Alcohol Binge-Drinking on the Transition to Alcohol Dependence: Relationship and Neurobiological Basis

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Alcohol Binge-Drinking on the Transition to Alcohol Dependence: Relationship and Neurobiological Basis

A Thesis submitted in partial satisfaction for the requirements for the degree

Master of Science

in

Biology

by

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2014
The Thesis of Sarah L. Kim is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2014
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ABSTRACT OF THE THESIS

Alcohol Binge-Drinking on the Transition to Alcohol Dependence: Relationship and Neurobiological Basis

by

Sarah L. Kim

Master of Science in Biology

University of California, San Diego, 2014

George F. Koob, Chair
William Kristan, Co-Chair

Intermittent access to alcohol in rats produces a pattern similar to alcohol binge drinking which has been shown to be associated with alcohol dependence in
humans, however direct causal evidence is missing. Moreover, the neuronal ensemble responsible for the excessive drinking behavior is currently unknown. The corticotropin-releasing factor (CRF) system in the central nucleus of the amygdala (CeA), an anti-reward stress brain system, has been speculated in playing a critical role during alcohol withdrawal. The aims of this set of studies were to test the causal effect of alcohol binge-drinking on the transition to alcohol dependence, and to test the causal role of recruitment of CeA neurons during withdrawal on alcohol drinking. To this end, we first measured the effect of a history of intermittent vs. continuous access to alcohol using a two-bottle choice paradigm on the motivation to drink in a model of alcohol dependence (chronic intermittent ethanol vapor exposure). We found that a history of intermittent access to alcohol accelerated the progression to alcohol dependence. We then tested whether withdrawal-dependent activation of Fos+ neurons in the CeA produces alcohol binge drinking behavior using the Daun02 inactivation method. Results showed that inactivation of Fos+ neurons decreased alcohol drinking both in binge-drinking rats and in dependent rats. These results demonstrate that a history of alcohol binge-drinking facilitates the transition to alcohol dependence and that recruitment of CeA neurons during withdrawal mediates excessive alcohol drinking.
Introduction

I. Binge Drinking, Addiction, and Alcoholism,

In 2012, 24.6% of people over the age of 18 reported they engaged in alcohol binge drinking behavior within the past month. The transition from casual binge drinking behavior to alcoholism is a critical issue that has become a heavy focus of study today. The National Institute of Alcohol Abuse and Alcoholism defines binge drinking as a pattern of drinking that brings blood alcohol levels to 0.08 g/dL (80 mg%). The Diagnostic and Statistical Manual of Mental Disorders (DSM) currently states that any person meeting any two of 11 criteria within a 12-month period receives a diagnosis of “alcohol use disorder” and the severity of the disorder is based on the number of criteria met (National Institute of Alcohol Abuse and Alcoholism, 2014). How does an individual transition from an occasional binge drinker to an alcoholic and what neurobiological mechanisms play a fundamental role in this transition?

Addiction, regardless of the drug, is conceptualized as a decreased function of brain reward systems and the recruitment of anti-reward stress systems, which provide powerful motivation to seek a drug. The anti-reward stress system conceptualizes the idea that there are brain systems that limit the reward when the reward system is triggered excessively. (Koob and Le Moal, 2001; 2008). Some of these anti-reward systems include the corticotropin release factor (CRF) system, dynorphin system, as well as the norepinephrine system. These systems contribute to the negative reinforcement of dependence by stress-like states (George and Koob, 2010). An increase in drug intake is then necessary to restore the decreased brain reward function. These changes worsen over
time and result in the compulsive use of drugs. The combination of decreased reward function and recruitment of the stress systems provide motivation for the compulsive drug-seeking behavior seen in addicts (Koob and Le Moal, 2008).

The addiction cycle is characterized by three stages: preoccupation-anticipation, binge-intoxication, and negative-withdrawal affect (Koob and Le Moal, 2001). The initial two stages, preoccupation-anticipation and binge-intoxication are important for the initiation of alcohol drinking, while the negative-withdrawal affect stage plays a critical role in the transition to drug dependence. Previous research on alcohol addiction has focused heavily on the first two stages, the focus of my research was mainly on the last stage pertaining to withdrawal and how withdrawal can increase the likelihood for the transition to addiction.

