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Palatable food affects HPA axis responsivity and forebrain neurocircuitry in an estrous cycle-specific manner in female rats

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Abstract

Eating palatable foods can provide stress relief, but the mechanisms by which this occurs are unclear. We previously characterized a limited sucrose intake (LSI) paradigm in which twice-daily access to a small amount of 30% sucrose (vs. water as a control) reduces hypothalamic-pituitary-adrenocortical (HPA) axis responses to stress and alters neuronal activation in stress-regulatory brain regions in male rats. However, women may be more prone to 'comfort feeding' behaviors than men, and stress-related eating may vary across the menstrual cycle. This suggests that LSI effects may be sex- and estrous cycle-dependent. The present study therefore investigated the effects of LSI on HPA axis stress responsivity, as well as markers of neuronal activation/plasticity in stress- and reward-related neurocircuitry in female rats across the estrous cycle. We found that LSI reduced post-restraint stress plasma ACTH in female rats specifically during proestrus/estrus (P/E). LSI also increased basal (non-stress) FosB/deltaFosB- and pCREB-immunolabeling in the basolateral amygdala (BLA) and central amygdala specifically during P/E. Finally, Bayesian network modeling of the FosB/deltaFosB and pCREB expression data identified a neurocircuit that includes the BLA, nucleus accumbens, prefrontal cortex, and bed nucleus of the stria terminalis as likely being modified by LSI during P/E. When considered in the context of our prior results, the present findings suggest that palatable food reduces stress responses in female rats similar to males, but in an estrous cycle-dependent manner. Further, the BLA may contribute to the LSI effects in both sexes, whereas the involvement of other brain regions appears to be sex-dependent.

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Sex differences; sucrose; ACTH; corticosterone; basolateral amygdala; nucleus accumbens

Introduction

Obesity is one of the largest public health issues in modern times. Over 68% of adults in the United States are overweight or obese, and this number continues to grow (Flegal et al., 2012; Ogden et al., 2015). There are many complex factors that interact to cause obesity, but one contributor may be daily life stressors. Approximately 40–70% of people report eating more when stressed (Weinstein et al., 1997; Oliver and Wardle, 1999; Epel et al., 2004), and the types of food typically chosen are highly-palatable, calorically-dense foods (Oliver and Wardle, 1999; Epel et al., 2001; Cartwright et al., 2003; Zellner et al., 2006, 2007; Laugero et al., 2011; Groesz et al., 2012; Kim et al., 2013). People may select these foods for their ability to reduce negative emotions or stressful feelings, a concept often thought of as ‘comfort feeding.’ Indeed, literature reports show that palatable food can decrease psychological and physiological measures of stress in people (Anderson et al., 1987; Markus et al., 2000; Dubé et al., 2005; Gibson, 2006; Macht and Mueller, 2007; Tomiyama et al., 2011; Tryon et al., 2013). Studies utilizing rodent models have also shown similar effects. During chronic stress, rodents preferentially shift their intake to more highly palatable foods when given a choice (Minor and Saade, 1997; Pecoraro et al., 2004; Ulrich-Lai et al., 2007; Packard et al., 2014), and a history of palatable food ingestion can reduce stress responses in rodents, including activation of the neuroendocrine hypothalamic-pituitary-adrenocortical (HPA) axis (Bell et al., 2002; Dallman et al., 2003; la Fleur et al., 2004, 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2007, 2010; Kinzig et al., 2008; Coccorello et al., 2009; Warne, 2009; Finger et al., 2011).

In order to study the neurobiological mechanisms underlying palatable-food mediated stress relief, our group developed a rodent model based on human snacking patterns (Ulrich-Lai et al., 2007, 2010). In this limited sucrose intake (LSI) paradigm, adult, male rats with *ad libitum* access to standard chow and water are also offered a limited amount (up to 4 ml/session; 8 ml/day) of a 30% sucrose drink (vs. water as a control) twice-daily. Two weeks of LSI reduces HPA axis responses to a subsequent acute restraint stress challenge in male rats. LSI also impacts brain circuits that regulate stress and reward in male rats. For example, LSI increases the mRNA and protein expression of numerous plasticity-related genes in the basolateral amygdala (BLA) in a basal, unstressed state (Ulrich-Lai et al., 2010; Christiansen et al., 2011; Egan and Ulrich-Lai, 2015; Packard et al., 2017). Advanced statistical analyses, including Bayesian modeling, indicate that LSI also modifies the predicted relationships among multiple stress- and reward-regulated brain regions (Ulrich-Lai et al., 2016). Collectively our findings suggest that LSI alters BLA functional connectivity in males.

Importantly, while the effects of LSI are well-characterized in male rats, the effects in female rats are unknown, despite the fact that there may be important sex and estrous cycle differences in stress-dampening by palatable foods. For instance, women may be more prone to emotional or ‘comfort feeding’ behaviors than men (Grunberg and Straub, 1992; Greeno

and Wing, 1994; Oliver and Wardle, 1999; Oliver et al., 2000; Wansink et al., 2003; Klein et al., 2004; Zellner et al., 2006). Emotional eating is also affected by the menstrual cycle, as measured by salivary hormone levels, with the greatest amount of emotional eating during the luteal phase, when estrogen levels are relatively high (Klump et al., 2013a, 2013b; Racine et al., 2013; Hildebrandt et al., 2015). Consistent with this idea, most reward- and feeding-related brain regions express estrogen receptors (ERs) (Shughrue et al., 1997, 1998; Osterlund et al., 1998; Shughrue and Merchenthaler, 2001; Merchenthaler et al., 2004). Likewise, many stress-regulatory brain regions express ER (Shughrue et al., 1997, 1998; Osterlund et al., 1998; Shughrue and Merchenthaler, 2001; Merchenthaler et al., 2004), and HPA responsivity/tone may also be impacted by the estrous cycle (Viau and Meaney, 1991, 2004; Carey et al., 1995; Walker et al., 2001), though not all papers report cycle-related HPA effects (Guo et al., 1994; Bland et al., 2005; Babb et al., 2013). Taking all of these factors into account, we hypothesized that the stress-blunting effects of LSI may differ between female and male rats, and may also be affected by estrous cycle phase.

