However, C57BL/6J mice have been reported to show elevated HICs 8-h after ethanol removal but only after 16-weeks of intermittent access to 20% ethanol in an escalation paradigm (Hwa et al., 2011). Furthermore, strains other than C57BL/6J mice were used to assess ataxia (Philibin et al., 2012) and anxiety-like behavior (Kliethermes et al., 2004). Importantly though, our goal was to address the potential criticism that models of binge-like ethanol drinking and dependence-like ethanol drinking trigger the same phenotypes and associated neuroplasticity, a criticism that might seem particularly relevant given evidence of similar involvement of CRF and NPY signaling in binge-like and dependence-like ethanol drinking (Gilpin et al., 2011; Lowery-Gionta et al., 2012; Roberto et al., 2010; Sparrow et al., 2012). However, because we found that binge-like ethanol drinking did not promote many phenotypes thought to be hallmarks of ethanol dependence, we argue that the binge-like drinking procedure that we employ models excessive ethanol intake prior to the onset of dependence. Consistent with this argument, we have found that a history of binge-like ethanol drinking blocks the ability of CRF to enhance GABAergic transmission in the central amygdala (CeA) of mice (Lowery-Gionta et al., 2012), while others have found that intermittent ethanol vapor exposure augments the ability of CRF to enhance GABAergic transmission in the CeA (Roberto et al., 2010). We would predict that more extensive experience with binge-like

drinking episodes would lead to the development of neuroplastic alterations in line with models of ethanol dependence.

While not assessed directly in the current set of experiments, it is of interest to consider the possible mechanisms by which a history of binge-like ethanol drinking promotes subsequent increases of voluntary ethanol intake. A previous report identified low sensitivity to the aversive effects of ethanol (Holstein et al., 2011) as a potential mechanism that modulates binge-like ethanol drinking, and thus blunted sensitivity to the aversive effects of ethanol following a history of binge-like ethanol drinking may promote subsequent increases of voluntary ethanol consumption. At the level of neurocircuitry, a growing number of neurochemicals have been implicated in binge-like ethanol drinking (Sprow and Thiele, 2012), and neuroadaptations in these neurochemical pathways following binge-like drinking could also be candidate mechanisms for increases of subsequent voluntary ethanol intake. CRF and NPY are two candidate pathways of particular interest. We have recently found that after 24-h of ethanol removal in mice with a 3-cycle history of binge-like ethanol drinking, CRF immunoreactivity was significantly elevated in the CeA, and consistently site-directed infusion of a CRF-1 receptor antagonist into the CeA protected against binge-like drinking (Lowery-Gionta et al., 2012). On the other hand, NPY immunoreactivity was significantly blunted in the CeA 24-h after ethanol removal in mice with a 3-cycle history of binge-like drinking (Sparrow et al., 2012). Importantly, in both of our previous experiments the effects of CRF or NPY on GABAergic transmission in the CeA were significantly altered for up to 24-h after ethanol removal (Lowery-Gionta et al., 2012; Sparrow et al., 2012), which together with immunoreactivity data suggest that binge-like ethanol drinking is associated with rigid neuroplastic alterations of CRF and NPY signaling in the CeA. We speculate that these

changes contribute to the transition to ethanol dependence. Evidence of increased voluntary ethanol drinking resulting from a history of binge-like ethanol drinking in the present report may reflect, in part, such changes in CRF and NPY signaling.

In summary, here we report that while repeated cycles of binge-like ethanol drinking promoted subsequent increases of voluntary ethanol intake, an effect that becomes more robust with increasing numbers of binge-like drinking episodes, a history of binge-like ethanol drinking did not impact other phenotypes thought to characterize a dependence-like state, including anxiety-like behavior, ataxia, and HICs. Together, these observations make a strong case that DID procedures (to model binge-like ethanol drinking) and ethanol vapor procedures (to model dependence-like drinking) do not lead to the same neurobiological end-point. Unlike ethanol vapor exposure procedures, excessive ethanol drinking stemming from DID procedures does not initially induce a dependence-like state. However, the subsequent increases of voluntary ethanol consumption and preference that become more robust following repeated episodes of binge-like ethanol drinking may reflect the early stages of ethanol dependence, suggesting that DID procedures may be ideal for studying the transition to ethanol dependence.

