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Sex differences and estrous influences on oxytocin control of food intake

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Abstract

Central oxytocin potently reduces food intake and is being pursued as a clinical treatment for obesity. While sexually dimorphic effects have been described for the effects of oxytocin on several behavioral outcomes, the role of sex in central oxytocin modulation of feeding behavior is poorly understood. Here we investigated the effects of sex, estrous cycle stage, and female sex hormones (estrogen, progesterone) on central oxytocin-mediated reduction of food intake in rats. Results show that while intracerebroventricular (ICV) oxytocin potently reduces chow intake in both male and female rats, these effects were more pronounced in males than in females. We next examined whether estrous cycle stage affects oxytocin's food intake-reducing effects in females. Results show that ICV oxytocin administration significantly reduces food intake during all estrous cycle stages except proestrous, suggesting that female sex hormones may modulate the feeding effects of oxytocin. Indeed, additional results reveal that estrogen, but not progesterone replacement, in ovariectomized rats abolishes oxytocin-mediated reductions in chow intake. Lastly, oxytocin receptor mRNA (*Oxtr*) quantification (via quantitative PCR) and anatomical localization (via fluorescent in situ hybridization) in previously established sites of action for oxytocin control of food intake revealed comparable *Oxtr* expression between male and female rats, suggesting that observed sex and estrous differences may be based on variations in ligand availability and/or binding. Overall, these data show that estrogen reduces the effectiveness of central oxytocin to inhibit food intake, suggesting that sex hormones and estrous cycle should be considered in clinical investigations of oxytocin for obesity treatment.

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Keywords

feeding; obesity; estrogen; progesterone; oxytocin receptor; hypothalamus

Introduction

Obesity is a growing national health concern in the United States with rates exceeding one third of the adult population (Dietz WH, 2015; Flegal KM et al., 2016). While reduced physical activity may be contributing, excessive caloric intake is the primary determinant driving the obesity epidemic (Bray GA et al., 2004; Gonzalez-Muniesa P et al., 2017; Hill JO and Peters JC, 1998). Significant advances have been made toward developing effective obesity pharmacotherapies that reduce the excess consumption of calories; however, the vast majority of preclinical research has been conducted in male rodents (Zakariaei Y et al., 2016; Zucker I and Beery AK, 2010). This is particularly problematic in light of emerging evidence that the neurobiology regulating feeding behavior is sexually dimorphic (Asarian L and Geary N, 2013; Sample CH and Davidson TL, 2018), and thus understanding the underlying mechanisms through which pharmacological treatments act in both males and females to affect feeding is important in developing effective obesity treatments. One neuropeptide currently under clinical investigation for the treatment of obesity is oxytocin. There is abundant evidence that central administration of oxytocin rapidly and potently reduces food intake (Altirriba J et al., 2014; Blevins JE and Baskin DG, 2015; Blevins JE et al., 2015; Ding C et al., 2019; Laby M et al., 2018; Lawson EA et al., 2015; Maejima Y et al., 2011; Morton GJ et al., 2012; Olszewski PK et al., 2017; Thienel M et al., 2016). Consistent with these results, oxytocin null (–/–) mice demonstrate enhanced initial and sustained sucrose consumption in comparison to WT mice (Amico JA et al., 2005; Leng G and Sabatier N, 2017). Additional preclinical evidence from animal model studies suggests that oxytocin reduces feeding without adverse side effects such as nausea and malaise (Herisson FM et al., 2016; Noble EE et al., 2014). While it is known that oxytocin plays an important role in many sex-specific behaviors (e.g., lactation, parturition) (Kendrick KM, 2000; Nishimori K et al., 1996) and has sexually dimorphic effects on various social behaviors (Borland JM et al., 2019; Scott N et al., 2015), it is poorly understood as to whether there are sex differences in oxytocin's regulation of feeding behavior.

The neuropeptide oxytocin is produced in the supraoptic nucleus and paraventricular nucleus of the hypothalamus (Lee HJ et al., 2009). Oxytocin has a multitude of behavioral effects, including modulating social behavior, stress responsivity, and energy balance (Campbell A, 2008; Dolen G et al., 2013; Ferguson JN et al., 2001; Ferguson JN et al., 2000; Hung LW et al., 2017; Leng G and Sabatier N, 2017; Reppucci C et al., 2018; Ross HE and Young LJ, 2009; Sabatier N et al., 2013) (see (Onaka T et al., 2012) for review). Early evidence for oxytocin's function in food intake control revealed that either intraperitoneal (IP) or intracerebroventricular (ICV) administration of oxytocin significantly reduces food intake in male rats (Arletti R et al., 1989). Additionally, oxytocin neurons are activated by food consumption and contribute to meal termination in male rats (Hume C et al., 2017; Johnstone LE et al., 2006), whereas either suppressing exocytosis of oxytocin or genetic reduction of oxytocin expression increases food intake (Kublaoui BM et al., 2008; Zhang G et al., 2011).

