Int J Physiol Pathophysiol Pharmacol 2019;11(4):163-176
www.ijppp.org /ISSN:1944-8171/IJPPP0100065

*Original Article*

# Differences between male and female rats in alcohol drinking, negative affects and neuronal activity after acute and prolonged abstinence

Jing Li, Pei Chen, Xiao Han, Wanhong Zuo, Qinghua Mei, Emily Yao Bian, Jennifer Umeugo, Jianghong Ye

*Department of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, Newark, NJ 07103, USA*

Received July 24, 2019; Accepted August 20, 2019; Epub August 25, 2019; Published August 30, 2019

**Abstract:** Alcohol consumption afflicts men and women differently. However, the underlying neuronal mechanisms that contribute to the difference are mostly unexplored. Although more men suffer from alcohol use disorders (AUD), women more frequently accelerate to dependence and develop adverse consequences of alcoholism sooner than men. Women also exhibit more significant negative emotions that cues more reactivity and alcohol-craving than men. Despite ample evidence that women are vulnerable to AUD, results of preclinical studies on sex differences in alcohol consumption and withdrawal-related behaviors are inconclusive. In this study, we trained adult male and female Sprague-Dawley rats to drink alcohol in the intermittent access to 20% ethanol two-bottle free-choice paradigm for two months. Their behaviors and Fos expression in related brain regions were measured at acute (24 h) and after prolonged (28 days) abstinence. We found that female rats drank more alcohol than males. After acute abstinence, rats of both sexes showed higher sensitivity to depressive, thermal, and mechanical stimuli. Females also displayed higher anxiety levels. After prolonged abstinence, rats of both sexes displayed depressive-like behaviors; the males displayed allodynia; the females showed higher anxiety levels and drank more alcohol upon reaccess to alcohol. Furthermore, during acute withdrawal, Fos-positive nuclei were increased in the prefrontal cortex, anterior cingulate cortex (ACC), nucleus accumbens (NAc), amygdala and lateral habenula (LHb) in the females, versus only in the ACC, amygdala, and LHb in the males. Conversely, after prolonged abstinence, Fos-positive nuclei were decreased in the prefrontal cortex, ACC, and NAc in the females, but fell in the ACC, NAc, and LHb of the males. Thus, adaptations in diverse brain regions may contribute to the sex differences in behaviors in ethanol-withdrawn rats.

**Keywords:** Ethanol, depression, anxiety, pain, Fos, female, Sprague-Dawley rats

## Introduction

Anxiety, depression, and hyperalgesia, which occur commonly during withdrawal from chronic, repeated and excessive alcohol consumption, have been considered crucial negative reinforcement factors leading to relapse [1-3]. Although more men suffer from alcohol use disorders (AUD), women more frequently accelerate to dependence and develop adverse consequences of alcoholism sooner than men [4-8]. Women also exhibit greater negative emotions such as anxiety, depression, and neuroticism [9-11]; they demonstrate greater stress that cues more reactivity and alcohol-craving than men. However, despite ample evidence that women are a population vulnerable to AUD, results of preclinical studies on sex differences in alcohol consumption and withdrawal-related behaviors are inconclusive. For example, in the two-bottle choice drinking paradigm [12-16], females of several strains of rodents consume more alcohol compared to their male counterparts; they also exhibit greater cue-induced and yohimbine-induced alcohol-seeking after chronic corticosterone exposure in adolescence [17]. Conversely, males had more severe physical signs of withdrawal, such as greater seizure susceptibility, hangover-like, and anxiety-like responses during acute removal from a liquid diet or intraperitoneal administration of ethanol [18, 19]. However, in some strains (e.g., P rats), the males drink more than the females [20]. Thus, significant gaps remain in our understanding of the role of sex on the effects of ethanol and its withdrawal/negative conse-

quences (e.g., anxiety, depression, and pain), particularly in animals withdrawn from the chronic intermittent-access, voluntary ethanol-drinking procedure, which mimics some aspects of the drinking pattern in human alcoholics [21].

Fos protein, an anatomical marker of neuronal activity [22, 23], has been used to identify brain regions that respond to experimental challenges [24-27]. By measuring Fos protein during acute ethanol withdrawal [28-30], several studies have revealed increased neuronal activity in multiple brain regions, most prominently in forebrain areas including many cortical regions, septum, accumbens, amygdala, paraventricular nucleus of the thalamus and hypothalamus, hippocampus, locus coeruleus, and lateral habenula (LHb). Emotional disturbances (anxiety, depression, and pain) can also stimulate specific brain regions such as the cerebral cortex (medial prefrontal cortex, ventrolateral orbital cortex, piriform cortex, and cingulate cortex), nucleus accumbens (NAc), amygdala, paraventricular hypothalamic nucleus (PV), and LHb [29, 31-39]. Overlapping patterns of Fos-induction in specific regions of the brain, activated by both ethanol withdrawal as well as the emotional-disturbance challenge, suggest that specific neuroanatomical sites in the brain are associated with the aversive effects observed during alcohol abstinence [40]. However, most such studies were conducted in male animals during acute ethanol withdrawal; hence, sex differences after acute and prolonged abstinence remain mostly unexplored.

To assess the role of sex in the withdrawal-related negative emotional behaviors and changes of neuronal activity in the brain, we examined the depressive- and anxiety-like behaviors, perceptive sensitivity, and Fos-expression in relevant brain regions in male and female Sprague-Dawley rats after acute (24 h) and prolonged (28 days) withdrawal from chronic, intermittent, voluntary alcohol drinking. We found significant differences between males and females in the related behaviors and brain regions in response to acute as well as prolonged withdrawal.

## Materials and methods

### *Animals*

The Animal Care and Utilization Committee of Rutgers, the State University of New Jersey, by National Institutes of Health guidelines, approved all procedures, minimizing the number of animals used and their suffering. All methods were performed following the relevant guidelines and regulations of NIH. Adult (2 months old) male and female Sprague-Dawley rats (Taconic Farms, NY) were housed individually in a climate-controlled room, following a 12 h light/dark schedule (lights off at 11:00 am), and given at least 1 week to acclimatize to the housing conditions and the handling prior to the actual experiments. Food and water were available *ad libitum*.

### *Intermittent access to 20% ethanol in two-bottle free choice (IA2BC) drinking procedure*

In the alcohol-drinking group, rats were first trained to drink ethanol for 12 weeks in the IA2BC schedule [30, 41-43]. Rats were given access to ethanol during three 24-hour sessions per week. On Monday, each rat was given access to one bottle of 20% (v/v) ethanol and one bottle of water. After 24 h, the ethanol bottle was replaced with a second water bottle that was available for the next 24 h. This pattern was repeated on Wednesdays and Fridays. The rats had unlimited access to two bottles of water over the weekend. The weight of each rat was measured on the Monday of each 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 (Pharmco, Brookfield, CT, USA). They were divided into two subgroups for behavioral tests: subgroup 1, at 24 hours of abstinence, n = 22/sex; subgroup 2, n = 14/sex, at 28 days of abstinence. After behavioral tests, eight rats/sex in subgroup 2 resumed alcohol drinking in the same IA2BC paradigm for other 2-weeks.

In the ethanol-naïve group (n = 14/sex, 8 for behavioral tests, 6 for immunohistology study), rats were age and sex-matched with the alcohol-drinking group; and had access to two bottles of water for 15 weeks. Behavioral tests and Fos study were conducted on week 16.

### *Blood ethanol concentration (BEC) measurements*

After drinking ethanol for 12 weeks, blood samples (approx. 0.2 ml) of both sexes (n = 8/sex) were collected from the lateral tail vein following 2 hours' access to 20% ethanol. The sam-

ples were centrifuged at room temperature (approximately 21-22°C) for 15 minutes at 8000 rpm, and 10 μl plasma from each blood sample was analyzed using the NAD-ADH enzyme spectrophotometric method [44].

