Ethanol preference is impacted by estrus stage but not housing or stress in female C57BL/6J mice.

Kimberly N. Williams, Anna L. Palmer, Katharine M. Cammack
The University of the South, Sewanee, Tennessee 37383

Vulnerability to maladaptive patterns of alcohol use, including dependence and relapse, is influenced by a combination of biological and environmental factors. A better understanding of how individual factors influence alcohol use is needed to help reduce alcohol dependence and relapse rates in the general population. This study explored how environmental enrichment (EE), stress and estrus cycle stage affect ethanol (ETOH) preference in female mice. Mice were housed in enriched or standard environments and exposed chronically to ETOH for two hours a day for twelve days, before entering a brief ETOH-free abstinence period. At the end of this abstinence period, mice were exposed to a series of mild stressors (forced swim tests) and anxiety was assessed via an elevated plus-maze. Preference was measured using a two-bottle choice test prior to ETOH exposure (baseline), after chronic ETOH exposure, and immediately following the abstinence period and stressor. Results revealed that mice preferred ETOH more strongly after chronic ETOH exposure, but that this increase was not affected by environment. ETOH preference was further increased after a brief abstinence period, but preference was not affected by environment or mild stress. However, mice in the proestrus/estrus stage of the estrus cycle preferred ETOH more strongly after a brief abstinence period than did mice in the metestrus/diestrus stage, suggesting that circulating levels of gonadal hormones may contribute to the incubation of drug preference. Anxiety- and despair-like behaviors were not impacted by estrus cycle stage. These findings suggest that estrus stage may affect ETOH preference, even after relatively short drug-free periods. Further research is needed to rectify the role of EE and stress in individual vulnerability or resilience to substance abuse. These findings also highlight a need for increased research into how gonadal hormones may influence ETOH preference in both mice and humans.

Abbreviations: abstETOH, time point after abstinence period; AUD, alcohol use disorder; EE, environmental enrichment; EPM, elevated plus maze; ETOH, ethanol; FST, forced swim test; M/D, metestrus/diestrus; P/E, proestrus/estrus; postETOH, time point post chronic ethanol exposure period; SE, standard environment.

Keywords: abstinence; alcohol use disorder; dependence; environmental enrichment; estrus cycle; incentive salience; social isolation; stress

---

Introduction

Alcohol use disorder (AUD) affects nearly 30% of US adults at some point over the course of their lives (Grant et al., 2015). AUD is characterized by compulsive alcohol drinking, loss of control over the amount consumed, and a negative affective state during abstinence, with most people with AUD experiencing significant distress and impairment in normal functioning as a result of their drinking behavior (National Institute on Alcohol Abuse and Alcoholism, 2017). In 2015, over 15 million adults were estimated to have AUD (National Institute on Alcohol Abuse and Alcoholism, 2017).

Globally, AUD increases risk for a number of physical and psychiatric issues and can be associated with serious social and economic costs (Rehm et al., 2009; Rehm, 2011; Grant et al., 2015). However, less than 10% of people with AUD seek treatment (National Institute on Alcohol Abuse and Alcoholism, 2017) and relapse rates for alcohol-dependent individuals who have sought treatment range from 40-80%, depending on the time of follow-up (Dawson et al., 2007). As those with AUD might lack access to treatment and/or be unaware of their disorder, it is increasingly important to identify factors that may contribute to an individual's susceptibility to developing AUD and the likelihood of relapse to improve clinical outcomes and reduce distress or impairment in normal daily functioning.

Many biological, psychological and environmental factors are known to influence vulnerability to drug abuse, dependence and relapse. In humans, environmental factors can include a wide range of pre- and postnatal experiences, including social relationships, socioeconomic status, and stress (for review, see Solinas et al., 2010). However, due to the high degree of variability (e.g., history and pattern of alcohol use) and unique ethical considerations inherent to human studies (e.g., Pratt & Davidson, 2005), it can be difficult to disentangle how various environmental factors affect the motivational/incentive value attributed to drugs of abuse, such as alcohol (Berridge & Robinson, 2016), at different stages of disordered use. Given the complexities of AUD, controlled research in experimental settings can be used to shed light on specific behavioral and neurobiological factors contributing to the trajectory of AUD.

Rodent models of alcohol drinking can provide important insight into the role that various biological and environmental factors play in alcohol preference and addiction-like behavior (Spanagel, 2017). While social aspects of drinking cannot be easily replicated in rodents, mice and humans share homologous neurological systems that allow researchers to investigate short- and long-term neuroadaptations in the rodent brain that arise in response to ethanol (ETOH) exposure. Rodents have similar brain circuitry involved in reward and reinforcement, such as the mesolimbic dopamine (DA) system (Berridge & Robinson, 2016), and executive function and decision-making circuits (e.g., Fuster & Bressler, 2015). Further, ETOH's primary pharmacological targets, GABAergic and glutamatergic systems (e.g., Vengeliene et al., 2008), are each present and fairly homologous across mammalian species. Some mice strains, such as the one used in the present study (C57BL/6J), voluntarily and readily drink ETOH (Belknap et al., 1993), increasing the face, criterion and even predictive validity of animal models (see Spanagel, 2017). While acute ETOH exposure is typically defined as a single and/or one-day exposure, no universal definition for chronic ETOH exposure exists. Instead, the frequency and length of chronic ETOH exposure can vary based on the experimental design, which is often driven, in part, by a consideration of drug pharmacokinetics and pharmacodynamics. It is worth emphasizing that chronic ETOH exposure does not necessitate physical dependence, the latter of which is marked by physical withdrawal in the absence of ETOH. With AUD, overt physical dependence may not be present, suggesting that relevant animal models of AUD incorporate repeated ETOH exposure over time.