Furthermore, oxytocin receptor (*Oxtr*)-deficient male mice show late-onset obesity, reduced thermogenesis, and decreased energy expenditure (Leng G et al., 2008; Wu Q et al., 2012). While together these data implicate oxytocin as a viable candidate for obesity pharmacotherapy in males, a dearth of female preclinical oxytocin data render it unclear as to whether oxytocin is similarly effective in females. Therefore, the current study examined the hypothesis that oxytocin differentially regulates food intake in male and female rats, and that observed sex differences are driven, in part, by the female sex hormones estrogen and progesterone.

Experimental Procedures

Animals

Male and female Sprague-Dawley rats (Envigo, Indianapolis, IN; weighing 320–450 g) were individually housed in wire hanging cages in a temperature controlled (22–23°C) vivarium with ad libitum access to water and chow (LabDiet 5001, LabDiet, St. Louis, MO; except where noted). Rats were either on a 12hr:12hr light/dark cycle (lights on at 0600h) or reverse light/dark cycle (lights off at 1000h). All procedures were approved by the Institute of Animal Care and Use Committee at the University of Southern California.

Surgery

For all surgical procedures, rats were anesthetized and sedated via intramuscular injections of ketamine (90mg/kg), xylazine (2.8mg/kg), and acepromazine (0.72mg/kg) and were given analgesic (a subcutaneous injection of ketaprofen [5 mg/kg] after surgery and once daily for 3 subsequent days thereafter). All animals were allowed to recover for at least one-week post-surgery prior to experiments.

Intracerebroventricular (ICV) cannulation surgery—Rats were surgically implanted with indwelling guide cannula (26-gauge, Plastics One, Roanoke, VA) targeting the lateral ventricle using the following stereotaxic coordinates, which are relative to the location of bregma, with dorsal/ventral (DV) coordinates relative to the skull surface at the cannula implantation site (Paxinos and Watson, 2007): –0.9 mm anterior/posterior (AP), +1.8 mm medial/lateral (ML), and –2.6 mm DV. Cannula were affixed to the skull as previously described using jeweler's screws and dental cement (Hsu TM et al., 2015). Following 7 days of recovery, cannula placement was evaluated via measurement of the cytoglucoopenia-induced sympathoadrenal-mediated glycemic effect that occurs from 210 µg (in 2µl) of 5-thio-D-glucose (5TG) (Ritter RC et al., 1981). Food was removed 1 hour prior to the placement test. Animals were injected with 5TG via injectors that extended 2 mm beyond the end of the guide cannula, and blood glucose was measured at 30, 50, 90, and 120 min post-injection. Placement was deemed correct if blood glucose doubled within the measurement period. For animals who did not pass the placement test the injector length was adjusted until the correct placement was achieved (based on the 5TG test) and the adjusted injector length was used for that animal in all subsequent experiments.

Ovariectomies and implantation surgeries—Immediately prior to ICV cannulation surgery, female rats (experiment 3) were bilaterally ovariectomized (OVX) via dorsal flank

incisions. Bilateral 2 mm incisions were made, ovaries were removed, and fallopian tubes were sutured, as previously described (Santollo J and Eckel LA, 2008; Wood RI and Rice R, 2013).

After a 1-week recovery period, females received either subcutaneous 5 mm empty sham Silastic implants or implants filled with crystalline 17 β -estradiol (Millipore, Burlington, MA) with the ends sealed with silicone adhesive (Schauwecker PE et al., 2009; Wood RI and Rice R, 2013). Estradiol is the major form of estrogen necessary for development and reproduction and has been shown to interact with oxytocin function in the brain to influence behavior (Jirikowski GF et al., 1988; Johnson AE et al., 1989). Females were allowed to recover from surgery for three weeks before testing.

Experiment 1: Effect of ICV oxytocin on food intake in male and randomly-cycling female rats

The effect of ICV oxytocin on standard chow intake was tested in males and randomly-cycling females (n=16/sex) using a within-subjects, counterbalanced design. Food was removed from the home cage 1 hr prior to injection, which occurred 10 min prior to the onset of the dark cycle. Rats were injected ICV with a volume of 1 μ l containing a dose of either 0.3, 1, or 2 μ g of oxytocin (Bachem, Torrance, CA) or artificial cerebrospinal fluid (aCSF; vehicle) via 33-gauge microsyringe injector. Injectors remained in place for 30 sec to allow for complete drug delivery. Food was weighed and returned to the home cage at dark onset and spill papers were placed underneath the suspended wire-bottom cages to collect crumbs for precise food intake measures. Food intake was measured at 30 min, 1 hr, 2 hr and 24 hr post injection, and cumulative food intake was calculated by subtracting the hopper weights at each time point from the food hopper weight at baseline (accounting for spillage).