*Forced swim test (FST)*

To measure general depressive-like behavior, we adopted a commonly used modified FST [42, 45, 46], with a transparent plastic tube (diameter = 24.5 cm, height = 51 cm) filled to 30 cm with water at 23-25°C. The pre-test was performed 24 h after ethanol withdrawal when the rat was placed individually into the FST apparatus for 15 minutes. The test was performed 24 h after the pre-test (i.e., at 48 h after ethanol withdrawal) when the rat was placed individually into the FST apparatus for 5 minutes, and its behaviors were recorded with video recorders. Immobility was defined as the rat floating in the water without struggling and only making movements necessary to keep its head above water.

*Sucrose preference test (SPT)*

The SPT was performed on a cohort of rats that had been drinking ethanol for 12 weeks in the IA2BC paradigm. Then these rats were habituated to two bottles of 1% sucrose of solution for 24 h before the 20 h fast and water deprivation. Following this, they were given one bottle of 1% sucrose solution and one bottle of water for 3 h. The preference ratio of 1% sucrose was calculated by the following formula: Preference ratio (%) = 1% sucrose solution intake (ml/3 h)/ total fluid intake (ml/3 h sucrose solution + ml/3 h water).

*Elevated plus maze test (EPM)*

Anxiety levels were evaluated with EPM [39, 47]. The maze consisted of four arms (two open without walls and two enclosed by 30 cm high walls) perpendicular to a central platform 50 cm above the floor, placed in an isolated, silent and dimly lit room (illumination of 100-140 lux). The time spent in the open arms, the number of entries into open arms and the total distance traveled were recorded using Smart 3.0 (Pan lab Harvard Apparatus, Barcelona, Spain).

*Open field test (OFT)*

The OFT apparatus consisted of chambers (TruScan Photobeam Activity Monitors, 16 × 16 × 16 inches, Coulbourn Instruments, Whitehall, PA) where each rat was gently placed in the center of the open field and allowed to explore for 60 minutes as its movements were recorded. The animal activities such as time spent in the center (14.4 × 14.4 cm), distance traveled, resting time, and vertical movement was tracked automatically by TruScan 2.0 software.

*Marbles burying test (MBT)*

Twenty 1.5 cm glass marbles were evenly spaced in five rows of four, on a 4-5 cm layer of corncob bedding material in a standard rat cage. After placing the rat in the test cage, the number of marbles buried in the bedding (to 2/3 their depth) after 30 min was counted [36, 47].

*Pain threshold test (PTT)*

An up-down testing paradigm was used to measure PTT in response to mechanical stimuli as described [48]. An up-down testing paradigm was used to measure paw withdrawal thresholds (PWT) in response to mechanical stimuli as described 1. Rats were placed in a Plexiglas chamber on an elevated mesh screen to acclimate the environment for 30 minutes. Then, a series of von Frey hairs (0.4, 0.6, 1, 1.4, 2, 4, 6, 8, 10, 15, and 26 g) were applied perpendicularly to the plantar surface of the hind paw for 3 s. In the current study, the 1-g von Frey hair was applied first. When rats suddenly withdrew the stimulated hind paw, it was considered as a positive response. Then the next smaller hair was applied; in the absence of a positive response, the next larger hair was applied. The test stopped when (1) 3 stimuli were applied after the first positive response, or (2) a negative response was obtained with the 26-g hair. According to the formula used in the previous studies, the paw withdrawal threshold was calculated by converting the pattern of positive and negative responses to the von Frey filament stimulation to a 50% threshold value.

Paw withdrawal latencies (PWL) to thermal stimuli were measured with an Analgesia Meter (Model 336, IITC Life Science Instruments, Woodland Hills, CA, USA). Briefly, each rat was placed in a Plexiglas chamber on a glass plate located above a light box. After 15-minute acclimation to the environment, these rats were subjected to radiant heat, which was applied by

aiming a light beam to the middle of the plantar surface of the hind paw. When the rat lifted its paw in response to the heat, the light beam was turned off. The PWL, measured in the unit of seconds, was defined as the length of time between the start of the light beam and the foot-lift. Each measure was repeated five times, allowing 5 minutes between successive tests to avoid sensitization to the stimulus. A cutoff time of 20 seconds was used to prevent paw tissue damage. The mean PWL of each rat was calculated by averaging the value of five tests.

*Estrous cycle phase identification*

To assess the influence of estrous cycle on ethanol intake, vaginal smears were collected to determine estrous cycle phases as described [49]. A cotton swab was moistened with sterile water and gently inserted approximately 4 mm into the vagina and slowly rotated clockwise to collect cell samples. The cells were transferred to slides, stained, and viewed under a light microscope. Four phases were used for categorization: 1. proestrus: almost exclusively clusters of round, well-formed, nucleated epithelial cells. 2. estrus: predominantly cornified squamous epithelial cells, present in densely packed clusters; 3. metestrus: predominantly small darkly-stained leukocytes; 4. diestrus: predominant leukocytes with rare cornified squamous epithelial cells which may still be present.

*Immunohistochemistry*

Naïve control rats (n = 6/sex) andalcohol-drinking rats from the 12-week IA2BC program were sacrificed for Fos immunostaining study at 24 hours withdrawal (acute, n = 6/sex) and at 28 days (prolonged, n = 6/sex) withdrawal. Rats were anesthetizeddeeply with sodium pentobarbital (50 mg/kg, i.p.), then transcardially perfused with saline followed by 4% paraformaldehyde (PFA). After post-fixation (overnight, at 4°C) in 4% PFA, cryoprotected tissue was sliced (at 30-μm) using a microtome (Microm HM550, Walldorf, German). Primary antibodies against c-Fos rabbit pAb (ABE457, 1:1,600, EMD Chemicals Inc. San Diego, CA) and biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA) were used. Two-dimensional counts of immunopositive cells from each image (0.1 $mm^2$ area) in each area were quantified using an image-analysis system comprising Nikon DS-Ri1 digital camera, Nikon Eclipse 80i microscope, and a computer with a NIS-Elements BR 3.0 software (Nikon, Melville, NY). An observer blinded to the treatment of the animals counted the number of positive nuclei in each slide.

*Data analysis*

Statistical analysis was performed using Sigma Plot (Systat Software Inc., San Jose, CA). Consumption data and BEC were subjected to Student's *t*-test. The consumption data during different estrous cycle phases were subjected to one-way analysis of variance (ANOVA). Behavioral and Fos data were statistically analyzed using two-way ANOVA with sex as a between-subjects factor and withdrawal as a within-subjects factor. The significant main effect was further analyzed using the *Tukey post hoc* test when appropriate. Statistical significance was declared at $P<0.05$.

## Results

*Sex differences in ethanol consumption of Sprague-Dawley rats in the IA2BC paradigm*

Alcohol drinking groups have access to 20% ethanol for 12 weeks (36 sessions). Average ethanol intake over 36 sessions (**Figure 1A**) indicated that female rats consumed significantly more alcohol than their male counterparts ($t$ = 9.39, $P$<0.0001), in keeping with a recent report [13]. Interestingly, females also drank significantly more water (in ml/kg) (**Table 1**). Therefore, there was no apparent sex difference in alcohol preference (**Table 1**).

Blood ethanol concentrations (BECs) measured immediately after 2 hours' access to ethanol were significantly higher in the females than in the males (**Figure 1B**, $t$=3.66, $P$=0.002). Average BEC in the females was 39.3±11.3 (from 8.5 to 80.00) mg/dl, with 71.4% (5/7) being above 20 mg/dl. Average BEC in the males was 13.1±2.5 (from 3.8 to 22.3) mg/dl, with only 25% (2/8) being above 20 mg/dl.

