

# Oestrogen-Dependent Oxytocin Dynamics in the Hypothalamus of Female Rats

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# Abstract

Oxytocin (OXT) is produced in the hypothalamic nuclei and is secreted into systemic circulation from the posterior pituitary gland (PP). In the central nervous system, OXT regulates behaviours including maternal and feeding behaviours. Our aim was to evaluate whether oestrogen regulates hypothalamic OXT dynamics. Herein, we provide the first evidence that OXT dynamics in the hypothalamus vary with sex and that oestrogen may modulate dynamic changes in OXT levels, using OXT-mRFP1 transgenic rats. The fluorescence intensity of OXT-mRFP1 in the hypothalamic nuclei and PP was most strongly expressed during the oestrus stage in female rats and decreased significantly in ovariectomised rats. Oestrogen replacement caused significant increases in the fluorescent intensities in the hypothalamic nuclei and PP in a dose-dependent manner. This was also demonstrated in feeding behaviour and hypothalamic Fos neurons using immunohistochemistry. Hypothalamic OXT expression was oestrogen dependent and could be enhanced centrally by the administration of oestrogen.

## Introduction

Oxytocin (OXT) is produced in the paraventricular (PVN) and the supraoptic nuclei (SON) of the hypothalamus<sup>1</sup>. Peripheral OXT is a neurohypophysial hormone that is originally synthesised in the magnocellular PVN (mPVN) and the SON and is secreted from the posterior pituitary gland (PP) into the systemic circulation. In the periphery, OXT regulates parturition and lactation<sup>2</sup>. Recent studies have suggested that in addition to its peripheral effects, hypothalamic OXT produced in the mPVN and the SON acts on the central nervous system to regulate many functions<sup>3</sup>, including social recognition and trust-building<sup>4,5</sup>. In addition, OXT is produced in the dorsal parvocellular PVN (dpPVN) and in this context, is involved in the modulation of stress and pain<sup>6,7</sup>. Interestingly, the OXT pathway from the PVN is involved in the control of feeding<sup>8</sup>. In particular, the hypothalamic OXT has an anorectic action, and therefore, modulating this pathway is anticipated to reduce obesity and high blood glucose levels<sup>9,10</sup>. However, while the peripheral actions of OXT in pregnant and lactating females are well known, the sex differences in hypothalamic OXT dynamics are still unclear.

Oestrogen is produced in the ovaries and placenta and binds to systemic oestrogen receptors (ER) via the blood to produce oestrogenic activity<sup>11</sup>. Oestrogen plays an important role in maintaining the physiological functions of systemic organs. Additionally, through the oestrus cycle, oestrogen further regulates female reproductive functions. In particular, oestrogen replacement therapy during menopause has been demonstrated to prevent various diseases as well as treat menopausal disorders in women<sup>12-14</sup>. Furthermore, the effects of oestrogen on food intake are thought to be mediated through ERs (both the ER $\alpha$  and ER $\beta$ ) within the central nervous system<sup>15-17</sup>. ER $\beta$ s are located on OXT neurons and are the predominant ER subtype in the PVN, a hypothalamic area involved in eating<sup>18</sup>. However, the details on the correlation between oestrogen and hypothalamic OXT dynamics are as of yet unknown.

In the present study, we used reporter OXT-monomeric red fluorescent protein 1 (mRFP1) transgenic rats to visualize OXT expression and clarify the relationship between hypothalamic OXT and the oestrus cycle<sup>19,20</sup>. To confirm the relationship between oestrogen and OXT, we assessed OXT-mRFP1 expression in bilaterally ovariectomised (OVX) rats with or without exogenous oestrogen replacement. We next assessed whether oestrogen replacement and OVX impact the regulation of OXT produced by hypothalamic neurons, thereby regulating central nervous system functions, including satiety. We also assessed food consumption and hypothalamic OXT Fos-neuronal activity in OVX rats with or without oestrogen replacement and with or without intraperitoneal (i.p.) administration of cholecystokinin (CCK)-8, an agent known to selectively activate OXT neurons<sup>21</sup>. Further, we assessed food consumption in OVX and oestrogen replacement rats with i.p. administration of CCK-8 and intracerebroventricular (i.c.v.) administration of OXT receptor antagonist (OXTR-A)<sup>22-24</sup>. Thus, we aimed to investigate whether oestrogen could regulate and control hypothalamic OXT dynamics.

## Results

### OXT-mRFP1 fluorescence differences between female and male rats

We used adult male and female OXT-mRFP1 Wistar transgenic rats that were maintained as described previously<sup>19,20</sup>. We first aimed to ascertain differences in OXT-mRFP1 fluorescence between 10-week-old female rats undergoing a normal oestrus cycle and 10-week-old male rats [first experiment (Exp. A)]. Female OXT-mRFP1 transgenic rats were further divided based on the four oestrus stages (prooestrus, oestrus, metoestrus, and dioestrus stages). As in a previous study, we observed the entire hypothalamus [SON, anterior parvocellular PVN (apPVN), dpPVN, and mPVN] and PP in OXT-mRFP1 transgenic rats using high-power fluorescence microscopy<sup>25</sup> (Figure 1a). OXT-mRFP1 fluorescence in the SON, apPVN, dpPVN, and mPVN was significantly different between reproductive males and females in the oestrus stage. OXT-mRFP1 fluorescence in the apPVN, dpPVN, and mPVN was significantly different among females depending on the oestrus stage. There was a significant difference in the OXT-mRFP1 fluorescence in the PP between male rats and female rats in the oestrus stage (Figure 1b). These results suggest that the presence of OXT expression in the hypothalamus and pituitary gland is influenced by sex.

