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Palatable food reduces anxiety-like behaviors and HPA axis responses to stress in female rats in an estrous-cycle specific manner

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Abstract

Eating tasty foods dampens responses to stress – an idea reflected in the colloquial term ‘comfort foods’. To study the neurobiological mechanisms by which palatable foods provide stress relief, we previously characterized a limited sucrose intake (LSI) paradigm in which male rats are given twice-daily access to 4 ml of 30% sucrose solution (vs. water as a control), and subsequently have reduced hypothalamic-pituitary-adrenocortical (HPA) axis responsivity and anxiety-related behaviors. Importantly, women may be more prone to ‘comfort feeding’ than men, and this may vary across the menstrual cycle, suggesting the potential for important sex and estrous cycle differences. In support of this idea, LSI reduces HPA axis responses in female rats during the proestrus/estrus (P/E), as opposed to the diestrus 1/diestrus 2 (D1/D2) estrous cycle stage. However, the effect of LSI on anxiety-related behaviors in females remains unknown. Here we show that LSI reduced stress-related behaviors in female rats in the elevated plus-maze and restraint tests, but not in the open field test, though only during P/E. LSI also decreased the HPA axis stress response primarily during P/E, consistent with prior findings. Finally, cFos immunolabeling (a marker of neuronal activation) revealed that LSI increased post-restraint cFos in the central amygdala medial subdivision (CeM) and the bed nucleus of the stria terminalis posterior subnuclei (BSTp) exclusively during P/E. These results suggest that in female rats, palatable food reduces both behavioral and neuroendocrine stress responses in an estrous-cycle dependent manner, and the CeM and BSTp are implicated as potential mediators of these effects.

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Keywords

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INTRODUCTION

The overall incidence of obesity among adults in the United States was 36% in 2014, and the Center for Disease Control reports the prevalence of obesity is slightly higher in women than men, affecting 38.3% and 34.3% of adult women and men, respectively (Flegal et al., 2012; Ogden et al., 2015). Daily life stress may be one contributor to these high obesity rates. In 40–70% of people, stress can increase food intake (Epel et al., 2004; Oliver and Wardle, 1999; Weinstein et al., 1997), particularly the intake of highly-palatable, calorically-dense foods that are often high in carbohydrates and/or fat (Cartwright et al., 2003; Epel et al., 2001; Groesz et al., 2012; Kim et al., 2013; Laugero et al., 2011; Oliver and Wardle, 1999; Zellner et al., 2006). These foods may be chosen for their ability to decrease feelings of stress or negative mood, an idea commonly thought of as 'comfort food.' In support of this idea, consumption of palatable foods can reduce stress responses both in humans (Anderson et al., 1987; Dube et al., 2005; Gibson, 2006; Macht and Mueller, 2007; Markus et al., 2000; Tomiyama et al., 2011; Tryon et al., 2013) and rodents (Bell et al., 2002; Dallman et al., 2005; Finger et al., 2011; Kinzig et al., 2008; la Fleur et al., 2005; Maniam and Morris, 2010; Pecoraro et al., 2004; Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007).

To investigate the mechanisms by which these highly-palatable 'comfort foods' can reduce stress responses, our lab has developed a limited sucrose intake (LSI) paradigm in rats (Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007). In this paradigm, adult, male rats with *ad libitum* access to standard chow are given twice-daily brief (up to 30 minutes (min)), limited (up to 4 ml/session; or 8 ml/day) access to a 30% sucrose solution (vs. water as a control). A history of LSI reduces numerous stress responses in male rats, including HPA axis responses to an acute stress, stress-induced tachycardia, and anxiety-like behavior (Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007).

Reports suggest that women may be more prone to 'comfort food' style eating than men (Grunberg and Straub, 1992; Klein et al., 2004; Oliver and Wardle, 1999; Oliver et al., 2000; Wansink et al., 2003; Wardle et al., 2000; Zellner et al., 2006), and this may vary across the menstrual cycle (Hildebrandt et al., 2015; Klump et al., 2013a; Klump et al., 2013b; Racine et al., 2013). Therefore, the effects of palatable food to reduce stress may differ between men and women, which could have important implications for prevention or treatment of obesity and its related metabolic disorders. While our LSI paradigm was initially developed and characterized in male rats, studying its effects in female rats could provide important insights into sex differences in palatable food-mediated stress relief. Indeed, an initial study of LSI in female rats showed that, while females have a similar metabolic response to the LSI paradigm as males, the ability of LSI to blunt the HPA axis stress response in females depends on estrous cycle stage (Egan et al., 2018). More specifically, LSI reduced HPA reactivity primarily during the proestrus/estrus (P/E) stage of their estrous cycle.

Importantly, altered HPA axis regulation is often accompanied by changes in mood and behavior (Gold, 2015; Jacobson, 2014; Packard et al., 2016; Spiga et al., 2014; Strohle and Holsboer, 2003). For example, LSI decreases HPA axis reactivity in male rats, in addition to reducing indices of anxiety-like behaviors in the elevated plus-maze, social interaction, and open field tests (Ulrich-Lai et al., 2010). This suggests that LSI-mediated HPA dampening may be concomitant with reduced anxiety-related behaviors in female rats. As LSI-induced HPA blunting primarily occurs during P/E, and anxiety-related behaviors can also vary throughout the estrous cycle (Frye et al., 2000; Frye and Walf, 2002; Galeeva and Tuohimaa, 2001; Gray and Cooney, 1982; Marcondes et al., 2001; Meziane et al., 2007; Mora et al., 1996; Pare and Redei, 1993; Walf et al., 2009), the present studies therefore test the hypothesis that LSI attenuates anxiety-related behaviors in females exclusively during P/E. Behavior was tested in the open field and elevated plus-maze – two widely-used tests of anxiety-related behaviors (Cruz et al., 1994; File et al., 2004; McCarthy et al., 1995; Pellow et al., 1985; Prut and Belzung, 2003; Ulrich-Lai et al., 2010; Walsh and Cummins, 1976). Struggling behavior was also assessed during a restraint test. Evidence indicates that the amount of struggling during restraint correlates with the degree of HPA activation (Grissom et al., 2008; Weinberg et al., 2010), and that the anxiolytic drug diazepam decreases behavioral struggling (Ushijima et al., 1986), suggesting the restraint test may be a useful approach to simultaneously measure stress-related behavior and HPA axis activation. Finally, the effect of LSI on post-restraint cFos immunolabeling, a common indirect marker of recent neuronal activation (Chan et al., 1993; Cullinan et al., 1995; Curran and Morgan, 1995; Kovacs and Sawchenko, 1996; Ulrich-Lai et al., 2007), was determined in multiple stress-activated brain regions to identify those that may contribute to the cycle-dependent anxiolytic and HPA-blunting effects of LSI.

MATERIALS AND METHODS

Animals

Adult, female Long-Evans rats weighing approximately 150–175 g (~7 weeks of age at time of arrival) were acquired from Harlan Laboratories (Indianapolis, IN). Rats were individually housed in a temperature- and humidity-controlled environment with a 12 hour (6:00–18:00) light-dark cycle. Rats were given *ad libitum* access to standard rat chow (LM-485; Harlan Teklad, Madison, WI) and water throughout the experiment. All animals were acclimated to the housing facilities for at least one week before beginning experimental protocols. Housing facilities were accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). All protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee (IACUC) and are consistent with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. To evaluate the overall effects of LSI on energy balance, food intake and body weight were monitored throughout the experiment, and body composition was measured with nuclear magnetic resonance (NMR, EchoMRI, Houston, TX) on experiment days 0 and 22. We have previously seen that both male (Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007) and female (Egan et al., 2018) LSI rats eat slightly less chow, presumably to compensate for the calories provided by the sucrose, and as a result LSI does not alter body weight and percent body fat.

Experimental overview

A schematic of the experimental timeline is shown in Figure 1. The LSI paradigm was performed as previously described (Christiansen et al., 2011; Egan et al., 2018; Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2016; Ulrich-Lai et al., 2011; Ulrich-Lai et al., 2007). Briefly, rats with free access to food and water were given additional twice-daily (approximately 8:30 h and 15:30 h), brief (up to 30 min), limited (up to 4 ml per session; maximum of 8 ml per day) access to a second drink bottle containing 30% sucrose solution (MP Biomedical, Solon, OH), or water as a control. Rats underwent the LSI paradigm for a total of 23 days, and received behavioral testing (as detailed below) in the open field test (OFT) and elevated plus-maze (EPM) on days 12 and 16 of LSI exposure, respectively. The OFT and EPM were performed midday between 10:00 h and 14:30 h, so as not to overlap with the times of LSI exposure. Rats also received a restraint stress (as detailed below) on the morning following the last LSI exposure (experiment day 24). Vaginal lavage was performed on each animal immediately following the OFT (day 12), EPM (day 16) and restraint (day 24) tests to determine estrous cycle stage as described below.