Experiment 2: Estrous cycle effects on oxytocin-induced inhibition of food intake

Estrous cycles were monitored daily in a cohort of female rats (n=15) for one week prior to the start of the experiment. To identify estrous stage, vaginal cytology was investigated by vaginal lavage (Becker JB et al., 2005; Ekambaram G et al., 2017; McLean AC et al., 2012). Cytology samples were collected in 0.9% sterile saline (Teknova) 1.5 hr prior to the start of the dark cycle. A standard laboratory microscope (AmScope) was used to examine vaginal smears at 10X. Stages were assigned at the same time each day to minimize the incidence of transitional and 'missed' stages and were based on vaginal cell morphology (presence or absence of epithelial cells, cornified cells, and leucocytes) as described in (Becker JB, Arnold AP, Berkley KJ, Blaustein JD, Eckel LA, Hampson E, Herman JP, Marts S, Sadee W, Steiner M, Taylor J and Young E, 2005).

Rats received a 1 μ l ICV injection of either 1 μ g dose of oxytocin or aCSF using a within-subjects design such that each rat received each treatment once during each estrous stage (8 total treatments per animal). Treatments were separated by at least 2 days and counterbalanced such that no two treatments per stage occurred in a row. As in Experiment 1, food hoppers were removed from the home cage 1 hr prior to injections, and injections occurred 10 min prior to dark onset. Following injection, food hoppers were returned at dark

onset and 30 min, 1 hr, 2 hr, and 24 hr cumulative chow intake (accounting for spillage) was measured as described in Experiment 1.

Experiment 3: Effect of estrogen and progesterone on oxytocin-induced inhibition of food intake

Female rats (n=16) were ovariectomized via bilateral dorsal flank incision, and indwelling cannula targeting the lateral ventricle were implanted as described in surgical methods above. Following a one-week surgical recovery period, rats received subcutaneous 5 mm Silastic implants filled with 17 β -estradiol (Millipore, Burlington, MA) or empty (sham) implants (n=8/group). These chronic Silastic implants slowly release physiological levels of estrogen (Wood RI and Rice R, 2013). After three weeks of surgical recovery, we utilized a counterbalanced mixed design, where ovariectomized female rats with either estrogen (n=8) or sham (n=6) implants were subdivided to receive 4 days of progesterone or vehicle injections (n=3 or 4/implant type) using a protocol modified from (Leibowitz SF et al., 2007). Using a within-subjects design, progesterone (Sigma-Aldrich, St. Louis, MO) in sesame oil (0.5 mg/0.2 mL) or sesame oil alone (vehicle) was subcutaneously injected 2 hrs prior to dark cycle onset. On days 2 and 4 of progesterone or vehicle injections, rats received 1 μ L ICV injection of 1 μ g dose of oxytocin or vehicle 10 min prior to dark cycle onset. Food intake was measured at 30 min, 1 hr, 2 hr, and 24 hrs (accounting for spillage) as described above.

Experiment 4a: Quantification of oxytocin receptor mRNA (*Oxtr*) expression in nucleus accumbens (ACB), ventromedial nucleus of the hypothalamus (VMH), and nucleus tractus solitarius (NTS) in male and female rats

To determine whether *Oxtr* expression varies as a function of sex or estrous stage, *Oxtr* gene expression was quantified in male (n=7) and female (n=8) rats. Vaginal cytology for female rats was examined daily for 1 week and on the day of tissue collection animals were categorized into either the proestrous/estrous stage (n=4) or metoestrous/diestrous stage (n=4). Prior to sacrifice, rats were 24 hr fasted and then given 5 g chow for re-feeding at the onset of the dark cycle. Thirty minutes after presentation of the meal, tissue was collected. Animals were first anesthetized and sedated via intramuscular injections of ketamine as described above, then decapitated. Brains were rapidly removed from the skull and flash frozen in -30°C isopentane on dry ice. Brains were then stored at -80°C until further analyses. Tissue punches of brain regions of interest (0.8 mm circumference) were collected using the Leica CM 1860 cryostat (Wetzlar, Germany) and anatomical landmarks were based on the Swanson rat brain atlas (Swanson LW, 2018). Tissue punches were collected from the nucleus accumbens (ACB; atlas levels 9–10), ventromedial nucleus of the hypothalamus (VMH; atlas levels 27–29), and nucleus tractus solitarius (NTS; atlas levels 69–70). Total RNA was extracted according to manufacturer's instructions using RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany). RNA was reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (Qiagen). qPCR was performed with TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) using the BIO-RAD CFX96 Optics Model realtime PCR machine. Negative reverse-transcribed samples were generated and all reactions were carried out in triplicate. The following TaqMan probes were used: *Oxtr*: Rn00563503_m1, *Gapdh*: Rn01775763_g1. To determine relative expression

values, the 2^{-Ct} method was used, where triplicate Ct values for each sample were averaged and subtracted from those derived from GAPDH as previously described (Davis EA et al., 2018).