To determine how prolonged abstinence affects ethanol consumption, a 28-day deprivation period was introduced after a 12-week (36 sessions) drinking in the IA2BC paradigm. Following the abstinence period, rats resumed drinking in the same IA2BC schedule; during this period, we observed a significant effect of sex ($F_{1, 26}$=21.04, $P$<0.001) and abstinence ($F_{2, 26}$=

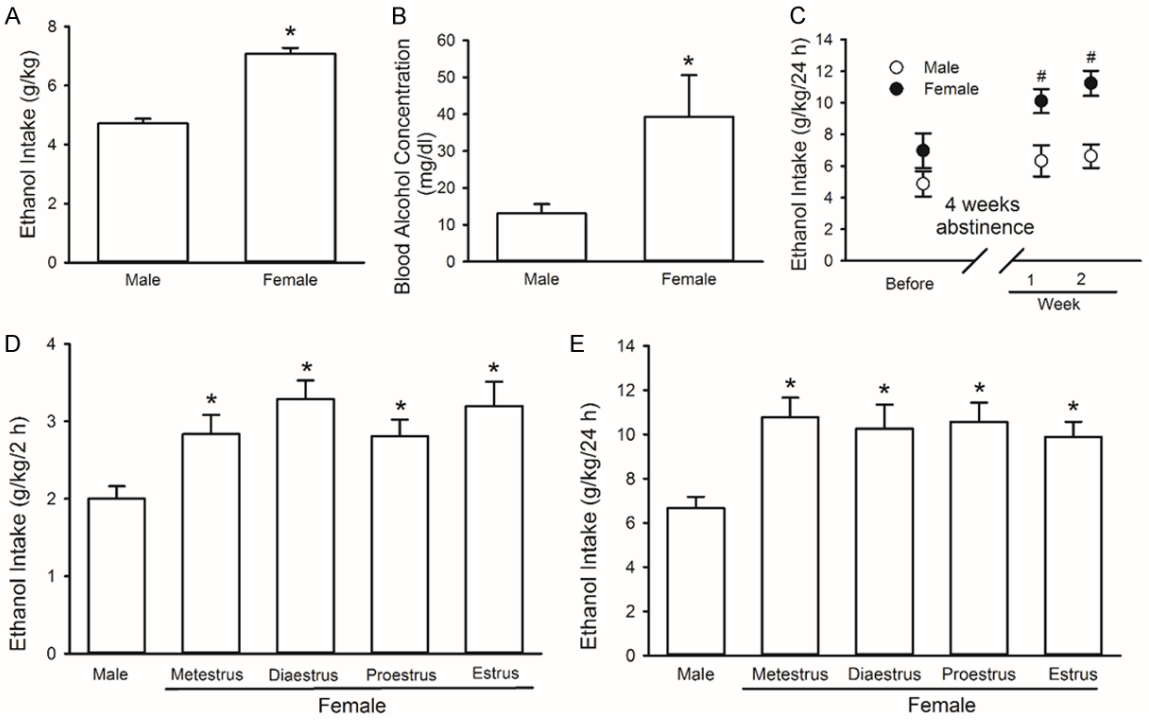


**Figure 1.** Ethanol intake of the male and female Sprague-Dawley rats in the IA2BC procedure. (A) The female rats drank significantly more ethanol than the male rats. (n = 12/sex, **P*<0.05, unpaired *t*-test. male *vs.* female). (B) Blood alcohol levels in the female rats were significantly higher than that in the male rats measured at two h after starting the ethanol drinking session. (n = 8/sex, **P*<0.05, unpaired *t*-test, male *vs.* female.). (C) Ethanol intake before and after abstinence when drinking resumed after the 28-day of abstinence, the female rats, but not the males, drank significantly more ethanol than before withdrawal. (n = 8/sex, two-way ANOVA, #*P*<0.05 *vs.* before withdrawal). Different menstrual cycles in female rats did not affect the results at 2 h (D) and 24 h (E) time points after starting ethanol drinking. The females in all menstrual cycles drank more than the males did. (n = 16 rats/estrus phase, n = 10 rats/metestrus, diestrus and proestrus phase; n = 36 rats/male. **P*<0.05 *vs.* males, one-way ANOVA).

**Table 1.** Water intake (ml/kg) and percent preference for ethanol

| Gender | Water intake (ml/kg) | Preference (%) |
|---|---|---|
| Male | 63.4 ± 1.7 | 32.6 ± 1.4 |
| Female | 87.8 ± 2.1* | 33.9 ± 1.1 |

Note: **P*<0.05 *vs.* males. n = 12 rats/sex, unpaired *t* test, male *vs.* female.

6.46, *P*=0.005) on ethanol consumption. The females, not the males, consumed significantly more alcohol than they did before abstinence (**Figure 1C**).

To determine whether hormonal fluctuations impacted alcohol consumption, we collected vaginal smears to monitor the estrous cycle for two weeks; their corresponding ethanol intake was also measured. The data of ethanol intake for each phase of the cycle were pooled. No significant difference was seen in ethanol consumption between various stages of the estrous cycle at both the two h and 24 h time points following access to ethanol (**Figure 1D**, **1E**). However, the females in all stages of the estrous cycle consumed significantly more ethanol than the males (**Figure 1D**, **1E**).

*Sex-differences in negative affective behaviors after abstinence*

*Anxiety-like behaviors:* Anxiety-like behavior was assessed after acute (24 h) and prolonged (28 days) abstinence, employing the elevated plus-maze test (EPM), open field test (OFT) and marbles buried test (MBT).

*EPM*

The alcohol-drinking females (AF) spent significantly shorter time and showed fewer entries in the open arms compared to water-drinking

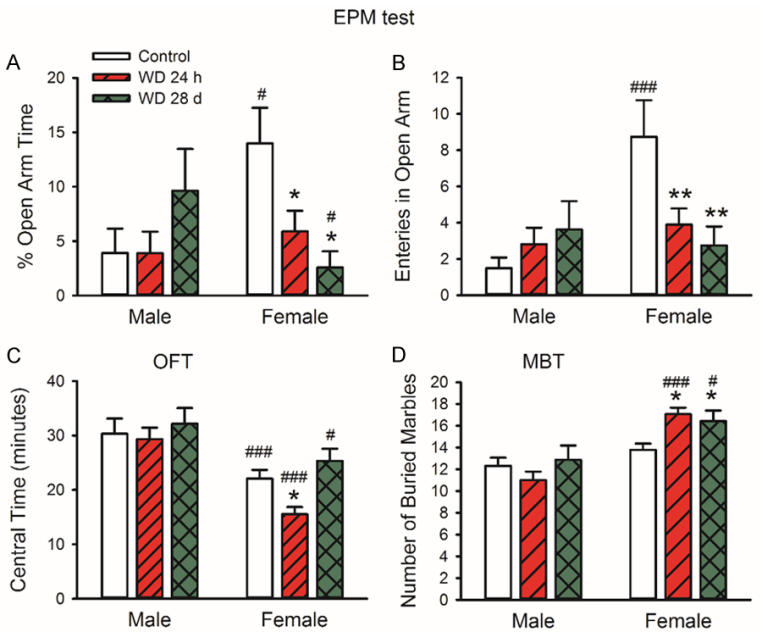


**Figure 2.** Sex differences in anxiety levels of rats during the 24-h and 28-days of abstinence. A. Percent of time in open arm in the elevated plus maze (EPM). After 24 h and 28 days of abstinence, the female rats spent a significantly shorter time in the open arms than the naïve control. B. Entries in open arm in EPM. After 24-h and 28-days of abstinence, females, but not males, entered fewer times into the open arms. C. Central time spent in the open field test (OFT). After 24 h of abstinence, the females spent a shorter time in the central area in OFT. D. Marbles buried in the marble-burying test (MBT). Females, but not the males, buried more marbles after 24-h and 28-days of abstinence. **P*<0.05, ***P*<0.01 *vs*. sex-matched water control, #*P*<0.05, ###*P*<0.001 *vs.* males. n = 8-10 rats/sex, two-way ANOVA.

female (WF) controls after acute and prolonged abstinence (**Figure 2A** and **2B**, all *P*<0.05). No difference was found in the time spent in open-arms between alcohol-drinking males (AM), and water-drinking male (WM) controls after acute and prolonged abstinence. There was no difference in entries in open arms between AM and WM controls after acute or prolonged abstinence (**Figure 2A** and **2B**, all *P*>0.05).

*OFT*

The AF spent a significantly shorter time in the central area of the open field chamber than WF controls after acute abstinence (**Figure 2C**, *P*<0.05). This difference was not observed after prolonged abstinence (**Figure 2C**). Also, no difference was found between AM and WM controls in the time spent in the central area of the open field chamber after acute and prolonged abstinence. Both male and female rats showed no difference in total distance traveled between alcohol and water controls after acute and prolonged abstinence in EPM or OFT (data not shown).