Alcoholism is defined as a chronic relapsing disorder with compulsive drinking, loss of control over intake, and the emergence of a negative emotional state during abstinence from the drug (Koob et al, 2004). Similar to other drugs of abuse, a severe emotional and somatic withdrawal syndrome characterizes alcoholism with an intense craving for alcohol/drug that is often driven by negative emotional states (Koob and Le Moal, 2008). Excessive alcohol intake is initially characterized by intoxication via binge drinking episodes that are positively reinforced by the pleasurable effects of alcohol. Periods of sobriety are found between these drinking episodes. During this initial binge drinking stage, environmental stimuli play an important role for setting "reward craving" in regards to the pleasurable alcohol effects that once drove the alcohol intake. Transition to alcohol dependence involves a switch from positive reinforcement (pleasurable alcohol
effects) to negative reinforcement (relief from a negative emotional state). Dysphoria, increased anxiety, and an increased sensitivity to stress during alcohol withdrawal, now motivate the search for alcohol. At this point, alcohol use is negatively reinforced and alcohol seeking takes place to obtain relief from the negative emotional states, which is also known as "relief craving." (Heiling and Koob, 2007)

II. Studying Alcohol Addiction with the Rat Model

Numerous studies have suggested that recruitment of the CRF stress systems may contribute to alcohol dependence but direct causal evidence is still scarce.

In a previous study, Wistar rats were found to have high levels of ethanol intake that was maintained over 5 months through repeated cycles of excessive drinking (binge drinking episodes) and abstinence via an intermittent two-bottle choice paradigm. The intermittent two-bottle choice method has shown remarkable face, construct and predictive validities for excessive alcohol drinking (Simms et al., 2008). While the low amount of alcohol consumed (blood alcohol levels <80 mg%) in this model limits its relevance to alcohol dependence (blood alcohol levels in the 150-300ng% range), it is however an excellent model of alcohol binge-drinking that may provide important information about a potential pre-dependence stage.

Chronic intermittent ethanol vapor exposure is an additional animal model utilized to induce alcohol dependence in rats with blood alcohol levels in the 150-250 mg% range. This model provides advantages for the experimenter in that the alcohol dose, duration of exposure, and exposure pattern can be easily controlled (Gilpin et al,
2008). Furthermore, this procedure is non-invasive for the animals compared to other drug induction procedures and long-term stable blood alcohol levels can be maintained. In addition, the animals show signs of tolerance and alcohol dependency when alcohol vapor exposure is terminated thus can be tested for behavior during abstinence (Gilpin et al, 2008).

III. The Gap in Research

While, alcohol binge drinking has been shown to be a risk factor for alcohol dependence in humans, causal evidence in animal models remains to be demonstrated. Furthermore, although previous research showed that withdrawal from alcohol activates neurons in the CeA, causal evidence that the neuronal ensemble recruited in the CeA during alcohol withdrawal is responsible for the excessive drinking behavior is missing. Thus, my research aimed to figure out the causal role of binge drinking in relation to the transition to dependence and focused on the neuronal ensemble in the CeA that is hypothesized to control the excessive drinking behavior during withdrawal.

IV. Evidence on Binge Drinking and Alcoholism

Studies have shown that an impairment of the medial prefrontal cortex (mPFC) and the over-activation of the brain stress system in the central nucleus of the amygdala (CeA) are key players in the transition to dependence (George et al, 2012; Koob, 2008). Binge drinking seems to play a critical role in the transition to alcohol dependence. Past studies have shown that chronic intermittent access to alcohol leads to the escalation of alcohol intake, which is a binge pattern that is also seen in humans. Rats that were given
intermittent access to alcohol demonstrated binge drinking behavior which increased in intake over time and ultimately stabilized at high levels while rats given continuous access maintained stable levels of drinking over the same time frame (George et al, 2012; Simms, 2008). Alcohol withdrawal was found to recruit a specific subpopulation of GABAergic and CRFergic neurons in the mPFC and a disconnection between the mPFC and CeA predicted an increase in binge drinking during acute abstinence for dependent animals (George et al, 2012).

V. The Corticotropin-Releasing Factor System

While the neurobiological mechanism for binge drinking is poorly known, converging evidence has shown that activation of the CRF-CRF1 receptor system is critical for excessive alcohol drinking. CRF neurons are found in the extended amygdala (CeA, BNST), prefrontal cortex (PFC) and paraventricular nucleus (PVN), as well as several brainstem regions. Activation of CRF1 receptors, particularly in the CeA, has been demonstrated to be involved with mediating behavioral responses to fear and anxiety, and to promote alcohol drinking in alcohol dependent rats (Koob and Le Moal, 2008; Funk et al, 2006). The extended amygdala contains a high density of CRF terminals, cell bodies, and CRF1 receptors. Blocking CRF1 function using a CRF1 specific antagonist attenuates the increased ethanol self-administration seen in dependent animals. Injections of CRF antagonists reduced the anxiety-like behaviors as well as the increased ethanol consumption associated with alcohol withdrawal. As ethanol dependence progresses, a dysregulation of the brain hypothalamic and extrahypothalamic
CRF stress systems is seen with ethanol withdrawal. The extrahypothalamic system becomes hyper-activated during withdrawal.