### Effects of OVX on OXT-mRFP1 fluorescence

OVX and sham operations were performed on the 11th week, and experiments were conducted on the 15th week. In order to further investigate the influence of sex on OXT expression, OVX (Exp. B) was conducted to induce an oestrogen-deficient state in reproductive female OXT-mRFP1 transgenic rats. These rats were compared to a sham-operated control group, which consisted of both male and female rats. As in Exp. A, OXT-mRFP1 fluorescence in the SON, apPVN, dpPVN, and mPVN revealed a significant difference between the male rats and female rats in the oestrus stage (Figure 2a). Moreover, OXT-mRFP1 fluorescence in the hypothalamus (SON, apPVN, dpPVN, and mPVN) and PP were significantly decreased in OVX rats when compared to females in the oestrus stage and were similar to the levels in male rats

(Figure 2b). Based on these results, we determined that OVX resulted in a decrease in the OXT expression in the hypothalamus and pituitary gland.

### **Effects of oestrogen replacement on OXT-mRFP1 fluorescence**

We performed OVX in 11-week-old female OXT-mRFP1 transgenic rats, conducted hormone replacement on week 15, and performed experiments on week 16. Given that oestrogen levels were expected to be affected by OVX, oestrogen supplementation experiments were performed (Exp. C). Oestrogen was supplemented in female OVX OXT-mRFP1 rats. Among the OVX groups, the rats in the groups with supplementation of low ( $\beta$ -oestradiol) E2 and high E2 elicited significant changes in OXT-mRFP1 levels in the hypothalamus (SON, apPVN, dpPVN, and mPVN) and the PP (Figure 3a). Interestingly, the high-dose E2 group demonstrated higher levels of OXT-mRFP1 fluorescence than the low-dose group in the hypothalamus (SON, apPVN, dpPVN, and mPVN) (Figure 3b). This represents the first evidence that oestrogen regulates OXT expression in hypothalamic OXT neurons in a dose-dependent manner.

### **Relationship between rat body weight and feeding**

We observed a significant change in rat body weight and feeding depending upon sex, OVX, and oestrogen replacement. Wistar rats were divided into five groups: sham-operated male, sham-operated female, only OVX, OVX plus low E2 replacement, and OVX plus high E2 replacement groups (Exp. D). We observed a significant change in rat weight depending upon sex and OVX. Female rats with OVX displayed a significant change in body weight compared to sham-operated reproductive female rats, and oestrogen supplementation affected body weight, depending on the dose (Figure 4a). We observed a significant change in rat feeding depending on sex, OVX, and oestrogen replacement (Figure 4b). This suggests that oestrogen regulates rat body weight and feeding. The difference in body weight is determined by body visceral and subcutaneous fat (Supplementary Figure 1). There was a difference in fat mass between 9<sup>th</sup> (Supplementary Figure 2), 13<sup>th</sup> (Supplementary Figure 3), and 16<sup>th</sup> (Supplementary Figure 4) week.

### **Effect of peripheral administration of CCK-8 on food intake with oestrogen replacement**

We performed CCK-8 administration experiments to investigate the relationship between oestrogen and food intake. All Wistar female rats with OVX were divided into four groups: oil only and high dose oestrogen in the subcutaneous tube with i.p. administration of saline or CCK-8 (Exp. E). Rats receiving high doses of oestrogen experienced significant weight loss (Figure 5a). Rats supplemented with high doses of oestrogen consumed significantly less food throughout the day. Cumulative food intake was significantly decreased in the OVX/High E2 group compared to the OVX/oil only group (Figure 5b). CCK-8 was administered to examine the amount of food consumed. Cumulative food intake was significantly decreased 0.5 h, 1 h, and 1.5 h after i.p. administration of CCK-8. There was a significant difference between OVX/oil only group and the OVX/High E2 group at 1.5 h after i.p. administration of saline and 3 h after i.p. administration of CCK-8. After 6 h, there was no significant difference in cumulative food intake for all groups (Figure 5c).

## Effect of oestrogen on Fos expression in OXT-ir neurons

We conducted immunohistochemistry to assess the levels of Fos and OXT in the hypothalamus (Exp. F). All Wistar female rats with OVX were divided into two groups: oil only and high dose oestrogen tubing. Tissues were harvested and evaluated for the expression of Fos and OXT through double-fluorescence immunohistochemistry (FIHC). We quantified immunofluorescently labelled OXT<sup>+</sup>, Fos<sup>+</sup>, and OXT<sup>+</sup>/Fos<sup>+</sup> double-labelled cells in the SON and PVN (Figure 5d). The number and percentage of OXT<sup>+</sup>/Fos<sup>+</sup> cells were significantly higher when CCK-8 was administered than when saline was administered. Among these rats, the number and percentage of OXT<sup>+</sup>/Fos<sup>+</sup> cells were significantly higher in the high E2 group than in the oil only group (Figure 5e).