A growing body of research in rodent models suggests that environmental enrichment (EE), typically containing a combination of complex inanimate and social stimuli (Sztainberg & Chen, 2010; Slater & Chao 2015), has a number of positive effects on brain and behavior (e.g., Nithianantharajah & Hannan, 2006; Olson et al., 2006; Hendershott et al., 2016), including a reduction in drug- and ETOH-seeking and intake (Alexander et al., 1978; Lopez et al., 2011; Li et al., 2015; Marianno et al., 2017) and ETOH-induced behavioral responses (Rueda et al., 2012). Specifically, Li and colleagues (2015) demonstrated that a rodent environment enriched with a large social group, novel toys, and exercise wheels reduced reinstatement of ETOH place preference in ETOH-exposed mice. However, it remains unclear which component(s) of the EE drove these findings. Lopez and colleagues (2015) housed adolescent mice in an enriched or standard environment but in social isolation, and found that mice

housed in the enriched environment consumed less ETOH later in life than did the standard-housed controls. It is unknown whether EE without social interaction also protects against ETOH drinking in adult mice. It is thought that EE may protect against drug-seeking because it (a) provides alternative stimuli with reward value and/or incentive salience (i.e., desirable stimuli that elicit motivation/effortful behavior; Salamone & Correa, 2012; Berridge, 2012) that consequently reduce the perceived reward value of the addictive substance (e.g., Alexander et al., 1978), which is consistent with the reward comparison hypothesis (Grigson, 2000), or (b) counteracts the negative effects of stress (Solinas et al., 2010), which is a major factor contributing to drug abuse and addiction (Koob, 2008).

Clinical evidence in humans suggests that stress contributes to motivation for alcohol and can thus drive maladaptive patterns of drinking behavior (Blaine & Sinha, 2017). Stress similarly affects ETOH consumption in rodents (Becker et al., 2012; Spanagel et al., 2014; Noori et al., 2014). Meta-analyses suggest that, in general, stress increases ETOH consumption in free-choice homecage drinking conditions (versus self-administration studies) and when tested after a few days of abstinence (Spanagel et al., 2014; Noori et al., 2014). However, the type and severity of the stressor may impact rodents' drinking behavior. The forced-swim test (FST) is a commonly used means of inducing stress in rodents (Can et al., 2012) and has been shown to increase ETOH intake and reinstate ETOH consumption in dependent rodents (Spanagel et al., 2014; Noori et al., 2014). However, in other studies, mice increased their ETOH consumption several days after exposure to predator odor, but not after other standard stressors, such as foot shock, tail pinch, tail suspension, and restraint stress (Cozzoli et al., 2014). These inconsistencies may be due, in part, to differences in experimental design (Crabbe, Wahlsten, & Dudek, 1999) and/or degree of activation of the hypothalamus-pituitary-adrenal (HPA) axis (Spanagel et al., 2014).

Finally, female rodents are relatively understudied in biomedical and neuroscience research (Beery & Zucker, 2011), including addiction biology (Becker et al., 2012; Sanchis-Segura & Becker, 2016; Becker & Koob, 2016). Female mice have a four-day estrus cycle consistent of proestrus, estrus, metestrus, and diestrus stages (Butcher, Collins, & Fugo, 1974). Each stage is marked by distinct levels of gonadal hormones, similar to the primate (human) menstrual cycle (Butcher et al., 1974). In female rats and mice, levels of ovarian hormones, such as estradiol and progesterone, alter the function of the mesolimbic dopamine system (Zhang et al., 2008; Becker et al., 2012; Calipari et al., 2017), a critical brain circuit that responds to motivationally significant stimuli (Salamone et al., 2015; Berridge & Robinson, 2016). Specifically, higher levels of estradiol are thought to enhance females' response to drugs of abuse, including alcohol (Carroll et al., 2004; Becker et al., 2012). Even across the cyclic fluctuations of the rodent reproductive cycle (i.e., estrus cycle; McLean et al., 2012), mesolimbic DA neurons fire more strongly and in different patterns at baseline when estradiol is high (estrus) versus low (diestrus/metestrus). These hormone-related differences are enhanced in the presence of drugs and drug-paired cues (Zhang et al., 2008; Calipari et al., 2017). Estrus-dependent changes in ETOH-induced DA release have been identified in the medial prefrontal cortex of rats (Dazzi et al., 2007). While these mesocortical DAergic neurons also originate in the ventral tegmental area (VTA), it is likely that mesolimbic DA responses to ETOH also change across the estrus cycle. It is also possible that, via these pathways, cycling gonadal hormones interact with stressors and/or environmental conditions to influence ETOH preference.

The purpose of the present study was to determine how EE in the absence of social interaction impacts ETOH preference in adult female mice with a history of chronic intermittent ETOH exposure. The study also investigates the effect of acute FST stress on ETOH preference following a brief ETOH abstinence period in a model of relapse-like behavior. Estrus stage was assessed after ETOH preference tests to identify potential effects of estrus status on ETOH preference. It was hypothesized that ETOH preference would be stronger in (a) ETOH-exposed mice housed in a standard environment compared to mice housed in an enriched environment, (b) mice exposed to

a series of acute stressors (FSTs) versus those that remained unstressed, (c) stressed mice housed in standard environments compared to stressed mice housed in enriched environments, and (d) mice with high levels of circulating gonadal hormones (proestrus/estrus) versus low levels of gonadal hormones (diestrus/metestrus).

Material and Methods

Animals

Female C57BL/6J mice (n= 26), eight weeks old and 18-20 g upon arrival (Jackson Laboratories, Bar Harbor, ME), were housed in standard cages (10.5 x 6 x 6 in.) with standard bedding (Aspen Shavings, Northeastern Products Corp), chow (Mouse Diet 5015, Lab Diet) and tap water ad libitum. C57BL/GJ mice readily consume larger quantities of alcohol relative to other genetic lines and are extensively used as a model in addiction literature, making it an ideal strain for the present study (Belknap et al., 1993; Rhodes et al., 2005). Mice were group-housed (3-4/cage) for two weeks while they acclimated to the colony, after which they were transferred to individual cages containing standard or enriched housing conditions (details below). The colony was maintained on a 12 h light-dark cycle throughout the experiment. All procedures were approved by the Institutional Animal Care and Use Committee at The University of the South.

Experimental Schedule

Mice were transferred to their respective housing environments four days prior to the start of the ETOH exposure. A series of ETOH preference tests were administered before and after mice were exposed chronically to ETOH. Once ETOH was discontinued, abstinent mice were exposed to a series of mild stressors to mimic conditions that might lead to relapse. Estrus samples were collected twice after key behavioral tests (Fig.1).

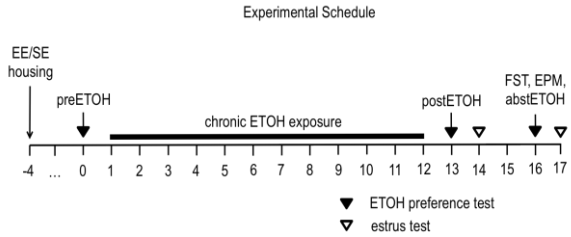

Figure 1. Timeline of experimental procedures. Mice were assigned to different housing conditions, given a preETOH test (closed arrowhead), chronically exposed to ETOH (black line), given a postETOH test, and underwent FST and EPM assays and the astETOH test after a brief abstinence period. Estrus stage was tested at two time points (open arrowhead).