An increase in extracellular CRF is seen within the CeA of dependent rats during withdrawal and is found to play a critical role in mediating increased ethanol self-administration to alleviate the anxiety like behaviors. A CRF antagonist injected directly into the CeA reduced the anxiety-like behaviors during withdrawal. In addition, lesions of the CeA also reduced the anxiety-like behaviors and voluntary ethanol drinking showing the importance of the CeA in regards to anxiety and ethanol consumption (Funk et al, 2006).

VI. GABAergic and CRFergic Neurons

While the role of CeA CRF neurons has been heavily studied, the role of PFC CRF neurons in alcohol drinking was unknown until recently. Our group showed that alcohol binge-drinking recruits a population of GABA and CRF neurons in the PFC during withdrawal and that it was associated with PFC-dependent cognitive impairment. Interestingly, abstinence-induced activation of the PFC predicted excessive alcohol intake (binge drinking). The intermittent group had an increase in Fos+ neurons during abstinence in both the PFC and CeA. c-fos is a proto-oncogene that is expressed upon neuronal recruitment and is used to identify neuronal ensembles associated with a specific behavior or state. A positive correlation was seen between the number of Fos+ neurons within the PFC and CeA during abstinence with the level of ethanol intake after 24 hours of abstinence. When access to alcohol was renewed, Fos expression was normalized in both the continuous and intermittent groups (George et al, 2012).
Colocalization of GABAergic neurons and Fos was also measured. GAD67 was the marker used for GABAergic interneurons. Within the PFC, Fos+ neurons were GAD67- for continuous access suggesting the recruitment of pyramidal glutamatergic neurons during abstinence rather than GABAergic interneurons. More importantly, Fos+ neurons were GAD67+ for a large proportion of neurons for intermittent access. This result suggests the recruitment of GABAergic interneurons during abstinence of the intermittent group (George et al, 2012).

Abstinence from alcohol was found to be associated with a functional disconnection between the mPFC and CeA. This disconnection may be an early index of neuroadaptation in alcohol dependence and may be critical for impaired executive control over motivated behavior during acute abstinence (George et al, 2012). Dysregulation of the PFC and CeA suggests that rats with intermittent access experience a more severe withdrawal leading to impaired executive function and increased alcohol craving that results in excessive alcohol intake in order to restore normal function. (George et al, 2012).

VII. Research Hypothesis

The over-activation of the brain stress system in the CeA and PFC is seen to be a key factor in the transition from goal-oriented to compulsive drinking in alcoholism and is the foundation of the research conducted. Intermittent access to alcohol results in an over-activation of the brain-stress systems within the CeA and PFC. We hypothesized that there is a recruitment of withdrawal-dependent CeA neurons that mediate the increased motivation to drink alcohol in intermittent access rats. Our study examined
whether a history of binge drinking facilitates the transition to alcohol dependence and furthermore, whether inactivation of CeA Fos+ neurons during withdrawal reduces alcohol binge drinking.

**VIII. Approach**

To test the hypothesis that history of binge drinking facilitates the transition to alcohol dependence, animals underwent a two-bottle choice paradigm with either continuous or intermittent alcohol access, and subsequent vapor exposure with fixed ratio and progressive ratio self-administration tests. Quinine adulteration tests were conducted to test alcohol drinking despite aversive consequences (a hallmark of alcohol dependence) during the two-bottle choice paradigm as well as during operant responding (Vendruscolo et al, 2012).

To test the hypothesis that inactivation of CeA Fos+ neurons during withdrawal reduces alcohol binge drinking we inactivated CeA Fos+ neurons using the Daun02 inactivation method in two separate cohorts of animals: (1) in animals that had a history of alcohol binge-drinking via intermittent access to 20% alcohol within a two-bottle choice model, and (2) in animals exhibiting dependence-induced drinking using the chronic intermittent ethanol vapor exposure. The Daun02 inactivation method allows specific inactivation of Fos+ neurons using cfos-LacZ transgenic rats expressing the beta-galactosidase under the control of the Fos promoter (Cruz et al, 2013; Koya et al, 2009). Daun02, upon injection, is converted to daunorubicin by beta-galactosidase to inactivate neurons expressing both beta-galactosidase and Fos at the time of injection.
Materials and Methods

Animals

All procedures were in accordance with the guidelines of the National Institutes of Health and The Scripps Research Institute Institutional Animal Care and Use Committee.