TABLE 1: Average (Mean ± SEM) behavior in the elevated plus maze (EMP) tests from Experiments 1 and 2.

	Time in Open Arms (s)	Percentage of Time in Open Arms (%)	Time in Closed Arms (s)	Percentage of Time in Closed Arms (%)
Experiment 1				
H_20	35.8 ± 5.58	11.9 ±1.86	136.3 ± 10.67	45.4 ± 3.56
1-cycle	41.3 ± 7.10	13.76 ± 2.36	150.6 ± 9.23	49.2 ± 3.15
6-cycle	38.2 ± 7.95	12.74 ± 2.65	155.0 ± 8.66	51.7 ± 2.89
Experiment 2				
H_20	70.9 ± 10.0	23.6 ± 3.35	164.2 ± 14.13	54.7 ± 4.71
1-cycle	62.9 ± 12.86	21.0 ± 4.28	187.9 ± 16.13	62.7 ± 5.38
3-cycle	41.3 ± 5.78	13.8 ± 1.93	214.3 ± 10.0	71.4 ± 3.33
6-cycle	80.8 ± 9.79	26.9 ± 3.26	164.9 ± 11.12	55.0 ± 3.71
10-cycle	54.3 ± 12.67	18.1 ± 4.22	189.6 ± 19.58	63.2 ± 6.53

Table 2: Average (Mean ± SEM) behavior in the open-field activity tests from Experiments 1 and 2.

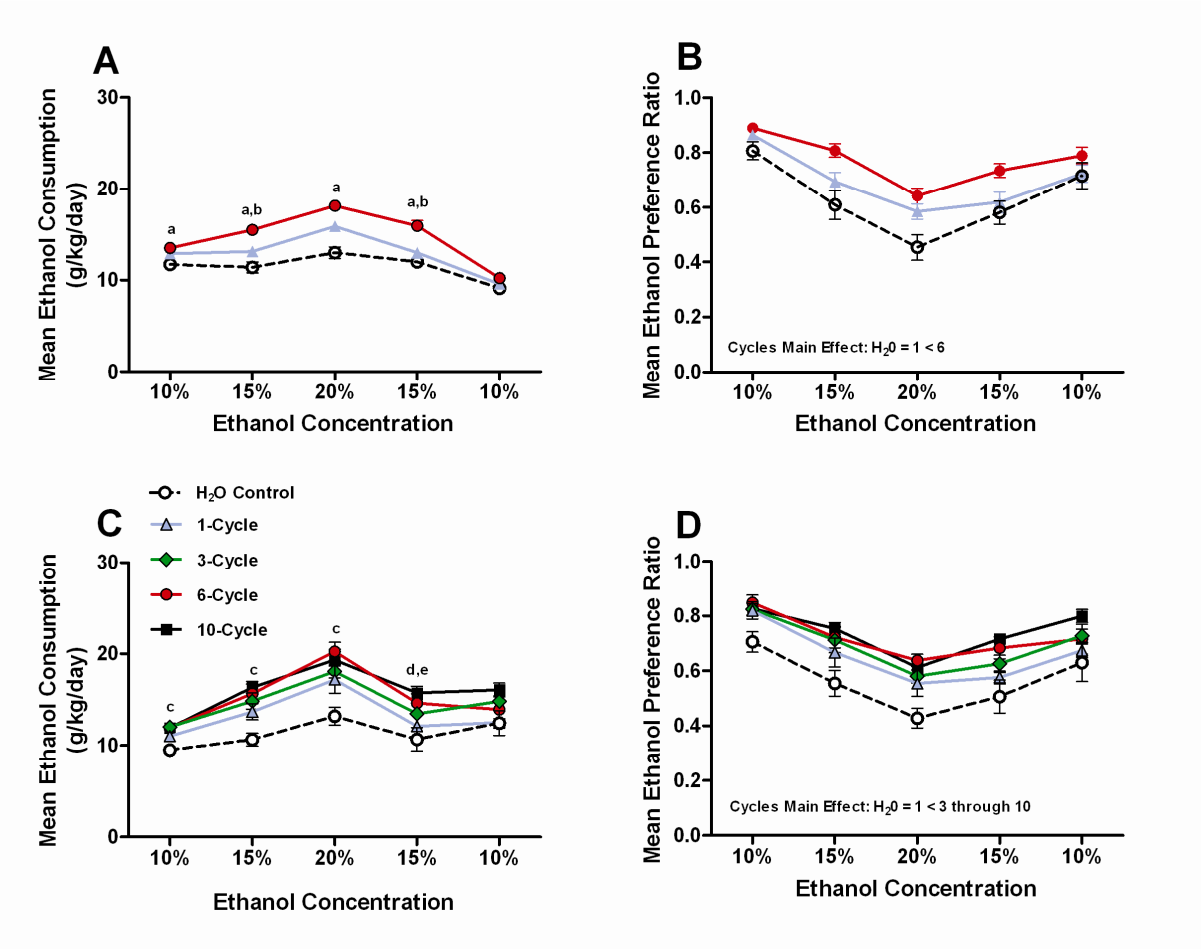
	Marginal Distance (cm^2/10min)	Time in Margin (s)	Center Distance (cm^2/10min)	Time in Center (s)
Experiment 1				
H_20	2271.4 ± 198.88	505.3 ± 5.58	711.0 ± 36.61	94.7 ± 5.58
1-cycle	2501.5 ± 119.17	510.5 ± 7.59	673.8 ± 68.14	89.5 ± 7.59
6-cycle	2325.9 ± 155.85	521.3 ± 5.53	609.2 ± 60.66	78.7 ± 5.53
Experiment 2				
H_20	2194.7 ± 163.56	507.6 ± 9.60	747.2 ± 66.37	92.4 ± 9.60
1-cycle	2351.8 ± 162.53	509.1 ± 8.49	742.6 ± 71.58	90.9 ± 8.49
3-cycle	2418.1 ± 163.64	500.1 ± 14.81	655.3 ± 101.75	99.9 ± 14.81
6-cycle	2311.0 ± 277.12	505.0 ± 7.90	678.4 ± 56.81	95.0 ± 7.90
10-cycle	2414.6 ± 125.60	500.9 ± 16.04	732.1 ± 86.19	99.1 ± 16.04