Housing

Mice were assigned pseudorandomly to individual cages containing environmental enrichment (EE) or standard enrichment (SE), so that equal numbers of mice (*n*= 13) were in each condition (EE=experimental group, SE=control group). Pseudorandom assignment consisted of mice first being randomly divided into groups of four, then subsequently divided into groups of two which were then randomly assigned to a housing condition. All mice were housed individually (social isolation) in both EE and SE cages. EE cages were designed to provide enhanced sensory, cognitive, and motor stimulation to mice, shown previously to cause neuroadaptive changes (Sztainberg & Chen, 2010; Nithianantharajah & Hannan, 2006). Each EE cage was large (17 x 8.5 x 8.5 in.) and contained a running wheel with attached dome-house, a scoop of cedar-scented bedding, soft nest material, a padded bolster that allowed access to water/ETOH bottles and provided a hiding space, and three objects (toilet paper roll, plastic cone, colored plastic disc, plastic conical tube, wooden block, or wooden chew stick). EE-housed mice were also given a spoonful of novel food each day, in addition to standard chow available *ad libitum.* SE cages were smaller (10.5 x 6 x 6 in.) and contained standard bedding, chow and water. All mice were housed individually, so that ETOH preference and ETOH consumption could be assessed accurately for each mouse (details below). Mice were transferred into EE and SE housing four days prior to the initial ETOH preference test to allow sufficient time to acclimate to the new

cage (Conour et al., 2006). The mice remained in their respective housing conditions for the remainder of the experiment.

Ethanol Preference Test

ETOH preference was assessed using a two-bottle choice procedure (e.g., Lopez et al., 2011). Briefly, water bottles were removed from cages two hours prior to each preference test to promote consumption. The preference test started 30-min prior to the start of the dark cycle (1800h), consistent with the timing of chronic ETOH exposure in the DiD (drinking-in-the-dark) procedure. Water bottles containing tap water and a 15% (v/v) ETOH solution diluted with tap water were inserted into each mouse's cage. Mice had access to both bottles for two hours. Bottles were weighed on a scale and the volume of liquid in each bottle was recorded to the closest milliliter (ml) before/after the test to reflect consumption. ETOH preference was assessed one day prior to chronic ETOH exposure (preETOH), one day after chronic ETOH exposure was discontinued (postETOH), and after a brief ETOH abstinence and exposure to a stressor (abstETOH).

Chronic Ethanol Exposure

Mice were exposed chronically to ETOH for two hours/day for 12 days in their home cage, using a limited-access drinking procedure (DiD) modified from previous reports (Rhodes et al., 2005). Thirty minutes before the start of the dark cycle (1800 h), water bottles were removed from each cage and replaced by a bottle containing a 15% (v/v) ETOH solution diluted with tap water. This ETOH concentration is readily consumed by C57BL/6J mice over short time periods (Lopez & Laber, 2015). The timing of ETOH exposure coincides with enhanced feeding/drinking in mice at the beginning of their active phase (Crabbe et al., 2009). ETOH bottles were weighed intermittently throughout the experiment, before/after the daily DiD exposures to confirm consumption. After twelve days, chronic ETOH exposure was discontinued and mice began a three-day ETOH abstinence period.

Forced-Swim Test (FST)

Mice were assigned pseudorandomly to stressed (SS) or no-stress (NS) conditions, so that approximately equal numbers of mice from each housing condition were in each stress condition (EE/SS n= 6, EE/NS n= 7, SE/SS, n= 7, SE/NS n= 6). The stressed condition consisted of a series of forced-swim tests (FSTs), based on existing protocols (Can et al., 2012). Briefly, mice are placed in a beaker of water and are forced to swim for several minutes. FSTs have been considered inescapable psychologicla stressors and increase HPA activity in rodents (Gesing et al., 2001; Sano et al., 2009), suggesting that the FST is a valid method for inducing stress. During each FST, mice were placed individually into glass beakers of room-temperature tap water, filled to a depth where their tails could not touch the bottom of the beaker. Each five-minute test was video-recorded for later analysis of despair-like behaviors. The presence of freezing and/or immobility reflect the mice's lack of escape attempts (Can et al., 2012) and have been argued to reflect either despair-like behavior (Porsolt et al., 1977) or an adaptive stress-coping strategy (Commons et al., 2017). The FSTs were performed a total of three times on a single day and were separated by at least an hour.

Elevated Plus Maze (EPM)

To determine if stress impacted anxiety, mice were transferred to a quiet testing room for an elevated plus maze (EPM) test. Each mouse was placed on the end of the open arm furthest from the room door and allowed to explore the maze for five minutes. Time spent in open and closed arms were recorded via stopwatch, in accordance to prior reports (Walf & Frye 2007; Komada et al., 2008). In these tests, higher percentages of time spent in the closed arms of the maze relative to the open arms of the maze reflect higher levels of anxiety, while more time spent in the open arms of the maze is indicative of lower anxiety (Walf & Frye 2007). Entrance into any maze arm was defined as both of the mouse's front paws on the arm. The maze was cleaned with 70% ETOH and allowed to dry between uses.

Estrus Testing

Estrus stage was identified via vaginal lavage and standard cytological procedures (McLean et al., 2012). Vaginal lavage was performed ~12 h after the postETOH and abstETOH preference tests. Samples were placed on glass slides, allowed to air-dry, and stained with 0.1% crystal violet. Cycle stage was identified via light microscopy with respect to existing literature (McLean et al., 2012).

Data Analysis

Statistical analyses were performed using SPSS. Two-tailed t-tests were used unless otherwise specified.

The volume of liquid consumed was measured before/after each ETOH preference test and select days of chronic ETOH exposure. These volumes were adjusted for drips lost from the bottles prior to analysis. Briefly, each mouse had its own dedicated bottles for ETOH or water. To mimic the process of introducing/removing the bottles during these tests, each bottle was inserted into/removed from a cage top over an empty cage, and drips from each bottle were collected in a beaker and measured to the closest ml. This assay was performed two to three times per bottle and the amount lost was averaged. This amount was subtracted from the difference in volume measured before and after each test to reflect the volume consumed by each mouse.