Ethanol Intake Procedures for Two-Bottle Choice

All alcohol was presented in 100 ml graduated cylinders with stainless steel drinking spouts while water was presented in 550 ml plastic bottles with stainless steel drinking spouts once the lights went off in the reversed light/dark cycle room. Bottles were weighed 24 hours after fluids were presented and the measurements were taken to the nearest gram. The weight of each rat was taken once a week to calculate the grams of ethanol intake per kilogram of body weight. Ethanol solutions were prepared in tap water from 95% (v/v) ethanol.

Daun02 Inactivation Method

The rats received a microinjection into the central amygdala of Daun02 (4 ug/ul in 5% DMSO, 5% Tween-80, ACSF) or vehicle. 0.5 ul volume was bilaterally infused over the course of 2 minutes. Injectors extended 2 mm beyond the end of the guide cannulae. 90 minutes after infusions, the animals were either given two-bottle choice or run in the operant boxes depending on the experiment.

Daun02 Microninjection into the Central Amygdala for cfos-lacZ Transgenic Rats Under the Two-Bottle Choice Paradigm
18 cfos-LacZ transgenic rats weighing 290 - 420 g were all assigned to intermittent alcohol self-administration. Each rat was singly housed and maintained on a 12 hour/day reverse light/dark cycle. The rats were given intermittent (24 hr/day for 3 days/week) access to alcohol (20% v/v) using a two-bottle choice procedure. The rats were then split into one of two groups of 9 animals: Daun02 or vehicle. Note that 4 animals from the Daun02 group and 6 animals from the vehicle group were removed from the study for improper cannula implantation.

**Daun02 Micronjection into the Central Amygdala for cfos-lacZ Transgenic Rats Upon Intermittent Alcohol Vapor Exposure**

18 cfos-LacZ transgenic rats were made dependent by intermittent exposure to alcohol vapors. They underwent cycles of 14 hours on and 10 hours off. The animals underwent behavioral testing for acute withdrawal during the off hours. Blood alcohol levels were tested weekly and ranged from 150 mg% to 250 mg%. Animals were kept in the reversed light/dark cycle room. Note that 7 animals were removed from the study due to clogged cannulas.

**Operant self-administration**

The self-administration sessions were conducted in standard operant conditioning chambers. A fixed ratio (FR) schedule of reinforcement was used. The rats were first trained to self-administer water with an overnight session and one lever only delivering water. Food was available during the 24-hour session. Training was followed with 1 hour sessions a day followed by 30 minute sessions
a day, both on the fixed ratio schedule but with two levers, one delivering alcohol and the other delivering water. Upon completed training, the rats were run twice a week and allowed to self-administer 10% v/v alcohol solution and water with two levers on a fixed ratio schedule of reinforcement which was considered a measurement of alcohol intake.

**Wistar Rats Under the Two-Bottle Choice Paradigm Followed by Alcohol Vapor Exposure**

24 Wistar rats weighing 330 - 560 g were randomly assigned to one of two conditions: continuous or intermittent alcohol self-administration. Each rat was singly housed and maintained on a 12-hour/day reverse light/dark cycle. The rats were given either continuous or intermittent alcohol access via the two-bottle choice paradigm and then were exposed to intermittent alcohol vapor only. They underwent cycles of 14 hours on and 10 hours off. The animals underwent behavioral testing for acute withdrawal during the off hours. Blood alcohol levels were tested weekly and ranged from 150 mg% to 250 mg%. Animals were kept in the reversed light/dark cycle room.

**Quinine Adulteration Test Two-Bottle Choice**

Quinine concentrations of 0.0125, 0.025, 0.05, 0.075, and 0.1 g/L of 20% ethanol were given to the animals on separate days following the same schedule of two-bottle choice. Note that 2 animals, one from continuous and one from intermittent, were removed from the study for lack of self-administration alcohol.

**Operant self-administration**
The self-administration sessions were conducted in standard operant conditioning chambers. Initially, a progressive ratio (PR) schedule of reinforcement was used for two times a week. The number of lever presses necessary for the next reinforcer (reward) progressively increased in the following progression: 1, 1, 2, 2, 3, 3, 4, 4, 5, 5, 7, 7, 9, 9, 11, 11, 13, 13, etc. The PR session terminated after 90 minutes or when 15 minutes passed without the rat obtaining a reinforcer. The final value of presses was determined by the break point at which each session terminated for each rat. PR sessions were executed for three different sets of two-bottle choice drinking schedules: 2 hours into drinking, 24-hour withdrawal for intermittent, and 24 hour withdrawal for both continuous and intermittent.