*MBT*

Marbles burying test (MBT) is based on the natural repertoire of rodent behavior whereby rats or mice will bury either harmful or harmless objects (such as glass marbles) in their bedding [50]. This test is proven to be a useful behavioral assay of anxiety or obsessive-compulsive disorder (OCD) behavior [51, 52]. The AF buried more marbles than the WF controls after acute and prolonged abstinence (**Figure 2D**, all *P*<0.05). Similarly, AF always buried more marbles than age-matched AM during acute or prolonged abstinence (**Figure 2D**, all *P*<0.05). However, no difference was found in the number of marbles buried by AM and WM controls after acute and prolonged abstinence.

*Depressive-like behavior*

Depressive-like behavior was assessed using the forced swimming test (FST). Both the AF and the AM had a longer immobile duration at acute and prolonged abstinence compared to their sex-matched water controls (**Figure 3A**, all *P*<0.05). Interestingly, the immobile time of both the WF and the AF was significantly longer than that of age-matched WM and AM (**Figure 3A**).

The measure of the latency to the first immobility has been shown to increase the sensitivity and improve the predictive validity of the FST [53]. In this study, only the AM, but not the AF, displayed a shorter latency to the first immobility after both acute and prolonged abstinence compared to their water controls (**Figure 3B**, *all P*<0.01). The latency to the first immobility in the WF was shorter than age-matched WM. This difference was not observed either after-acute and prolonged abstinence (**Figure 3B**).

Sucrose preference test (SPT) results showed that compared to their sex-matched Naive controls, sucrose preference was reduced in both the AF and the AM in acute abstinence (**Figure 3C**, all *P*<0.05), implying a depressive state.

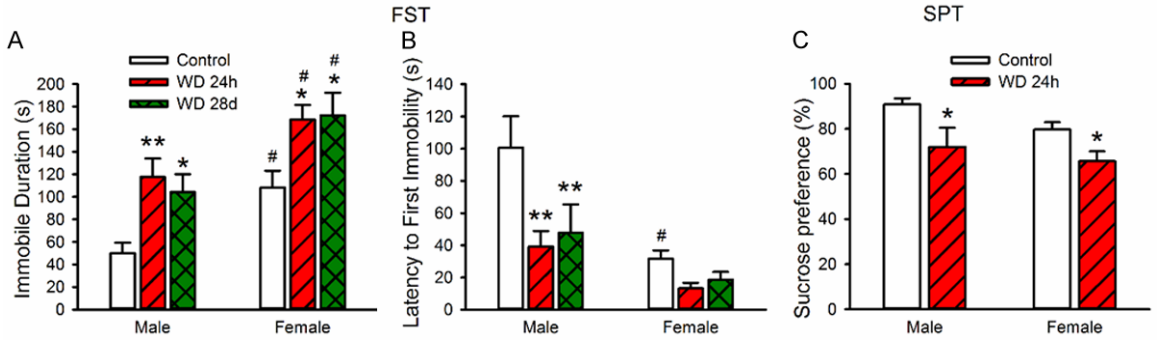


**Figure 3.** Depressive-like behaviors of rats during 24-h and 28-days of ethanol-withdrawal (WD). A. Both males and females showed longer immobility duration in forced swimming test (FST) after 24-h and 28-days of abstinence. B. Males, but not females, show shorter latency to the first immobility after 24-h and 28-days of abstinence in FST. C. The sucrose preference was lower in both male and female rats withdrawn from chronic ethanol than ethanol-naïve rats. The Sucrose Preference Test (SPT) was conducted during 24-h ethanol withdrawal. **P*<0.05, ***P*<0.01 *vs.* sex-matched water controls; #*P*<0.05 *vs.* males. n = 8-10 rats/sex, two-way ANOVA.

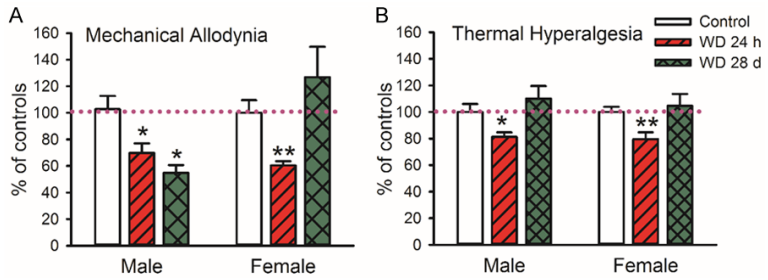


**Figure 4.** Changes in thermal and mechanical nociceptive thresholds in rats during 24-h and 28-days of ethanol withdrawal (WD). (A) Paw withdrawal latency response to mechanical stimuli. (B) Paw withdrawal latency response to thermal stimuli. Rats of both sexes show a decreased threshold to mechanical and thermal stimuli on their paws than that of their sex-matched controls after 24-h of abstinence (A, B). After 28-days of abstinence, the males, but not the females, displayed a lower paw withdrawal threshold to mechanical stimuli than that of their sex-matched controls. **P*<0.05, ***P*<0.01 compared with sex-matched water controls. n = 8-10 rats/sex, two-way ANOVA.

*Mechanical allodynia and thermal hyperalgesia*

Both AM and AF showed a lower threshold to mechanical stimulation on their paws than that of their sex-matched controls at acute abstinence (**Figure 4A**, all *P*<0.05). After the prolonged abstinence, the AM, but not the AF, still displayed a lower paw-withdrawal threshold than that of their sex-matched controls (**Figure 4A**, *P*<0.05).

Both the AM and the AF showed a shorter latency to thermal stimuli than that of their sex-matched controls after acute abstinence (**Figure 4B**, all *P*<0.05). However, there was no difference between naïve and alcohol drinking in both sexes after prolonged abstinence (**Figure 4B**).

*Sex differences in changes in neuronal activities of the brain after acute (24 h) and prolonged (28 days) abstinence*

Having discovered the sex difference in behaviors after acute as well as prolonged abstinence, we then investigated its neuronal basis using immunoreactivity with Fos protein, an anatomical marker of neuronal activity in the brain [54]. We analyzed Fos in multiple brain regions that play critical roles in conditioned drug behaviors and emotional dysregulation during ethanol withdrawal [40, 55-57], namely: the prefrontal cortex, anterior cingulate cortex, nucleus accumbens, amygdala, and lateral habenula.

*Prefrontal cortex (PFC)*

After both acute and prolonged abstinence, the AF, but not the AM, showed significant changes in Fos expression in the PFC, including infralimbic cortex, prelimbic cortex, orbitofrontal cortex (**Table 2**).

*Infralimbic cortex (IL)*

AF displayed a significant increase in Fos-positive (Fos+) cells in the IL after acute absti-

**Table 2.** Fos expression in multiple brain regions of male and female rats after acute withdrawal (24 h) and long-term withdrawal (28 days)

| Regions | Female | | | *P* value | *P* value | Male | | | *P* value | *P* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | Naïve | WD 24 h | WD 28 d | N. vs. 24 h | N. vs. 28 d | Naïve | WD 24 h | WD 28 d | N. vs. 24 h | N. vs. 28 d |
| IL | 230.1 ± 19.7 | 419.7 ± 26.2 | 103.1 ± 12.2 | <0.01 | <0.01 | 248.2 ± 26.8 | 254.2 ± 17.9 | 235.0 ± 41.5 | n.s. | n.s. |
| PrL | 268.3 ± 22.3 | 472.8 ± 22.5 | 154.4 ± 4.01 | <0.01 | <0.05 | 364.7 ± 31.1 | 341.8 ± 20.4 | 304.7 ± 41.6 | n.s. | n.s. |
| OFC | 409.1 ± 46.5 | 703.9 ± 41.6 | 114.1 ± 25.2 | <0.01 | <0.01 | 463.5 ± 45.2 | 339.0 ± 52.0 | 404.3 ± 60.7 | n.s. | n.s. |
| ACC | 214.9 ± 9.9 | 413.7 ± 31.2 | 85 ± 15.4 | <0.01 | <0.01 | 187.9 ± 10.0 | 253.9 ± 19.3 | 135.5 ± 14.5 | <0.01 | <0.01 |
| Nac core | 150.0 ± 14.8 | 226.0 ± 26.4 | 92.1 ± 15.7 | <0.01 | <0.01 | 82.8 ± 4.7 | 89 .2 ± 8.2 | 55.4 ± 3.4 | n.s. | <0.01 |
| Nac shell | 128.4 ± 10.6 | 297.8 ± 21.5 | 70.0 ± 13.8 | <0.01 | <0.01 | 70.4 ± 4.2 | 119.6 ± 17.8 | 49.8 ± 6.1 | <0.05 | n.s. |
| CEA | 29.0 ± 2.7 | 56.4 ± 13.0 | 24.0 ± 2.6 | <0.01 | n.s. | 11.7 ± 1.8 | 20.3 ± 8.8 | 16 .4 ± 1.8 | <0.01 | n.s. |
| BLA | 60.8 ± 5.4 | 121.4 ± 11.9 | 27.5 ± 3.0 | <0.01 | <0.01 | 19.1 ± 2.9 | 33.2 ± 4.2 | 21.0 ± 1.8 | <0.01 | n.s. |
| LHb | 16.0 ± 1.5 | 25.0 ± 1.6 | 16.5 ± 1.7 | <0.01 | n.s. | 15.4 ± 1.5 | 28.9 ± 1.7 | 8.4 ± 0.6 | <0.01 | <0.01 |