A preference score, calculated by dividing the amount of ETOH consumed (in ml) by the total amount of ETOH and water consumed during the two-bottle preference test (using adjusted volumes, as described above), was used to reflect ETOH preference. The impact of housing on ETOH preference measured before and after chronic ETOH exposure was assessed using a two-way ANOVA. The impact of housing and stress on ETOH preference measured before and after the brief abstinence period was measured using a three-way ANOVA.

Anxiety-like behavior was defined in two ways: time (s) spent in the open arms and difference in time (s) spent in the open arms versus closed arms. These measures were relatively consistent with each other, so time spent in the open arms was used for analyses. The impact of housing and stress on open-arm time was assessed using a two-way ANOVA.

Despair-like behavior was assessed through recording the total time spent immobile (floating or freezing), total number of freezes (defined as a float/freeze lasting at least 3 s), and latency to first freeze during each FST. The difference in time spent immobile between the first and last FST were calculated for each measure to assess behavioral changes over time. Data from mice in proestrus or estrus (P/E) were compared to data from mice in diestrus or metestrus (D/M), consistent with existing literature (e.g., Calipari et al., 2017). Independent t-tests were used to compare ETOH preference scores, EPM and FST data, measured after the brief abstinence period, between P/E and D/M estrus groups. As different mice were in different estrus categories, they had to be treated as independent groups and thus repeated-measures analyses could not be used. Given existing literature on the effect of higher estrogen levels on drug reward and preference (e.g., Zhang et al., 2008; Calipari et al., 2017), these t-tests were one-tailed. Comparisons within each estrus group across timepoints (e.g., postETOH and abstETOH) were not performed because each group contained some (but not all) of the same animals. Chi-square goodness-of-fit tests were conducted to evaluate whether housing or stress impacted estrus stage.

Results

Ethanol preference increased after chronic ethanol exposure but was unaffected by housing

To assess whether housing impacted the incentive value of ETOH, ETOH preference was compared between mice living in enriched and standard environments, both before and after chronic ETOH exposure. There was a significant main effect of preference test [$F(1,24)=52.068$, $p<0.000$], indicating that mice preferred ETOH more strongly after chronic exposure (postETOH: $M=0.59$, $SD=0.15$) than they did prior to exposure (preETOH: $M=0.25$, $SD=0.21$) (Fig.2). There was no interaction

between housing and ETOH preference [$F(1,24)=0.783$, $p=0.385$], suggesting that preference increased similarly in EE and SE mice. These findings suggest that 12 consecutive days of chronic (two hours/day) ETOH exposure was sufficient to increase ETOH preference and that the increased preference did not depend on housing condition.

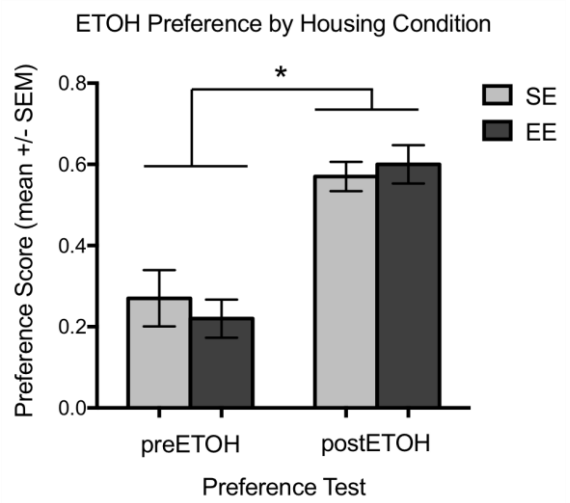

Figure 2. Ethanol preference increased after chronic ETOH exposure, regardless of housing. SE, standard environment, n=13; EE, enriched environment, n=13; preETOH and postETOH, ETOH preference test before and after chronic ETOH, respectively. * denotes $p<0.05$

Ethanol preference increased after a brief ethanol abstinence period, but was unaffected by housing or mild stress

To assess the impact of stress and/or housing on relapse-like behavior, ETOH preference was compared across a brief, three day abstinence period with respect to housing and stress conditions. There was a significant main effect of preference test [$F(1,22)=43.712$, $p<0.000$], indicating that mice preferred ETOH more strongly after brief ETOH abstinence (abstETOH: $M=.8627$, $SD=0.15$) than right after chronic ETOH exposure (postETOH: $M=0.59$, $SD=0.15$) (Fig.3). There were no significant interactions between preference test and housing ($p=0.412$), preference test and stress ($p=0.458$), or preference test, housing and stress ($p=0.787$), suggesting that housing and stress conditions used in the present study did not impact ETOH preference after a brief abstinence period.

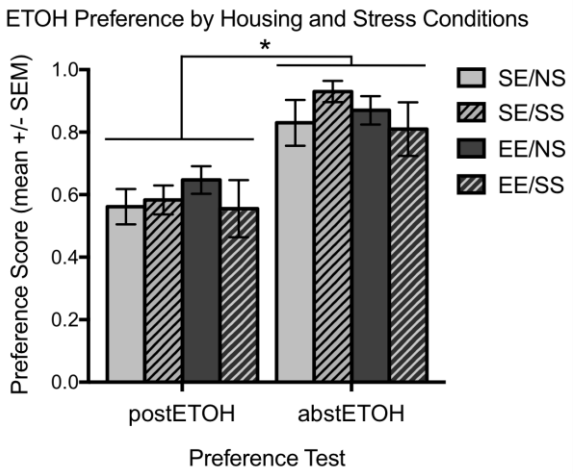

Figure 3. Ethanol preference increased after a brief abstinence period, regardless of either housing or stress condition. postETOH= preference test following chronic exposure, abstETOH= preference test following abstinence and stress period, SE=standard environment, EE=enriched environment, NS=not stressed, SS=swim stress. SE/NS n=6, SE/SS n=7, EE/NS n=7, EE/SS n=6. * denotes $p<0.05$

Anxiety after a brief ethanol abstinence period was unaffected by housing or mild stress

To assess the impact of stress and/or housing on anxiety-like behavior, time (s) spent in the open arms of the EPM after a brief, the three-day abstinence period was compared with respect to housing and stress conditions. There were no main effects of housing ($p=0.192$) or stress ($p=0.962$), or an interaction between the factors ($p=0.293$), suggesting that neither stress nor housing affected anxiety-like behavior.

