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Abstract

Among women with Alcohol Use Disorders (AUDs), women have higher rates of anxiety-disorders and are more influenced by early life stress compared to men. Preclinical models have been used to study the relationships between early life stress, anxiety-like behavior, and alcohol intake and preference. However, fewer studies have been done with female rats than male rats. To that end, chronic social instability was utilized as a model of early life stress in females. In this model Long Evans rats are placed in different pairs every day, and this has previously produced anxiety-like behavior in female rats (McCormick et al., 2008). This study extended the McCormick et al. model by including an extra experimental group and including an alcohol self-administration paradigm. Experimental groups were: (1) chronic social instability (pair-housed for 17 days with a novel cage mate introduced every 24h); (2) Social stability; pair-housed with same cage mate daily); (3) Isolated; single housed for entire study); (4) Acute social instability; pair-housed with same cage mate for 16 days but novel cage mate for 24h on last day). A well-validated model for assessment of anxiety-like behavior, the elevated plus maze, was utilized to evaluate anxiety-like behavior after the housing manipulation, and plasma corticosterone (CORT) levels were measured. Following these measures, rats were given a two bottle choice and intermittent access between alcohol and water, and alcohol consumption and preference was measured. Following these measures, no significant differences were found between experimental groups for anxiety-like behavior, CORT levels, or alcohol intake/preference. Thus, further study is needed to find a preclinical model of early life stress that promotes anxiety-like behavior and alcohol consumption.

Acknowledgements

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Introduction

Studies in animals and humans have shown that the brain is particularly sensitive to stress in early childhood, and early life stress exposure has been related to increased reactivity to stress in adulthood. Negative psychological and brain function effects from early-life stress in humans appear to be long lasting (Lupien et al., 2009). Repetitive and cumulative stressful life events happening prior to puberty are associated with problems related to ethanol consumption and drug dependence during adolescence and into early adulthood (Enoch, 2011), and are also related to major depression, posttraumatic stress disorder, and anxiety disorders (Heim & Nemeroff, 2001; Pine et al., 1998; Widom et al., 2007). Women appear to have a more consistent association between early life stress/trauma, anxiety disorders, and ethanol abuse than men (Enoch, 2011; Widom et al., 2007). Ethanol-dependent women are also more likely to have a comorbid anxiety disorder than men (Kessler et al., 1997, 2005). Because anxiety and ethanol dependence have common neural substrates (Heilig et al., 2007), it is imperative to explore these shared neural substrates in male and female subjects.

Despite the prevalent connection among early life stress, anxiety, and ethanol-dependence in women, the majority of preclinical research focusing on these relationships has utilized male subjects. Male animal models have provided great insight into how early life stress, anxiety, and ethanol consumption relate to each other and changes in the brain. For example, social isolation during adolescence serves as an early life stressor and imparts behavioral risk factors in male rats (Butler et al., 2014a). However, adolescent social isolation does not do so for females (Butler et al., 2014b). It has proven challenging to model stress and the relationship between anxiety and

increased ethanol self-administration in female rats. This may be because sex differences are evident in the type of stressor that elicits enduring behavioral changes. For example, defeat is seen as a plausible stressor that increases plasma corticosterone for males where social stress is seen as a stressor that increases plasma corticosterone for females (Haller, 1999). Males have been used preferentially in many models to study anxiety and ethanol preference. Some examples of this include studies examining innate anxiety in male rats (Ohl et al., 2001), anxiolytic and anxiogenic drug effects on elevated plus-maze (EPM) exploration in male rats (Pellow, 1985), and measures of anxiety-like behavior in male rats bred for high ethanol preference (Möller, 1997). The disproportionate focus on male subjects in preclinical studies, in spite of data suggesting higher comorbidity rates of anxiety and ethanol dependence in women, suggests a need for a reliable animal model of anxiety, ethanol dependence, and stress in females.

A model of chronic social instability, in which rats are given different cage partners every 24 hours following an hour of isolation, is utilized in this study as a model of chronic early life stress, and has been shown to engender anxiety-like behaviors in female rats. That is, chronic social instability engenders sex specific effects on anxiety-like behavior and corticosterone response, in that female rats under chronic social instability had greater locomotor sensitization to nicotine compared to non-stressed controls and male rats under chronic social instability (McCormick, 2004). Females kept under chronic stress display anxiety-like behaviors as adults (McCormick, 2007). It has also been demonstrated that high social anxiety-like behavior is linked with higher social drinking in female rats (Varlinskaya et al., 2015). This model may be promising in

further examining the relationship between early life stress, anxiety-like behavior, and self-administered ethanol intake in female rats.