Note: n = 6 rats/sex, two-way ANOVA. Abbreviation: IL, infralimbic cortex; PrL, prelimbic cortex; OFC, orbital cortex; ACC, anterior cingulate cortex; Nac, Nucleus accumbens; CEA, central nucleus of the amygdala; BLA, basolateral nucleus of the amygdala; LHb, lateral habenula.

nence ($P$<0.01), and a dramatic *decrease* after prolonged abstinence, compared with sex-matched ethanol-naïve rats (**Table 2**). However, there was no significant change in Fos expression in IL of the AM after acute or prolonged abstinence.

*Prelimbic cortex (PrL)*

Like the IL, the AF displayed a significant increase in Fos+ cells in the PrL after acute abstinence ($P$<0.01) and a dramatic *decrease* after prolonged abstinence, compared to sex-matched ethanol-naïve rats (**Table 2**). There was no change in Fos+ cells in the AM after acute and protracted abstinence (**Table 2**).

*Orbitofrontal cortex (OFC)*

The amount of Fos+ cells in AF dramatically increased after the acute abstinence ($P$<0.01) and then significantly decreased after the prolonged abstinence ($P$<0.01), compared to WF rats in the OFC (**Table 2**). However, there was no significant change in Fos expression in OFC of the AM following acute and prolonged abstinence (**Table 2**).

*Anterior cingulate cortex (ACC)*

Fos+ cells in the ACC increased considerably-after acute abstinence (all $P$<0.01) and dramatically decreased after the prolonged abstinence (all $P$<0.05) in both sexes compared to sex-matched ethanol-naïve rats (**Table 2**).

*Nucleus accumbens (NAc)*

*NAc core*: AF displayed a remarkable increase of Fos+ cells in the NAc core after acute abstinence ($P$<0.001) and a significant decrease after the prolonged abstinence ($P$=0.01) compared with sex-matched controls (**Table 2**). Though the number of Fos+ cells in the AM did not differ from that in the sex-matched controls after acute abstinence, it significantly decreased after the prolonged abstinence compared to sex-matched controls (**Table 2**, $P$=0.001).

*NAc shell*: Both the AF and the AM displayed a significant increase in Fos+ cells in the NAc shell after the 24 h withdrawal compared to their sex-matched controls (**Table 1**, $P$<0.05). After 28 days' withdrawal, the number of Fos+ cells decreased significantly in the AF, while the levels of Fos+ cells in the WM controls were like those in AM (**Table 2**).

*Amygdala*

*Central nucleus of the amygdala (CEA)*: Both the AF and the AM ($P$<0.01) displayed a significant increase of Fos+ cells in the CEA after-acute abstinence, compared to their sex-matched controls (**Table 2**, $P$<0.01). After prolonged abstinence, the number of Fos+ cells in both sexes returned to the level of their sex-matched controls (**Table 2**).

*The basolateral nucleus of the amygdala (BLA)*: Both the AF and AM ($P$<0.01) displayed a remarkable increase of Fos+ cells in the BLA after acute abstinence, compared to their sex-matched controls (**Table 2**, $P$<0.01). After prolonged abstinence, the number of Fos+ cells significantly decreased in the AF, while the

number of Fos+ cells in the AM returned to the levels of WM (**Table 2**).

*Lateral habenula (LHb)*

Both AM and AF displayed a significant (all $P<0.01$) increase of Fos+ cells in the LHb after acute abstinence, compared to their sex-matched controls (**Table 2**). In AF, this level eventually returned to the baseline level of the sex-matched controls; whereas, in the AM the number of Fos+ cells in the LHb remained significantly decreased after prolonged abstinence (**Table 2**).

## Discussion

The female SD rats drank more alcohol and had higher BECs than the males, and this was not affected by the phases of the estrous cycle. Rats of both sexes displayed depressive-like behavior after the 24-h (acute), and 28-days (prolonged) abstinence; they also exhibited thermal hyperalgesia and mechanical allodynia after acute abstinence. The males displayed only mechanical allodynia after protracted abstinence. Notably, only the females showed higher levels of anxiety after both acute and prolonged abstinence, and a strong alcohol deprivation effect, as indicated by the significantly elevated alcohol consumption upon resuming drinking after the prolonged abstinence. Moreover, after acute abstinence, Fos+ nuclei increased widely in the cerebral cortex (IL, PrL, OFC, ACC), NAc, amygdala, and LHb in the females but was limited to the ACC, amygdala, and LHb in the males. After prolonged abstinence, the number of Fos+ cells was substantially decreased in the cerebral cortex and NAc of the females and the ACC, LHb, and NAc in the males.

Despite compelling evidence showing female rats drink more alcohol than the males of many strains, few studies concerning sex differences have been done on SD rats. This may be because SD rats consume low to moderate levels of ethanol [58-60] and have a lower ethanol preference than P rats [61, 62]. The IA2BC schedule has been considered as a useful model mimicking some drinking patterns in alcoholics. Several studies have found that some SD rats in the IA2BC paradigm drank excessive amounts of ethanol [48, 63, 64] and showed signs of physical dependence and hyperalgesia during abstinence [48, 64]. In keeping with a previous report [13], we observed that female SD rats drank more than their male counterparts in the IA2BC paradigm. Estrous cycle stage may not be a significant player in the difference since the female rats consumed a similar amount of alcohol in different estrous cycle stages.

In addition to alcohol consumption, we noted a significant sex difference in withdrawal-related behaviors. Previous rodent studies, including our own, have shown depressive- and anxiety-like behaviors in rodents after acute [42, 47, 65, 66] and prolonged abstinence [65]; however, most of these studies were done on males. In the current study, although depressive-like behavior was found in rats of both sexes, immobility time was much longer in the females, in keeping with a previous report [67]. A previous study has demonstrated that the measure of the latency to the first immobility improves the predictive validity of the Forced Swim Test [53]. However, the AM, but not the AF had a shorter latency to the first immobility after both acute and prolonged abstinence compared to Naive controls. This result suggests that males have a higher propensity to develop depressive-like behavior than females during abstinence from chronic alcohol. Another possibility is those naive females are more likely to develop depressive-like behavior than naïve males, as indicated by the naive females who had a much shorter latency to the first immobility (males: 110.6 ± 19.42 s; females: 31.6 ± 5.2 s). This short latency makes it difficult to detect the difference between naïve female control and AF.

Moreover, we found the females had higher levels of anxiety (less time and number of entries in open arm in Elevated Plus Test) after both acute and prolonged abstinence, in general agreement with a previous clinical study [68]. We also noted that male controls spend a very short time (around 5%) in the open arms. Thus, we cannot exclude a possible floor effect, which makes it difficult to detect the difference between the naïve male control and the AM. However, the females but not males, buried more marbles in Marble Burying Test (MBT), indicating high anxiety levels after both acute and prolonged abstinence. MBT has been used successfully to characterize anxiety or obsessive-compulsive behavior [52]. Since withdraw-

al symptoms have been linked to a vulnerability for relapse in drinking [1, 3] and were stronger in the females, it is not surprising that the females consumed more alcohol than the males in our and other investigators' studies [13].