The strength of ethanol preference after a brief abstinence period was affected by estrus stage

A few samples for postETOH ($n=1$) and abstETOH ($n=3$) were insufficient and could not be analyzed. Of the remaining females, most (59.1%) were identified as proestrus/estrus (P/E) at both test points (postETOH and abstETOH), one female was identified as metestrus/diestrus (M/D) at both test points, and the remainder (36.4%) were identified as P/E during one test and M/D during the other. These latter data suggested

that at least some of the females were still cycling actively.

To determine if estrus cycle stage increased the incentive value of ETOH, ETOH preference scores for P/E mice were compared to M/D mice. ETOH preference scores calculated after chronic ETOH exposure (postETOH) were similar in P/E mice (*M*=0.59, *SD*=0.16, *n*=20) and M/D mice (*M*=0.54, *SD*=0.09, *n*=5). However, ETOH preference scores calculated after a brief abstinence period (abstETOH) differed significantly between P/E mice (*M*=0.92, *SD*=0.12, *n*=17) and M/D mice (*M*=0.77, *SD*=0.14, *n*=6) [$t(21)= 2.014$; $p<0.05$] (Fig.4). These findings suggest that elevated levels of estrogen and progesterone may increase the incentive value of ETOH across even brief abstinence periods.

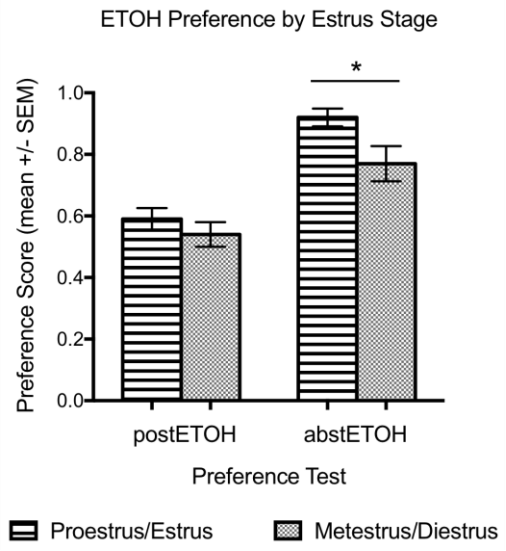

Figure 4. Ethanol preference by estrus cycle stage before and after the brief abstinence period. There was a significant difference in ETOH preference after the brief abstinence between mice in proestrus/estrus (n=17) and metestrus/diestrus (n=6) stages of the estrus cycle. * denotes p<0.05

Affective behaviors did not change as a result of estrus status

To identify if estrus cycle stage altered affective behaviors, both elevated plus-maze (EPM) and forced-swim test (FST) data were compared between P/E and M/D mice. Estrus stage was identified about twelve hours after the abstETOH test. There was no difference in the time spent in the open arms of the EPM expressed by mice in P/E (*M*=47.9, *SD*=25.3, *n*=17) versus M/D (*M*=52.37, *SD*=31.2, *n*=6), suggesting that estrus cycle stage did not impact anxiety-like behaviors in these mice. There was only one mouse assigned to the stress condition (and thus with FST data) with a sufficient sample for estrus identification, so FST data could not be analyzed with respect to estrus status.

Discussion

The present results suggest that ETOH preference increased following chronic ETOH exposure and again after a brief abstinence period, but this increase was not affected by housing condition or a series of acute mild stressors. Estrus cycle stage affected the strength of ETOH preference after abstinence, with females in P/E expressing stronger preference than those in metestrus/diestrus.

These data do not support the initial hypothesis that mice housed in an enriched environment would be protected against the escalation of drinking behavior or relapse-like responses. This finding contrasts with those of Li et al. (2015), who found that C57BL/6 mice housed in an enriched environment showed less reinstatement of ETOH place preference compared to mice in standard housing, suggesting that an enriched environment may reduce the incentive value attributed to ETOH. However, it is important to note that conditioned place preference (CPP; used by Li et al., 2015) and two-bottle choice preference (used here) are not equivalent, as CPP utilizes experimenter-administered ETOH during conditioning and the two-bottle choice procedure is more equivalent to a self-administration procedure. Further, the EE protocol used in Li et al. (2015) included social housing with conspecifics, unlike the social isolation used in the current study, which was necessary to accurately gauge the volumes drank in the ETOH preference tests. It is plausible that EE must include social interaction to assert a protective effect against relapse-like behaviors in adult mice.

Additional considerations regarding the EE used in the current study may explain

discrepancies with the literature. Mice in the SEs were housed in clear plastic cages, allowing them to see the colony room, whereas EE cages were opaque and did not permit as much visibility. While experimenters' presence in the room throughout the study could hypothetically provide a unique source of stimulation to mice in standard (clear) cages, mice's vision is very poor (Baker, 2013) and likely provided limited stimulation. Additionally, the toys present in enriched housing were not alternated/replaced during the study and it is possible that the enriching effect of these objects only lasted a few days. A follow up study could address the role of these variables in ETOH preference.

Interestingly, the series of acute mild stressors (FSTs) did not impact subsequent ETOH preference in ETOH-abstinent mice, regardless of housing condition. These findings did not support our second and third hypotheses. A possible explanation for these findings is that ETOH consumption was variable between individuals and thus obscured group differences in FST response. While correlations were assessed, a lack of power may have contributed to these results. Another possible explanation is that the FSTs were a weak or insufficient manipulation to induce stress. The FST was originally developed as a model of despair-like behavior, due to its sensitivity to antidepressant medications (Porsolt et al., 1977). The FST typically elicits two coping responses from the subject: active strategies (swimming, climbing) and passive strategies (floating, immobility). Conditions that increased the duration of active strategies and/or decreased the duration of passive strategies were considered to be anti-depressant in nature, and the FST has been used extensively to measure depression-like behavior in rodents. However, recent work has questioned the construct and face validity of this model (Commons et al., 2017) and argued that, instead of the inference of despair-like behavior, these behaviors instead more accurately represent strategies to cope with stress (de Kloet & Molendijk, 2016; Commons et al., 2017).