Table 3: Average scores (mean ± SEM) from the rotarod and HIC test in Experiment 2.

	Rotarod: Latency to Fall (s)	Rotarod: RPM at Fall	HIC Score
H_20	175.4 ± 13.12	25.9 ± 1.82	0.23 ± 0.12
1-cycle	168.0 ± 13.66	25.2 ± 1.96	0.14 ± 0.04
3-cycle	142.9 ± 18.00	25.5 ± 2.46	0.27 ± 0.07
6-cycle	151.8 ± 17.39	25.3 ± 2.54	0.18 ± 0.06
10-cycle	151.7 ± 17.23	26.6 ± 2.50	0.13 ± 0.06

HIC = Handling-induced convulsion (scored on a scale from 1 to 7).

Fig. 1. A history of repeated cycles of binge-like ethanol (20%, v/v) drinking significantly augments subsequent 2-bottle choice voluntary ethanol consumption. Data points at each concentration of ethanol (v/v) represent an 8-day average, and data are expressed as mean g of ethanol consumed per kg of body weight per day (g/kg/day; A and C) or mean ethanol preference (ethanol consumed / total fluid intake; B and D). The top row represents data collected from Experiment 1 (A and B) and the bottom row represents data collected from Experiment 2 (C and D). Data are expressed as mean ± SEM. Significance legend (based on Bonferroni tests): **a** = H_20 < 6-cycles; **b** = 1-cycle < 6-cycles; **c** = H_20 < 3-, 6-, and 10-cycles; **d** = H_20 < 6- and 10-cycles; **e** = 1-cycle < 10-cycles. Significant differences between groups on the cycles main effect (indicated in B and D) are based on Bonferroni tests.



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