In order to test this hypothesis, the current study investigated the effects of LSI on HPA axis responses to acute stress, as well as whether these effects vary with estrous cycle stage. The impact of LSI on FosB/deltaFosB- and phospho-cyclic AMP response element binding protein (pCREB)-immunolabeling was later assessed in multiple stress- and reward-regulatory brain regions across the estrous cycle in the basal, unstressed state. This immunolabeling approach was selected for four primary reasons. First, prior experiments in male rats demonstrate that FosB/deltaFosB- and pCREB-immunolabeling are increased by a history of LSI in the basal, unstressed state in several brain regions that regulate stress and reward (Ulrich-Lai et al., 2010, 2016; Christiansen et al., 2011; Egan and Ulrich-Lai, 2015). Second, the expression of these transcription factors can enable the assessment of prolonged effects that accompany the chronic, repeated sucrose intake pattern of the LSI paradigm. For instance, while the phosphorylation of CREB to form activated pCREB occurs rapidly during neuronal activation, pCREB expression can also be prolonged, particularly after chronic, repeated or sustained activation (Bito et al., 1996; Laifenfeld et al., 2005; Rybnikova et al., 2008; Kreibich et al., 2009). Likewise, FosB is a member of the Fos immediate early gene family that is rapidly and transiently expressed following neuronal activation (Hope et al., 1992; Nestler, 2008), while deltaFosB is a truncated form of the full-length FosB that resists degradation and accumulates with chronic or repeated stimulation (Nestler et al., 1999). Consistent with this idea, pCREB and FosB/deltaFosB immunolabeling are increased in the BLA for at least 18 hours after the last sucrose exposure in male LSI rats (Ulrich-Lai et al., 2010, 2016; Christiansen et al., 2011). Third, while these transcription factors are well-established markers of neuronal activation, they are also associated with neuroadaptation and/or neural plasticity. pCREB is thought to be critical for long-term potentiation and learning and memory processes (Silva et al., 1998; Huang et al., 2000; Miyamoto, 2006). FosB/deltaFosB expression is also linked with long-term changes in neural plasticity and neuroadaptation, including following chronic treatment with pharmacological rewards like drugs of abuse, and natural rewards like sexual activity and palatable food intake (Chen et al., 1997; Nestler et al., 1999; McClung et al., 2004; Wallace et al., 2008; Vialou et al., 2010; Christiansen et al., 2011; Nestler, 2013). Finally, exploratory Bayesian network analyses can be performed on the immunolabeling data to discover the

most likely neural network whose functional relationships are altered by sucrose in an estrous cycle-specific manner in female rats.

Experimental procedures

Animals

Adult, female Long-Evans rats (~175 g body weight, and ~8–10 weeks of age) were acquired from Envigo (formerly Harlan Laboratories, Indianapolis, IN). Rats were individually housed in a temperature- and humidity-controlled facility that is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC). Rats were maintained on a 12/12-h light/dark cycle (with lights on at 06:00 h) and acclimated to the housing facility for at least one week before experimental onset. Experimental procedures 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.

Limited sucrose intake (LSI) paradigm

Rats were given *ad libitum* access to standard rat chow (LM-485; Envigo Teklad, Madison, WI) and their normal water bottle throughout the experiment. In addition, twice a day (at ~09:30 h and ~15:30 h) they were provided brief access (up to 30 min) to a second bottle containing 30% sucrose solution dissolved in water (MP Biomedical, Solon, OH), while controls were offered a second bottle containing water. Rats were allowed to drink up to 4 ml/session (or up to 8 ml/day) from the second drink bottle, as described previously (Ulrich-Lai et al., 2007, 2010). Rats received this LSI paradigm for 14 days (d). To test HPA axis responsivity to acute stress, rats received a restraint stress challenge on d15, as described below, and did not receive LSI that day. To assess the impact of chronic LSI on brain pCREB and FosB/deltaFosB expression in a basal, unstressed state, LSI resumed on d16–18, prior to perfusion and tissue collection on d19, as described below. Prior work indicates that a single brief stress exposure does not cause prolonged increases in pCREB and FosB/deltaFosB expression (Kovács and Sawchenko, 1996; Stanciu et al., 2001; Perrotti et al., 2004), suggesting that the expression measured on d19 primarily reflects the effects of the LSI paradigm, with minimal impact of the prior acute restraint. A schematic of the experimental timeline is shown in Figure 1. Body weight, food intake, and drink intake from the second drink bottle were monitored throughout the experiment, and body composition was measured with NMR (EchoMRI, Houston, TX).

Restraint stress and plasma hormone measurement

On the morning of d15, rats (n = 26/group) were exposed to a 20-min restraint stress challenge. Rats were placed into clear, well-ventilated restraint tubes and tail-clip blood samples (200 µl) were quickly collected into ice-cold EDTA-coated tubes. Care was taken to ensure that this first blood collection (i.e., at 0 min) was completed within 3 min of first handling each rat's cage to ensure assessment of basal, pre-stress hormone levels (Vahl et al., 2005). Blood samples were again collected at 20, 40, and 60 min after the onset of restraint and were each completed within 3 minutes; these post-stress collection time points were selected to keep them comparable to our prior HPA restraint stress testing in LSI-

treated male rats (Ulrich-Lai et al., 2007, 2010). After the completion of all blood sample collection (i.e., immediately after the 60 min time point), vaginal lavage was performed to determine estrous cycle stage by cytology (see below) (Marcondes et al., 2002; Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015). Blood samples were centrifuged ($3000 \times g$, 15 min, 4°C) and plasma was stored at -80°C until measurement of plasma ACTH and corticosterone by radioimmunoassay (RIA), as described below.

Tissue collection and immunohistochemistry

On the morning of d19 (i.e., approximately 18 hours after the last LSI presentation), animals were injected with an overdose of pentobarbital and vaginal lavage samples were taken to determine estrous cycle stage. Next, a cardiac puncture blood sample was quickly collected, and plasma was stored at -80°C until later measurement of plasma estradiol by RIA. Rats were then perfused transcardially with 0.9% saline, followed by 3.7% paraformaldehyde in phosphate-buffered saline (PBS). Adrenal and thymus glands were isolated, cleaned and weighed as indirect indices of historically elevated plasma ACTH and corticosterone levels, respectively (Martí et al., 1993; Blanchard et al., 1995, 1998; Herman et al., 1995; Avishai-Eliner et al., 2001; Ulrich-Lai et al., 2007; Ulrich-Lai and Herman, 2009). Brains were removed, post-fixed overnight in 3.7% paraformaldehyde-PBS at room temperature, and then stored in 30% sucrose in PBS at 4°C. Brains were later sectioned (Leica Biosystems microtome, Wetzlar, Germany) into a 1-in-12 series of 25 µm tissue slices. Brain slices were stored at -20°C in cryoprotectant (1% polyvinylpyrrolidone (Sigma-Aldrich, Perth, WA), 30% ethylene glycol (Fisher Scientific, Pittsburgh, PA), and 30% sucrose (Amresco, Solon, OH) in PBS).

Determination of estrous cycle stage by cytology

In order to determine the impact of estrous cycle on HPA and brain measures, vaginal lavage samples were taken at the end of the restraint stress on d15, and again just prior to perfusion and tissue collection on d19. Vaginal cytology was then used to determine estrous cycle stage (Marcondes et al., 2002; Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015). In particular, diestrus 1 (D1) cytology was characterized by a prevalence of white blood cells and mucous, with smaller amounts of epithelial cells that were primarily of the cornified type (Marcondes et al., 2002; Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015). Diestrus 2 (D2) also had large numbers of white blood cells and mucous, but was distinguished by having more non-cornified vs. cornified epithelial cells (Marcondes et al., 2002; Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015). In contrast, proestrus (P), late proestrus (LP) and estrus (E) each had little to no white blood cells and mucous. P was characterized by large numbers of non-cornified epithelial cells, E had large numbers of cornified epithelial cells, and LP had a mixture of both cornified and non-cornified epithelial cells (Marcondes et al., 2002; Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015). Animals were then classified into two different estrous cycle sub-groups: D1/D2, when females would be expected to have lower levels of gonadal hormones, and P/E, when females would be expected to have higher levels of gonadal hormones (Butcher et al., 1974; Smith et al., 1975; Nequin et al., 1979; Cecchini et al., 1983; Lu et al., 1985; Asarian and Geary, 2013). As our primary focus was to evaluate the impact of estrous cycle on HPA axis and brain immunolabeling responses, these two estrous cycle sub-groups were then

compared to each other in all analyses (see below) to test for potential cycle-specific effects. Note that since the HPA testing and brain immunolabeling measures were assessed 4 days apart (i.e., d15 vs d19), and the rat estrous cycle typically varies between ~4–5 days in length (Becker et al., 2005; Goldman et al., 2007; Cora et al., 2015), many (~65%) of the rats were determined to be in the same estrous cycle sub-group on days 15 and 19.