Experiment 4b: Fluorescence *in situ* hybridization for *Otxr* expression in ACB, VMH, and NTS in male and female rats

Representative images for the relative anatomical locations within brain regions of interest (ACB, VMH, mNTS) were obtained using fluorescence *in situ* hybridization (FISH). Briefly, male and female rats were anesthetized and transcardially perfused with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borate buffer (pH 9.5). Brains were post-fixed for 24 hours at 4°C in 12% sucrose in 4% paraformaldehyde, then flash-frozen in isopentane prior to sectioning on a freezing stage microtome at 30 µm. Serial sections were collected and stored in antifreeze solution at -20°C until further analyses. FISH protocols were conducted as previously described (Noble EE et al., 2018; Suarez AN et al., 2018) and utilized an *Otxr* (Advanced Cell Diagnostics; Newark, CA) probe. Briefly, tissue sections were mounted on subbed glass slides (Fisher brand Superfrost Plus) and placed in a vacuum desiccant chamber overnight (~16 hrs). Slides were postfixed in 4% PFA for 1hr 45 min, washed 5 x for 5 min in KPBS, placed in solution of 100 mM Tris (pH 8), 50 mM EDTA (pH 8), and 0.1% Proteinase K (10mg/mL, Sigma P2308) in water for 30 min, and then rinsed for 3 min in the same Tris and EDTA solution without Proteinase K all at 37°C. Slides were rinsed in 100mM triethanolamine (pH 8) in water for 3 min, incubated in 0.25% acetic anhydride in 100mM triethanolamine for 10 min at room temperature, and rinsed twice in 10% 20X saline-sodium citrate buffer for 2 min. Slides were then dehydrated in increasing concentrations of ethanol (50%, 70%, 95%, 100%, 100%) and air dried. Probes were applied to slides and incubated for 2 hrs for hybridization. Reagents from RNAscope® Fluorescent Multiplex Detection Reagent Kit V2 (ACD, 323119) were used to amplify the probe. *Otxr* signal was developed by incubating with the appropriate channel probe for 15 min, with the Cy3 fluorophore diluted 1:500 in TSA Buffer (ACD, 322809) for 30 min, and then with the horseradish peroxidase (HRP) blocker for 30 min. Between each incubation, slides were washed with RNAscope® Wash Buffer 1X (ACD, 320058) for 2 min. Lastly, slides were coverslipped with ProLong® Gold Antifade Reagent (Cell Signaling, 9071S). Representative images for *Otxr* expression in the ACB, VMH, and NTS were confined to Swanson Atlas levels 9–10, 27–29, and 69–70, respectively.

Statistical analyses

Except where noted, experiments were analyzed using repeated measures two-way analysis of variance (ANOVA) or mixed design two-way ANOVA. To adjust for multiple comparisons, post-hoc analyses were conducted with Tukey's Honestly Significant Difference when significant main effects or interactions were obtained. For experiment 1, intake data were also analyzed with a repeated measures three-way ANOVA including time as a repeated-measures variable, and a linear regression for magnitude of 30-min food intake suppression of oxytocin (1 µg vs. vehicle) against body weight was conducted. For experiment 4, qPCR data were analyzed using an unpaired two-sample Student's t-test. Statistical analyses were performed using Statistica (V7; StatSoft, Inc.) using an α level for significance of $p < .05$.