Open field test (OFT)

On experiment day 12, rats were individually placed into an open field apparatus (~1 m x ~1 m) for 5 min under dim light, and their behavior was video recorded for later analysis. Clever TopScan (CleverSys Inc., Reston, VA) software was used to determine the time spent in the center of the open field and total distance traveled. Grooming and rearing behaviors were scored by two observers unaware of the treatment groups, and the observers' scores were averaged to obtain the mean value of each behavior for each rat. The values between the observers were typically within ~10% of each other. In rare instances, larger discrepancies occurred between the two observers' scores, in which case the video for that rat was rescored to reach a consensus.

Elevated plus-maze (EPM)

On experiment day 16, rats were individually placed into the center of an EPM apparatus (approximate arm size of 4 in. wide x 40 in. long, 14-in. high walls on the closed arms) in a dark room under red lights, and were allowed to explore the maze for 5 minutes. The resulting behavior was video recorded for later analysis. Clever TopScan (CleverSys Inc., Reston, VA) software was used to measure the total time spent in the open arms, the total number of entries into the open arms, the total number of entries into all arms, and the total distance traveled. Head dipping behavior, defined as any time the animal moved its head off the EPM apparatus and looked over the edge of an open arm (Cruz et al., 1994), was scored by two observers unaware of treatment groups and the average of these scores was taken as the value for each rat. The observers' values were typically within ~10% of each other. In the rare instance when larger discrepancies occurred, the video for that rat was rescored to reach consensus. Moreover, behavioral patterns in the EPM are known to vary across the duration of the test, with most open arm activity occurring during the first 1–2 minutes of the test (Arabo et al., 2014; Bertoglio and Carobrez, 2002; Carobrez and Bertoglio, 2005; Casarrubea et al., 2013; Holmes and Rodgers, 1998; Rodgers et al., 1996). Thus, to consider

the temporal dynamics of each rat's response to the test, behaviors were grouped into consecutive 1-min bins for analysis.

Restraint stress for behavioral struggling, HPA reactivity, and brain cFos responses

On the morning of experiment day 24, rats were given a 20-minute restraint stress challenge, and did not receive LSI on that day. Prior to restraint testing, rats were left undisturbed in their home cages in their normal housing room. The home cage for each rat was then individually carried to an adjacent procedure room, where each rat was placed into a clear, well-ventilated plastic restraint tube (i.e., a cylinder that is approximately 20 cm long, and 7 cm in diameter) with rapid collection of tail-clip blood samples (200 μl) into ice-cold EDTA-coated tubes. Care was taken to ensure this first blood collection (i.e., at 0 min) was completed within 3 min of first touching/moving each rat's home cage, thereby ensuring assessment of basal, pre-stress hormone levels (Vahl et al., 2005). Rat behavior while in the restraint tube was video recorded for later analysis. Blood samples were again collected at 20, 40, 60, and 90 min after the onset of restraint, with each sample collection being completed within 3 min. Note that in our previous experiment assessing the impact of LSI on HPA reactivity to restraint stress in female rats (Egan et al., 2018), blood samples were collected through 60 min after stress onset, as we routinely use for analogous studies in male rats (Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2011). In that experiment, LSI reduced the plasma adrenocorticotropic hormone (ACTH) response at the 60-min time point, but did not impact the corresponding plasma corticosterone levels. However, this did not preclude the possibility that plasma corticosterone may have been affected at later post-stress time points, as there is an inevitable time-lag between changes in plasma ACTH and subsequent changes in plasma corticosterone. In the present experiment, therefore, post-stress blood collection sampling was extended to 90 min after stress onset to increase the opportunity to detect potential changes in plasma corticosterone in female rats. Blood samples were centrifuged (3000 x *g*, 15 min, 4°C) and plasma was stored at −80°C until measurement of plasma ACTH and corticosterone by radioimmunoassay (RIA). Struggling behaviors, defined as any behaviors that represent the animal trying to make or find a way out of the restrainer (e.g., biting, chewing, scratching or pushing at the walls of the restrainer, and/or attempting to turn or spin around in the restrainer (Grissom et al., 2008; Weinberg et al., 2010)), was scored by two observers unaware of the treatment groups. The values between observers were typically within 10% of each other, and in the rare instance in which larger discrepancies occurred, the video for that rat was rescored to reach a consensus. Since struggling behavior varies across the duration of the restraint, with most vigorous struggling occurring during the first 5 min of the test (Grissom et al., 2008; Weinberg et al., 2010), data were grouped into consecutive 5-min bins for analysis.

Immediately after the 90-min time point blood collection, rats were injected with an overdose of pentobarbital and vaginal lavage samples were taken to determine estrous cycle stage. Next, a cardiac blood sample was quickly collected and resulting plasma was stored at − 80°C until later measurement of plasma estradiol by RIA. Rats were then transcardially perfused with 0.9% saline followed by 3.7% paraformaldehyde for collection of brains for cFos immunolabeling. The 90-min post-stress time point was selected for brain collection as it approximates the peak of the cFos protein response to acute stress (Kovacs and

Sawchenko, 1996; Sonnenberg et al., 1989). Brains were post-fixed in 3.7% paraformaldehyde overnight at room temperature, and were then stored in 30% sucrose in phosphate-buffered saline (PBS) at 4°C. Brains were sectioned (25 μm) in a 1-in-12 series on a freezing-stage microtome (Leica Biosystems, Wetzler, Germany) and slices were stored in cryoprotectant (PBS with 1% polyvinylpyrrolidone (PVP-40, Sigma Chemical, Perth, WA), 30% ethylene glycol (Fisher Scientific, Pittsburgh, PA) and 30% sucrose (Amaresco, Solon, OH)) at −20°C until immunolabeling for cFos protein expression.

Assessment of cFos immunolabeling in stress-related brain regions

Immunohistochemistry was performed as previously described (Ryan et al., 2018). Sections were washed in 50 mM potassium phosphate-buffered saline (KPBS), incubated in a 20% hydrogen peroxide solution for 20 min, and rinsed again in KPBS. Sections were then blocked in a solution of 0.1% bovine serum albumin (BSA, Sigma-Aldrich) with 0.2% Triton X-100 (Sigma-Aldrich) in KPBS for 1 hr at room temperature, and incubated overnight at 4°C with primary antibody (primary rabbit antisera against cFos, 1:200, product # sc-52, Santa Cruz, Dallas, TX) diluted in blocking solution. The following morning, sections were rinsed in KPBS, incubated in biotinylated goat anti-rabbit secondary antibody (1:500, Vector Laboratories, Burlingame, CA) for 1 h, rinsed in KPBS, and incubated in avidin-biotin-peroxidase (Vectastain ABC solution, Vector Laboratories, Burlingame, CA) for 1 hr. Following another rinse in KPBS, sections were reacted with 3,3'-diaminobenzidine (Sigma Chemical, Perth, WA). Immunolabeling for cFos was visualized using brightfield light microscopy on an Axio Imager.M2 microscope, with an AxioCam camera, and using Zen 2012 software (Carl Zeiss Microscopy, Jena, Germany).

The density of cFos-positive cells was measured bilaterally in all available, intact sections that contained the regions of interest, as defined by standard rat brain atlases (Paxinos and Watson, 1998; Swanson, 1998), using Scion Image software (Scion Corp, Frederick, MD). Immunolabeling for cFos was assessed in the following regions: basolateral amygdala (BLA); bed nucleus of the stria terminalis, posterior subnuclei (BSTp) (principal, transverse, and interfascicular); central amygdala (CeA), medial (CeM) and lateral (CeL) subdivisions; dorsomedial hypothalamus (DMH), dorsal and ventral subdivisions; medial amygdala, posterodorsal nucleus (MeApd); nucleus accumbens (NAc),core and shell subdivisions; prefrontal cortex (PFC), prelimbic (PL), infralimbic (IL) and anterior cingulate (Ant Cing) subdivisions; and paraventricular nucleus of the hypothalamus (PVN). All analyses were performed by an observer unaware of the treatment groups.

Hormone assays

Plasma ACTH levels were measured via RIA using a specific antiserum generously donated by Dr. William Engeland (University of Minnesota, Minneapolis, MN) as described previously (Jasper and Engeland, 1991). The minimum detection of this assay is 15.6 pg/ml, and the intra-assay and inter-assay coefficients of variance are both 13%. Plasma corticosterone levels were measured via an ^{125}I corticosterone double antibody RIA kit (product #07120103, MP Biomedicals, Solon, OH). The minimum detection of this assay is 7.7 ng/ml, and the intra-assay and inter-assay coefficients of variance are both 7%, as indicated by the manufacturer. Plasma estradiol levels were measured via an ^{125}I 17B-

estradiol (E2) double antibody RIA kit (product # 07138102, MP Biomedicals, Solon, OH). The minimum detection of this assay is 10 pg/ml, and the intra-assay and inter-assay coefficients of variance are 5 and 9%, respectively, as indicated by the manufacturer.