Hormone measurements

Plasma ACTH concentrations were determined by an RIA using a specific antiserum graciously donated by Dr. William Engeland (University of Minnesota, Minneapolis, MN) (Engeland et al., 1981, 1989). Plasma corticosterone levels were determined using an ¹²⁵I corticosterone double antibody RIA kit (product # 07120103, MP Biomedicals, Solon, OH); this kit measures total corticosterone levels (per technical support at the manufacturer). Plasma estradiol levels were determined using an ¹²⁵I 17 β -estradiol (E2) double antibody RIA kit (product # 07138102, MP Biomedicals, Solon, OH). The intrassay coefficient of variation was less than 10% for all assays.

Brain immunolabeling

Brain immunohistochemistry was performed for pCREB and FosB/deltaFosB. Briefly, brain sections were washed in 50 mM potassium phosphate-buffered saline (KPBS), incubated for 20 min in hydrogen peroxide (2% in KPBS) solution at room temperature, and rinsed again in KPBS. Sections were then incubated in blocking solution for 1 h at room temperature. The blocking solution was composed of 0.1% bovine serum albumin (Sigma-Aldrich, Perth, WA) and 0.2% Triton X-100 (Sigma-Aldrich, Perth, WA) in KPBS. Brain sections were then incubated with primary antibodies (diluted in blocking solution) overnight at 4°C. The primary antibodies used included rabbit antisera against FosB/deltaFosB (1:300, product #sc-7203, Santa Cruz, Dallas, TX) or rabbit antisera against pCREB (1:500, product #06-519, Millipore, Darmstadt, Germany). The following day, sections were rinsed in KPBS and incubated in biotinylated goat anti-rabbit secondary antibody (1:500 for pCREB, 1:250 for FosB/DeltaFosB, product #BA1000, Vector Laboratories, Burlingame, CA) for 1 h. Following rinsing with KPBS, sections were incubated in avidin-biotin-peroxidase (Vectastain ABC solution, Vector Laboratories, Burlingame, CA) for 1 h, rinsed again in KPBS, and reacted with 3,3'-diaminobenzidine (Sigma-Aldrich, Perth, WA). Sections were dehydrated through a graded series of ethanol and coverslipped with DPX mountant (Sigma-Aldrich, Perth, WA).

FosB/deltaFosB and pCREB immunolabeling were each measured in eight brain regions known to be involved in stress and/or reward regulation (Swanson and Sawchenko, 1983; Park and Carr, 1998; Dayas et al., 1999; LeDoux, 2000; Figueiredo et al., 2003; Herman et al., 2003; Bhatnagar et al., 2004; Grippo et al., 2004; Radley et al., 2006; Choi et al., 2007; Ulrich-Lai and Herman, 2009; Ulrich-Lai et al., 2010; Myers et al., 2014): BLA, bed nucleus of the stria terminalis, anterodorsal subdivision (BSTad) and principal subdivision (BSTpr), central amygdala (CeA), posterodorsal subdivision of the medial amygdala (MeApd), nucleus accumbens (NAc), prefrontal cortex (PFC), and paraventricular nucleus of the hypothalamus (PVN). Anatomical regions were defined using standard rat brain atlases (Paxinos and Watson, 1998; Swanson, 1998). Immunolabeling was captured using a

brightfield microscope (Axio Imager.M2) with an AxioCam camera and Zen 2012 software (Carl Zeiss Microscopy, Jena, Germany). The density of pCREB- or FosB/DeltaFosB-positive cells was measured using Scion Image software (Scion Corp, Frederick, MD). Analyses were performed in all available, intact sections that contained the regions of interest. Analyses were performed by lab personnel that were blinded to group assignments.

Bayesian network modeling

While immunohistochemical analyses can provide insight into changes within specific brain regions, brain regions do not generally work in isolation, but rather function within a dynamic, interconnected network. To simultaneously consider the influence of both proteins (FosB/deltaFosB and pCREB) across all examined brain regions, we therefore performed an exploratory Bayesian network analysis using R 3.3.2 software with bnlearn package version 4.0. The hc function in the bnlearn package uses a hill-climbing greedy search strategy with random restarts to screen all possible Bayesian networks. The network with the highest possible score is then identified as the ‘best fit’ for the water- and sucrose-fed rats alone, and together, for each cycle stage. Briefly, this procedure initially places all the data into an ‘empty’ network that assumes no connections among the various brain regions in terms of their extent of FosB/deltaFosB and pCREB protein expression. The greedy search algorithm then adds, removes, and reverses a single connection between one specific protein in one specific brain region, and another specific protein in another specific brain region. The network score of this new network is then calculated, and represents the degree that the model’s theoretical distribution overlaps with the actual distribution of the data. The greedy search algorithm then continues to try all possible combinations of connectivity (i.e., across the expression of both FosB/deltaFosB and pCREB proteins, and among all of the different brain regions) and the network score of each is calculated. Ultimately, the ‘best fit’ model is determined as the model that gives the highest network score, since this is the one whose theoretical distribution has the highest likelihood of explaining the actual data distribution. Of note, the exploratory manner of the Bayesian network analysis did not assume any prior information on the network structure, and therefore provides an overall (though very rough) picture of the likely underlying relationships among both immunolabeled proteins across all 8 brain regions.

Statistical analysis

Data are presented as mean $\pm$ SEM. Drink intake, food intake, and body weight data were each analyzed via two-way ANOVA using the factors DRINK and TIME, with TIME as a repeated measures factor. Percent body fat, adrenal weight, and thymus weight data were each analyzed via two-tailed t-test comparing sucrose vs. water. Plasma ACTH and corticosterone were each analyzed by three-way ANOVA using the factors DRINK, CYCLE, and TIME, with TIME as a repeated measures factor. FosB/deltaFosB and pCREB immunolabeling data were each analyzed via two-way ANOVA with the factors DRINK and CYCLE. The Bayesian network models were analyzed via the Pearson’s Chi-squared test for homogeneity comparing D1/D2 vs. P/E. Plasma estradiol levels were analyzed via one-way ANOVA across the estrous cycle stages. ANOVAs were followed by a protected Newman-Keuls post-hoc analysis. Data sets with non-homogenous variance underwent a square root transformation prior ANOVA. Potential outliers were identified as described previously

(McClave and Dietrich, 1994; Ulrich-Lai et al., 2010). Briefly, we used two criteria and both had to be met for a value to be removed as an outlier: (1) values were greater than 1.96 times the standard deviation from the mean, and (2) values were below the lower quartile or above the upper quartile by more than 1.5 times the interquartile range. Statistical significance was taken as $p < 0.05$. All ANOVA main or interactive effects that reached significance are described in the Results section.