Results

ICV oxytocin's anorexigenic effects are more pronounced in male vs. female rats

The efficacy of oxytocin to reduce food intake was compared in male and randomly-cycling female rats. When time was included as a variable, a repeated measures, 3-way ANOVA of non-cumulative intake revealed that there was no significant time x dose x sex interaction ($F_{(9,261)} = 0.44$; $p = 0.911$). Since there was no time x oxytocin dose x sex interaction, cumulative intake at separate time points was examined for all other analyses. At the 30-min time point, a 2-way ANOVA with dose as a repeated measures variable revealed a significant dose x sex interaction ($F_{(3,84)} = 4.55$, $p < 0.01$) and a significant main effect of dose ($F_{(3,84)} = 30.72$, $p < 0.0001$). Post-hoc analyses reveal that ICV injection of oxytocin reduced 30 min food intake in male rats at all three doses given (0.3, 1, and 2 μg), but only the 1 and 2 μg dose reduced chow intake in randomly-cycling females (Fig. 1A; p s<0.05). Furthermore, the food intake suppressive effects of the 1 and 2 μg doses were sustained at 1 hr in male rats (p s<0.05), but not in randomly-cycling females (Fig. 1B). At 2 hrs post injection, ICV oxytocin was no longer effective at reducing intake in either males or females (Fig. 1C and 1D). A linear regression of the magnitude of suppression of oxytocin (1 μg ; 1 hr vehicle intake minus 1 hr oxytocin intake) against body weight revealed no significant relationship between the magnitude of food intake reduction by oxytocin and body weight at the time of treatment (adjusted $R^2=0.07$, $p>0.05$ for both sexes, adjusted $R^2=-0.04$, $p>0.05$ males only, adjusted $R^2=-0.03$, $p>0.05$ females only). Collectively, these results indicate that the efficacy of central oxytocin to reduce chow intake is not dependent on body weight and is greater in males than in female rats, as evident from results revealing that higher doses of oxytocin are required to reduce chow intake in females vs. males, as well as data revealing a longer duration of intake reduction in males vs. females following administration of doses that were effective in both sexes.

Effects of ICV oxytocin on chow intake differ throughout the female estrous cycle

Since the efficacy of oxytocin was lower in female rats than in males, we reasoned that female sex hormones may affect oxytocin signaling. To investigate whether estrous stage alters the capacity of ICV oxytocin to reduce chow intake, female rats were injected with ICV oxytocin during each of the 4 stages of the rodent estrous cycle using a within-subjects design. A 2-way ANOVA of 30-min cumulative intake shows that there was no stage x oxytocin interaction ($F_{(1,13)} = 0.72$, $p > 0.05$). There was a significant main effect of oxytocin ($F_{(1,13)} = 19.91$, $p < 0.01$), but no main effect of stage ($F_{(1,13)} = 3.37$, $p > 0.05$). However, post hoc analyses revealed that ICV oxytocin reduced 30-min chow intake in the estrous, metoestrous, and diestrous stages ($p < 0.05$), but not in the proestrous stage (Fig. 2A). There was no main effect of oxytocin for 1-hr cumulative intake.

Estrogen, but not progesterone replacement reduces the efficacy of central oxytocin to reduce chow intake

As estrogen and progesterone both surge together during the proestrous phase of the rat estrous cycle (Bauer-Dantoin AC et al., 1995), which is the stage in which the capacity for oxytocin to reduce chow intake in randomly cycling females was diminished in Exp. 2, we next tested whether estrogen in combination with progesterone alters the effectiveness of

oxytocin to reduce chow intake in OVX female rats (timeline in Fig. 3). Results revealed that at 30 min post injection there was a significant oxytocin x estrogen interaction ($F_{(1,12)} = 7.56$, $p < 0.05$), but neither the oxytocin x progesterone nor the oxytocin x estrogen x progesterone interactions were significant. These data suggest that estrogen modulates the effects of oxytocin on feeding, but progesterone does not, nor does progesterone influence estrogen's effects on oxytocin-mediated food intake reduction (Fig. 4A). There was a significant main effect of both estrogen and oxytocin for food intake at 30 min (main effect of implant: $F_{(1,12)} = 9.25$, $p < 0.05$; main effect of oxytocin ($F_{(1,12)} = 55.51$; $p < 0.0001$) (Fig. 4A). Post hoc analyses revealed that ICV oxytocin reduced 30-min food intake for the OVX (sham implant + oil) and for the OVX+P (sham implant + progesterone treatment) treatments ($p < 0.05$), but did not reduce intake for OVX+E (estrogen + oil) or OVX+EP (estrogen + progesterone) treatments (Fig. 4C). Oxytocin had no effect at 1 hr post injection for any treatment (Fig. 4B). Overall, estrogen, but not progesterone, interacts with oxytocin in the reduction of food intake.