Early life stress and alcoholism correlate with hypothalamic-pituitary-adrenal (HPA) axis dysfunction in humans (Lovallo, 2012). HPA axis dysfunction may entail reduced or increased responsiveness to stressors as measured by circulating levels of corticosterone (CORT) and impaired dexamethasone (DEX) suppression of CORT, which are often observed in dependent individuals and individuals at risk for ethanol dependence (Zimmermann et al., 2004). In animals, similar effects exist, as seen in rat models. For example, chronic mild stress may cause a rise in corticotropin-releasing hormone in male rats (Duncko, 2001).

The brain is particularly sensitive to stress in early childhood, and women have greater prevalence of comorbidities between early life stress, anxiety, and ethanol abuse than men. However, while research has looked far into the relationship of these factors in male rat models, a reliable female model is necessary for further understanding of common neural substrates. Chronic social instability has been demonstrated to elicit anxiety-like behaviors in female rats, resulting in corticosterone irregularities, which is a sign of HPA axis dysfunction associated with early life stress in humans. This experiment explores the relationship between anxiety, stress, and ethanol-dependence in a female rat animal model in chronic social instability. It is hypothesized that female Long-Evans rats placed in chronic social instability in adolescence (Postnatal day (PND) 30-46) will show greater anxiety-like behavior and will have greater ethanol intake than control groups.

METHODS

Subjects

32 female Long Evans rats were used in this study, acquired from Envigo Laboratories (Indianapolis, IN) post-weaning (~40 grams upon arrival, approximately PND 21). Rats were given nine days to acclimate to the laboratory after arrival before testing. Rats were maintained in the same room for the duration of the study, and cage bedding was changed weekly and food and water was always available *ad libitum*. Rats were weighed at the time of cage change and before behavioral procedures. All animal care procedures were in accordance with the NIH Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) and approved by the University of Dayton Institutional Animal Care and Use Committee (protocol #014-03-A1).

Procedural Outline

A timeline of the complete experimental design is included (Figure 1). Following 16 days of the chronic social instability housing procedure, subjects were housed in social isolation for the remainder of the study. The rats were tested for anxiety-like behavior on the elevated plus maze at PND 49. Dependent measures in the elevated plus maze included time spent in open arms and closed arm entries. Blood was then taken via tail nick for measurement of plasma corticosterone level (PND 51). Following these procedures, groups began four week ethanol self-administration (detailed below) at PND 57, after which blood was taken again via tail nick for a second measure of plasma corticosterone level.

Stress Paradigms:

Chronic social instability stress

Four experimental groups existed in this study. Groups included, 1. Control: isolation (Iso): rats raised 1/cage and handled only for routine maintenance; 2. Acute social instability stress (aSI): rats raised 2/cage and exposed to isolation stress once for 1 hour followed by re-pairing with a novel partner for 24 hours only on the last day; 3. Social stability (SS): rats raised 2/cage and handled only for routine maintenance. In the fourth group (group of greatest interest), Chronic Social Instability (CSI), rats received a new cage partner systematically. Rats in the chronic social instability group, and, for the final day, the acute social instability group, were put in social isolation for 1 hour in a standard laboratory cage, and then put into a novel cage with a novel rat for the remainder of the 24 hour period (in a standard sized laboratory cage). Though rats were exposed to each rat of its group three or four times (due to N value), it was never on consecutive days. This procedure was repeated 16 times for Chronic Social Instability rats (McCormick et al., 2008). Following 16 days of the procedure, all rats were single housed on day 17 for the duration of the studies.

General Behavioral Measures

Elevated plus maze

The elevated plus maze is a widely used and well-validated model in preclinical research to measure anxiety-like behavior in rats and mice (Pellow et al., 1985). A standard apparatus is elevated 72.4 cm from the floor with four radial arms (10.2 × 50.8 cm) of which two opposing arms are enclosed by walls (40.6 cm high; apparatus built by UD Engineering Dept.) and the other two arms are open. Animal behavior was recorded using

EthoVision Tracking software (Noldus Information Technology, Wageningen, Netherlands). Subjects were placed at the central junction, facing an open arm, and activity was recorded for 5 min. Anxiety-like behavior was assessed as the time spent on the open arms, and total number of entries into the closed arms is used as a measure of general locomotor activity (Holmes and Rodger, 1999). That is, rats generally prefer dark, enclosed spaces over open, brightly lit spaces (Butler et al., 2014a, 2014b; Chappell et al. 2013; McCool and Chappell 2009; Silberman et al., 2010), so less time on the open arms is indicative of greater anxiety-like behavior.