Results

Effects of LSI on energy balance

Rats given access to sucrose quickly began to drink close to the daily maximum amount permitted. In contrast, water controls drank little from the second drink bottle; this finding is expected as these rats have *ad libitum* access to their normal water bottle, and therefore have little motivation to drink extra water. Total fluid intake from the second bottle showed main effects of DRINK ($F_{1,935} = 936.39$, $p < 0.01$) and TIME ($F_{17,935} = 61.03$, $p < 0.01$), as well as a DRINK $\times$ TIME interaction ($F_{17,935} = 31.10$, $p < 0.01$). Post-hoc analyses revealed that sucrose-fed rats drank more at every day of LSI exposure (Fig. 2A).

Food intake also showed main effects of DRINK ($F_{1,259} = 44.62$, $p < 0.01$) and TIME ($F_{4,259} = 13.16$, $p < 0.01$). Post-hoc analyses revealed that sucrose-fed rats reduced their food intake by approximately 10% compared to water-fed rats (Fig. 2B), which is roughly isocaloric to the amount consumed in sucrose drink. This resulted in no differences in body weight (Fig. 3C) or percent body fat (Fig. 2D) between the drink groups.

HPA axis response to acute stress

Analysis of the plasma ACTH response to acute stress showed a main effect of TIME ($F_{3,187} = 595.25$, $p < 0.01$), as well as DRINK $\times$ TIME ($F_{3,187} = 3.84$, $p < 0.05$) and DRINK $\times$ CYCLE $\times$ TIME ($F_{3,187} = 3.04$, $p < 0.05$) interactions. Post-hoc analyses revealed that among rats in the diestrus 1/diestrus 2 (D1/D2) stage of the estrous cycle, there were no differences in plasma ACTH between drink groups (Fig. 3A). However, among rats in the proestrus/estrus (P/E) stage of the estrous cycle, sucrose-fed rats had reduced post-restraint plasma ACTH specifically at the 60-min time point (Fig. 3B).

Analysis of the plasma corticosterone response to acute stress showed a main effect of DRINK ($F_{1,207} = 6.30$, $p < 0.05$), a main effect of TIME ($F_{3,207} = 652.82$, $p < 0.01$), and a DRINK $\times$ TIME interaction ($F_{3,207} = 3.71$, $p < 0.05$). However, post-hoc analyses revealed no specific group differences at any time point among rats in D1/D2 (Fig. 3C) or P/E (Fig. 3D). Together, the ACTH and corticosterone results suggest that sucrose decreased the HPA axis response to stress specifically during the P/E stage, and that this effect occurred primarily for plasma ACTH. Though, given the fact that corticosterone secretion is temporally delayed relative to circulating ACTH levels, and the observation that the drink effects on ACTH occurred at the last sample time point (60 min), this does not preclude the possibility that drink-induced differences in plasma corticosterone could have occurred at later post-stress time points.

Of note, among water-fed controls, there was no significant effect of estrous cycle alone on either post-stress plasma ACTH or corticosterone, though the plasma ACTH levels tended to be slightly higher in P/E. This finding is in line with reports that HPA reactivity/tone is inconsistently elevated during proestrus/estrus versus diestrus (Viau and Meaney, 1991, 2004; Guo et al., 1994; Carey et al., 1995; Walker et al., 2001; Bland et al., 2005; Figueiredo et al., 2007; Babb et al., 2013).

Adrenal and thymus weights

Sucrose (vs. water) intake did not affect either raw adrenal weight, or adrenal weight normalized to body weight (both two-tailed t-test, $p > 0.05$). Similarly, raw and normalized thymus weights did not differ between drink groups (both two-tailed t-test, $p > 0.05$). These results suggest that limited, intermittent sucrose intake did not induce adrenal hypertrophy or thymic involution. These results are summarized in Table 1.

FosB/deltaFosB and pCREB immunolabeling

Immunohistochemistry was performed to measure labeling of FosB/deltaFosB and pCREB in seven brain regions known to be involved in stress and/or reward pathways: BLA, BSTad, BSTpr, CeA, MeApd, NAc, PFC, and PVN.

FosB/deltaFosB—Several brain regions showed interactive effects of sucrose drink and estrous cycle stage on the density of FosB/deltaFosB-positive cells (Fig. 4, Table 2). In the BLA (Fig. 4A,B), there were main effects of DRINK ($F_{1,47} = 7.91$, $p < 0.01$) and CYCLE ($F_{1,47} = 6.44$, $p < 0.05$) as well as a DRINK $\times$ CYCLE interaction ($F_{1,47} = 4.46$, $p < 0.05$). Post-hoc analyses showed that FosB/deltaFosB labeling was increased by sucrose drink specifically during P/E. In the CeA (Fig. 4C,D), there was a main effect of DRINK ($F_{1,51} = 8.54$, $p < 0.01$) and a DRINK $\times$ CYCLE interaction ($F_{1,51} = 4.20$, $p < 0.05$). Similar to the BLA, post-hoc analyses revealed that FosB/deltaFosB labeling in the CeA was increased specifically during the P/E stage. Since there is some evidence that the medial and lateral subdivisions of the CeA can be differentially regulated by stress and reward (Martina et al., 1999; Fudge and Haber, 2000; Cioocchi et al., 2010; Haubensak et al., 2010; Duvarci et al., 2011; Gilpin et al., 2015), we also analyzed the FosB/deltaFosB immunolabeling for each of these CeA subdivisions. Both subdivisions showed a similar pattern of labeling as the total CeA (with statistical analyses indicating that sucrose increased FosB/deltaFosB-positive cells specifically in P/E for both the medial and lateral CeA, data not shown). There was also a DRINK $\times$ CYCLE interaction ($F_{1,47} = 7.67$, $p < 0.01$) in the PVN (Fig. 4E,F), where sucrose decreased FosB/deltaFosB immunolabeling specifically during D1/D2.

In contrast, for the MeA (Fig. 4G,H, Table 2) there was a main effect of sucrose to increase the density of FosB/deltaFosB-positive cells (DRINK, $F_{1,47} = 6.36$, $p < 0.05$) and a main effect of P/E to lower them (CYCLE, $F_{1,47} = 4.47$, $p < 0.05$), with no interaction between drink and cycle.

Finally, there were no main or interactive effects of either sucrose drink or estrous cycle on the density of FosB/deltaFosB-positive cells in the BSTad, BSTpr, NAc, or PFC (Table 2). As the core and shell subdivisions of the NAc are differentially involved in reward regulation

(Deutch and Cameron, 1992; Bassareo and Di Chiara, 1999; Bassareo et al., 2011; Corbit and Balleine, 2011; Cacciapaglia et al., 2012), we also analyzed the FosB/deltaFosB immunolabeling separated into these NAc subdivisions. Both subdivisions showed a similar pattern of labeling as the total NAc (data not shown), with statistical analyses indicating that there were no main or interactive effects of sucrose drink or estrous cycle. Collectively, these results indicate that there are differential effects of sucrose drink and estrous cycle stage on FosB/deltaFosB protein expression across multiple brain regions involved in stress and/or reward processing.

pCREB—There was a significant interaction between the effects of sucrose drink and estrous cycle stage ($F_{1,49} = 4.25$, $p < 0.05$) on the density of pCREB-positive cells in the CeA (Fig. 5A,B, Table 3). Post-hoc analyses revealed that sucrose increased pCREB labeling compared to water specifically in the P/E stage. However, when the CeA was divided into medial and lateral subdivisions for analysis, neither of these sub-regions alone reached statistical significance (data not shown).