While a summary of this rationale is beyond the scope of this manuscript, it is worth noting two important FST-induced changes to the hypothalamic-pituitary-adrenal (HPA) axis, which activates in response to physically or psychologically stressful events. First, plasma levels of corticosterone (CORT), a glucocorticoid released by the adrenal glands in response to stressful events, was elevated by a 15min FST in mice (Sano et al., 2009); elevations in CORT are also seen in response to standard stressors (e.g., footshock; Cozzoli et al., 2014). Second, a 15min FST increased levels of mineralocorticoid receptors in the hippocampus when measured 8-24hrs later (Gesing et al., 2001); these receptors are one of the primary molecular targets of CORT. It is possible that three, 5min FSTs did not affect HPA activity as strongly as one 15min FST; however, it is also possible that the mice found multiple returns to the FST more stressful. Due to experimental scope and logistical considerations, physiological measures could not be used in the present experiment, but further studies could use physiological outcomes to inform behavioral data. Regardless, the lack of FST-induced stress effect on ETOH preference was intriguing and warrants further study.

The impact of stress depends not only on the type of stressor but also the time elapsed between the delivery of the stressor and assessment of ETOH preference. It is possible that longer ETOH abstinence periods may have produced stronger ETOH preferences in the stressed mice, consistent with existing literature (e.g., Cozzoli et al., 2014). Finally, all mice were housed in the same colony room, regardless of stress condition, and it is possible that ultrasonic vocalizations made by stressed mice between FSTs may have caused distress to the unstressed mice, though the degree to which this could have affected subsequent ETOH preference is unknown.

While others have identified sex- and estrus-cycle-dependent differences in a number of FST metrics, including immobility, swimming and climbing durations (Bogdanova et al., 2013; Kokras et al., 2015), no estrus-dependent differences emerged in the present study, likely due to a lack of power. EE and social isolation have also had inconsistent effects on FST performance (Bogdanova et al., 2013), though most of these studies were done in rats. It is also worth noting that unconditioned anxiety levels, measured using

the elevated plus maze (EPM), did not differ between stressed and non-stressed groups, suggesting that the FSTs did not elevate anxiety consistent with previous work that stress and anxiety are separable (Andreatini & Bacellar, 1999).

The strength of ETOH preference following abstinence did differ as a result of estrus stage. Females in proestrus/estrus showed stronger preference for ETOH (versus water) than those in metestrus/diestrus. Females are more sensitive to drugs of abuse compared to males and ovariectomized females, and when estradiol levels in intact females are high versus low (Carroll et al., 2004; Becker et al., 2012), though estrus cycle findings are not always consistent (e.g., Forger & Morin, 1982). It is important to note that the present study did not find an effect of estrus cycle stage on affective behaviors (FST or EPM), suggesting that estrus-related differences in ETOH preference are specific to circuits involved in motivation/incentive salience, rather than emotion/affect.

The mesolimbic dopamine (DA) system helps attribute incentive value to a variety of evolutionarily important stimuli, such as food and offspring, with rewarding and/or reinforcing properties (Salamone et al., 2015; Berridge & Robinson, 2016). In female rats and mice, higher levels of ovarian hormones, particularly estradiol, affect the firing rate of mesolimbic dopamine (DA) neurons and amount of synaptic DA (Becker, 1999; Zhang et al., 2008; Becker et al., 2012; Calipari et al., 2017). Because of this, estradiol is thought to enhance the incentive salience attributed to drugs of abuse and, via Pavlovian association, their predictive cues (Carroll et al., 2004; Becker et al., 2012). For instance, DA levels in the nucleus accumbens in response to entering a cocaine-paired chamber were higher in females during estrus than diestrus, a difference thought to be due, in part, to the function of DA reuptake transporters on the presynaptic membrane of mesolimbic DA terminals (Calipari et al., 2017). It is possible that the elevated estradiol levels in proestrus/estrus females enhanced the incentive value ("drive") attributed to ETOH and thus increased their preference score in the two-bottle choice test. Further, drugs of abuse can act synergistically with estradiol to modulate DA firing (e.g., Zhang et al., 2008). It is possible that ETOH, by modulating multiple molecular targets (e.g., GABAergic and glutamatergic receptor function), may be altering DA function, either directly or indirectly, in unique ways across the estrus cycle.

Importantly, the enhanced ETOH preference was specific to the brief abstinence period, suggesting that estrus stage may influence the incubation of ETOH craving, which is an increase in drug-seeking with increasing time spent abstinent (Bienkowski et al., 2004). Indeed, reinstatement of cocaine preference following extended abstinence (craving incubation) is stronger in female rats during estrus relative to other phases of the cycle and compared to males (Feltenstein & See, 2007; Kerstetter et al., 2008; Pickens et al., 2011). As the incubation of drug craving can continue for weeks of drug abstinence (Pickens et al., 2011), it is possible that estrus-related differences would be more dramatic after a longer ETOH-free period.

Indirect evidence for this hypothesis comes from a study on mesocortical DA, a separate circuit arising from similar sets of neurons in the ventral tegmental area. Dazzi et al. (2007) demonstrated that there are estrus-related changes in ETOH-induced dopamine (DA) output of mesocortical neurons in rats. ETOH administration elevated prefrontal DA levels in females during estrus but not in proestrus or diestrus stages. Ovariectomy eliminated these changes and replacement of estrus-like hormones reinstated the ETOH-induced elevation of DA levels, leading the authors to conclude that ovarian hormones directly influenced the changes in DA output. By altering how ETOH impacts neurotransmitter release in the brain, estrus stage may influence ETOH preference.

The results of this study indicate that ETOH preference was enhanced by chronic ETOH exposure but not EE or exposure to a series of mild stressors. Estrus stage may impact ETOH preference after brief abstinence periods, suggesting that estrus-induced changes to mesolimbic DA may alter the severity and/or time course of incubation of ETOH craving. To our knowledge, this is the first study to explore

these questions in mice. Our findings highlight a continued need for research on the types of environmental factors that contribute to drug vulnerability and the role that gonadal hormones and reproductive cycle stage might play in ETOH preference, particularly after prolonged abstinence. For instance, given the positive effects of social and emotional support on health in general (Reblin & Uchino, 2008), it is plausible that including social interaction in this study's EE protocol could impact ETOH consumption and relapse-like behavior. Further studies could also test the molecular hypotheses set forth in this manuscript. Additional consideration should be given to the role of gonadal hormones in ETOH craving, particularly in relapse models of addiction, as findings may inform the efficacy of treatments for women with alcohol use disorder. Ultimately, a better understanding of how extended ETOH exposure can alter ongoing behaviors and cause behavioral changes that underly relapse-like behavior will inform potential directions for future neurobiological and physiological studies in animal models, as well as shed light on some of the complex neuroadaptations occurring in humans struggling with AUD.