Homecage two-bottle choice, intermittent access, ethanol self-administration

To accurately measure ethanol consumption of each individual rat, rats were single housed for the duration of ethanol self-administration studies. Ethanol self-administration was conducted using a homecage intermittent access, two-bottle choice design (Chappell & Weiner, 2008; Simms et al., 2008; Wise, 1973), which has been shown to engender behaviorally/pharmacologically relevant blood ethanol levels in Long Evans rats (approx. 40 mg/dl in chronically stressed male rats, Chappell et al., 2013). In this model, rodents are given two bottles in their home cage, one containing 20% ethanol (190 proof ethanol diluted in water) and one containing water, on Mondays, Wednesdays, and Fridays. Water and ethanol consumption are measured after 30min and 24hours of access to ethanol by weighing bottles before putting them on the cages and at each time point. Ethanol intake (g/kg) and an ethanol preference ratio (ethanol drank/total fluid intake) was calculated at each time point. Water and ethanol were given in graduated drinking tubes, and the position of the bottles was alternated on each drinking day to control for potential side preferences. Rats were maintained on this schedule for 4 weeks. Rats were given free *ad libitum* access

to food throughout the drinking paradigm and were weighed at the beginning of each ethanol drinking day.

Blood collection via tail nick and corticosterone measurements

Blood was collected for testing of corticosterone levels, a possible sign of HPA axis dysfunction correlated with stress (Zimmermann et al., 2004). Each animal is placed on a clean bench top and lightly restrained while the tail tip is snipped for collection of blood from the lateral tail vein in a microhematocrit tube. Two-three microhematocrit tubes/animal were taken, equaling approximately 50-75µL. The entire procedure takes approximately 1-2 minutes. The tail nick procedure is commonly employed in studies measuring ethanol intake and stress hormone levels (e.g., Butler et al., 2014; Hayes et al., 2013; Sharrett-Field et al., 2013). Blood was spun in a centrifuge, plasma was extracted, and plasma was stored at -80°C until use in the corticosterone assay. Bloods were taken following the EPM (Time point 1) and ethanol drinking paradigm (Time point 2). CORT was measured using a competitive enzyme immunoassay containing a polyclonal CORT antibody (Immunodiagnostic Systems, Scottsdale, AZ). Briefly, CORT from the samples and standards compete with enzyme-labeled CORT for binding in a 96-well plate. The enzyme-labeled CORT reacts with a chromogenic substrate, creating color that is inversely proportional to the CORT concentration of the sample. Color intensity was detected using a microplate reader (BioTek Synergy 4), with absorbance measured at 450nm. CORT concentrations were derived from a standard curve fit to a four parametric logistic equation. This assay required a 1:20 dilution of plasma samples.

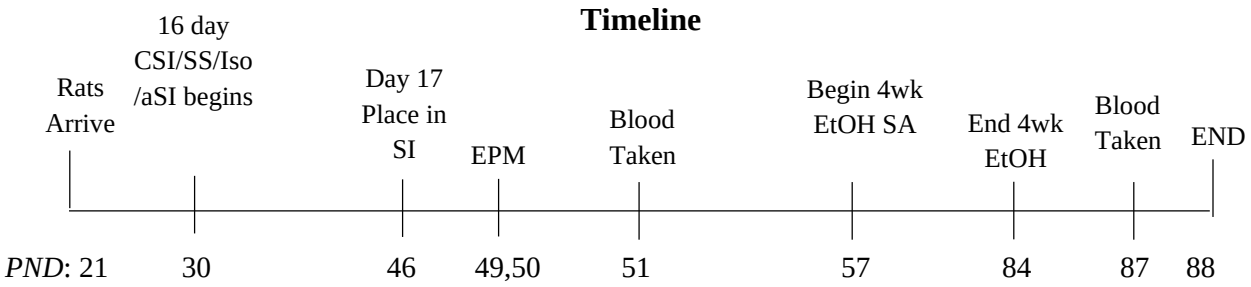

Figure 1. Experimental Design. aSI, acute social instability; CSI, chronic social isolation; EPM, elevated plus maze; EtOH, alcohol; Iso, isolation; PND, post natal day; SS, social stability; SA, self-administration.

Statistics

Data for anxiety-like behavior on the EPM was measured using a one-way ANOVA to compare the four groups' time on the open arms and number of closed arm entries. CORT data was analyzed using a one-way ANOVA to compare differences across groups in levels of CORT, with Tukey's multiple comparison post-hoc tests interpreted when appropriate. EtOH drinking and preference data were analyzed using repeated measures two-way ANOVAs. All analyses were set at significance level of $p < 0.05$.

Results

Elevated Plus Maze

Following the 16 day housing procedure, anxiety-like behavior was assessed on the EPM. On the EPM, CSI, SI, GH, and ASI did not differ on time spent on open arms (Fig. 2a.); that is, a one-way ANOVA conducted for open arm time indicated no differences across groups ($F(3, 28) = 0.484$, $p = 0.696$). Closed arm frequency, a measure

of locomotion, showed no difference across groups ($F(3, 28) = .0305$, $p = 0.821$), suggesting no difference in overall locomotion (Fig. 2b).