Once the animals underwent alcohol vapor exposure, 30-minute FR sessions were conducted followed by PR sessions.

*Quinine Adulteration Test Operant Self-Administration*

The quinine adulteration test was performed with 30-minute FR operant sessions. Quinine concentrations of 0.005, 0.01, 0.025, 0.05, 0.1, 0.2, 1.0, and 2.0 g/L of 10% ethanol were adding to the alcohol solution used in operant sessions. The procedure for the FR sessions was the same as listed above.
Results

Experiment 1:

The effect of a history of intermittent access to alcohol (two-bottle choice) on compulsive alcohol drinking and dependence-induced drinking.

We first tested the effect of intermittent vs. continuous access to alcohol on progressive ratio response and the quinine adulteration test. After stabilization of escalation (5 weeks) rats were tested under a progressive ratio responding schedule of reinforcement (PR) after either 2 hour access to alcohol (both groups), 24 hours into withdrawal (both groups), or when only the intermittent access group was 24 hours into withdrawal (to evaluate the motivation using the same protocol used in the two-bottle choice procedure) (Fig. 1). A difference in lever pressing is seen two hours into drinking when alcohol access was reinstated following a 24-hour withdrawal for the intermittent access group. The continuous access group had higher responding for alcohol compared to the intermittent access group during the PR session 2hr into drinking (P ≤ 0.1) (Fig. 1). For both the 24-hour withdrawal schedule (both groups) and 24-hour intermittent withdrawal, similar response levels are seen between the two groups with the continuous access pressing slightly more.
Figure 1. Average progressive ratio lever presses. Continuous access showed more pressing for a PR session two hours into drinking compared to the intermittent access group but with a $P = 0.14$. Similar pressing levels were seen for both groups when the intermittent group was in 24-hour withdrawal while the continuous group was given alcohol access, $P = 0.74$. Very similar results were seen when both groups were in 24-hour withdrawal, $P = 0.67$.

A possible explanation for the lower PR responding in the intermittent group 2hr into drinking is that the intermittent group already binged on alcohol before being tested on PR. To test this hypothesis, we measured the amount of alcohol intake during the 2hr preceding the PR testing (Fig. 2). Within the two hours of access to alcohol following a 24-hour abstinence for the intermittent access group, the intermittent significantly consumed more than twice the amount of the continuous group ($***P < 0.001$).
Figure 2. Volume of ethanol intake during 2-hour two-bottle choice alcohol access. Intermittent access had a higher intake of alcohol during the 2hr two-bottle choice access following a 24-hour abstinence (***(P ≤ 0.001) between the continuous and intermittent drinking levels during the 2 hours of access.

We then performed a quinine adulteration test (after X weeks of intermittent vs. continuous access to alcohol), a measure of compulsive alcohol drinking, to measure alcohol drinking despite the aversive effect of quinine, (Vendruscolo et al., 2012). Increasing concentrations of quinine were added to the two-bottle choice model (Fig. 3). A decrease in alcohol intake was seen at all doses of quinine compared to baseline values for the intermittent and the continuous access group (P ≤ 0.05, Fig. 3).
Figure 3. Quinine addition to 20% ethanol two-bottle choice bottles for continuous and intermittent access. A significant decrease in ethanol intake is seen at 0.0250 g/L for continuous access (**P < 0.0005). The highest sensitivity to quinine is seen at 0.050 g/L for the intermittent access compared to baseline values (***p < 0.0005).

After completion of the PR and quinine adulteration testing rats were stabilized under fixed ratio (FR) for 1 week and then given intermittent exposure to alcohol vapor for 8 weeks with FR testing twice a week to measure alcohol response. A history of intermittent access to alcohol (5 months) facilitated escalation of alcohol intake compared to continuous access (Fig. 4A). The animals underwent FR testing upon exposure to alcohol vapor (Fig. 4B). An increase in lever pressing was seen for both groups, however intermittent access rats showed an acceleration of escalation of alcohol intake with plateau levels reached after 4 weeks while it took 8 weeks to the continuous group to reach similar levels (Fig. 4B).
Figure 4. Intermittent access to alcohol induces escalated intake compared to continuous access for the two-bottle choice model. A.) Intermittent access to alcohol exhibits escalated intake for two-bottle choice. B.) Continuous access increase to similar alcohol levels as intermittent access under fixed ratio testing four weeks behind intermittent.