Acknowledgements

The authors would like to acknowledge the students in PSYC359 (Spring 2017) at the University of the South for study design and data collection, and the laboratory TA for their help maintaining the rodent colony. The authors would also like to thank the Department of Psychology and the Center for Teaching at The University of the South for funding and support.

Corresponding Author

Katharine Cammack, Ph.D.
The University of the South
735 University Avenue, Sewanee, TN, 37383
kmcammac@sewanee.edu

References

Alexander BK, Coambs RB, Hadaway PF (1978) The effect of housing and gender on morphine self-administration in rats. Psychopharmacology, 58, 175-179.

Andreatini R, Bacellar LF (1999) The relationship between anxiety and depression in animal models: a study on the forced swimming test and elevated plus-maze. Braz J Med Biol Res 32(9):1121-1126

Baker M (2013) Neuroscience: Through the eyes of a mouse. Nature 502: 156-158

Becker JB (1999) Gender differences in dopaminergic function in striatum and nucleus accumbens. Pharmacol Biochem Behav 64(4): 803-812

Becker JB, Perry AN, Westenbroek C (2012) Sex differences in the neural mechanisms mediating addiction: A new synthesis and hypothesis. Biol Sex Differ 3(1):14

Becker JB, Koob GF (2016) Sex differences in animal models: Focus on addiction. Pharmacol Rev 68(2): 242-263

Beery AK, Zucker I (2011) Sex bias in neuroscience and biomedical research. Neurosci Biobehav Rev 35: 565-572.

Belknap JK, Crabbe JC,Young ER (1993) Voluntary consumption of ethanol in 15 inbred mouse strains. Psychopharmacology, 112 (4), 503-510

Berridge KC (2012) From prediction error to incentive salience: mesolimbic computation of reward motivation. European Journal of Neuroscience 35: 1124-1143.

Berridge KC, Robinson TE (2016) Liking, wanting, and the incentive-sensitization theory of addiction. American Psychologist, 71(8), 670-679.

Bienkowski P, Rogowski A, Korkosz A, Mierzejewski P, Radwanska K, Kaczmarek L, Bogucka-Bonikowska A, Kostowski W (2004) Time-dependent changes in alcohol-seeking behavior during abstinence. Eur Neuropsychopharm 14(5), 355-360.

Blaine SK, Sinha R (2017) Alcohol, stress, and glucocorticoids: From risk to dependence and relapse in alcohol use disorders. Neuropharmacology 122:136-147.

Butcher RL, Collins WE, Fugo NW (1974)

Plasma Concentration of LH, FSH, Prolactin, Progesterone, and Estradiol-17b Throughout the 4-Day Estrous Cycle of the Rat. Endocrinology 94 (6): 1704-1708.

Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF (2013) Factors influencing behavior in the forced swim test. Physiol Behav 118:227-239

Calipari ES, Juarez B, More C, Walker DM, Cahill ME, Ribeiro E, Roman-Ortiz C, Ramakrishnan C, Deisseroth K, Han MH, Nestler EJ (2017) Dopaminergic dynamics underlying sex-specific cocaine reward. Nat Comm 8: 13877.

Can A, Dao DT, Arad M, Terrillion CE, Piantadosi SC, Gould TD (2012) The mouse forced swim test. J Vis Exp. 59, e3638

Carroll ME, Lynch WJ, Roth ME, Morgan AD, Cosgrove KP (2004) Sex and estrogen influence drug abuse. Trends Pharmacol Sci 25(5): 273-279

Commons KG, Cholanians AB, Babb JA, Ehlinger DG (2017) The rodent forced swim test measures stress-coping strategy, not depression-like behavior. ACS Chem Neurosci 8(5): 955-960

Conour LA, Murray KA, Brown MJ (2006) Preparation of animals for research--Issues to consider for rodents and rabbits. ILAR J. 47 (4), 283-293.

Cozzoli DK, Tanchuck-Nipper MA, Kaufman MN, Horowitz CB, Finn DA (2014) Environmental stressors influence limited-access ethanol consumption by C55BL/6J mice in a sex-dependent manner. Alcohol. 48, 741-754.

Crabbe JC, Wahlsten D, Dudek BC (1999) Genetics of Mouse Behavior: Interactions with Laboratory Environment. Science Magazine 284: 1670-1672.

Crabbe JC, Metten P, Rhodes JS, Yu CH, Brown LL, Phillips TJ, Finn DA (2009) A line of mice selected for high blood ethanol concentrations shows drinking in the dark to intoxication. Biol Psychiatry 65:662–670.

Dawson DA, Goldstein RB, Grant BF (2007) Rates and Correlates of Relapse Among Individuals in Remission From DSM-IV Alcohol Dependence: A 3-Year Follow-Up. Alcoholism Clinical and Experimental Research. 31(12), 2036-2045.

Dazzi L, Seu E, Cherchi G, Barbieri PP, Matzeu A, Biggio G (2007) Estrous cycle-dependent changes in basal and ethanol-induced activity of cortical dopaminergic neurons in the rat. Neuropsychopharmacology, 32, 892-901.

de Kloet ER, Molendijk ML (2016) Coping with the forced swim stressor: Towards understanding an adaptive mechanism. Neural Plast 2016:6503162

Feltenstein MW, See RE (2007) Plasma progesterone levels and cocaine-seeking in freely cycling female rats across the estrous cycle. Drug and Alcohol Dependence. 89 (2-3), 183-189.

Forger NG, Morin LP (1982) Reproductive state modulates ethanol intake in rats: Effects of ovariectomy, ethanol concentration, estrous cycle, and pregnancy. Pharmacol Biochem Behav 17(2): 323-331

Fuster JM, Bressler SL (2015) Past makes future: role of pFC in prediction. J Cogn Neurosci 27(4): 639-654.

Gesing A, Bilang-Bleuel A, Droste SK, Linthorst AC, Holsboer F, Reul MJM (2001) Psychological stress increases hippocampal mineralocorticoid receptor levels: involvement of corticotropin-releasing hormone. J Neurosci 21(13): 4822.4829

Grant BF, Goldstein RB, Saha TD, et al. (2015) Epidemiology of DSM-5 alcohol use disorder: Results from the National Epidemiologic Survey on Alcohol and Related Conditions III. JAMA Psychiatry 72: 757–766

Grigson PS (2000) Drugs of abuse and reward comparison: A brief review. Appetite 35(1): 89-91.