***Oxtr* expression does not differ in male and female rats in the ACB, VMH, or NTS**

The nucleus accumbens (ACB) (Herisson FM, Waas JR, Fredriksson R, Schioth HB, Levine AS and Olszewski PK, 2016), ventromedial nucleus of the hypothalamus (VMH) (Noble EE, Billington CJ, Kotz CM and Wang C, 2014), and nucleus tractus solitarius (NTS) (Ong ZY et al., 2015; Ong ZY et al., 2017) have previously been shown to be sites of action for oxytocin-induced inhibition of food intake. Thus, we hypothesized that relative levels and anatomical distribution of *Oxtr* expression in these brain regions might differ between male and female rats, and by estrous cycle stage. To assess whether there are quantitative differences in *Oxtr* expression in males and females we performed quantitative polymerase chain reaction (qPCR) in tissue punches from these brain regions. qPCR relative *Oxtr* mRNA expression did not significantly differ across female estrous stages in the ACB, VMH, and NTS, and thus all further analyses combined all female estrous stages for comparison with males. Results revealed no quantitative differences in *Oxtr* mRNA expression in the ACB, VMH, or NTS between males and females (Fig. 5A–5C). To further examine possible anatomical differences in *Oxtr* expression between males and females within these brain regions, fluorescence *in situ* hybridization (FISH) analyses revealed that both males and females express *Oxtr* in the medial core of the ACB (figure 5D), as well as in central part of the VMH (VMHc) (Fig. 5E). Furthermore, *Oxtr* expression was confined to the ventral portion of the medial NTS (mNTS) (immediately dorsal to the dorsal motor nucleus of the vagus [DMX]) in both males and females (figure 5F). Overall, *Oxtr* expression was quantitatively and qualitatively similar across female estrous stages, as well as between sexes.

Discussion

Oxytocin has potent inhibitory effects on food intake in both humans and experimental animal models (Arletti R et al., 1990; Blevins JE, Graham JL, Morton GJ, Bales KL, Schwartz MW, Baskin DG and Havel PJ, 2015; Herisson FM, Waas JR, Fredriksson R, Schioth HB, Levine AS and Olszewski PK, 2016; Iwasaki Y et al., 2015; Klockars A et al., 2017; Klockars OA et al., 2018; Klockars OA et al., 2017; Lawson EA, Marengi DA, DeSanti

RL, Holmes TM, Schoenfeld DA and Tolley CJ, 2015; Maejima Y, Iwasaki Y, Yamahara Y, Kodaira M, Sedbazar U and Yada T, 2011; Maejima Y et al., 2015; Maejima Y et al., 2018; Mullis K et al., 2013; Noble EE, Billington CJ, Kotz CM and Wang C, 2014; Ott V et al., 2013; Sclafani A et al., 2007; Seelke AM et al., 2018; Striepen N et al., 2016). Similarly, data from oxytocin null rodent models reveal that the absence of oxytocin leads to the development of obesity, hyperleptinemia, and decreased peripheral insulin sensitivity (Camerino C, 2009). Thus, oxytocin is a promising target for pharmacotherapy development for the treatment of obesity. However, few preclinical studies have directly examined whether sex differences should be a consideration for the efficacy of oxytocin as an obesity pharmacotherapy. Specifically, there is a paucity of research directly examining the food intake suppressive effects of oxytocin in male compared to female animals. Furthermore, sex differences in oxytocin's behavioral effects have been reported for the regulation of social behavior (Benelli A et al., 1995; Engelmann M et al., 1998), anxiety-related behavior (Lukas M and Neumann ID, 2014; Lukas M et al., 2011) and stress (Borland JM et al., 2018; Bredewold R and Veenema AH, 2018; Minhas S et al., 2016), and these effects stem in part from interactions with sex hormones (estrogen, progesterone, testosterone (Amico JA et al., 2002; Johnson AE, 1992; Sloan DK et al., 2018; Yamashita M et al., 2013)). While these studies provide evidence that oxytocin has sexually dimorphic effects on behavior and that sex hormones influence oxytocin signaling in the brain, the interaction between female sex hormones and oxytocin in the control of food intake is not well understood. To address this gap in the literature, the present study sought to investigate potential sex differences in chow intake in male vs. randomly cycling female rats following central (ICV) oxytocin administration. Consistent with previous reports, our results reveal robust, short-term reduction of food intake in both male and female rats. We also found that the capacity for oxytocin to reduce food intake was attenuated in females compared with males, where lower doses were effective at reducing food intake in males, and doses that were effective in both sexes reduced intake for a longer duration in males.

The observed sex differences in oxytocin's anorexigenic effects in the present study were not based on body weight differences between males and females, as there was no significant correlation between the magnitude of central oxytocin-induced food intake reduction and body weight at the time of the treatment. We note, however, that previous findings have observed differences in oxytocin's food intake reducing efficacy in female rats when comparing two different strains of rats with varying growth rates (Bjorkstrand E and Uvnas-Moberg K, 1996), suggesting that there may be interactions between oxytocin and growth factors, and/or rodent strain. In humans, oxytocin administration also has a greater effect on food intake reduction in obese compared to normal-weight men (Thienel M, Fritsche A, Heinrichs M, Peter A, Ewers M, Lehnert H, Born J and Hallschmid M, 2016). Our study examined food intake in healthy lean rats, and thus it is important for future work to examine sex differences in oxytocin's hypophagic effects in obese vs. lean rats.

Sex differences in oxytocin's anorexigenic effects may be in part due to the modulatory effects of the estrous cycle and interactions between sex hormones and oxytocin expression, signaling, and/or receptor binding. Indeed, the presence of an estrogen response element in the promotor region of the oxytocin gene (Adan RA et al., 1993; Peter J et al., 1990; Quinones-Jenab V et al., 1997) suggests that these two systems may interact.