In the BLA, there was a main effect of P/E to lower the density of pCREB-positive cells (CYCLE, $F_{1,47} = 21.28$, $p < 0.01$), with no main or interactive effects of sucrose drink (Fig. 5C,D, Table 3). Post-hoc analyses showed that pCREB immunolabeling was decreased during P/E in both drink groups.

Finally, there were no main or interactive effects of either sucrose or estrous cycle stage on the density of pCREB-positive cells in the BSTad, BSTpr, MeApd, NAc (or its core and shell subdivisions, data not shown), PFC, or PVN (Table 3).

Bayesian network modeling

Because brain regions do not work in isolation, but rather function as an interconnected, dynamic network, we wanted to perform an analysis that considered both FosB/deltaFosB and pCREB expression across all eight brain regions at once. In order to do this, we performed exploratory Bayesian network analyses across the estrous cycle. In this approach, all possible networks were considered, and the optimal network (i.e., the one that best fit the data) was developed for sucrose- vs. water-fed rats in D1/D2 and P/E.

The exploratory Bayesian analyses revealed that the expression of pCREB and FosB/deltaFosB proteins were likely related either to each other, and/or to themselves, across multiple brain regions (Fig. 6). Moreover, there were striking differences between the identified networks in D1/D2 (Fig. 6A) versus P/E (Fig. 6B). During D1/D2, there were a total of 30 protein associations that were predicted to occur only in water controls, 23 predicted to occur only in sucrose-fed rats, and 28 predicted to occur in both water- and sucrose-fed rats. However, during P/E, the total number of water-only associations was markedly reduced to 14, while the sucrose-only associations increased to 33, and those predicted for both water and sucrose rats were reduced to 19. Statistical comparison of the Bayesian networks in D1/D2 versus P/E with a Pearson's Chi-squared test for homogeneity indicated that these networks are significantly different ($p < 0.01$).

Upon closer visual inspection, it becomes further apparent that specific changes in the relationships among particular brain regions vary markedly across estrous cycle stage. The marked decrease in water-only associations from D1/D2 to P/E occurred for most of the examined brain regions, including the BLA (which went from 10 to 0 water-only relationships, or 100% less), BSTpr (from 7 to 2, or 71% less), PFC (from 7 to 2, or 71% less), NAc (from 6 to 2, or 67% less), MeA (from 12 to 6, or 50% less), and PVN (from 7 to 5, or 29% less). Cycle stage did not noticeably alter the number of water-only associations for the CeA (4 in D1/D2, and 5 in P/E) or BSTad (5 in D1/D2, 4 in P/E). These findings imply that for water controls, functional relationships among the BLA, PFC, NAc, MeA, BSTpr, and PVN predominate during D1/D2. This supports the idea that stress- and reward-processing may be broadly distributed across a large, multimodal brain network that is impacted by estrous cycle.

In contrast, the observed increase in sucrose-only associations from D1/D2 to P/E was primarily due to increases for the PFC (from 6 to 12, or 100% more), BSTad (from 5 to 10, or 100% more), and NAc (from 7 to 12, or 71% more). Cycle stage had less impact on the number of sucrose-only associations for the PVN (7 in D1/D2, and 7 in P/E), BLA (5 in D1/D2, and 6 in P/E), BSTpr (9 for both stages), MeA (3 in D1/D2, and 4 in P/E), and CeA (3 in D1/D2, and 4 in P/E). However, the gain (or maintenance) of sucrose-only relationships from D1/D2 to P/E occurred in several of the same regions that experienced a loss of water-only relationships. Many regions therefore had a marked increase in the proportion of predicted relationships that were sucrose-only from D1/D2 to P/E, including for the BSTad (from 28% to 63%), NAc (from 30% to 67%), BLA (from 26% to 60%), PFC (from 26% to 57%), and BSTpr (from 45% to 75%). In contrast, the proportion of sucrose-only relationships was less affected by estrous cycle in the PVN (41% in D1/D2 and 4% in P/E), CeA (17% in D1/D2 and 29% in P/E), and MeA (17% in D1/D2 and 20% in P/E). Collectively, these results suggest that a history of sucrose drink promotes functional relationships among the NAc, BLA, PFC, BSTpr, and BSTad, primarily in P/E, suggesting the intriguing possibility that sucrose recruits this network to mediate stress relief in a P/E-specific manner.

Plasma estradiol

Plasma estradiol was measured on experiment d19 as an independent means to corroborate the d19 estrous cycle staging by lavage cytology. While the experiment was designed (and statistically powered) to analyze HPA and brain immunolabeling results after division into two broad estrous cycle stages (i.e., D1/D2 and P/E), plasma estradiol peaks specifically in proestrus (Butcher et al., 1974; Smith et al., 1975; Nequin et al., 1979; Cecchini et al., 1983; Lu et al., 1985; Asarian and Geary, 2013). As a result, in order to use plasma estradiol to confirm cytological staging, the rats were further subdivided into diestrus day 1, diestrus day 2, proestrus, late proestrus/estrus, and estrus stages based upon their lavage cytology. This analysis (Table 4) revealed a significant main effect of estrous stage, as determined by cytology, on plasma estradiol levels (one-way ANOVA; $F_{4,49} = 7.83$, $p < 0.01$). Moreover, post-hoc analyses showed that rats staged in proestrus by cytology had increased plasma estradiol compared to all other groups, consistent with the known variations of estradiol across the estrous cycle (Butcher et al., 1974; Smith et al., 1975; Nequin et al., 1979;

Cecchini et al., 1983; Lu et al., 1985; Asarian and Geary, 2013). These positive control data support our ability to use vaginal lavage cytology to determine estrous cycle stage.

Discussion

In this study, we found that a history of limited, intermittent sucrose intake reduced the plasma ACTH response to a subsequent acute stress in female rats. Moreover, this HPA blunting only occurred during the P/E stage and not the D1/D2 stage of the estrous cycle. Markers of neuronal activation that are linked to plasticity and neuroadaptation were altered by sucrose drink and estrous cycle in the basal, non-stressed state, including sucrose-induced increases in CeA FosB/deltaFosB and pCREB-immunolabeling, as well as BLA FosB/deltaFosB-immunolabeling, that occurred specifically in P/E. Bayesian network analyses predicted a large number of water-only associations during D1/D2 (vs. P/E), while more sucrose-only associations were predicted for P/E (vs. D1/D2). Moreover, the P/E-specific shift towards a greater proportion of sucrose-only associations occurred among the BLA, NAc, PFC, BSTad, and BSTpr, implicating these structures as members of a network whose inter-relationships are altered by LSI in a cycle-dependent manner in females. Collectively, these data support the hypothesis that palatable food reduces HPA axis reactivity and alters stress/reward-related neurocircuitry in an estrous-cycle specific manner in female rats.