After stabilization of alcohol drinking escalation (after week 8), the groups underwent the quinine adulteration test and their responses were compared to historical data obtained previously with rats exposed to air or alcohol vapor in a similar manner but without a history of intermittent or continuous access to two-bottle choice. Results show that both the continuous and intermittent groups exhibited persistent responding for alcohol despite high concentration of quinine. Indeed, the intermittent and continuous groups required a dose 10 times higher than the dose required to decrease alcohol drinking in non-dependent rats and 2 times higher than the dose required to decrease alcohol in dependent rats without a history of intermittent or continuous access to alcohol (Vendruscolo et al., 2012) (Fig. 5). Moreover, the continuous access group had higher lever responses compared to the intermittent group at the highest concentration of quinine (2.0 g/L), suggesting that despite a slower escalation of alcohol intake and reaching
similar levels of alcohol drinking after 8 weeks, the continuous rats exhibited increased resistance to quinine compare to the intermittent group (Fig. 5).

Figure 5. Dose-response effect of quinine on alcohol drinking in continuous and intermittent rats after 8 weeks of exposure to alcohol vapor. Note that data is compared to historical data obtained in non-dependent rats and in regular dependent rats without access to two-bottle choice, but with similar exposure to alcohol vapor (~9 weeks). Continuous rats showed greater motivation to drink alcohol despite the bitter taste of quinine for almost all concentrations. A greater difference between the two groups was seen starting at 0.1g/L of quinine in 10% ethanol, 0.01 < P < 0.05. At 0.2 g/L, P = 0.057.

Experiment 2

Administration of Daun02 in the CeA partially decreases alcohol drinking in rats with intermittent access to alcohol.

Rats given intermittent access in a two-bottle choice paradigm increased their intake of alcohol over the course of 70 days and had stable levels of drinking thereafter (Fig. 6A). To test the hypothesis that intermittent binge drinking recruits a subpopulation
of CRF neurons within the CeA, Daun02 was injected into the CeA and the changes in alcohol drinking levels was evaluated. Daun02 administration in the CeA specifically decreased alcohol drinking in rats that had intermittent access (***P < 0.05) but not water drinking (Fig. 6B). Within-subject analysis shows that rats treated with Daun02 exhibited an average of 26.3% decrease (***P < 0.05) in alcohol intake that was not observed in the vehicle group (Fig. 6C, 6D).

![Figure 6](image_url)

**Figure 6.** Effect of intra-CeA Daun02 on alcohol drinking. A.) Intermittent access to alcohol induces escalated intake. B.) A significant decrease in alcohol drinking is seen Daun02 injected animals. C.) A within subject analysis in alcohol intake measured in g/kg of alcohol seen in Daun02 injected animals. D.) Nonsignificant pattern of alcohol drinking in g/kg seen in vehicle-injected animals.

Intra-CeA Daun02 administration decreased the number fos-positive neurons within the CeA by 23.8% (*P < 0.05) (Fig. 7) suggesting CRF neurons play a partial role in binge-drinking behavior.

![Figure 7](image_url)

**Figure 7.** Effect of intra CeA Daun02 on Fos-positive neurons in the CeA.
Experiment 3:

Administration of Daun02 in the CeA reversed dependence-induced alcohol drinking in rats with intermittent exposure to alcohol vapor.

Intra-CeA Daun02 microinjection attenuated lever pressing for alcohol (#P < 0.05 vs. post-vapor) down to pre-vapor levels (P >>> 0.1 vs. pre-vapor) while the vehicle group did not have a significant decrease in lever presses compared to pre-vapor pressing levels (*P ≤ 0.1) (Fig. 8). Intermittent alcohol vapor exposure for at least 8 weeks on a different cfos-lacz transgenic rat group exhibited an increase in lever pressing (**P < 0.05) for all animals pre-Daun02 administration (Fig. 8).

![Graph](image-url)

Figure 8. Effect of intra-CeA Daun02 on responding for alcohol in alcohol vapor dependent rats. An increase in drinking post-alcohol vapor exposure seen for all animals (**P < 0.05). Significant decrease in lever pressing was observed for the Daun02 group, (#P < 0.05) but not the vehicle group (*P ≤ 0.1).
Discussion

Previous work has shown that intermittent access to alcohol using a two-bottle choice paradigm produces an escalation of alcohol-drinking associated with blood alcohol levels close to the legal limit in humans (80 mg%) and produces a pattern of compulsive alcohol drinking and impaired executive functions (Simms et al., 2008; Hopf et al., 2010; George et al., 2012). Two important unanswered questions were 1.) Which neuronal system mediates alcohol binge-drinking, and 2.) Does a history of escalation of binge-drinking facilitate the transition to alcohol dependence. Here, we provide causal evidence that recruitment of withdrawal-dependent Fos+ neurons in the CeA contributes to alcohol binge-drinking particularly in dependent rats. We also provide evidence that a history of alcohol binge-drinking accelerates the transition to alcohol dependence and increases compulsive alcohol drinking.