Furthermore, sequential exposure to long-term (2 week) estradiol and progesterone treatment followed by withdrawal of progesterone treatment increases oxytocin mRNA in the paraventricular and supraoptic nucleus of the hypothalamus in OVX female rats, as well as in castrated male rats (Amico JA et al., 1995; Amico JA et al., 1997; Thomas A and Amico JA, 1996). Based on these findings, as well as our results that central oxytocin was more effective in reducing food intake in males compared to randomly-cycling females and the fact that the female ovarian hormones estrogen and progesterone are highest during the proestrous phase (Eckel LA, 2011; Geary N, 1998), we hypothesized that oxytocin would differ in effectiveness in reducing food intake during proestrous. Indeed, the present study found that ICV oxytocin significantly reduced chow intake in all stages, except the proestrous stage. Consistent with these findings, oxytocin's food intake suppressive effects in OVX female rats were reduced by estrogen replacement, whereas progesterone did not have affect estrogen's modulation of oxytocin.

Whether there are sex-differences and interactions with sex hormones in oxytocin-mediated inhibition of food intake in humans is poorly understood. A recent meta-analysis concluded that peripheral oxytocin levels increase from the early follicular phase to ovulation, followed by a decrease in levels from ovulation to the mid-luteal phase (Engel S et al., 2019). These results suggest that oxytocin may also interact with sex hormones in humans, however, the extent to which these effects are relevant to feeding behavior is unknown. Moreover, another study revealed that the pattern of oxytocin concentrations in circulation was minimally influenced by menstrual cycle or by sex (Challinor SM et al., 1994). Thus, further research is clearly required to systematically understand the influence of menstrual cycle and sex on oxytocin's pharmacological food intake reduction in humans.

Collectively our results raise several possible questions. Firstly, like oxytocin, estrogen rapidly reduces food intake (Eckel LA, 2011; Eckel LA and Geary N, 2001; Geary N et al., 1995), and thus it could be possible that estrogen and oxytocin act through disparate (vs. convergent or interacting) mechanisms to reduce intake. Along these lines, it may be the case that the reduced effectiveness of oxytocin during proestrous and in OVX rats with estrogen replacement is based on lower baseline intake when estrogen levels are high, thus yielding a floor effect in which CNS oxytocin cannot augment an already low level of intake. However, a previous study showed that vehicle- or estrogen-treated OVX rats refed after 48 hr of fasting demonstrated increased activation in oxytocin-producing neurons, but that expression was lower in estrogen-treated animals in comparison with vehicle-treated controls (Lucio-Oliveira F and Franci CR, 2012). These results are consistent with the present findings and further suggest that estrogen may act by directly modulating oxytocin peptide expression at the neuronal level. Further argument against a floor effect vs. a direct interaction between central oxytocin and estrogen is that the 2 µg dose of oxytocin yielded a near complete suppression of 30 min intake in randomly cycling females, suggesting that baseline intake during proestrous (and with estrogen replacement) was not at a floor, but rather, that oxytocin under these conditions was less effective at reducing intake.

Another consideration is that additional sex hormones, such as prolactin, may contribute to sex differences in the effectiveness of oxytocin to control food intake. While the present results do not support a role for progesterone in interacting with oxytocin's feeding effects,

Yamashita *et al.* found that prolactin-releasing peptide-deficient mice had reduced refeeding-induced oxytocin overexpression (Takayanagi Y et al., 2008; Yamashita M, Takayanagi Y, Yoshida M, Nishimori K, Kusama M and Onaka T, 2013), highlighting a possible role for oxytocin and prolactin interaction in food intake control. Future studies are needed to determine the extent to which prolactin interacts with estrogenic systems to modulate the efficacy of oxytocin to reduce feeding.

Another consideration for the sex-differences in the observed oxytocin-mediated feeding effects could be due to shifts in focus in behavior, such as the stimulatory effects of oxytocin on maternal behavior in female rodents. For example, oxytocin acts as a stimulator of maternal behavior in virgin female rats (Pedersen CA et al., 1982; Pedersen CA and Prange AJ, Jr., 1979), where central administration of oxytocin to an estrogen-primed adult reduces response latencies towards fostering pups in virgin female rats. Thus, it is possible that oxytocin administration during proestrous or in OVX female rats with estrogen replacement led to increased maternal or female-specific behaviors, and that this drive competed with food-motivated behavior. However, we find this interpretation unlikely given that the female rats used in this study had never been exposed to pups. Further, the anorexigenic effects of central oxytocin were reduced in females vs. males, which is inconsistent with the interpretation that elevated competing behavioral drives in females (increased maternal drive) is the basis for the observed sex differences in oxytocin-mediated feeding effects.