Determination of estrous cycle stage by cytology

To determine the impact of estrous cycle on behavioral, HPA, and brain measures, vaginal lavage samples were taken at the end of each behavioral test (on experiment days 12 and 16), and again just prior to animal perfusion and tissue collection (on experiment day 24). Estrous cycle stage assessments were limited to the days of sample collection due to concerns that the stress associated with a daily lavage procedure could impact the primary experimental outcomes (i.e., stress-related behavioral and HPA axis reactivity, and brain activation patterns). Vaginal cytology was then used to determine estrous cycle stage (Becker et al., 2005; Cora et al., 2015; Goldman et al., 2007; Marcondes et al., 2002). More specifically, diestrus 1 (D1) cytology was characterized by large numbers of leukocytes, mucous, and smaller amounts of non-nucleated epithelial cells. Diestrus 2 (D2) was very similar to D1, but could be distinguished by having a predominance of leukocytes. In contrast, proestrus (P) and estrus (E) had predominant clusters of nucleated non-cornified and non-nucleated cornified epithelial cells, respectively, with little-to-no leukocytes and mucous. Late proestrus was defined as a transitional stage between P and E, in which large numbers of nucleated non-cornified epithelial cells were accompanied by the appearance of some non-nucleated cornified epithelial cells. Animals were then classified into two different estrous cycle sub-groups: 1) those in D1 or D2 (D1/D2), when females would be expected to have lower levels of gonadal hormones, and 2) those in proestrus, late proestrus, or estrus (P/E), when females would be expected to have higher levels of gonadal hormones (Asarian and Geary, 2013; Butcher et al., 1974; Cecchini et al., 1983; Lu et al., 1985; Nequin et al., 1979; Smith et al., 1975). These two estrous cycle sub-groups were then compared to each other in all analyses (see below) to test for potential cycle-specific effects.

Statistical analysis

Data are presented as mean ± SEM. Intake from the second drink bottle, food intake, body weight, and percent body fat data were each analyzed by two-way repeated measures ANOVA with the factors DRINK and TIME. OFT data were analyzed via two-way ANOVA with the factors DRINK and CYCLE. EPM, restraint struggling, plasma ACTH and plasma corticosterone data were each analyzed by three-way repeated measures ANOVA with the factors DRINK, CYCLE, and TIME. Plasma estradiol levels were analyzed by one-way ANOVA with estrous cycle stage as the factor. Data sets with non-homogenous variance underwent a square-root transformation prior to ANOVA. ANOVAs were followed by a protected Neuman-Keuls post-hoc analysis. Potential outliers were tested as described previously (McClave and Dietrich, 1994; Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007) using two criteria: 1) outliers were values that were more than 1.96 times the standard deviation from the mean, and 2) outliers were values that were below the lower quartile or above the upper quartile by more than 1.5 times the interquartile range. Both criteria had to be met for a value to be removed as an outlier. Statistical significance was taken as $p < 0.05$. All main or interactive effects in the ANOVAs that reached statistical significance are

described in the Results section; any main or interactive effects that are not discussed were $p > 0.05$.

When statistical differences were observed, effect size estimates were performed to indicate the strength of the treatment effects. For two-way and three-way ANOVAs, eta squared (η^2, the proportion of total variation that can be attributed to each main or interactive effect) was determined using the formula: $\eta^2 = SS_{effect} / SS_{total}$. In addition, as eta squared decreases as more variables are included in the experimental design, this complicates comparisons of effect sizes across experiments. To circumvent this difficulty, partial eta squared, (η_p^2, the proportion of variation attributed to each main or interactive effect after excluding the variance explained by the other effects) was also determined using the formula: $\eta_p^2 = SS_{effect} / (SS_{effect} + SS_{error(effect)})$.

RESULTS

Effects of LSI on energy balance

Rats given access to a 30% sucrose solution began to drink it in amounts approaching the maximum allowed within the first few days of LSI (Figure 2A), in accordance with its known high palatability to rats (Sclafani, 1987, 1991). In contrast, controls given water in the second drink bottle drank very little, as they had *ad libitum* access to water throughout, and thus had little-to-no incentive to drink additional water. Statistical analysis of drink intake showed a main effect of DRINK ($F_{1,1103} = 476.22$, $p < 0.01$, $\eta^2 = 0.78$, $\eta_p^2 = 0.91$), a main effect of TIME ($F_{22,1103} = 10.28$, $p < 0.01$, $\eta^2 = 0.024$, $\eta_p^2 = 0.18$), and a DRINK x TIME interaction ($F_{22,1103} = 3.76$, $p < 0.01$, $\eta^2 = 0.009$, $\eta_p^2 = 0.075$). Post-hoc analyses revealed greater drink intake among sucrose-fed rats on every day of LSI exposure.

Food intake also showed a main effect of DRINK ($F_{1,239} = 74.27$, $p < 0.01$, $\eta^2 = 0.44$, $\eta_p^2 = 0.62$), and post-hoc analysis revealed that sucrose-fed rats reduced their food intake compared to water-fed controls (by ~15–20%) (Figure 2B). Body weight increased for both sucrose- and water-fed rats throughout the experiment (main effect of TIME $F_{5,287} = 226.62$, $p < 0.01$, $\eta^2 = 0.56$, $\eta_p^2 = 0.83$), with no difference between drink groups (Figure 2C). Percent body fat also showed a main effect of TIME ($F_{1,95} = 57.16$, $p < 0.01$, $\eta^2 = 0.61$, $\eta_p^2 = 0.69$), with increased body fat on experiment day 22 compared to experiment day 0 (Figure 2D); moreover, there were no differences between drink groups at either time point. Notably, the overall effects of LSI on energy balance are similar to those seen previously using both male (Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007) and female (Egan et al., 2018) rats.

Open field test

The percent of time spent in the center of the open field, a traditional measure of anxiety-like behavior in the OFT (File et al., 2004; McCarthy et al., 1995; Prut and Belzung, 2003; Walsh and Cummins, 1976), showed no differences among drink or cycle groups (Figure 3A). Similarly, grooming, an ethological index that positively correlates with anxiety-like behavior (Prut and Belzung, 2003; Walsh and Cummins, 1976), also showed no differences among groups (Figure 3B), nor did rearing, which can be used as a measure of vertical

locomotion (Prut and Belzung, 2003) (Figure 3C). In contrast, total distance traveled showed a main effect of CYCLE ($F_{1,45} = 4.65$, $p < 0.05$, $\eta^2 = 0.082$, $\eta_p^2 = 0.010$), and a DRINK x CYCLE interaction ($F_{1,45} = 5.85$, $p < 0.05$, $\eta^2 = 0.10$, $\eta_p^2 = 0.12$). Post-hoc analyses revealed that sucrose-fed rats had increased total locomotion during the D1/D2 estrous cycle stage (Figure 3D). For consistency with the other behavioral tests utilized in this study, grooming and rearing were also analyzed as a time course after subdividing the data into consecutive 1-min intervals. That analysis also showed no effects of DRINK or CYCLE for either grooming or rearing (data not shown). Taken together, these data suggest that neither LSI nor estrous cycle affect anxiety-like behaviors in the OFT in female rats.

Elevated plus-maze

Anxiety-like behaviors in the EPM were assessed via the time spent in open arms and the number of entries into open arms (Cruz et al., 1994; File et al., 2004; McCarthy et al., 1995; Pellow et al., 1985; Ulrich-Lai et al., 2010), as well as the number of head dips, which is considered an ethological index of exploratory behavior indicative of reduced anxiety (Cruz et al., 1994). Time spent in the open arms showed a main effect of TIME ($F_{4,184} = 19.53$, $p < 0.01$, $\eta^2 = 0.22$, $\eta_p^2 = 0.37$), with the highest amounts of open arm time occurring during the first minute of the EPM test. Open arm time also showed a DRINK x CYCLE x TIME interaction ($F_{4,184} = 4.14$, $p < 0.01$, $\eta^2 = 0.047$, $\eta_p^2 = 0.11$), and post-hoc analyses revealed that during minute 1, sucrose increased open arm time only when rats were in the P/E estrous cycle stage at the time of testing (Figure 4A).

The number of entries into open arms also showed a main effect of TIME ($F_{4,189} = 23.37$, $p < 0.01$, $\eta^2 = 0.26$, $\eta_p^2 = 0.41$), with the greatest number occurring during the first minute of the test, as well as DRINK x TIME ($F_{4,189} = 5.17$, $p < 0.01$, $\eta^2 = 0.058$, $\eta_p^2 = 0.13$) and DRINK x CYCLE x TIME ($F_{4,189} = 6.69$, $p < 0.05$, $\eta^2 = 0.076$, $\eta_p^2 = 0.16$) interactions. Post-hoc analyses revealed that during minute 1, sucrose increased the number of open arm entries exclusively when rats were in the P/E estrous cycle stage at the time of testing (Figure 4B).