Increased sensitivity to painful stimuli has been detected in rats consuming ethanol and during acute abstinence [48, 69]. We observed increased sensitivity to both thermal and mechanical stimuli in SD rats of both sexes after acute abstinence. A previous rat study showed a further increase in mechanical allodynia after 5-week abstinence [70]. Consistent with this, we observed increased sensitivity to mechanical stimuli in the males after 28-days abstinence. These observations are consistent with clinical studies showing persistent or even worsened peripheral neuropathy in alcohol withdrawal patients [71, 72]. We, however, did not detect significant changes in perceptive sensitivity to either mechanical or thermal stimuli in the females after 28-day abstinence, although these rats showed higher levels of anxiety and depression.

The mechanisms underlying sex difference in ethanol withdrawal syndrome are poorly understood [73]. Analyses of immediate early genes, such as c-Fos, suggest it involves certain brain regions [74, 75]. Previous studies have shown increased Fos protein expression during acute ethanol withdrawal in multiple areas, including cerebral cortex (medial prefrontal cortex (mPFC), ventrolateral orbital cortex, piriform cortex, and cingulate cortex), nucleus accumbens, amygdala, paraventricular hypothalamic nucleus (PV) and the LHb [28-30]. In keeping with these studies, we observed a widespread and robust Fos-induction during acute abstinence in mPFC (including IL, PrL), OFC, ACC, NAc core and shell, CEA, BLA, and LHb in the AF; but only in the CEA, BLA, and LHb in the AM. A previous mouse study, using the transcription factor Egr1 as an index of cellular activity, had found that protracted withdrawal symptoms (anxiety and depression) are associated with increased activity in the CEA and bed nucleus of the stria terminalis [65]. In contrast, in the current rat study, we detected a dramatic reduction of Fos+ nuclei in most tested brain regions [such as IL, PrL, OFC, ACC, NAc (core and shell), and BLA] of the AF after prolonged abstinence. Additionally, we found high levels of Fos-induction during acute abstinence, but a significant decrease in Fos+ nuclei in the ACC, NAc core and LHb of the AM. Thus, changes in Fos expression depend on brain regions, sex, and the duration of abstinence.

It is unclear why Fos protein was suppressed after prolonged abstinence. However, Fos desensitization may be one of the mechanisms [28]. Fos protein, a putative transcriptional factor which forms the activator protein-1 (AP-1) complex with c-jun, binds DNA at AP-1 specific sites at the promoter and enhancer regions of target genes and converts extracellular signals into corresponding gene expression [76]. In response to chronic ethanol use and long-term abstinence, Fos desensitization occurs, indicating cellular adaptation or neuronal plasticity at the level of gene regulation; however, the proteins whose expression is regulated by Fos in the nervous system remain unknown. They may be linked to a dysfunctional hypothalamic-pituitary-adrenal (HPA) axis activity, which has been associated with an enhanced negative emotional state and susceptibility to relapse [77-79]. The vulnerability of the HPA axis to excess alcohol and abstinence also differs between sexes [80, 81]. After withdrawal from chronic alcohol consumption, the females had a lower circulating level of corticosterone and dampened CRH (corticotropin-releasing hormone) and vasopressin expression in the medial parvocellular division of the PVN (PVNmp) neurons without a change in the serum estrogen. Conversely, the males had normal circulating levels of corticosterone and decreased CRH and vasopressin expression in PVNmp neurons with lower testosterone levels. These data indicate the adaptation of the whole CNS (including the HPA axis) to alcohol exposure and withdrawal [82]. The blunted stress-induced cortisol response has been associated with higher anxiety levels in individuals with alcohol use disorders [83]. Therefore, it is not surprising that the females showed more pronounced anxiety- and depressive-like behaviors and alcohol deprivation effects than the males and exhibited Fos-depression in more brain regions than in the males after long-term abstinence.

The current study also showed that Fos could be used as a marker to map the specific brain regions where adaptation has occurred, chang-

ing the "set point" for drug reward and instigating further drug use [84]. Also, considering the differential intensity of Fos activation and reduction in various brain regions in both sexes, as shown in the current study, the induced gene regulation appears to vary between different brain regions. In other words, the molecular fingerprinting of each brain region after chronic ethanol exposure and withdrawal seems to be different.

In summary, the current study describes that acute and prolonged withdrawal from chronic ethanol consumption (under the IA2BC paradigm) produced negative effects in a sex-dependent manner: female SD rats exhibited depression-like behavior, apparent anxiety, and a stronger susceptibility to relapse. The male SD rats showed depressive-like behavior and allodynia. Furthermore, following acute and prolonged abstinence, the immediate early gene *c-fos* expression in specific brain regions also indicates sex differences. The brain regions which were activated have particular roles in mediating aspects of ethanol-driven behavior related to reward, dependence, emotion, and withdrawal. This information may elucidate the mechanisms underlying the sex differences in tolerance, physical and psychological dependence on and withdrawal from chronic alcohol consumption.

## Acknowledgements

NIH funded this work (grants AA021657 and AA022292), and a grant from the New Jersey Health Foundation. The authors appreciate Dr. Somdatta Gupta and Miss Alija Rypien reading over the manuscript. The authors declare no conflict of interest.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Jianghong Ye, Department of Anesthesiology, New Jersey Medical School, Rutgers, The State University of New Jersey, 185 South Orange Avenue, Newark, NJ 07103, USA. Tel: 973-972-1866; E-mail: ye@njms.rutgers.edu

## References

[1] Gilpin NW and Koob GF. Neurobiology of alcohol dependence: focus on motivational mechanisms. Alcohol Res Health 2008; 31: 185-195.

[2] Koob GF. Alcoholism: allostasis and beyond. Alcohol Clin Exp Res 2003; 27: 232-243.

[3] Becker HC. Alcohol dependence, withdrawal, and relapse. Alcohol Res Health 2008; 31: 348-361.

[4] Ashley MJ, Olin JS, le Riche WH, Kornaczewski A, Schmidt W and Rankin JG. Morbidity in alcoholics. Evidence for accelerated development of physical disease in women. Arch Intern Med 1977; 137: 883-887.

[5] Diehl A, Croissant B, Batra A, Mundle G, Nakovics H and Mann K. Alcoholism in women: is it different in onset and outcome compared to men? Eur Arch Psychiatry Clin Neurosci 2007; 257: 344-351.

[6] Erol A and Karpyak VM. Sex and gender-related differences in alcohol use and its consequences: Contemporary knowledge and future research considerations. Drug Alcohol Depend 2015; 156: 1-13.

[7] Mann K, Ackermann K, Croissant B, Mundle G, Nakovics H and Diehl A. Neuroimaging of gender differences in alcohol dependence: are women more vulnerable? Alcohol Clin Exp Res 2005; 29: 896-901.

[8] Randall CL, Roberts JS, Del Boca FK, Carroll KM, Connors GJ and Mattson ME. Telescoping of landmark events associated with drinking: a gender comparison. J Stud Alcohol 1999; 60: 252-260.

[9] King AC, Bernardy NC and Hauner K. Stressful events, personality, and mood disturbance: gender differences in alcoholics and problem drinkers. Addict Behav 2003; 28: 171-187.

[10] Hartwell EE and Ray LA. Sex moderates stress reactivity in heavy drinkers. Addict Behav 2013; 38: 2643-2646.

[11] Rubonis AV, Colby SM, Monti PM, Rohsenow DJ, Gulliver SB and Sirota AD. Alcohol cue reactivity and mood induction in male and female alcoholics. J Stud Alcohol 1994; 55: 487-494.

[12] Cailhol S and Mormede P. Sex and strain differences in ethanol drinking: effects of gonadectomy. Alcohol Clin Exp Res 2001; 25: 594-599.

[13] Priddy BM, Carmack SA, Thomas LC, Vendruscolo JC, Koob GF and Vendruscolo LF. Sex, strain, and estrous cycle influences on alcohol drinking in rats. Pharmacol Biochem Behav 2017; 152: 61-67.