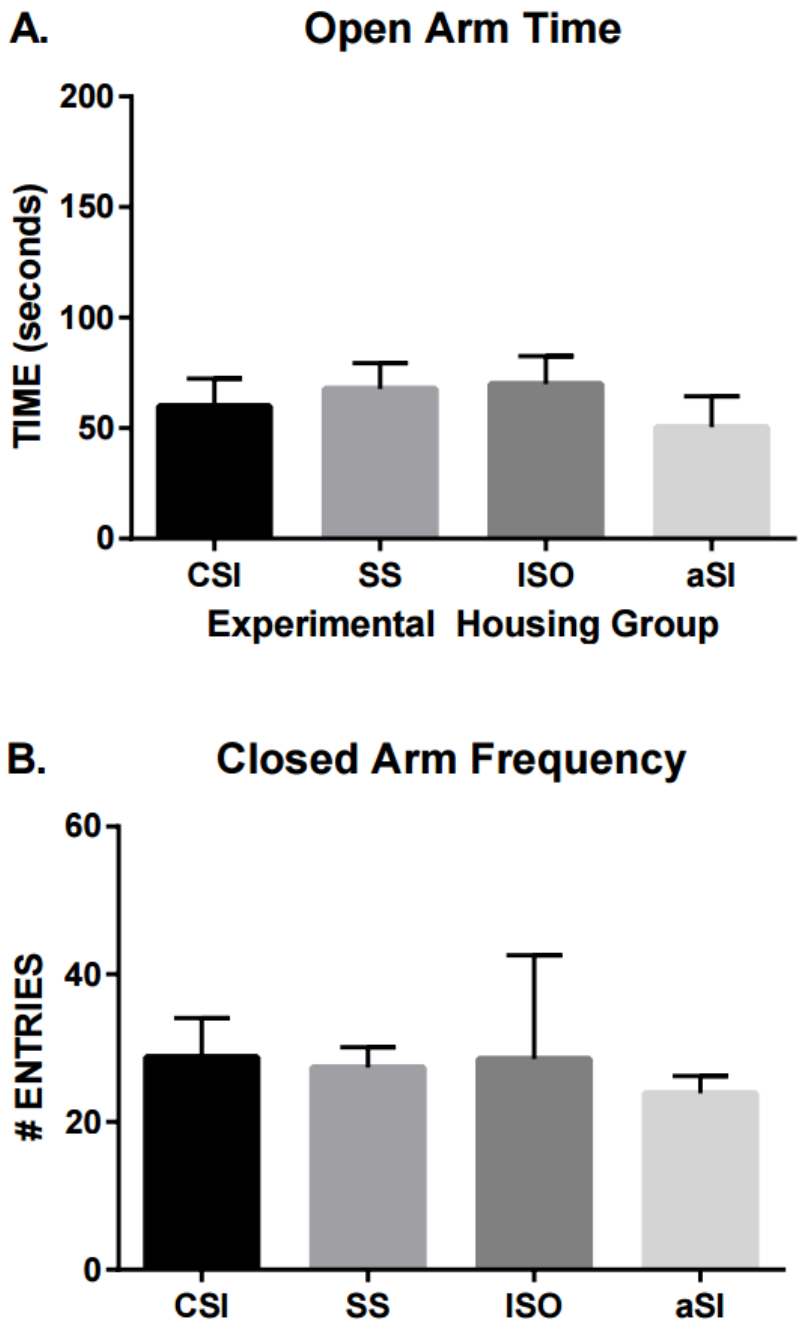

Figure 2. Anxiety-like behavior on Elevated Plus Maze (EPM). There was no significant difference in anxiety-like behavior (Fig. 2a) or general locomotion (Fig. 2b) on the EPM.

CORT Measurements

Following behavioral testing (Time point 1) and following ethanol drinking (Time point 2), blood was taken via tail nick to obtain plasma for CORT measurements. For time point 1, a one-way ANOVA indicated a significant difference across groups in plasma level of CORT ($F(3, 28) = 3.971$, $p = 0.0178$). Tukey's multiple comparisons post hoc tests showed that ISO CORT levels were significantly lower compared to the CSI group ($p < 0.05$) and the SS group ($p < 0.05$) (Fig. 3). For time point 2, following the EtOH drinking paradigm, a one-way ANOVA showed that there was no significant difference across groups in plasma level of CORT ($F(3, 27) = 0.3251$, $p = 0.807$) (Fig. 4).