Impact of LSI on energy balance in female rats

We first examined the effects of twice-daily, limited access to a sucrose drink on metabolic-related properties in female rats. Within the first few days of the paradigm, female rats began to drink sucrose in amounts approaching the maximum allowed, while concurrently reducing their chow intake, resulting in no differences in body weight or percent body fat. Collectively, these data support that a 30% sucrose drink is highly palatable to female rats, as shown previously (Deshaies et al., 1983; Sclafani, 1987a, 1987b). Moreover, the findings indicate that female rats are able to fully compensate for the sucrose-derived calories, thereby preventing both increased body weight and adiposity in this paradigm. Of note, this metabolic response pattern mirrors that which occurs in male rats during the LSI paradigm (Ulrich-Lai et al., 2007, 2010), suggesting that the LSI effects on energy balance are comparable between males and females.

Impact of LSI and estrous cycle on basal and post-stress HPA axis responsivity

We previously characterized the effects of LSI on basal and stress-induced HPA axis functioning in male rats. In male rats, LSI does not alter basal HPA axis functioning, as there are no differences between sucrose- and water-fed rats in plasma ACTH or plasma corticosterone prior to the acute stressor exposure (Ulrich-Lai et al., 2007, 2010). Furthermore, neither adrenal nor thymus weights are affected by a history of LSI in male rats (Ulrich-Lai et al., 2007), indicating comparable historical levels of plasma ACTH and corticosterone. In contrast, stress-induced HPA reactivity is consistently blunted by LSI in male rats. This invariably includes reduced post-stress plasma corticosterone following LSI, though this is accompanied by reduced post-stress plasma ACTH somewhat less consistently (Ulrich-Lai et al., 2007, 2010; Christiansen et al., 2011). Such dissociations between ACTH and corticosterone are quite common (Dempsher and Gann, 1983; Engeland et al., 1989;

Kiss et al., 1994; Pecoraro et al., 2004; Ostrander et al., 2006; Ulrich-Lai et al., 2007, 2010; Bornstein et al., 2008), and since we have previously shown that LSI does not alter adrenal responsivity to ACTH (Ulrich-Lai et al., 2010), this likely occurs because of the timing of sample collection relative to the peak ACTH and corticosterone responses, and/or differences between bioactive vs. immunoreactive ACTH (Engeland et al., 1989).

The present work extended this ongoing line of research into a new and clinically-relevant direction by asking whether LSI alters basal and post-stress HPA axis activity in female rats. Indices of basal HPA axis functioning (i.e., pre-stress plasma ACTH and corticosterone, and adrenal and thymus gland weights) were not affected by LSI in female rats. LSI reduced the plasma ACTH response to restraint stress among rats in P/E, and not among those in D1/D2. This finding suggests that LSI may blunt post-stress hypothalamic-pituitary responses in a cycle-dependent manner. In contrast, post-stress plasma corticosterone was not affected by sucrose in either the D1/D2 or P/E stages. As seen previously with male rats, it is not uncommon to observe dissociations between plasma ACTH and corticosterone responses (Dempsher and Gann, 1983; Engeland et al., 1989; Kiss et al., 1994; Pecoraro et al., 2004; Ostrander et al., 2006; Ulrich-Lai et al., 2007, 2010; Bornstein et al., 2008). There are several possible explanations for this dissociation. For instance, since circulating ACTH is a primary factor that induces corticosterone synthesis and secretion by the adrenal cortex, changes in circulating ACTH typically precede the downstream changes in plasma corticosterone by a marked amount of time (e.g., up to 30–60 min) (Herman et al., 2016). Treatment effects on plasma ACTH are therefore not expected to produce corresponding variations in plasma corticosterone levels until a considerable amount of time later. The blood collection time points utilized in the present work were selected to facilitate comparisons to our prior experiments in male rats. However, since LSI effects on plasma ACTH occurred at a later time point in females relative to males (e.g., at 60 min), our blood collection schedule did not capture plasma corticosterone levels at a later time point (e.g., 90 min) when the variations in plasma ACTH would be predicted to impact corticosterone. It seems inappropriate then to definitely conclude that LSI does not alter post-restraint plasma corticosterone since we cannot exclude the possibility that the corticosterone effect may have occurred at later post-stress time points in the females. The findings also strongly suggest that the blood collection time course should be extended for future LSI studies in female rats.

Additionally, it has previously been shown that estrogen enhances adrenal responsivity to ACTH (Figueiredo et al., 2007), which may contribute towards the maintenance of post-stress plasma corticosterone levels in sucrose-fed rats during P/E despite blunted ACTH responses. Moreover, while the effects of estrogen on HPA axis responses have been extensively studied, plasma progesterone is also increased in P/E compared to D1/D2 (Butcher et al., 1974; Smith et al., 1975; Nequin et al., 1979; Cecchini et al., 1983; Lu et al., 1985; Asarian and Geary, 2013), and progesterone has been shown to both increase (Redei et al., 1994; Patchev et al., 1996; Keller-Wood, 1998; Figueiredo et al., 2007) or decrease (Viau and Meaney, 1991) HPA axis responsivity to stress. We therefore cannot exclude a role for progesterone in the differences in HPA axis responses and neuronal activation (as discussed below) seen in P/E compared to D1/D2.

It could be the case that the LSI paradigm may itself influence the estrous cycle. To evaluate this possibility, we would need to perform daily lavages for many days prior to and throughout the LSI paradigm. Since the stress associated with the daily lavage procedure could have impacted our primary experimental outcomes (i.e., HPA responsivity, brain activation patterns), we opted to limit assessments of the estrous cycle to the days of sample collection (e.g., days 15 and 19).

Impact of LSI and estrous cycle on stress- and reward-regulatory brain regions

To identify the potential neurobiological mechanisms in female rats, FosB/deltaFosB and pCREB immunolabeling were assessed in several regions known to be involved in stress and/or reward regulation. These data were then subjected to two different types of statistical analysis in order to (1) investigate the effects of LSI and estrous cycle, and (2) identify the most likely neural circuitry whose inter-relationships are altered by LSI in an estrous cycle-specific manner in female rats. With the first analysis, standard ANOVAs evaluated group differences for each protein within each individual brain region. With the second analysis, exploratory Bayesian modeling simultaneously evaluated the relationships between the two proteins and across all the examined brain regions in either D1/D2 or P/E. These two different types of analysis each have their own advantages and disadvantages. The primary advantage of ANOVA is that it is a relatively simple analysis that is easily conducted using common statistical software packages. In contrast, while Bayesian modeling is much more complicated to perform, it has the advantage of being able to simultaneously consider a large number of comparisons without the need for an *a priori* model, and with relatively small group sizes, as commonly occur in biological data sets (Gelman et al., 2003; Needham et al., 2007; Ulrich-Lai et al., 2016). In this manner, it is well suited for modeling multi-nodal neuronal circuits to identify the one most likely to mediate a specific process (Gelman et al., 2003; Needham et al., 2007; Ulrich-Lai et al., 2016).