[14] de la Torre ML, Escarabajal MD and Aguero A. Sex differences in adult Wistar rats in the voluntary consumption of ethanol after pre-exposure to ethanol-induced flavor avoidance learning. Pharmacol Biochem Behav 2015; 137: 7-15.

[15] Dhaher R, McConnell KK, Rodd ZA, McBride WJ and Bell RL. Daily patterns of ethanol drinking in adolescent and adult, male and female, high alcohol drinking (HAD) replicate lines of

rats. Pharmacol Biochem Behav 2012; 102: 540-548.
[16] Maldonado-Devincci AM, Alipour KK, Michael LA and Kirstein CL. Repeated binge ethanol administration during adolescence enhances voluntary sweetened ethanol intake in young adulthood in male and female rats. Pharmacol Biochem Behav 2010; 96: 476-487.
[17] Bertholomey ML, Nagarajan V and Torregrossa MM. Sex differences in reinstatement of alcohol seeking in response to cues and yohimbine in rats with and without a history of adolescent corticosterone exposure. Psychopharmacology (Berl) 2016; 233: 2277-2287.
[18] Devaud LL and Chadda R. Sex differences in rats in the development of and recovery from ethanol dependence assessed by changes in seizure susceptibility. Alcohol Clin Exp Res 2001; 25: 1689-1696.
[19] Varlinskaya EI and Spear LP. Acute ethanol withdrawal (hangover) and social behavior in adolescent and adult male and female Sprague-Dawley rats. Alcohol Clin Exp Res 2004; 28: 40-50.
[20] Rezvani AH, Levin ED, Cauley M, Getachew B and Tizabi Y. Ketamine differentially attenuates alcohol intake in male versus female alcohol preferring (P) rats. J Drug Alcohol Res 2017; 6.
[21] Carnicella S, Ron D and Barak S. Intermittent ethanol access schedule in rats as a preclinical model of alcohol abuse. Alcohol 2014; 48: 243-252.
[22] Sagar SM, Sharp FR and Curran T. Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 1988; 240: 1328-1331.
[23] Curran T and Morgan JI. Fos: an immediate-early transcription factor in neurons. J Neurobiol 1995; 26: 403-412.
[24] Matsumoto I, Leah J, Shanley B and Wilce P. Immediate early gene expression in the rat brain during ethanol withdrawal. Mol Cell Neurosci 1993; 4: 485-491.
[25] Simonato M, Hosford DA, Labiner DM, Shin C, Mansbach HH and McNamara JO. Differential expression of immediate early genes in the hippocampus in the kindling model of epilepsy. Brain Res Mol Brain Res 1991; 11: 115-124.
[26] Yount GL, Ponsalle P and White JD. Pentylenetetrazole-induced seizures stimulate transcription of early and late response genes. Brain Res Mol Brain Res 1994; 21: 219-224.
[27] McCown TJ, Duncan GE, Johnson KB and Breese GR. Metabolic and functional mapping of the neural network subserving inferior collicular seizure generalization. Brain Res 1995; 701: 117-128.
[28] Chang SL, Patel NA and Romero AA. Activation and desensitization of Fos immunoreactivity in the rat brain following ethanol administration. Brain Res 1995; 679: 89-98.
[29] Knapp DJ, Duncan GE, Crews FT and Breese GR. Induction of Fos-like proteins and ultrasonic vocalizations during ethanol withdrawal: further evidence for withdrawal-induced anxiety. Alcohol Clin Exp Res 1998; 22: 481-493.
[30] Li J, Zuo W, Fu R, Xie G, Kaur A, Bekker A and Ye JH. High frequency electrical stimulation of lateral habenula reduces voluntary ethanol consumption in rats. Int J Neuropsychopharmacol 2016; [Epub ahead of print].
[31] Li Y, Wang Y, Xuan C, Li Y, Piao L, Li J and Zhao H. Role of the lateral habenula in pain-associated depression. Front Behav Neurosci 2017; 11: 31.
[32] Li B, Piriz J, Mirrione M, Chung C, Proulx CD, Schulz D, Henn F and Malinow R. Synaptic potentiation onto habenula neurons in the learned helplessness model of depression. Nature 2011; 470: 535-539.
[33] Barthas F, Sellmeijer J, Hugel S, Waltisperger E, Barrot M and Yalcin I. The anterior cingulate cortex is a critical hub for pain-induced depression. Biol Psychiatry 2015; 77: 236-245.
[34] Gill MJ, Ghee SM, Harper SM and See RE. Inactivation of the lateral habenula reduces anxiogenic behavior and cocaine seeking under conditions of heightened stress. Pharmacol Biochem Behav 2013; 111: 24-29.
[35] Li J, Fu C, Liu H, Fu R, Zuo W, Kang S, Chen P, Gregor D, Paulose R, Bekker A and Ye JH. Electroacupuncture attenuates hyperalgesia in rats withdrawn from chronic alcohol drinking via habenular Mu opioid receptors. Alcohol Clin Exp Res 2017; 41: 637-643.
[36] Li W, Zuo W, Wu W, Zuo QK, Fu R, Wu L, Zhang H, Ndukwe M and Ye JH. Activation of glycine receptors in the lateral habenula rescues anxiety- and depression-like behaviors associated with alcohol withdrawal and reduces alcohol intake in rats. Neuropharmacology 2019; 157: 107688.
[37] Kang S, Li J, Zuo W, Chen P, Gregor D, Fu R, Han X, Bekker A and Ye JH. Downregulation of M-channels in lateral habenula mediates hyperalgesia during alcohol withdrawal in rats. Sci Rep 2019; 9: 2714.
[38] Gregor DM, Zuo W, Fu R, Bekker A and Ye JH. Elevation of transient receptor potential vanilloid 1 function in the lateral habenula mediates aversive behaviors in alcohol-withdrawn rats. Anesthesiology 2019; 130: 592-608.
[39] Kang S, Li J, Bekker A and Ye JH. Rescue of glutamate transport in the lateral habenula alleviates depression- and anxiety-like behaviors