Hendershott TR, Cronin ME, Langella S, McGuinness PS, Basu AC (2016) Effects of environmental enrichment on anxiety-like behaviors, sociability, sensory gating, and spatial learning in male and female C57BL/6J mice. Behavioral Brain Research. 314, 215-225.

Kerstetter KA, Aguilar VR, Parrish AB, Kippin TE (2008) Protracted time-dependent increases in cocaine-seeking behavior during cocaine withdrawal in female relative to male rats. Psychopharmacology, 198(1), 63-75.

Kokras N, Antoniou K, Mikail HG, Kafetzopoulos V, Papadopoulou-Daifoti Z, Dalla C (2015) Forced swim test: What about females? Neuropharmacology 99:408-421

Koob GF (2008) A role for brain stress systems in addiction. Neuron 59: 11-34.

Komada M, Takao K, Miyakawa T (2008) Elevated plus maze for mice. J Vis Exp. 22, e1088

Li X, Meng L, Huang K, Wang H, Li D (2015) Environmental enrichment blocks reinstatement of ethanol-induced conditioned place preference in mice. Neuroscience letters. 599, 92-96.

Lopez MF, Laber K (2015) Impact of social isolation and enriched environment during adolescence on voluntary ethanol intake and anxiety in C57BL/6J mice. Physiol Behav. 148, 151-6.

Lopez MF, Doremus-Fitzwater TL, Becker HC (2011) Chronic social isolation and chronic variable stress during early development induce later elevated ethanol intake in adult C57BL/6J mice. Alcohol. 45 (4), 355-364.

Marianno P, Abrahao KP, Camarini R (2017) Enrivonmental enrichment blunts ethanol consumption after restraint stress in C57BL/6 mice. PLoS ONE 12(1): e0170317.

McLean AC, Valenzuela N, Fai F, Bennett SAL (2012) Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp. 67, e4389

National Institute on Alcohol Abuse and Alcoholism (2017) Alcohol use disorder. Department of Health and Human Services: USA. Retrieved from https://www.niaaa.nih.gov/alcohol-health/overview-alcohol-consumption/alcohol-use-disorders

Nithianantharajah J, Hannan AJ (2006) Enriched environments, experience dependent plasticity and disorders of the nervous system. Nature Reviews Neuroscience. 7, 697-709.

Noori HR, Helinski S, Spanagel R (2014) Cluster and meta-analyses on factors influencing stress-induced alcohol drinking and relapse in rodents. Addict Biol 19, 225–232 20

Olson AK, Eadie BD, Ernst C, Christie BR (2006) Environmental enrichment and voluntary exercise massively increase neurogenesis in the adult hippocampus via dissociable pathways. Hippocampus 16: 250-260

Pratt WM, Davidson D (2005) Does participation in an alcohol administration study increase risk for excessive drinking? Alcohol 37(3): 135-141

Pickens CL, Airavaara M, Theberge F, Fanous S, Hope BT, Shaham Y (2011) Neurobiology of the incubation of drug craving. Trends in Neuroscience. 34(8), 411-420.

Porsolt RD, Le Pichon M, Jalfre M (1977) Depression: A new animal model sensitive to antidepressant treatments. Nature 266(5604): 730-732.

Reblin M, Uchino BN (2008) Social and Emotional Support and its Implication for Health. Current Opinion in Psychiatry 21 (2): 201-205.

Rehm J (2011) The risks associated with alcohol use and alcoholism. Alc Res Health 34(2): 135-143

Rehm J, Mathers C, Popova S, Thavorncharoensap M, Teerawattananon Y, Patra J (2009) Global burden of disease and injury and economic cost attributable to alcohol-use disorders. Lancet 373(9682): 2223-2233.

Rhodes JS, Best K, Belknap JK, Finn DA, Crabbe JC (2005) Evaluation of a simple model of ethanol drinking to intoxication in C57BL/6J mice. Physiol Behav. 84 (1): 53-63.

Rueda AVL, Teixeira AMA, Yonamine M, Camarini R (2012) Environmental enrichment blocks ethanol-induced locomotor sensitization and decreases BDNF levels in the prefrontal cortex in mice. Addiction Biology, 17, 736–745

Salamone JD, Correa M (2012) The Mysterious Motivational Functions of Mesolimbic Dopamine. Neuron Review 76: 470-485.

Salamone JD, Pardo M, Yohn SE, Lopez-Cruz L, SanMiguel N, Correa M (2015) Mesolimbic dopamine and the regulation of motivated behavior. Curr Top Behav Neurosci 27: 231-257.

Sanchis-Segura C, Becker JB (2016) Why we should consider sex (and study sex differences) in addiction research. Addict

Biol 21(5): 995-1006.

Sano K, Koushi E, Irie K, Higuchi S, Tsuchihashi R, Kinjo J, et al. (2009) Delta(9)-tetrahydrocannabinol enhances an increase of plasma corticosterone levels induced by forced swim-stress. Biol Pharm Bull 32(12): 2065-2067

Slater AM, Cao L (2015) A protocol for housing mice in an enriched environment. J Vis Exp. 100, e52874

Spanagel R (2017) Animal models of addiction. Dialogues Clin Neurosci 19(3): 247-258.

Spanagel R, Noori HR, Heilig M (2014) Stress and alcohol interactions: animal studies and clinical significance. Trends Neurosci 37(4): 219-227.

Solinas M, Thiriet N, Chauvet C, Jaber M (2010) Prevention and treatment of drug addiction by environmental enrichment. Progress in Neurobiology, 92, 572-592.

Sztainberg Y, Chen A (2010) An environmental enrichment model for mice. Nat Protoc, 5(9), 1535-1539.

Vengeliene V, Bilbao A, Molander A, Spanagel R (2008) Neuropharmacology of alcohol addiction. Br J Pharmacol, 154(2): 299-315.

Walf AA, Frye CA (2007) The use of the elevated plus maze as an assay of anxiety-induced behavior in rodents. Nat Protoc 2: 322-328.

Zhang D, Yang S, Yang C, Jin G, Zhen X (2008) Estrogen regulates responses of dopamine neurons in the ventral tegmental area to cocaine. Psychopharmacology 199: 625-635