Analysis of FosB/deltaFosB- and pCREB-immunolabeling by ANOVA identifies the BLA and CeA as key sites—Individual analysis of FosB/deltaFosB and pCREB protein expression within each brain region by ANOVA implicated the BLA and CeA as potential candidates for mediating LSI stress-dampening, as both showed sucrose-induced changes that occurred specifically in P/E. This pattern mirrors the blunted ACTH response seen during P/E in sucrose-fed rats. The BLA is generally thought to promote HPA axis and behavioral responses to stress (Coover et al., 1973; Feldman et al., 1983; Goldstein et al., 1996; Shekhar et al., 2003; Bhatnagar et al., 2004; Ulrich-Lai and Herman, 2009; Wang et al., 2014). Though the BLA contains both local excitatory and inhibitory influences, so a history of LSI may alter the excitatory/inhibitory balance resulting in reduced stress responses. For example, we have previously shown in male rats that LSI specifically increases plasticity in parvalbumin interneurons in the BLA (Packard et al., 2017), and an intact BLA is necessary for LSI-induced HPA blunting (Ulrich-Lai et al., 2010). This suggests that LSI may blunt stress responses, at least in part, by increasing intra-BLA inhibitory tone, thereby reducing the BLA's stress-excitatory output. The CeA is thought to regulate behavioral and autonomic nervous system responses to stress (Rooszendaal et al., 1990, 1991; Davis, 1992; Ulrich-Lai and Herman, 2009), whereas its contribution to HPA axis regulation is less clear, in part due to discrepant reports in the literature. For instance,

axon-sparing lesions of the CeA inconsistently reduce HPA axis activation by restraint stress, suggesting that the CeA's role may depend on other factors, such as prior experiences or housing conditions (Van de Kar et al., 1991; Prewitt and Herman, 1994, 1997; Dayas et al., 1999; Xu et al., 1999). Furthermore, there are also strong functional and anatomical interactions between the BLA and CeA that regulate stress-related behavioral end points (Krettek and Price, 1978; Janak and Tye, 2015). For example, optogenetic stimulation of direct BLA-to-CeA projections reduces anxiety-related behavior (Tye et al., 2011), though the potential contribution of this particular pathway to HPA axis regulation is not yet known. Finally, it is important to note that prior investigations of HPA axis regulation by the BLA and CeA have primarily used only male subjects, so the extent to which these structures may contribute to sex- and estrous cycle-dependent HPA effects is not clear. However, both the CeA and BLA express estrogen receptors (ER) (Shughrue et al., 1997; Osterlund et al., 1998; Shughrue and Merchenthaler, 2001; Merchenthaler et al., 2004), and BLA neuronal activity varies between males and females, across the estrous cycle, and in response to estradiol treatment (Womble et al., 2002; Blume et al., 2017), supporting this possibility. When taken together, these findings implicate the BLA and CeA as potential key sites where LSI and the estrous cycle may interact to affect stress responses in females.

Analysis of pCREB and FosB/deltaFosB-immunolabeling by Bayesian modeling implicates a BLA-NAc-PFC-BST network—Bayesian analysis identified a multi-nodal BLA-NAc-PFC-BSTpr-BSTad circuit in which the proportion of predicted sucrose-related associations were increased for these structures specifically in P/E. Notably, the BLA was also implicated by the ANOVA analysis approach, as discussed above, further increasing confidence that it may contribute to LSI's effects in females, and as it does in males (Ulrich-Lai et al., 2010; Christiansen et al., 2011; Packard et al., 2017). Moreover, the Bayesian analyses indicate that the BLA is likely not working alone, but rather via relationships among the NAc, PFC, BSTpr, BSTad, and itself. This is striking, as a similar modeling approach indicated that sucrose may act in male rats by altering relationships in a network that includes the BLA, PFC, and BSTpr (Ulrich-Lai et al., 2016). Collectively, this suggests that modulation of a BLA-PFC-BSTpr circuit may be a common consequence of 'comfort feeding' that is utilized by both males, and females in P/E.

It is interesting to note though that prior modeling did not identify a role for the NAc following LSI in males (Ulrich-Lai et al., 2016), as it did for females in the present work. The NAc is critical for reward processing (Wise and Rompre, 1989; Berridge, 1996; Park and Carr, 1998; Kelley and Berridge, 2002; Grippio et al., 2004; Norgren et al., 2006), and estrogen can potentiate the rewarding properties of drugs of abuse (Hecht et al., 1999; Lynch et al., 2000, 2001; Jackson et al., 2005; Hu and Becker, 2008), suggesting that the rewarding properties of sucrose intake may similarly vary with estrous cycle. As the hedonic/rewarding properties of sucrose are necessary for stress-dampening in male rats (Ulrich-Lai et al., 2010), this provides a potential mechanism by which cycle-dependent changes in sucrose reward could contribute to the cycle-dependence of sucrose stress-blunting in females. Thus, the NAc may contribute to LSI's effects in a sex-dependent manner, perhaps playing a role primarily in females during P/E.

Finally, while the CeA was strongly implicated by the statistical ANOVA analyses, it was not also identified through Bayesian network modeling. On the one hand, this could imply that the CeA works alone following LSI, and is thus not identified through an approach that focuses on the relationships among multiple brain regions. On the other hand, the CeA may be a component of a distinct LSI-recruited network whose other members were not assessed. For example, the CeA can regulate stress responses via anatomical and functional connections with brainstem structures, like the nucleus of the solitary tract and the lateral parabrachial nucleus (Schwaber et al., 1982; van der Kooy et al., 1984; Swanson and Petrovich, 1998; Herman et al., 2003). This is a novel possibility, as it suggests for the first time that the CeA may contribute to the stress relieving properties of ‘comfort foods’ via its ability to influence a distinct stress-regulatory circuit in an estrous cycle-specific manner. Future empirical studies can directly address this intriguing hypothesis.

Summary

Prior mechanistic studies of the stress-relieving properties of palatable foods have been almost exclusively performed using male rodents (Bell et al., 2002; Dallman et al., 2003; la Fleur et al., 2004, 2005; Pecoraro et al., 2004; Ulrich-Lai et al., 2007, 2010; Kinzig et al., 2008; Coccorello et al., 2009; Warne, 2009; Finger et al., 2011), despite observations that women may be more prone to stress-related eating (Grunberg and Straub, 1992; Greeno and Wing, 1994; Oliver and Wardle, 1999; Oliver et al., 2000; Wansink et al., 2003; Klein et al., 2004; Zellner et al., 2006), and that stress-related eating varies across the menstrual cycle (Klump et al., 2013a, 2013b; Racine et al., 2013; Hildebrandt et al., 2015). The present work therefore explored the stress-relieving properties of a limited, intermittent sucrose intake paradigm in female rats across the estrous cycle. The results indicate that LSI reduces post-stress plasma ACTH specifically during P/E, and also increases basal, non-stress pCREB- and/or FosB/deltaFosB-immunolabeling in the BLA and CeA specifically during P/E. Finally, Bayesian network modeling of the pCREB and FosB/deltaFosB expression data implicates a neurocircuit that includes the BLA, NAc, PFC, BSTpr, and BSTad as the most likely to be altered by LSI during P/E. When considered in the context of our prior results, the present findings suggest that palatable food reduces stress responses in both male and female rats, with the effect in females varying across estrous cycle stage. Moreover, the BLA may contribute to LSI stress-dampening in both males and females, whereas the involvement of other brain regions, like the nucleus accumbens, may be sex-dependent.

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Highlights

- Limited sucrose intake (LSI) blunts the plasma ACTH response to restraint stress in female rats during proestrus/estrus
- LSI does not blunt post-restraint plasma ACTH during the diestrus phase of the estrous cycle
- Bayesian modeling is used with neuronal activation/plasticity markers to identify a brain network modified by LSI
- The modeled network includes the BLA, NAc, PFC, and BST as likely being modified by LSI during proestrus/estrus

Figure 1.