The number of head dips also showed a main effect of TIME ($F_{4,184} = 27.61$, $p < 0.01$, $\eta^2 = 0.29$, $\eta_p^2 = 0.46$), with the greatest number occurring during the first minute of the test, as well as DRINK x TIME ($F_{4,184} = 4.33$, $p < 0.01$, $\eta^2 = 0.045$, $\eta_p^2 = 0.12$) and DRINK x CYCLE x TIME ($F_{4,184} = 3.25$, $p < 0.05$, $\eta^2 = 0.033$, $\eta_p^2 = 0.090$) interactions. Similar to other measures, post-hoc analyses revealed that during minute 1, sucrose increased the number of head dips only when rats were in P/E (Figure 4C).

In contrast, when the total number of entries into the open and closed arms was used as a measure of locomotor activity (Cruz et al., 1994; File et al., 2004; McCarthy et al., 1995; Pellow et al., 1985), there were main effects of DRINK ($F_{1,184} = 6.04$, $p < 0.05$, $\eta^2 = 0.043$, $\eta_p^2 = 0.16$) and TIME ($F_{4,184} = 8.99$, $p < 0.01$, $\eta^2 = 0.019$, $\eta_p^2 = 0.21$), but no individual group differences were identified by post-hoc analysis at any time point (Figure 4D). Total distance traveled over the entire five minutes was also analyzed, with no main or interactive effects of either drink or cycle (data not shown). Collectively, these data suggest that LSI decreases anxiety-like behaviors in the EPM during the P/E stage of the estrous cycle. Moreover, open arm and head dipping behaviors were expressed at their highest levels

during the first minute of the EPM test, and the LSI effects were most notable at this same time point.

Struggling during the restraint test

Time spent struggling during an acute restraint stress test showed main effects of DRINK ($F_{1,140} = 4.11$, $p < 0.05$, $\eta^2 = 0.034$, $\eta_p^2 = 0.087$) and TIME ($F_{2,140} = 21.02$, $p < 0.01$, $\eta^2 = 0.16$, $\eta_p^2 = 0.33$), with the time spent struggling generally decreasing over the duration of the test. Struggling time also showed DRINK x CYCLE ($F_{1,140} = 4.63$, $p < 0.05$, $\eta^2 = 0.038$, $\eta_p^2 = 0.097$), CYCLE x TIME ($F_{2,140} = 4.02$, $p < 0.05$, $\eta^2 = 0.030$, $\eta_p^2 = 0.086$) and DRINK x CYCLE x TIME ($F_{2,140} = 3.21$, $p < 0.05$, $\eta^2 = 0.024$, $\eta_p^2 = 0.069$) interactions. Post-hoc analyses revealed that during the first five minutes of the restraint test, sucrose decreased the time spent struggling only among rats tested in the P/E stage of the estrous cycle (Figure 5).

HPA axis response to restraint

To assess the HPA axis response to acute stress, we measured plasma ACTH and corticosterone prior to and following the restraint test. For the plasma ACTH response (Figure 6A,B), there were main effects of DRINK ($F_{1,234} = 7.92$, $p < 0.01$, $\eta^2 = 0.031$, $\eta_p^2 = 0.16$) with sucrose generally lowering plasma ACTH, and TIME ($F_{4,234} = 197.48$, $p < 0.01$, $\eta^2 = 0.63$, $\eta_p^2 = 0.82$) with restraint stress elevating plasma ACTH levels, as well as a DRINK x TIME interaction ($F_{4,234} = 3.84$, $p < 0.01$, $\eta^2 = 0.012$, $\eta_p^2 = 0.082$). Post-hoc analyses revealed sucrose decreased plasma ACTH primarily at 20, 40, and 60 min after restraint onset during the P/E estrous cycle stage.

The plasma corticosterone response (Figure 6C,D) showed a main effect of TIME ($F_{4,234} = 402.48$, $p < 0.01$, $\eta^2 = 0.85$, $\eta_p^2 = 0.90$) with restraint stress increasing plasma corticosterone levels, and a DRINK x TIME interaction ($F_{4,234} = 3.33$, $p < 0.05$, $\eta^2 = 0.007$, $\eta_p^2 = 0.072$). Post-hoc analyses revealed that sucrose decreased plasma corticosterone primarily at 90 min after restraint onset during the P/E stage of the estrous cycle.

Plasma estradiol

Plasma estradiol levels were measured on day 24 to corroborate the day 24 estrous cycle staging by lavage cytology, as in our prior study (Egan et al., 2018). While the experiment was designed to analyze behavioral, HPA, and immunolabeling endpoints in two broad estrous cycle stages (i.e., D1/D2 vs. P/E), plasma estradiol peaks specifically in proestrus (Asarian and Geary, 2013; Butcher et al., 1974; Cecchini et al., 1983; Lu et al., 1985; Nequin et al., 1979; Smith et al., 1975). Plasma estradiol levels were therefore compared after further subdividing rats into diestrus 1, diestrus 2, proestrus, late proestrus/estrus, and estrus based on their lavage cytology. This analysis (Table 1) revealed no differences between the cycle stages (one-way ANOVA, $p > 0.05$). However, unlike previous experiments (Egan et al., 2018), no rats in this particular cohort were identified as being in proestrus on experiment day 24. Thus, there was no opportunity to observe the specific stage characterized by peak estradiol levels, resulting in roughly equivalent plasma estradiol levels across the other stages (though plasma estradiol did trend higher during late proestrus/estrus

relative to diestrus 2 when these two stages were directly compared ($p = 0.05$ by 2-tailed Mann-Whitney U test)).

Post-restraint cFos immunolabeling in stress-associated brain regions

Quantification of cFos-positive cells was used as an index of restraint-induced activation in several brain regions known to be involved in stress regulation (Ulrich-Lai and Herman, 2009), including the BLA, BSTp, CeM, CeL, DMH (dorsal and ventral subdivisions), MeApd, NAc (core and shell subdivisions), PFC (PL, IL, Ant Cing subdivisions), and PVN.

As the medial and lateral subdivisions of the central amygdala can be differentially regulated by stress and reward (Ciocchi et al., 2010; Duvarci et al., 2011; Fudge and Haber, 2000; Gilpin et al., 2015; Haubensak et al., 2010; Martina et al., 1999), analysis of the CeA was sub-divided into its medial and lateral subneuclei. For the CeM, there was a main effect of RINK ($F_{1,44} = 4.31$, $p < 0.05$, $\eta^2 = 0.088$, $\eta_p^2 = 0.095$), with post-hoc analysis revealing that sucrose primarily increased cFos-positive immunolabeling among rats in P/E (Figure 7A,D). Of note, this sucrose effect appears to occur specifically in the medial subdivision of the CeA, since cFos-positive immunolabeling in the CeL was not altered by either drink or cycle (Figure 7B,D).

For the BSTp, there was a main effect of DRINK ($F_{1,35} = 4.34$, $p < 0.05$, $\eta^2 = 0.10$, $\eta_p^2 = 0.12$) and a DRINK x CYCLE interaction ($F_{1,35} = 4.60$, $p < 0.05$, $\eta^2 = 0.11$, $\eta_p^2 = 0.13$). Post-hoc analyses revealed that sucrose increased cFos-positive immunolabeling only among rats in the P/E stage of the estrous cycle (Figure 7C,E).

In contrast, neither sucrose nor estrous cycle stage impacted post-restraint cFos immunolabeling in any of the other brain regions examined (Table 2), including the BLA, DMH (dorsal and ventral subdivisions), MeApd, NAc (core and shell subdivisions), PFC (PL, IL, and anterior cingulate subdivisions), and PVN.

DISCUSSION

In this study, we found that a history of limited, intermittent sucrose reduces stress-related behaviors during the EPM and restraint tests during the P/E stage of the estrous cycle. This was accompanied by reduced HPA axis responses to stress in sucrose-fed rats during P/E. Furthermore, post-restraint cFos immunolabeling in the CeM and BSTp was also increased by sucrose during P/E. Collectively, these data support the hypothesis that palatable foods reduce anxiety-like behaviors in female rats in an estrous cycle-dependent manner.

Impact of LSI on stress-related behaviors

As there is evidence that there may be sex differences in anxiety-related behaviors (Frye et al., 2000; Frye and Walf, 2002; Johnston and File, 1991; Pare and Redei, 1993), and that anxiety-related behaviors may vary with estrous cycle (Frye et al., 2000; Frye and Walf, 2002; Galeeva and Tuohimaa, 2001; Gray and Cooney, 1982; Marcondes et al., 2001; Meziane et al., 2007; Mora et al., 1996; Pare and Redei, 1993; Walf et al., 2009), the present study tested whether LSI blunts anxiety-related behaviors in female rats, as seen previously for male rats (Ulrich-Lai et al., 2010). Furthermore, as we have previously shown that LSI

dampens HPA axis responsivity primarily during the P/E stage of the estrous cycle (Egan et al., 2018), we hypothesized that LSI would similarly attenuate anxiety-like behaviors during P/E in female rats. We found that a history of LSI increased time in the open arms of the EPM and decreased struggling during restraint, but had no effect on time in the center of the OFT, in female rats during P/E. These findings generally support our hypothesis, and suggest that LSI reduces stress-related behaviors in female rats in an estrous cycle-dependent manner. It should be noted as a limitation that estrous cycle assessments by vaginal lavage were restricted to the days of sample collection, as opposed to occurring daily throughout the entire experiment. We chose to limit the number of vaginal lavages to avoid the possibility that the stress associated with repeated daily lavage could confound the behavioral and HPA axis responses to stress. Nonetheless, it is intriguing to speculate that reduced anxiety-related behavior during P/E may have ethological relevance for rodents, as it could promote exploration and the seeking of a mate. Moreover, the specificity of the LSI effects to the P/E estrous cycle stage may have important clinical implications, as it suggests that stress relief by ‘comfort foods’ may also vary across the menstrual cycle in women.