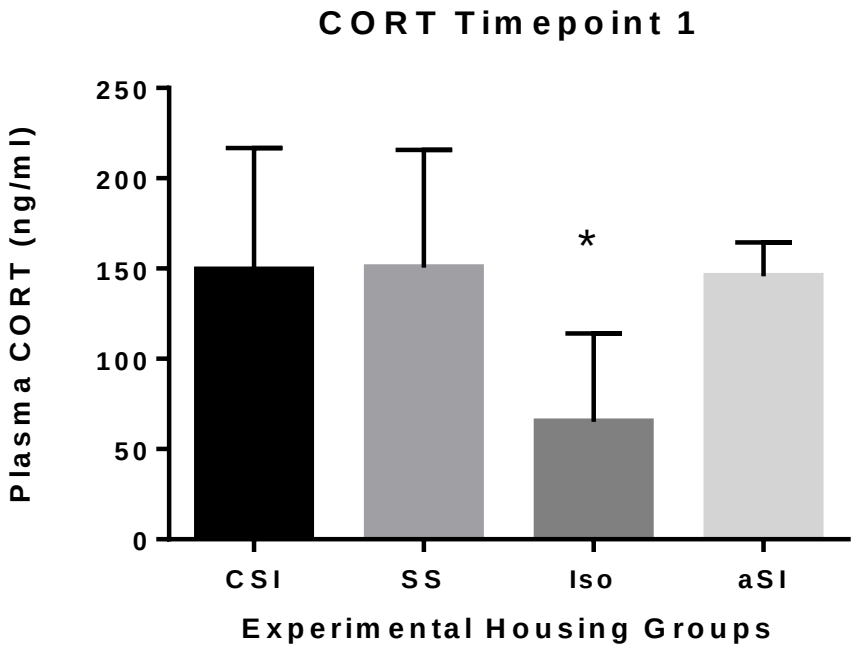

Figure 3. Plasma CORT levels. A one-way ANOVA indicated a significant difference across groups in plasma CORT level. Tukey posthoc tests showed that ISO CORT levels were significantly lower compared to the CSI and SS groups (*$p<0.05$).

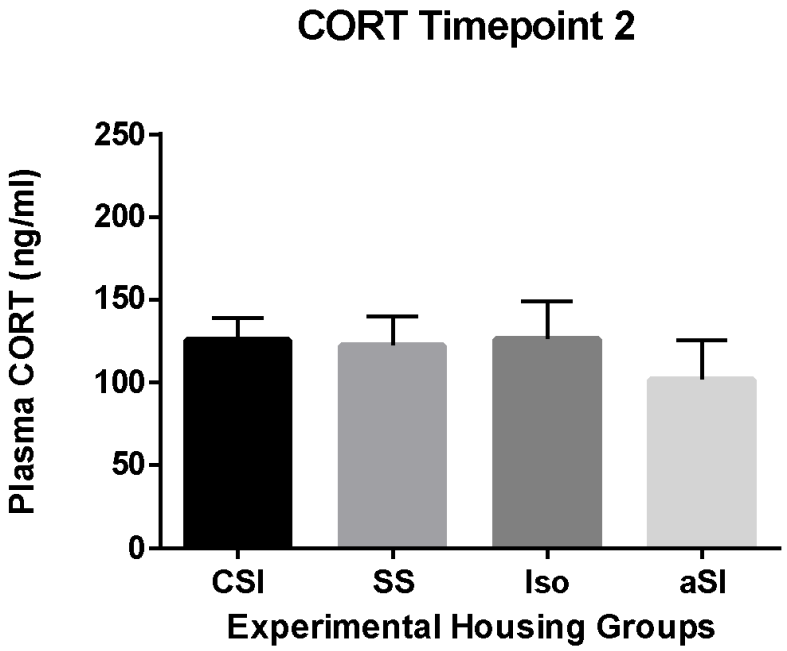

Figure 4. Plasma CORT levels at time point 2. A one-way ANOVA shows that there was no significant difference across groups in plasma level of CORT.

Home Cage Ethanol Drinking

Home Cage ethanol drinking began following behavioral testing and the first CORT testing. A RM 2-way ANOVA (group x day) showed no significant interaction, nor main effect of group for 30 minute intake ($F(33, 308) = 1.098$, $p = 0.3323$; $F(3, 28) = 0.2023$, $p = 0.8939$) (Fig. 5A). For 24 hour intake, there was no significant interaction nor main group effect ($F(33, 308) = 1.354$, $p = 0.0993$; $F(3, 28) = 0.5175$, $p = 0.6736$) (Fig. 5B). For 30 minute EtOH preference, there was no significant main effect of group ($F(2, 28) = 1.358$, $p = 0.0974$), and no significant interaction ($F(33, 308) = 1.216$, $p = 0.3221$) (Fig. 5C). For 24 hour preference, there was no significant interaction ($F(33, 308) = 0.8783$, $p = 0.6632$) or group effect ($F(3, 28) = 2.345$, $p = 0.0944$) (Fig. 5D).