I. Effect of intra-CeA Daun02 on alcohol drinking in non-dependent rats with intermittent access to alcohol and in dependent rats with chronic exposure to alcohol vapor

Research has shown that alcohol binge-drinking recruits neuronal ensembles in the PFC and CeA 24 h into withdrawal. While these neurons showed activation during withdrawal, the number of Fos+ neurons was normalized when access to alcohol was renewed, suggesting that recruitment of PFC and CeA neurons may contribute to the motivation to drink alcohol (George et al, 2012). Moreover, only rats exhibiting escalation of binge-drinking (intermittent rats) showed recruitment of these neuronal ensembles in the CeA and PFC (George et al, 2012). Rats with continuous access to
alcohol did not show escalation of alcohol drinking and exhibited very little if any changes in the number of Fos+ neurons in the CeA. Therefore we hypothesized that recruitment of withdrawal-dependent CeA neurons mediates the increased motivation to drink alcohol in intermittent rats. Utilizing the Daun02 inactivation method, the Fos+ neurons were inactivated within the CeA and the effect on ethanol intake was analyzed in 2 different cohorts of rats given either intermittent access to two-bottle choice (non-dependent rats) or exposed to alcohol vapor for 8 weeks (dependent rats).

The intermittent rats following the two-bottle choice model showed an escalation of alcohol intake (Fig. 6A) as shown in a previous study using the same procedure (Simms et al, 2008). This escalation in drinking supports the hypothesis that intermittent access increased the risk for binge drinking in rats. Upon bilateral Daun02 or vehicle administration within the CeA, a decrease in drinking was seen for the Daun02 group compared to its baseline value (Fig. 6B, 6C). Although a decrease in intake was observed, Daun02 only partially produced a small decrease in Fos+ neurons within the CeA suggesting that these Fos+ neurons may only partially support binge-drinking (Fig. 7).

A separate cohort of rats was made dependent on alcohol through intermittent vapor exposure. Operant self-administration fixed ratio sessions were performed with the animals and similar levels of baseline lever pressing was seen pre-vapor exposure. Post-vapor exposure (8 weeks) exhibited significant lever responses in both groups (Daun02 and vehicle). Upon bilateral CeA administration of Daun02 or vehicle, a significant decrease in lever pressing was seen for the Daun02 group in comparison to the vehicle
group suggesting that Fos+ inactivation reverses dependence-induced alcohol drinking in rats that were exposed to alcohol vapor (Fig. 8).

Based on these results, the inactivation of the Fos+ neurons by Daun02 seems to alleviate binge drinking behavior for both non-dependent animals under two-bottle choice and dependent animals with chronic exposure to alcohol vapor during acute abstinence.

II. Effect of intermittent access to two-bottle choice on the motivation for alcohol measured using a progressive ratio and the quinine adulteration test

The animals were separated into two separate groups of either continuous access or intermittent access to two-bottle choice alcohol. When the animals were tested using progressive ratio operant self-administration during the two-bottle choice paradigm, a difference in lever pressing response was seen two-hours into drinking when alcohol access was renewed. The intermittent access group was previously in a 24-hour withdrawal prior to being given alcohol while the continuous group had access to alcohol prior to the 2-hours. The intermittent access group binge-drunk when two-bottle alcohol access was reinstated and drank about two times the alcohol volume of the continuous (Fig. 2). Due to the alcohol binge during the two-bottle choice reinstatement, the data suggests that the intermittent access group lost motivation for their progressive ratio session explaining the decreased response in lever presses compared to their continuous counterparts (Fig. 1).

When the intermittent access group was in 24-hour withdrawal and the continuous access group previously had access to alcohol prior to their PR session, there seems to be
no significant difference in lever pressing frequency. The binge-pattern associated with intermittent access seen in two-bottle choice models does not seem to apply under progressive ratio (Fig. 1, 4A). The difference in motivation may lie in the difficulty obtaining alcohol where working memory is needed for the progressive ratio model compared to two-bottle choice access. A previous study found that working memory was impaired in intermittent access rats in a spontaneous alternation Y-maze compared to a continuous access group during acute withdrawal (George et al., 2012). This might suggest why the intermittent access group did not have a higher lever pressing response as expected during withdrawal. An alternative hypothesis is that PR responding is not sensitive enough to detect the increased motivation associated with alcohol binge-drinking in this model.