LSI reduced anxiety-related behaviors during the EPM in both male and female P/E rats (Ulrich-Lai et al., 2010), indicating that LSI may have similar anxiolytic effects in males and females. In contrast, LSI did not affect anxiety-related behaviors in the OFT in female rats, as it previously did in male rats (Ulrich-Lai et al., 2010). Moreover, LSI increased the distance travelled in the OFT during D1/D2, though vertical locomotor activity (rearing) in the OFT was not affected by drink or cycle, nor was total distance travelled in the EPM. This suggests LSI does not have widespread effects on overall locomotor activity, and complicates interpretation of the female OFT results. However, as LSI decreases anxiety-related behaviors in the OFT in males (with no effects on total locomotor activity) (Ulrich-Lai et al., 2010), the effects of LSI on behaviors in the OFT may differ between male and female rats. For example, a duration of LSI longer than 12 days may be needed to alter anxiety-related behaviors in the OFT in females. In support of this idea, reduced anxiety-related behaviors were observed in the EPM on day 16 of LSI exposure, indicating that a longer duration of LSI may be required for anxiolytic effects in females.

Finally, the effect of LSI on struggling during the restraint test has not been previously characterized in either male or female rats. As such, our finding that LSI reduced struggling only during P/E is a novel finding that supports the use of struggling as an approach to simultaneously measure stress-related behavior and HPA axis activation. Additionally, preliminary data shows LSI similarly reduces struggling during the restraint test in male rats (unpublished data), suggesting that LSI may impact stress coping behaviors during the restraint test in both sexes.

LSI reduces HPA axis reactivity to restraint stress

As we previously reported (Egan et al., 2018), LSI reduced plasma ACTH responses to restraint stress during the P/E stage of the estrous cycle. In the current study, we also found that LSI reduced plasma corticosterone during P/E at the 90-min post-stress time point. We had previously collected blood samples through 60-min post-restraint, as is routinely used in analogous studies in male rats, and as such, failed to detect LSI effects on post-restraint

plasma corticosterone (Egan et al., 2018). By extending the sampling time out to 90 minutes post-restraint in the current experiment, we were able to detect changes in plasma corticosterone that are not apparent at earlier time points. This supports the need for extended sampling times in female rats, and suggests the time course of LSI effects of HPA axis reactivity may differ between male and female rats. For example, it is intriguing to speculate that LSI may act in P/E females, at least in part, by shortening the duration of the post-stress glucocorticoid response. This could occur via direct actions on HPA-regulatory brain regions, increased glucocorticoid negative feedback, and/or a faster rate of corticosterone metabolism and clearance. It is also interesting to note that plasma ACTH is reduced by LSI as early as 20 minutes after stress onset, whereas plasma corticosterone is not reduced until 90 minutes. It is possible that early after stress onset, plasma ACTH levels are above the threshold required to evoke maximal adrenal corticosterone release, and as a result, reduced plasma corticosterone by LSI can only be observed after plasma ACTH levels fall below this threshold at later post-stress time points. In support of this idea, estrogen enhances adrenal responsivity to ACTH (Figueiredo et al., 2007), implying that less plasma ACTH may be needed to evoke maximal rates of adrenal corticosterone production during P/E. Thus, during P/E, despite a clear reduction in plasma ACTH by sucrose at 20–60 min after stress onset, the reduction in plasma corticosterone may not be observed until plasma ACTH levels have waned (e.g., at 90 min).

Potential brain mechanisms underlying P/E-specific effects of LSI in female rats

We previously measured the effect of LSI on immunolabeling for two proteins that are associated with long-term changes in neural plasticity and neuroadaptation (pCREB and FosB/deltaFosB) in multiple stress- and reward-regulatory brain regions in the basal, unstressed state of female rats (Chen et al., 1997; Huang et al., 2000; McClung et al., 2004; Miyamoto, 2006; Nestler et al., 1999; Silva et al., 1998)). A Bayesian network analysis was then performed on this dataset, identifying a neurocircuit that includes the BLA, NAc, PFC and BST as likely being modified by LSI in female rats during P/E (Egan et al., 2018). As the BLA, NAc, and PFC do not have significant direct projections to the PVN (Ulrich-Lai and Herman, 2009), this suggests that they may act through one or more intermediary structures, such as the BSTp, to influence HPA reactivity. Importantly, the present post-stress cFos data extend and support this possibility, showing that restraint-induced cFos activation in the BSTp is enhanced by prior LSI only during P/E, thereby corroborating the BSTp as a potential candidate for this intermediary brain site. The BSTp provides direct inputs to the PVN that are largely GABAergic in nature (Boudaba et al., 1996; Choi et al., 2007; Dong and Swanson, 2004; Herman et al., 2003; Myers et al., 2014; Ulrich-Lai and Herman, 2009), and thus increased activation of the BSTp during P/E may act to blunt HPA axis responsivity by increasing inhibition of the PVN. The BLA, NAc, and PFC all have direct or indirect projections to the BSTp (Dong et al., 2001; McDonald, 1991; Myers et al., 2014; Pitkanen et al., 1997; Vertes, 2004), so there is anatomical evidence to support the idea of the BSTp as this intermediary. Moreover, the BSTp expresses both estrogen receptor alpha (ERα) and beta (ERβ) (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001; Shughrue et al., 1998), and ER signaling is linked with altered BST neuronal activation (cFos) and anxiety-related behavior (Kudwa et al., 2014; Lund et al., 2005; Oyola et al., 2012; Walf and Frye, 2005). Therefore, a possible scenario is that LSI and estrous cycle interact to alter the

functional relationships within a BLA-NAc-PFC circuit in the basal, unstressed state. Subsequently, when a stressor occurs during P/E, signals from the BLA, NAc, and PFC converge on the BSTp, which integrates these signals to provide greater inhibition of the PVN, therefore resulting in an overall blunted HPA axis response. Importantly, this same neurocircuit is well-positioned to modulate the effects of LSI on stress-related behaviors, including struggling during restraint and open arm exploration in the EPM (Dong and Swanson, 2004; Felix-Ortiz et al., 2016; Weinberg et al., 2010).

The present analysis of post-stress cFos immunolabeling indicated the CeM as another potential region mediating P/E-specific stress-dampening by LSI. The CeM is the main output nucleus of the CeA, and activation of this region is typically thought of as stress-excitatory (Beaulieu et al., 1986; Ciocchi et al., 2010; Duvarci et al., 2011; Feldman et al., 1994; Haubensak et al., 2010; Xu et al., 1999). Thus, the observed increase in cFos immunolabeling, coupled with decreased behavioral and HPA axis stress responses, seems contradictory. However, the role of the CeA in stress regulation may be more complex than originally thought. For instance, sucrose ingestion increases neuronal activation (cFos) in the CeA, (particularly the medial portion) (Yamamoto et al., 1997) as does administration of the anxiolytic drug diazepam (Lkhagvasuren et al., 2014), and LSI increases pCREB- and FosB/deltaFosB-immunolabeling in the CeA of unstressed female rats (Egan et al., 2018). This suggests the CeA may contribute to palatable feeding and/or anxiolytic responses. In support of this, rats given *ad libitum* sucrose in addition to chow had increased post-restraint corticotropin-releasing hormone (CRH) mRNA in the CeA, which was accompanied by decreased HPA axis responsivity to restraint (Foster et al., 2009). Furthermore, Logrip et al. showed that synaptic responses in the CeM can vary across the estrous cycle (Logrip et al., 2017). Taking this information together, we speculate that differential functioning in the CeM during P/E may make it more sensitive to the effects of previous sucrose intake, thus promoting greater stress-blunting effects of LSI during P/E.