in ethanol-withdrawn rats. Neuropharmacology 2018; 129: 47-56.
[40] Koob GF and Le Moal M. Drug addiction, dysregulation of reward, and allostasis. Neuropsychopharmacology 2001; 24: 97-129.
[41] Li J, Bian W, Dave V and Ye JH. Blockade of GABA(A) receptors in the paraventricular nucleus of the hypothalamus attenuates voluntary ethanol intake and activates the hypothalamic-pituitary-adrenocortical axis. Addict Biol 2011; 16: 600-614.
[42] Li J, Kang S, Fu R, Wu L, Wu W, Liu H, Gregor D, Zuo W, Bekker A and Ye JH. Inhibition of AMPA receptor and CaMKII activity in the lateral habenula reduces depressive-like behavior and alcohol intake in rats. Neuropharmacology 2017; 126: 108-120.
[43] Simms JA, Steensland P, Medina B, Abernathy KE, Chandler LJ, Wise R and Bartlett SE. Intermittent access to 20% ethanol induces high ethanol consumption in Long-Evans and Wistar rats. Alcohol Clin Exp Res 2008; 32: 1816-1823.
[44] Kristoffersen L, Skuterud B, Larssen BR, Skurtveit S and Smith-Kielland A. Fast quantification of ethanol in whole blood specimens by the enzymatic alcohol dehydrogenase method. Optimization by experimental design. J Anal Toxicol 2005; 29: 66-70.
[45] Slattery DA and Cryan JF. Using the rat forced swim test to assess antidepressant-like activity in rodents. Nat Protoc 2012; 7: 1009-1014.
[46] Fu R, Zuo W, Shiwalkar N, Mei Q, Fan Q, Chen X, Li J, Bekker A and Ye JH. Alcohol withdrawal drives depressive behaviors by activating neurons in the rostromedial tegmental nucleus. Neuropsychopharmacology 2019; 44: 1464-1475.
[47] Kang S, Li J, Zuo W, Fu R, Gregor D, Krnjevic K, Bekker A and Ye JH. 1813-1824. Neuropsychopharmacology 2017; 42: 1813-1824.
[48] Fu R, Gregor D, Peng Z, Li J, Bekker A and Ye J. Chronic intermittent voluntary alcohol drinking induces hyperalgesia in Sprague-Dawley rats. Int J Physiol Pathophysiol Pharmacol 2015; 7: 136-144.
[49] McLean AC, Valenzuela N, Fai S and Bennett SA. Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. J Vis Exp 2012; e4389.
[50] De Boer SF and Koolhaas JM. Defensive burying in rodents: ethology, neurobiology and psychopharmacology. Eur J Pharmacol 2003; 463: 145-161.
[51] Njung'e K and Handley SL. Evaluation of marble-burying behavior as a model of anxiety. Pharmacol Biochem Behav 1991; 38: 63-67.
[52] Kedia S and Chattarji S. Marble burying as a test of the delayed anxiogenic effects of acute immobilisation stress in mice. J Neurosci Methods 2014; 233: 150-154.
[53] Castagne V, Porsolt RD and Moser P. Use of latency to immobility improves detection of antidepressant-like activity in the behavioral despair test in the mouse. Eur J Pharmacol 2009; 616: 128-133.
[54] Cruz FC, Javier Rubio F and Hope BT. Using c-fos to study neuronal ensembles in corticostriatal circuitry of addiction. Brain Res 2015; 1628: 157-173.
[55] Koob G and Kreek MJ. Stress, dysregulation of drug reward pathways, and the transition to drug dependence. Am J Psychiatry 2007; 164: 1149-1159.
[56] Koob GF and Le Moal M. Drug abuse: hedonic homeostatic dysregulation. Science 1997; 278: 52-58.
[57] Shah A, Zuo W, Kang S, Li J, Fu R, Zhang H, Bekker A and Ye JH. The lateral habenula and alcohol: role of glutamate and M-type potassium channels. Pharmacol Biochem Behav 2017; 162: 94-102.
[58] Khanna JM, Kalant H, Shah G and Sharma H. Comparison of sensitivity and alcohol consumption in four outbred strains of rats. Alcohol 1990; 7: 429-434.
[59] Linseman MA. Alcohol consumption in free-feeding rats: procedural, genetic and pharmacokinetic factors. Psychopharmacology (Berl) 1987; 92: 254-261.
[60] Melchior CL and Myers RD. Genetic differences in ethanol drinking of the rat following injection of 6-OHDA, 5,6-DHT or 5,7-DHT into the cerebral ventricles. Pharmacol Biochem Behav 1976; 5: 63-72.
[61] Martinetti MP, Lowery EG, Vona SR, Wichnick AM, Adler RA and Finch DG. Limited-access consumption of ascending ethanol concentrations in alcohol-preferring, nonpreferring, and Sprague-Dawley rats. Alcohol Clin Exp Res 2006; 30: 836-843.
[62] Martinetti MP, Khan Z and Lewis MJ. Matching law choice analyses of ethanol and sucrose consumption in alcohol-preferring (P), nonpreferring (NP), and Sprague-Dawley (SD) rats. Alcohol Clin Exp Res 2007; 31: 1338-1348.
[63] Bito-Onon JJ, Simms JA, Chatterjee S, Holgate J and Bartlett SE. Varenicline, a partial agonist at neuronal nicotinic acetylcholine receptors, reduces nicotine-induced increases in 20% ethanol operant self-administration in Sprague-Dawley rats. Addict Biol 2011; 16: 440-449.
[64] Li J, Zou Y and Ye JH. Low frequency electroacupuncture selectively decreases voluntarily ethanol intake in rats. Brain Res Bull 2011; 86: 428-434.
[65] Lee KM, Coehlo M, McGregor HA, Waltermire RS and Szumlinski KK. Binge alcohol drinking

elicits persistent negative affect in mice. Behav Brain Res 2015; 291: 385-398.
[66] Warnock KT, Yang AR, Yi HS, June HL Jr, Kelly T, Basile AS, Skolnick P and June HL. Amitifadine, a triple monoamine uptake inhibitor, reduces binge drinking and negative affect in an animal model of co-occurring alcoholism and depression symptomatology. Pharmacol Biochem Behav 2012; 103: 111-118.
[67] Kokras N, Antoniou K, Mikail HG, Kafetzopoulos V, Papadopoulou-Daifoti Z and Dalla C. Forced swim test: what about females? Neuropharmacology 2015; 99: 408-421.
[68] Karpyak VM, Biernacka JM, Geske JR, Abulseoud OA, Brunner MD, Chauhan M, Hall-Flavin DK, Lewis KA, Loukianova LL, Melnyk GJ, Onsrud DA, Proctor BD, Schneekloth TD, Skime MK, Wittkopp JE, Frye MA and Mrazek DA. Gender-specific effects of comorbid depression and anxiety on the propensity to drink in negative emotional states. Addiction 2016; 111: 1366-1375.
[69] Gatch MB. Effects of benzodiazepines on acute and chronic ethanol-induced nociception in rats. Alcohol Clin Exp Res 1999; 23: 1736-1743.
[70] Dina OA, Barletta J, Chen X, Mutero A, Martin A, Messing RO and Levine JD. Key role for the epsilon isoform of protein kinase C in painful alcoholic neuropathy in the rat. J Neurosci 2000; 20: 8614-8619.
[71] Yokoyama A, Takagi T, Ishii H, Muramatsu T, Akai J, Kato S, Hori S, Maruyama K, Kono H and Tsuchiya M. Impaired autonomic nervous system in alcoholics assessed by heart rate variation. Alcohol Clin Exp Res 1991; 15: 761-765.
[72] Spahn TW, Lohse AW, Otto G, Tettenborn B, Hopf HC and Meyer zum Buschenfelde KH. Remission of severe alcoholic polyneuropathy after liver transplantation. Z Gastroenterol 1995; 33: 711-714.
[73] Charness ME, Simon RP and Greenberg DA. Ethanol and the nervous system. N Engl J Med 1989; 321: 442-454.
[74] Dave JR, Tabakoff B and Hoffman PL. Ethanol withdrawal seizures produce increased c-fos mRNA in mouse brain. Mol Pharmacol 1990; 37: 367-371.
[75] Wilce P, Beckmann A, Shanley B and Matsumoto I. Gene expression during ethanol withdrawal. Alcohol Alcohol Suppl 1994; 2: 97-102.
[76] Chiu R, Boyle WJ, Meek J, Smeal T, Hunter T and Karin M. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. Cell 1988; 54: 541-552.
[77] Breese GR, Sinha R and Heilig M. Chronic alcohol neuroadaptation and stress contribute to susceptibility for alcohol craving and relapse. Pharmacol Ther 2011; 129: 149-171.
[78] Junghanns K, Backhaus J, Tietz U, Lange W, Bernzen J, Wetterling T, Rink L and Driessen M. Impaired serum cortisol stress response is a predictor of early relapse. Alcohol Alcohol 2003; 38: 189-193.
[79] Junghanns K, Tietz U, Dibbelt L, Kuether M, Jurth R, Ehrenthal D, Blank S and Backhaus J. Attenuated salivary cortisol secretion under cue exposure is associated with early relapse. Alcohol Alcohol 2005; 40: 80-85.
[80] Silva SM, Santos-Marques MJ and Madeira MD. Sexually dimorphic response of the hypothalamo-pituitary-adrenal axis to chronic alcohol consumption and withdrawal. Brain Res 2009; 1303: 61-73.
[81] Silva SM and Madeira MD. Effects of chronic alcohol consumption and withdrawal on the response of the male and female hypothalamic-pituitary-adrenal axis to acute immune stress. Brain Res 2012; 1444: 27-37.
[82] Spencer RL and McEwen BS. Adaptation of the hypothalamic-pituitary-adrenal axis to chronic ethanol stress. Neuroendocrinology 1990; 52: 481-489.
[83] Dai X, Thavundayil J, Santella S and Gianoulakis C. Response of the HPA-axis to alcohol and stress as a function of alcohol dependence and family history of alcoholism. Psychoneuroendocrinology 2007; 32: 293-305.
[84] Koob GF and Nestler EJ. The neurobiology of drug addiction. J Neuropsychiatry Clin Neurosci 1997; 9: 482-497.