Experimental timeline of limited sucrose intake (LSI) exposure. Female rats received twice-daily access to a limited amount (4 ml/session, or 8 ml/day) of a 30% sucrose solution (vs. water as a control) on d1–14. On d15, rats did not receive LSI and instead were given a 20-minute acute restraint stress challenge. LSI resumed on d16–18, and rats were sacrificed for tissue collection on the morning of d19.

Figure 2.

The impact of limited sucrose intake on metabolic parameters 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 all days of LSI exposure. * $p < 0.05$ vs. water. $n = 26/\text{group}$.

Figure 3.

A history of limited sucrose intake (LSI) blunts the plasma ACTH response to restraint stress specifically during proestrus/estrus. The impact of prior sucrose (vs. water) on the plasma ACTH (A,B) and corticosterone (C,D) responses to an acute restraint stress during diestrus, D1/D2 (A,C) and proestrus/estrus, P/E (B,D). * $p < 0.05$ vs. water. $n = 8-18/\text{group}$. Note that for each hormone, data were analyzed by 3-way ANOVA with repeated measures comparing drink, cycle, and time, but are shown separated by estrous cycle to aid visualization.

paraventricular hypothalamic nucleus; opt, optic tract; MeA, medial amygdala; III, third ventricle.

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Figure 5.

A history of limited sucrose intake (LSI) increases pCREB-immunolabeling in the central amygdala specifically in proestrus/estrus. The impact of prior sucrose (vs. water) on the density of pCREB-positive cells in the CeA (A,B) and BLA (C,D). Representative images shown in (b) and (d) were taken at 50× magnification from a water D1/D2 rat; scale bar = 100 μ m. * $p < 0.05$ vs. water, # $p < 0.05$ vs. D1/D2. $n = 7-17$ /group. Abbreviations: BLA, basolateral amygdala; CeA, central amygdala; ec, external capsule (denoted by dashed line); ic, internal capsule; ITC, intercalated nuclei of the amygdala

Figure 6.

Exploratory Bayesian network considered the contribution of both FosB/deltaFosB and pCREB in all the brain regions at once. Predicted significant relationships between proteins within and across brain regions are shown in *blue* for water control rats, in *red* for sucrose-fed rats, and in *yellow* when predicted in both water control and sucrose-fed. Separate Bayesian networks were created for D1/D2 (A) and P/E (B), and a Pearson's Chi-squared test for homogeneity indicated that the D1/D2 and P/E networks were significantly different ($p < 0.01$). As an example, for the predicted network during D1/D2, MeA pCREB expression is related to PVN pCREB expression for rats drinking water, but not sucrose

(denoted by the circled blue box in panel A). In contrast, for the predicted network during P/E, MeA pCREB is related to PVN pCREB for rats drinking sucrose, but not water (denoted by the circled red box in panel B). Abbreviations: PVN, paraventricular hypothalamic nucleus; PFC, prefrontal cortex; NAc, nucleus accumbens; MeA, medial amygdala; CeA, central amygdala; BSTpr, bed nucleus of the stria terminalis, principal subdivision; BSTad, bed nucleus of the stria terminalis, anterodorsal subdivision; BLA, basolateral amygdala; FosB, FosB/deltaFosB

Table 1

Raw (un-normalized) adrenal and thymus weights, and tissue weights normalized to body weight, were not altered by prior LSI. n = 26/group.

	Water	Sucrose
Adrenal weight (mg)	48.5 ± 1.8	45.2 ± 1.5
Adrenal weight (mg) per 100 g body weight	20.7 ± 0.7	19.6 ± 0.7
Thymus weight (mg)	397.2 ± 17.7	399.4 ± 18.7
Thymus weight (mg) per g body weight	1.7 ± 0.1	1.7 ± 0.1

n = 26/group.

Table 2

The impact of LSI and/or estrous cycle stage on the density of FosB/deltaFosB-positive cells (number of cells/mm²) in multiple stress- and reward-regulatory brain regions.

Brain region	Water D1/D2	Water P/E	Sucrose D1/D2	Sucrose P/E
BLA	80.4 ± 9.4	83.0 ± 4.8	84.6 ± 4.5	112.9 ± 5.2 ^{*#}
BSTad	87.3 ± 6.7	98.9 ± 6.5	105.5 ± 10.8	90.1 ± 5.5
BSTpr	15.3 ± 2.3	11.5 ± 0.9	11.3 ± 1.0	11.7 ± 1.8
CeA	82.1 ± 9.8	69.5 ± 5.6	88.3 ± 6.5	105.0 ± 7.2 [*]
MeA	56.0 ± 5.8	51.7 ± 2.4	68.1 ± 3.4	57.5 ± 3.1 [#]
NAc	224.3 ± 18.8	215.2 ± 11.9	203.3 ± 9.6	200.2 ± 18.1
PFC	115.3 ± 11.4	124.1 ± 9.4	112.3 ± 14.0	105.1 ± 12.2
PVN	45.0 ± 9.0	35.5 ± 5.2	23.5 ± 2.5 [*]	45.3 ± 4.9 [#]

^{*} p<0.05 vs. water,

[#] p<0.05 vs. D1/D2, n = 7–17/group.

Table 3

The impact of LSI and/or estrous cycle stage on the density of pCREB-positive cells (number of cells/mm²) in multiple stress- and reward-regulatory brain regions.

Brain region	Water D1/D2	Water P/E	Sucrose D1/D2	Sucrose P/E
BLA	624.3 ± 6.7	566.2 ± 6.2 [#]	602.3 ± 7.6	576.6 ± 12.2 [#]
BSTad	832.3 ± 24.1	834.8 ± 18.4	817.9 ± 18.4	780.8 ± 29.1
BSTpr	571.4 ± 37.1	600.3 ± 14.0	582.8 ± 24.8	588.5 ± 23.3
CeA	826.9 ± 9.5	800.5 ± 9.2	824.6 ± 9.4	844.5 ± 15.2 [*]
MeApd	918.5 ± 16.7	902.6 ± 17.8	927.7 ± 15.0	988.0 ± 32.1
NAc	240.0 ± 4.7	241.2 ± 5.3	238.6 ± 3.8	241.7 ± 5.2
PFC	707.8 ± 22.9	711.3 ± 13.5	710.3 ± 10.0	698.6 ± 11.4
PVN	198.7 ± 7.7	200.8 ± 5.3	204.2 ± 11.4	217.6 ± 8.8

^{*} p<0.05 vs. water,

[#] p<0.05 vs. D1/D2. n = 7–17/group.

Table 4

Plasma estradiol is elevated specifically during proestrus. Estradiol levels on experiment d19 are shown for each estrous cycle stage, as determined by vaginal lavage cytology on d19.

Estrous cycle stage	Plasma estradiol (pg/ml)
Diestrus 1 (n = 12)	120.0 ± 12.7
Diestrus 2 (n = 8)	101.2 ± 8.2
Proestrus (n = 4)	183.6 ± 14.2 *
Late proestrus/estrus (n = 6)	126.6 ± 22.2
Estrus (n = 19)	100.8 ± 5.8

*
p < 0.05 vs. all other stages.