Finally, the BLA generally promotes stress responses (Bhatnagar et al., 2004; Coover et al., 1973; Feldman et al., 1983; Goldstein et al., 1996; Szafarczyk et al., 1986), suggesting that LSI may act by decreasing its stress-excitatory output. Consistent with this idea, in male rats LSI alters indices of neuronal plasticity within the BLA in the basal, unstressed state, and also reduces post-stress BLA cFos expression (Christiansen et al., 2011; Ulrich-Lai et al., 2010; Ulrich-Lai et al., 2007). In female rats, LSI increases BLA FosB/deltaFosB immunolabeling in the basal, unstressed state during P/E, and Bayesian analyses identify the BLA as a member of the most likely neurocircuit that is modified by LSI in both males and female P/E rats (Egan et al., 2018; Ulrich-Lai et al., 2016). However, the present data indicate that LSI does not alter the number of post-stress cFos-positive cells in the BLA of female rats (Table 2). Thus, while our prior results strongly implicate a role for the BLA in the LSI stress-blunting in male rats, a role for the BLA in the LSI effects in female rats appears to be more nuanced. As there are strong functional relationships among the BLA, CeA and BST (Dong et al., 2001; Myers et al., 2014; Pitkanen et al., 1997; Swanson and Petrovich, 1998; Tye et al., 2011), this may indicate that for female rats, the BLA contributes to LSI stress-dampening primarily via its ability to influence the function of these other brain regions.

Conclusions

Women may be more prone to eating 'comfort food' than are men, and emotional eating can vary across the menstrual cycle (Greeno and Wing, 1994; Grunberg and Straub, 1992; Hildebrandt et al., 2015; Klein et al., 2004; Klump et al., 2013a; Klump et al., 2013b; Oliver and Wardle, 1999; Racine et al., 2013; Wansink et al., 2003; Wardle et al., 2000; Zellner et al., 2006). This suggests the potential for important sex- and cycle-dependent effects of 'comfort feeding.' We have previously shown that a limited, intermittent sucrose intake paradigm reduces anxiety-like behaviors in male rats (Ulrich-Lai et al., 2010), and the present work extended this line of research to examine the effects of LSI on anxiety-related behaviors in female rats across the estrous cycle. The results indicate that LSI reduces stress-related behaviors in the EPM and restraint tests (but not the OFT), while also blunting HPA axis activation in female rats during P/E. Furthermore, LSI increases post-restraint cFos immunolabeling in the CeM and BSTp only during P/E. Collectively, the results support the hypothesis that palatable foods reduce anxiety-related behavior and HPA axis responsivity, with the effects in females varying across the estrous cycle stage. Moreover, neuronal signaling in the CeM and BSTp may contribute to the stress-blunting effects of LSI in females during P/E.

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HIGHLIGHTS

- Stress relief by “comfort foods” may vary across the estrous cycle.
- Sucrose blunts anxiety-like behaviors in a cycle-specific manner.
- Sucrose decreases the HPA axis response to restraint in a cycle-specific manner.
- Sucrose increases post-restraint cFos in forebrain in a cycle-specific manner.

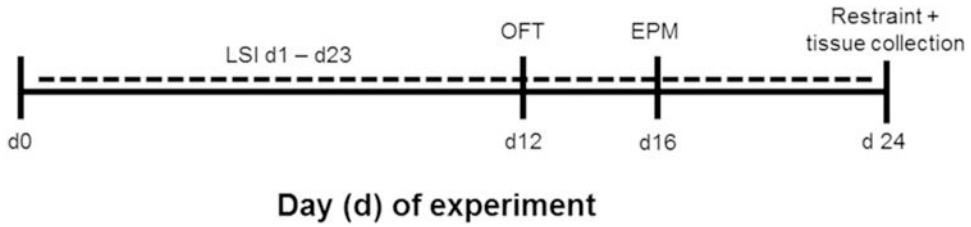

Figure 1. Experimental timeline of LSI exposure.

Female rats received twice-daily access to a limited amount of sucrose drink (vs. water as a control) on days 1–23. Rats were tested in the open field test (OFT) and elevated plus-maze (EPM) between the morning and afternoon LSI sessions on days 12 and 16, respectively. On the morning of day 24, rats received a restraint challenge with subsequent collection of brain tissue for cFos immunolabeling.

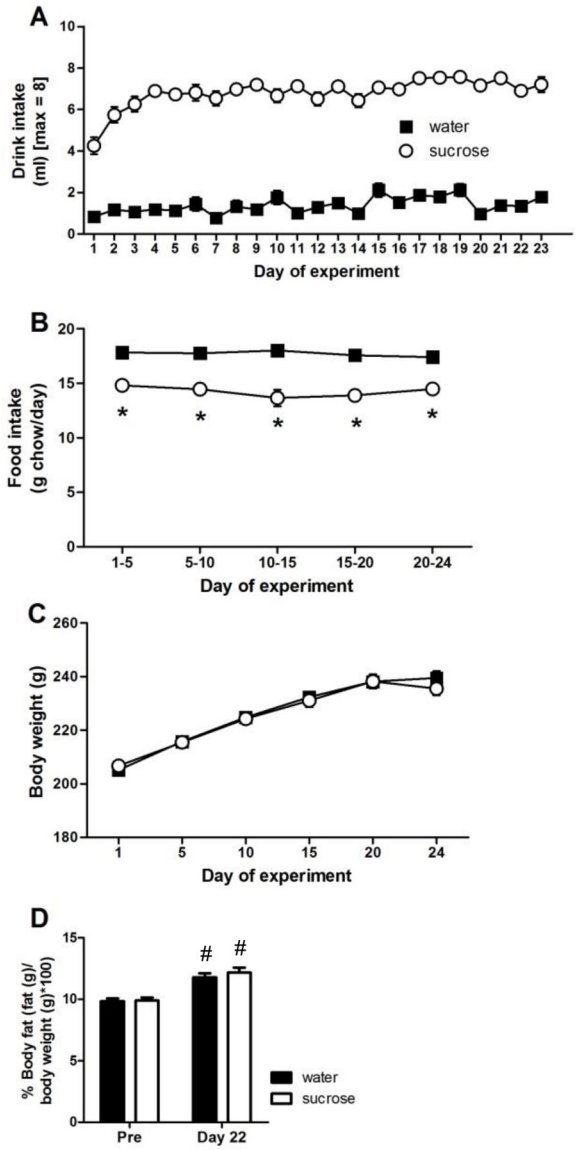

Figure 2. The impact of LSI on energy balance in female rats.

(A) Drink intake from the second drink bottle, (B) food intake, (C) body weight, and (D) percent body fat. Not shown on panel (A) – all sucrose are greater ($p < 0.05$) than their respective water for every day of LSI exposure. *$p < 0.05$ vs. water, #$p < 0.05$ vs. Pre (experimental day 0). n = 23–24/group.

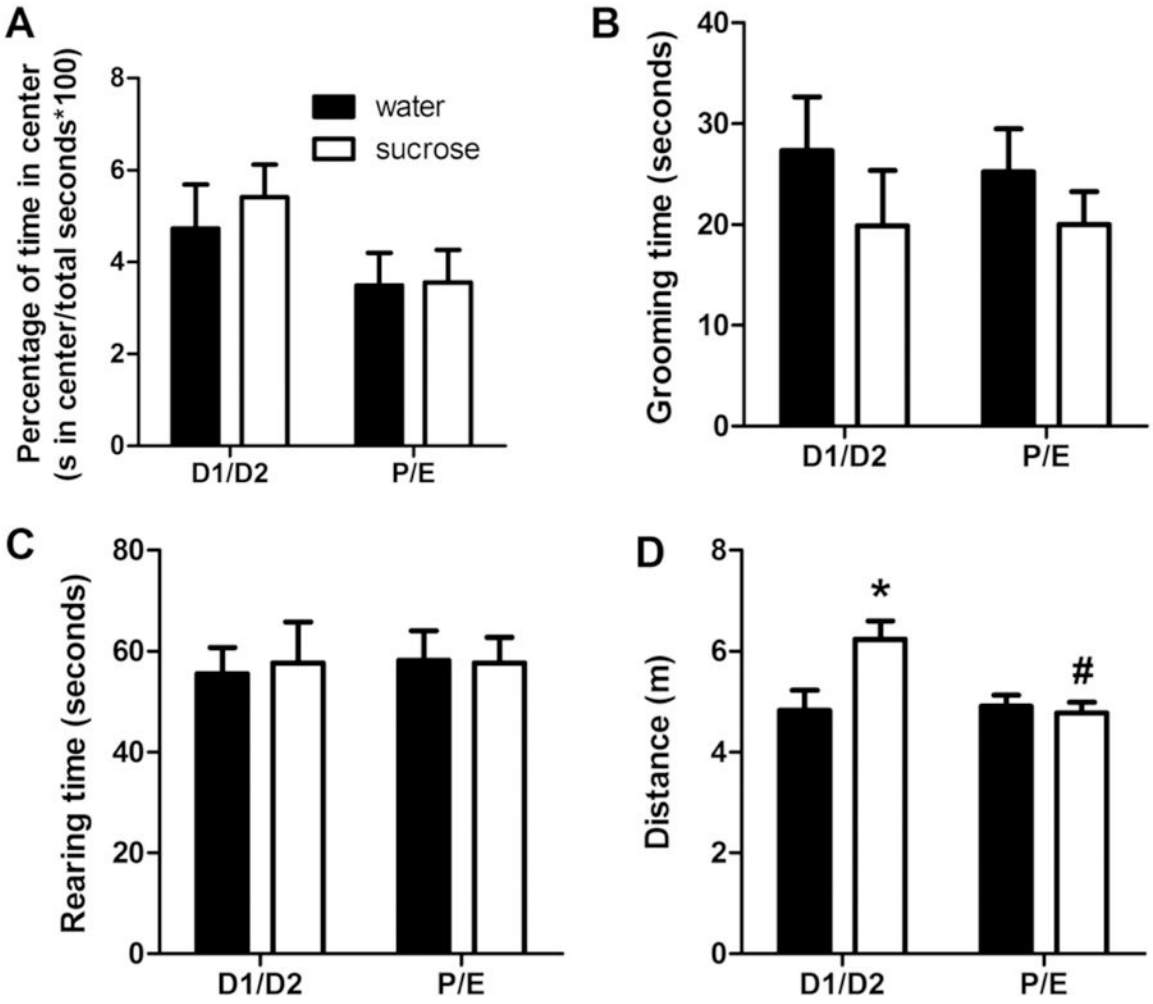

Figure 3. A history of LSI increases total locomotor activity but not anxiety-related behaviors in the OFT.