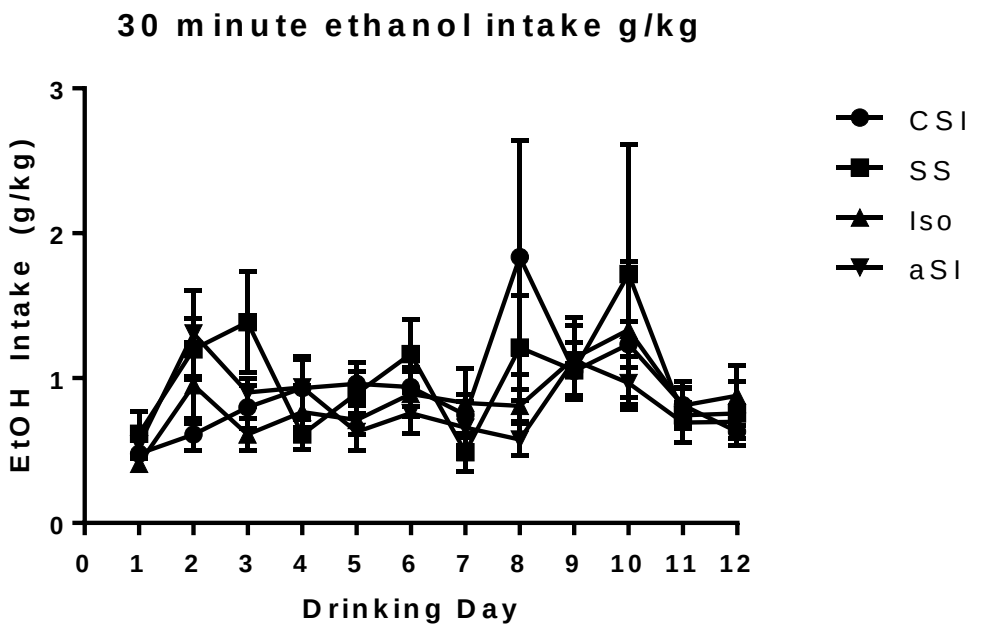

Figure 5A.

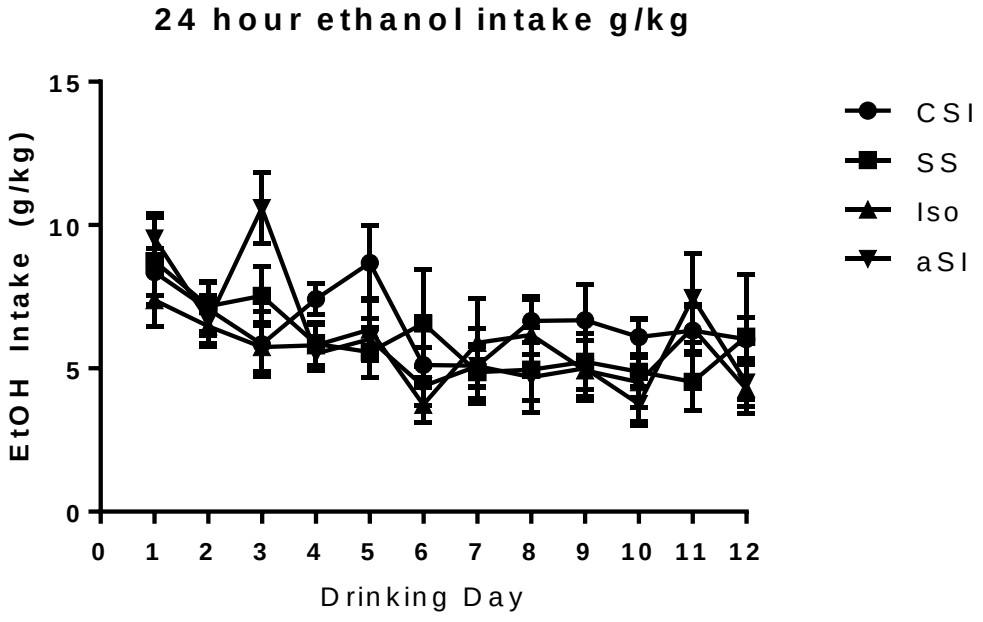

Figure 5B.