The quinine adulteration test was used to test for compulsive alcohol drinking. Upon increasing concentrations of quinine addition to the two-bottle choice ethanol, a decrease in drinking was seen for both groups starting at 0.0250 g/L quinine in 20% ethanol. Both the intermittent access group and continuous access group significantly decreased alcohol intake at 0.0250 g/L compared to baseline although there was a slightly greater decrease seen for the continuous access group (Fig. 3). These results obtained before exposure to vapor contradict results obtained in a previous study (Hopf et al., 2011), where they observed increased resistance to quinine in intermittent access rats with decreased alcohol intake at 0.01g/L in the continuous group and 0.1g/L in the intermittent group. While difference in the protocols between studies may explain the different results, it suggest that the escalation of alcohol intake in intermittent vs.
continuous rats can occur without increased compulsive-like alcohol drinking as measured by the quinine test.

**III. Effect of intermittent access to two-bottle choice on the dependence-induced drinking using the chronic intermittent ethanol vapor model and fixed ratio quinine adulteration test**

A clear distinction in alcohol intake with the two-bottle choice model is seen with an escalation and stabilization of intake for the intermittent access followed by steady intake with the continuous access group (Fig. 4A) paralleling results seen in a previous study (Simms et al., 2011; George et al, 2012). Subsequent exposure to alcohol vapor facilitated an increase in fixed ratio lever presses for both intermittent and continuous access groups. While both groups ultimately reached similar lever response levels by week 8, the intermittent access group increased in responses at week 4 and stabilized thereafter while the continuous took 4 more weeks to reach the same level (Fig. 4B). These results suggest that intermittent two-bottle choice (binge-drinking) played a fundamental role in the motivation to induce drinking more quickly with chronic intermittent ethanol vapor: a history of binge drinking behavior facilitated the transition to dependence.

A quinine adulteration test was used in FR operant self-administration testing. The continuous and intermittent access groups exhibited persistent responses to lever pressing despite the aversive effects of quinine (Fig. 5). Based on historical data from a previous study, both groups needed a higher concentration of quinine to decrease alcohol drinking compared to non-dependent (10 times) and dependent animals (2 times)
(Vendruscolo et al., 2012) (Fig. 5). At 2.0 g/L quinine, the highest concentration, the continuous access group was more motivated to obtain alcohol compared to the intermittent group suggesting that the continuous group had an increased resistance to quinine despite taking longer to catch up to similar levels of alcohol intake during vapor exposure (Fig. 4B, 5).

In the Hopf et al., study, a difference in sensitivity towards quinine between the two groups was observed. The intermittent group was more resistant to quinine and decreased ethanol intake at a higher quinine concentration compared to the continuous but at concentrations much higher than our study; 0.01g/L for continuous access and 0.1 g/L quinine for intermittent access (Hopf et al., 2011). Our results showed that post-vapor the quinine dose required to decrease alcohol was similar for the intermittent access rats are (0.1g/L) suggesting that the difference between studies stem from the higher resistance to quinine of the continuous rats (0.01g/L in Hopf study vs. 0.1 g/L in our study). Difference in the duration of exposure 3-4 months (Hopf et al) vs. 7 months (our study: 5 months of intermittent access + 2 months of vapor) may explain the higher compulsive intake in the continuous access group post vapor.

IV. Future Directions

Our research showed that a history of 5 months of binge drinking with the two-bottle choice paradigm facilitates the transition to alcohol dependence and increases compulsive alcohol drinking. Further studies will investigate whether shorter exposure to alcohol binge drinking (2-4 weeks) is enough to facilitate the transition to alcohol dependence. We found that the inactivation of Fos+ neurons within the CeA during
withdrawal attenuates binge drinking behavior in animals with a history of binge drinking within a two-bottle choice paradigm (non-dependent) and alcohol vapor model (dependent rats). The decrease in drinking seen with Daun02 administration coincided with a decrease in Fos+ neurons but the small percentage decrease suggests that in addition to CeA neurons, other neuromolecular mechanisms within the brain play a fundamental role in initiating binge drinking behavior. Research into these other mechanisms will broaden the understanding of alcohol addiction and aid in the research for therapeutic remedies.
References


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