We investigated the possibility that there may be sex differences in *Oxtr* expression in sites of action previously established to be relevant for oxytocin's control of food intake (Herisson FM, Waas JR, Fredriksson R, Schioth HB, Levine AS and Olszewski PK, 2016; Noble EE, Billington CJ, Kotz CM and Wang C, 2014; Ong ZY, Alhadeff AL and Grill HJ, 2015; Ong ZY, Bongiorno DM, Hernando MA and Grill HJ, 2017). Similar to previous reports, our data show that *Oxtr* is expressed in the ACB, VMH, and the NTS in adult male and female rats. However, we did not observe any sex or estrous stage differences in *Oxtr* expression ACB, VMH, and mNTS. In addition, anatomical FISH analyses revealed that *Oxtr* is expressed in similar subregions within these nuclei across estrous stage and in males and females. More specifically, *Oxtr* was expressed predominantly within the medial core of the ACB, the central part of the VMH (VMHc), and the ventromedial portion of the mNTS. One additional consideration is that from the current data set, we cannot say whether sex hormones differentially affect binding affinity of the oxytocin receptor. Estrogen and progesterone have been previously shown to regulate *Oxtr* binding in human cell cultures (Amico JA, Rauk PN and Cai HM, 2002), as well as in the rodent brain in a site-specific manner (Tribollet E et al., 1990; Worley NB et al., 2019). Thus, when considering the present findings with these previous studies revealing female sex hormone influences on oxytocin binding, it is likely that oxytocin binding affinity and/or oxytocin neuropeptide expression mediate the sex differences observed in the present study. Future work is needed to directly investigate this hypothesis.

With rising obesity trends, there is an increased need to develop effective pharmacotherapies that curb food intake and inhibit excessive caloric intake. While significant advances have been made in ingestive behavior research in the past 30 years, there is a paucity of data in the neuropeptidergic regulation of food intake control in females, specifically (Zakariaeiz

Y, Cosgrove KP, Potenza MN and Mazure CM, 2016; Zucker I and Beery AK, 2010). Here we show that oxytocin differentially affects food intake in male and female rodents, and that circulating estrogen interacts with oxytocin by attenuating the effectiveness of oxytocin at reducing food intake.

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Abbreviations

ICV	Intracerebroventricular
OXTR	Oxytocin receptor
E	Estrogen
P	Progesterone
OVX	Ovariectomized
ACB	Nucleus accumbens
VMH	Ventromedial nucleus of the hypothalamus
NTS	Nucleus tractus solitarius

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Figure 1:

Sex differences comparison for the effect of oxytocin on food intake for normal dark onset feeding. Effect of intracerebroventricular injection of 0 (aCSF), 0.3, 1, or 2 μg of oxytocin on cumulative chow intake in grams at 30 min (a), 1 hr (b), 2 hrs (c) and 24 hrs (d) post injection. Data are means ± SEM; *p<0.05.

Figure 2:

Comparison of the effects of oxytocin on dark onset food intake during different stages of the estrous cycle in naturally-cycling females. Effect of intracerebroventricular injection of 0 (aCSF), or 1 µg of oxytocin on cumulative chow intake in grams at 30 min (a), or 1 hr (b) post injection. Data are means $\pm$ SEM; * $p < 0.05$.

Figure 3:

Timeline of oxytocin treatment in ovariectomized female rats with and without estrogen (E) and progesterone (P) replacement. All females received bilateral ovariectomies, and after one-week recovery, females received either sham or estrogen implants. After an additional three-week recovery period, females underwent 4 days of progesterone or oil treatment, and effect of ICV oxytocin on food intake was measured. After an additional 1-week washout, progesterone or oil treatment groups were switched and effect of ICV oxytocin on chow intake was examined.

Figure 4:

Comparison of the effects of oxytocin on dark onset food intake in ovariectomized female rats with and without estrogen (E) and progesterone (P) replacement. Effect of intracerebroventricular injection of 0 (aCSF), or 1 µg of oxytocin on cumulative chow intake in grams at 30 min (a), or 1 hr (b) post injection. Data are means $\pm$ SEM; * $p < 0.05$.

Figure 5:

Expression of *Oxt* in feeding-relevant neural sites. Relative *Oxt* mRNA expression (top panels) in tissue punches from the nucleus accumbens (ACB; a), ventromedial hypothalamus (VMH; b), and nucleus tractus solitarius (NTS; c) from male and female rat brains.

Representative images of coronal brain sections (bottom panels) showing fluorescence in situ hybridization for *Oxt* (red) in the ACB (a), VMH (b), and NTS (c) with dapi counterstain (blue). Data are means $\pm$ SEM.