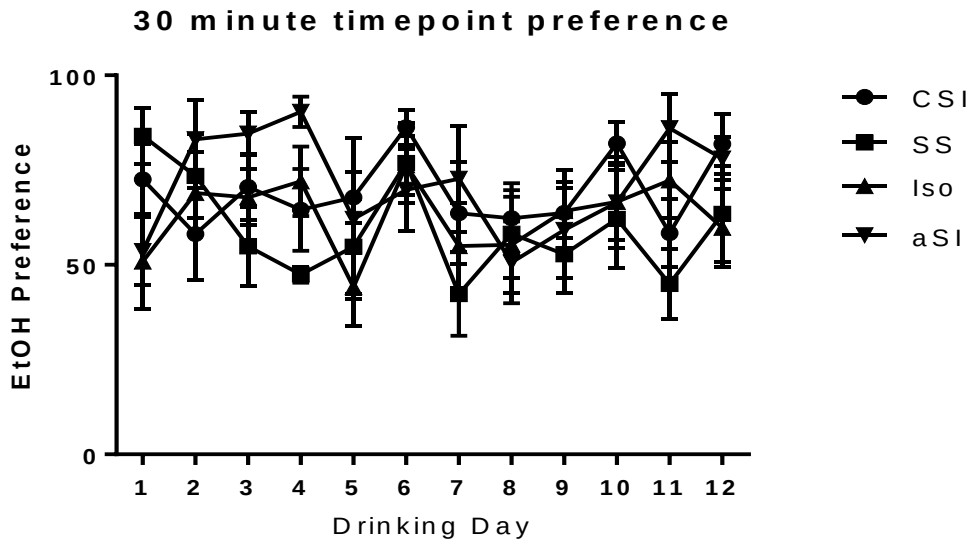

Figure 5C.

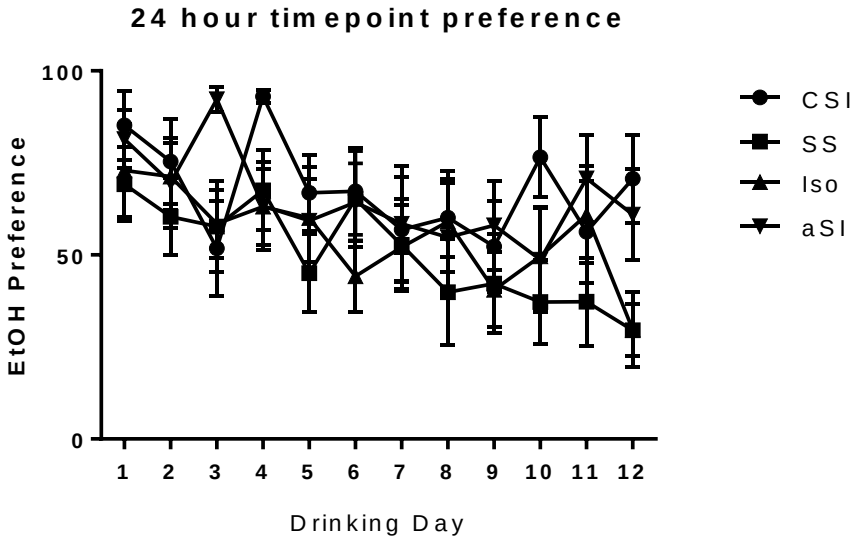

Figure 5D.

Figure 5A-D. Ethanol intake and preference. For each dataset A-D, a two-way RM ANOVA revealed no significant interaction or main effect of group for intake and preference measures at the 30 minute and 24 hour time points. Data presented as mean +/- SEM.

Discussion

The current data suggests that chronic social instability (CSI) does not produce a significant increase in anxiety-like behaviors and EtOH drinking in female rats, bringing into question the chronic social instability model for females as a model of early life stress that elicits increased EtOH drinking and anxiety-like behavior later in life. While chronic social instability has produced an increase in anxiety-like behavior and EtOH drinking in males in this lab previously, and produced a female stress response in other studies (McCormick et al. 2008), this model failed to produce changes in anxiety-like behavior or EtOH intake or preference in females. Finding a female model is vital, as preclinical data is necessary to develop new treatments for humans. More studies are finding that male and female rats have different physiological, behavioral, and neurobiological responses to stress (Balog et al. 2015; Lu et al. 2015), as do male and female humans. (Bao and Swaab 2011; Desantis et al. 2011; Goldstein et al., 2010; Teicher et al.; Wang et al., 2007). For example, hormonal levels, such as testosterone and estrogen, have sex specific impacts on the serotonergic system, and women and men have significant differences in neural activity in response to stress (Wang et al., 2007; Robichaud et al., 2006; Hiroi et al, 2006). Though a reproducible female model of early life stress engendering increased EtOH consumption and anxiety-like behavior has yet to be found, the data of the current study may be utilized to further study and understand possible female models that produce anxiety-like and addiction-like behavior in response to early life stress.

The chronic social instability model was utilized mainly due to data collected by McCormick et al. (2004), which showed that female rats under CSI had greater