The impact of prior sucrose (vs. water) on the percentage of time spent in the center (A), time spent grooming (B) and rearing (C), and total distance traveled (D) in female rats tested during diestrus (D1/D2) and proestrus/estrus (P/E). *$p < 0.05$ vs. water, #$p < 0.05$ vs. D1/D2. n = 8–13/group.

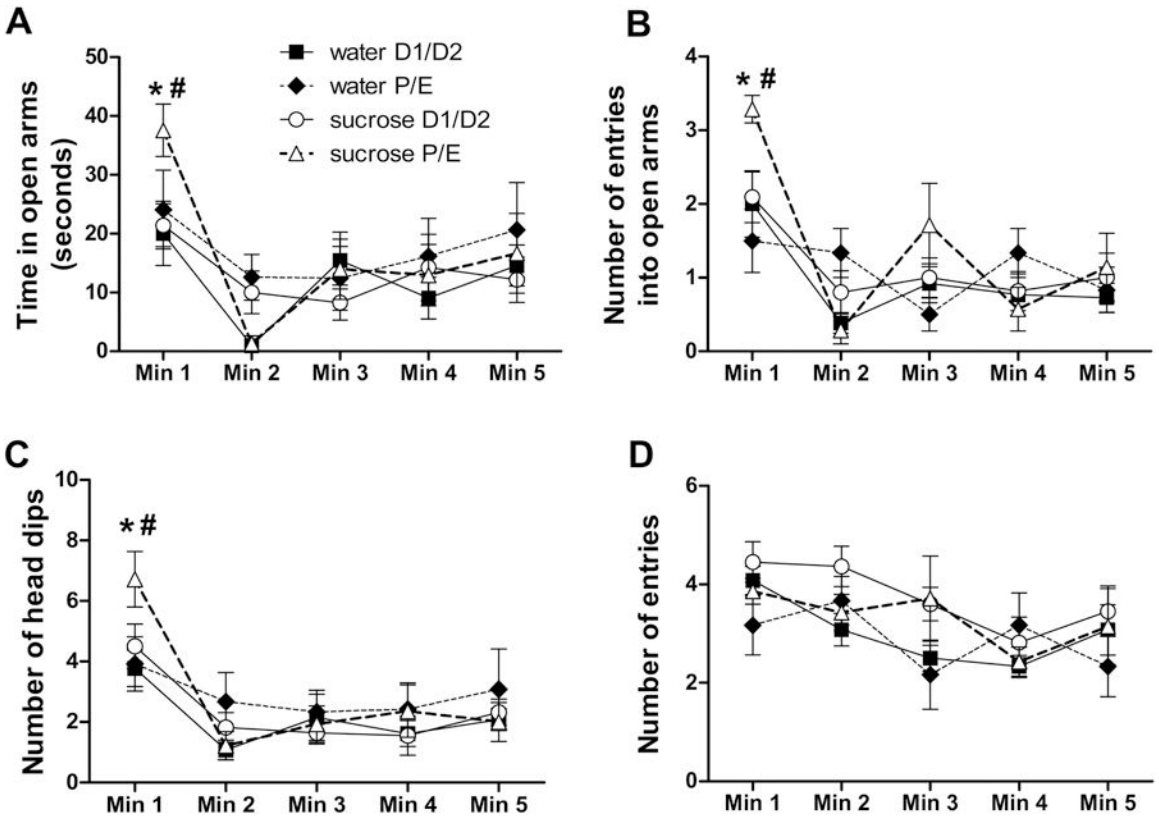

Figure 4. A history of LSI decreases anxiety-like behaviors in the EPM only during P/E.

The impact of prior sucrose (vs. water) on the time spent in the open arms (A), and the number of entries into the open arms (B), head dips (C), and total (open and closed) arm entries (D) in female rats tested during diestrus (D1/D2) and proestrus/estrus (P/E). *p < 0.05 vs. water, #p < 0.05 vs. D1/D2. n = 6–12/group.

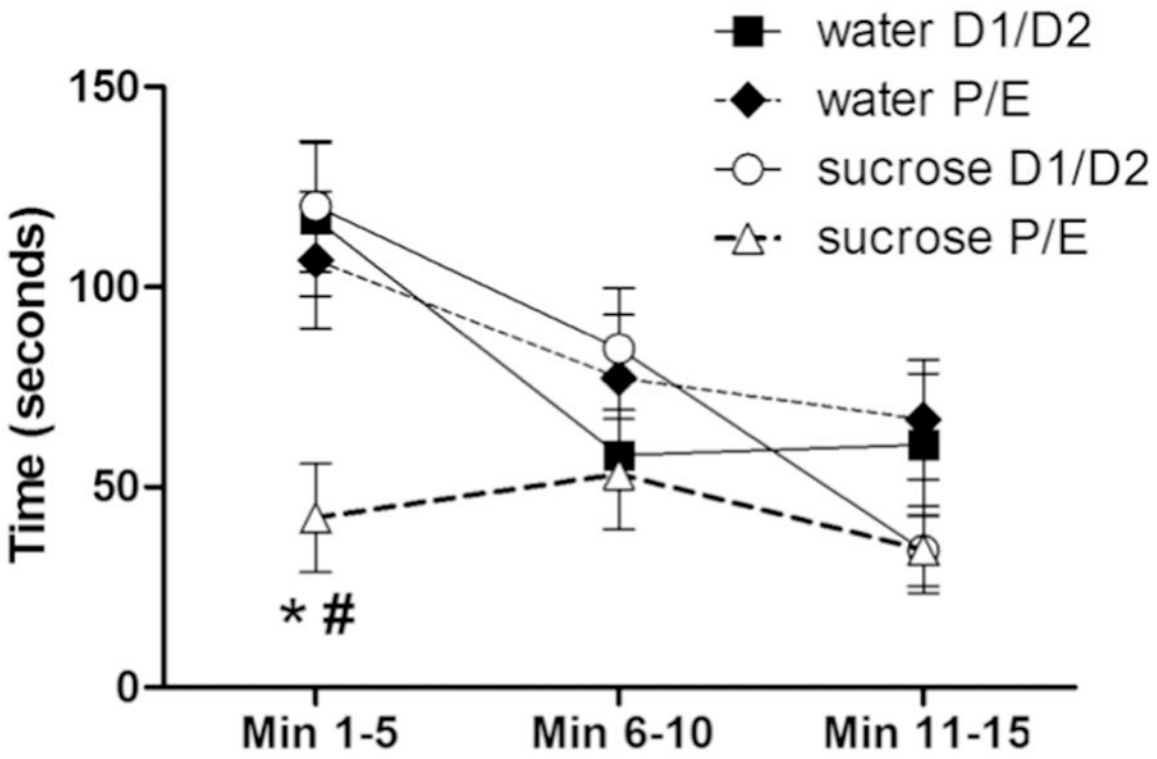

Figure 5. A history of LSI decreases struggling behavior during the restraint test only during P/E.

The impact of prior sucrose (vs. water) on the time spent struggling by female rats tested during diestrus (D1/D2) and proestrus/estrus (P/E). *p < 0.05 vs. water, #p < 0.05 vs. D1/D2. n = 9–15/group.

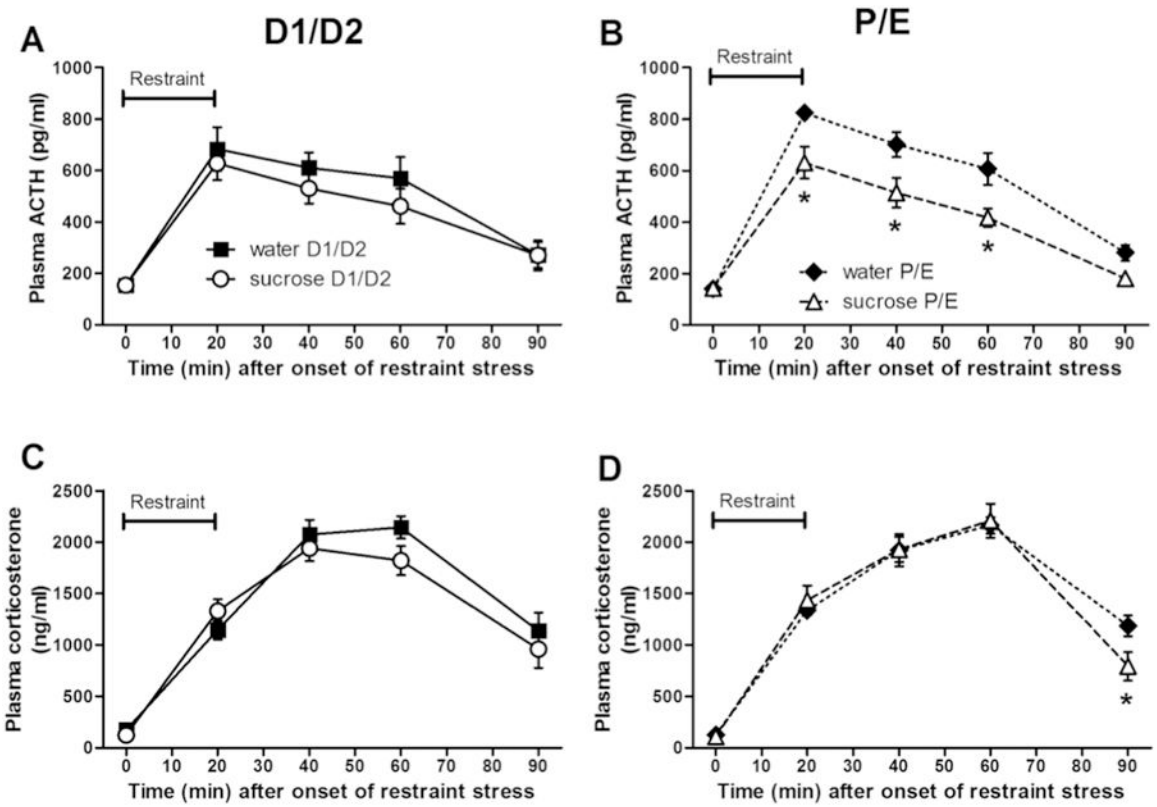

Figure 6. A history of LSI dampens the plasma ACTH and corticosterone responses to restraint only during P/E.

The impact of prior sucrose (vs. water) on the plasma ACTH (A, B) and corticosterone (C, D) responses to an acute restraint stress in female rats tested during diestrus (D1/D2) (A, C) and proestrus/estrus (P/E) (B, D). *$p < 0.05$ vs. water. n = 9–15/group. Note that for each hormone, data were analyzed via 3-way repeated measures ANOVA comparing DRINK, CYCLE, and TIME, but are graphed separated by estrous cycle stage to aid visualization.

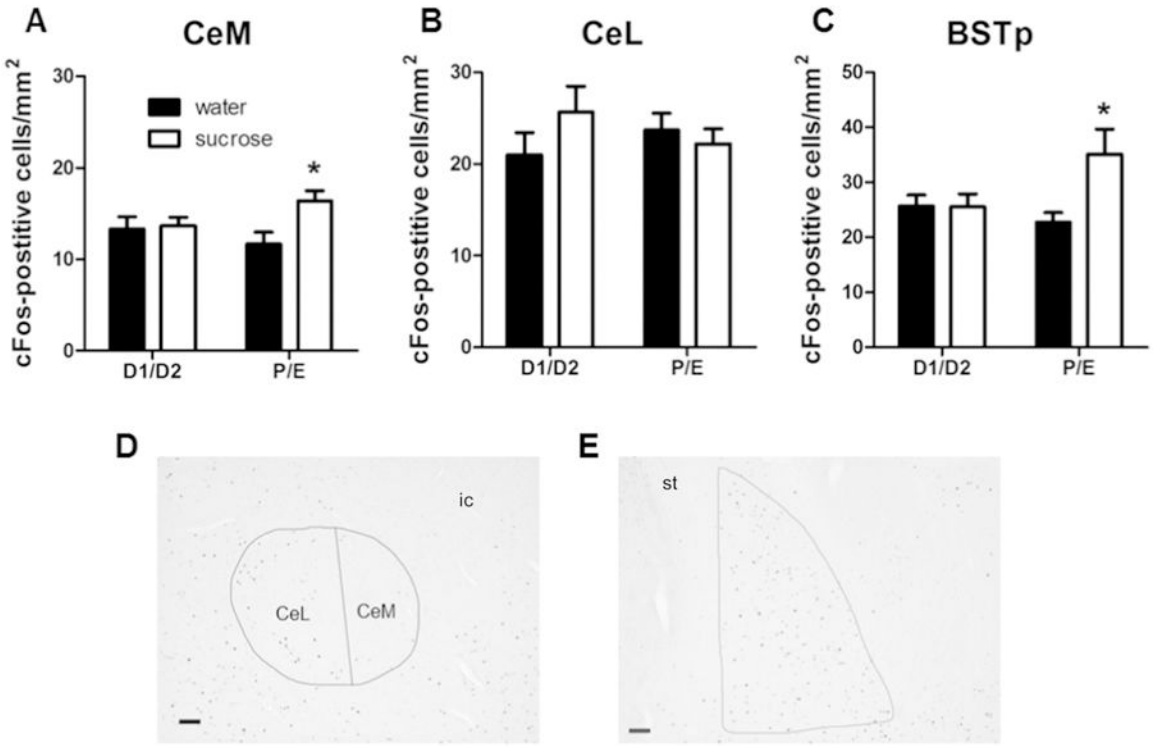

Figure 7. A history of LSI increases post-restraint cFos-immunolabeling in the CeM and BSTp only during P/E.

The impact of prior sucrose (vs. water) on the density of post-restraint cFos-positive cells in the CeM (A, D), CeL (B, D), and BSTp (C, E) in female rats tested during diestrus (D1/D2) and proestrus/estrus (P/E). Representative images shown in (D) and (E) are taken at 50x magnification from a water D1/D2 rat; scale bar = 100 μm. *$p < 0.05$ vs. water. n = 7–15/group. Abbreviations: BSTp, posterior subnuclei of the bed nucleus of the stria terminalis; CeM, medial subdivision of the central amygdala; CeL, lateral subdivision of the central amygdala; ic, internal capsule; st, stria terminalis.

Table 1.

Rat plasma estradiol levels.

Rat plasma estradiol levels on experiment day 24 are shown divided by their respective estrous cycle stage, as determined by vaginal lavage cytology on experiment day 24. N/A= not available as no rats were in this estrous cycle stage at the time of assessment.

Estrous cycle stage	Plasma estradiol (pg/ml)
Diestrus 1 (n = 14)	133.5 ± 12.70
Diestrus 2 (n = 7)	109.9 ± 4.86
Proestrus (n = 0)	N/A
Late proestrus/estrus (n = 6)	161.5 ± 22.69
Estrus (n = 19)	145.9 ± 13.76

Table 2.

Post-restraint cFos-positive immunolabeling in stress- and reward-regulatory brain regions.

The impact of prior sucrose (vs. water) on the density of post-restraint cFos-positive cells (number of cells/ mm^2) in multiple stress- and reward-regulatory brain regions of female rats tested during diestrus (D1/D2) and proestrus/estrus (P/E). n = 7–15/group.

Brain region	Water D1/D2	Water P/E	Sucrose D1/D2	Sucrose P/E
BLA	78.06 ± 2.67	80.69 ± 3.77	86.08 ± 4.33	86.98 ± 4.28
DMH dorsal	83.94 ± 14.45	85.46 ± 8.53	70.68 ± 7.24	90.18 ± 4.18
DMH ventral	104.85 ± 9.90	118.58 ± 9.06	101.67 ± 9.88	116.09 ± 6.92
MeApd	118.93 ± 12.01	130.92 ± 9.82	133.16 ± 8.93	133.56 ± 9.91
NAc core	55.00 ± 6.57	50.86 ± 5.25	49.57 ± 4.66	46.89 ± 5.55
NAc shell	60.62 ± 5.48	55.10 ± 4.60	50.19 ± 2.16	50.34 ± 5.46
PFC PL	128.16 ± 9.78	147.41 ± 9.35	156.32 ± 10.98	139.73 ± 7.42
PFC IL	102.78 ± 5.82	111.49 ± 6.66	115.76 ± 6.89	112.77 ± 5.29
PFC Ant Cing	112.94 ± 8.43	128.65 ± 9.86	122.29 ± 9.02	123.35 ± 6.79
PVN	158.69 ± 11.14	163.72 ± 8.74	148.08 ± 9.09	143.34 ± 9.50