locomotor sensitization to nicotine compared to non-stressed controls and male rats under CSI. Enhanced locomotor sensitization is an accepted indicator of enhanced drug abuse vulnerability (Abrahao et al., 2013), leading to the hypothesis that CSI could provoke an addiction-like response to ethanol. McCormick et al. (2008) also demonstrated that male and female rats both experienced anxiety-like behavior in response to CSI, though females in estrous may express reduced anxiety-like behavior. Other studies support the CSI paradigm as a stressor for female rats as well, in that female rats can be remain under CSI without habituating and have elevated CORT levels (Haller et al. 1999; Herzog et al. 2009). Thus, CSI was adapted and slightly modified in the current study to examine it as a possible paradigm that engenders addiction-like behaviors for ethanol. CSI was applied during PND 30-46, an adolescent time point in female Long Evans rats (Crews et al., 2007). Further, the ISO experimental group and ethanol self-administration paradigm was added. It is unclear what variables may have produced differing results. Differences between McCormick et al. (2004; 2008) and the current study include that McCormick et al. utilized stimulant drugs that were not self-administered, conducted a vaginal swab measure of the estrous cycle, measured CORT after confinement to the open arm of the EPM, and measured anxiety-like behavior on the EPM at a slightly different time point. These factors may bridge the gap between the varying results, in that timing of CORT measurements for females may be vital, and that the administration and type of drug may have an influence on the results.

CORT levels were taken as a measure of HPA axis activity. During time point one, but not time point two, female rats in the ISO group had significantly lower levels of CORT. The importance of this result is unclear, but it's important to note that we

measured plasma CORT at a single moment. While a single measure such as this may be beneficial, another approach such as a dexamethasone suppression test may provide a wider scope of measurement of HPA axis function. In addition, human studies demonstrate that women also appear to have less correlation between CORT levels and limbic activity (Wang et al., 2007), which may translate to animal models and affect the validity of a single CORT measurement as an index of stressor effect in female animal models as well.

In regard to ethanol drinking, there were no group differences for ethanol intake or preference. There was high preference and intake, however, with the g/kg values over 24 hours equating with behaviorally relevant blood ethanol concentrations (Chappell et al., 2013; Simms et al. 2008). A model of addiction is needed, however, in which early life stress motivates female rats to drink with higher intake and/or preference than others. Such a model would allow better research on the effect of early life stress on ethanol consumption and preclinical testing of pharmacological drugs, such as anxiolytics, which can be applied to humans.

The elevated plus maze is a validated paradigm for male rats indicative of anxiety-like behavior. Anxiolytic drugs have been shown to reliably increase time on the open arms of the EPM in male rodents, and anxiogenic drugs are shown to reliably decrease time on the open arms (Pellow et al., 1985; Troelsen et al., 2005). However, the effects of anxiolytic drugs on the EPM for female rats are not consistent (Ravenelle et al., 2014; Simpson et al., 2012), with some studies pointing towards the effects of the estrous cycle on anxiety-like behavior. Levels of estrogen and progesterone, which vary at

different levels of the estrous cycle, may or may not change how female rats behave on the elevated plus maze (Marcondes et al., 2001; Molina-Hernandez et al., 2013).

For male rats, several models of chronic stress during adolescence demonstrate reproducibility and consistency, in that they continually produce anxiety-like behavior and addiction-like behavior (Butler et al., 2014b; Chappell and Weiner, 2008; Möller, 1997). That reliable models have not been found in female rats indicates physiological and neurobiological differences that impact the effects of stress on males and females. In understanding the differences between male and female preclinical models, the influence of the estrous cycle offers one possible explanation. However, collective data on the influence of the estrous cycle is inconclusive. HPA axis activity is known to be higher in proestrous (Carey et al., 1995), and diestrous females are known to have lower corticotropin releasing hormone gene expression (Patchev et al., 1995). However, while some studies have found that the estrous cycle may regulate and reduce anxiety-like behavior (McCormick, 2007), other data have demonstrated that ethanol drinking patterns may vary across the estrous cycle without changing total ethanol (g/kg) intake (Ford et al., 2002b) and that ethanol intake in an operant self-administration paradigm is similar across the estrous cycle (Roberts et al., 1998), indicating a lack of connection between the estrous cycle and ethanol drinking. While certain studies suggest a connection between estrous stage and anxiety-like behavior (Bitran & Dowd, 1996; Mora et al., 1996), others indicate that estrous cycle does not affect anxiety-like behavior on the elevated plus maze (Bitran et al., 1991; Nomikos & Spyraki, 1988). Overall, it cannot be concluded that the estrous cycle has a uniform effect on anxiety-like or overall ethanol drinking behavior in female rats, but is something that requires further study. The estrous

cycle was not assessed in this study, and further research would be needed to evaluate if the estrous cycle impacts CSI results.

Overall, these results support that a different model is needed to induce an ethanol addiction-like phenotype in female Long Evans rats, in which early life stress elicits increased anxiety-like behavior and ethanol drinking later in life. The profile of responses in females was not similar to that observed in males in a separate study of this lab, suggesting that female and male rats may respond differently (behaviorally and physiologicall) to stressors than males, comparable to differences seen in female and male humans. By addressing the lack of a replicable model of an early life stressor that causes addiction-like phenotype later in life, further preclinical studies may use such a model to study possible treatments and variables that influence addiction-like and anxiety-like behavior. Future studies may increase understanding of how female behavior and physiology differs from males in response to stress.

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