## Effect of pre-treatment with OXT receptor antagonist (OXTR-A) on food intake

In the previous experiment (Exp. E), there was a significant difference between the OVX / oil-only group and the OVX / high E2 group at 3 hours after i.p. administration of CCK-8. Therefore, we assessed food intake for 3 hours after i.p. administration of CCK-8 and i.c.v. administration of OXTR-A. All Wistar female rats with OVX were divided into four groups: oil only and high dose oestrogen in the subcutaneous tube with i.p. administration of CCK-8 and i.c.v. administration of saline or OXTR-A (Exp. G). Rats receiving high doses of oestrogen experienced significant weight loss (Figure 6a). Cumulative food intake for 3 hours was significantly increased in i.c.v. administration of OXTR-A group compared to i.c.v. administration of saline group (Figure 6b).

## Discussion

The present study provides the first evidence that hypothalamo-neurohypophysial OXT is oestrogen-dependent and shows dynamic changes during the oestrus cycle. OXT-mRFP1 fluorescence intensity in the SON and PVN was expressed most strongly among adult oestrous female rats and was significantly reduced in OVX rats. Oestrogen supplementation restored fluorescence intensity in the SON and PVN in OVX rats in a dose-dependent manner. Thus, the dynamics of hypothalamic OXT expression is regulated by oestrogen. As one of the physiological meanings, we confirmed that feeding suppression induced by the peripheral administration of CCK-8 resulted in the activation of OXT neurons, and this was enhanced among oestrogen-replaced female rats.

OXT is mainly produced in neurosecretory neurons located in the SON and PVN in the hypothalamus. We successfully generated transgenic rats bearing an OXT-mRFP1 fusion gene, which resulted in the visualization of OXT expression<sup>19,20,25-28</sup>. The PVN is divided into regions such as apPVN, dpPVN, and mPVN, and previous studies with the OXT-mRFP1 transgenic rats reported different effects<sup>29</sup>. OXT<sup>+</sup> neurons in the SON and mPVN project their axons to the PP, where OXT is thereby secreted into the systemic circulation and elicits activity peripherally<sup>30</sup>. There are reports that in males, OXT is involved in sexual behaviour, ejaculation, and transport of spermatozoa. In females, the peripheral effects of OXT are related to labour and lactation<sup>31</sup>. With respect to the central functions, OXT is also somatodendrically

released from neurons in the SON and mPVN and acts directly on the brain<sup>32</sup>. Neurons expressing OXT receptors are ubiquitous in the brain and have a wide range of functions<sup>33</sup>. It has been reported that OXT is not only associated with confidence and bond formation but is also strongly associated with autism<sup>34,35</sup>. In the present study, the observed differences related to the sex of OXT-mRFP1 rats suggest that the production of OXT in the hypothalamus differs according to sex. This may further indicate that the central actions of OXT can also vary with sex.

A pathway that projects OXT from the apPVN and dpPVN to the medulla and spinal cord has been identified<sup>29</sup>. This is known to affect the autonomic nervous system and has been described to induce analgesic effects and regulate pain<sup>36</sup>, gastrointestinal motility and cardiovascular responses<sup>37</sup>. There are clinical reports that the pain threshold is high during pregnancy and postpartum<sup>38</sup>. Chronic pain after caesarean section is reported to be 1/10 as severe as that after other open surgeries<sup>39</sup>. We speculate that these observations could be explained by hypothalamic OXT dynamics. In our study, OXT-mRFP1 expression was increased in the apPVN and dpPVN throughout the oestrus cycle, and this may, in turn, regulate pain thresholds.

Spontaneous ovulator animals have an ovulation cycle. Humans have an oestrus cycle of approximately 28 days while rats have a shorter cycle of 4-5 days. The blood levels of hormones secreted from the ovaries also fluctuate periodically, and these dynamics are common between humans and rats. Oestrogen acts on the mucous membrane epithelium and changes its histology. Therefore the oestrus cycle can be monitored through a vaginal liquid smear examination<sup>40</sup>. Based on this examination, the oestrus cycle in rats can be classified into prooestrus, oestrus, metoestrus, and dioestrus cycles. The highest increase in blood oestrogen levels occurs when the LH surge coincides with the oestrus stage. Ovulation subsequently occurs several hours later<sup>41</sup>. In OXT-mRFP1 transgenic rats, the fluorescence intensity of mRFP1 has been shown to be delayed by several hours after stimulation<sup>19,29,42</sup>. It is thought that the fluorescence intensity of mRFP1 increases several hours after the point of the highest prooestrus production of oestrogen by the ovary. This, therefore, indicates that fluorescence intensity peaks during the oestrus stage.

In females, oestrogen levels are reduced as a result of age-related reductions in ovarian function and when the ovaries are removed due to gynaecological surgery or treatment. In addition to mammary glands and genital organs, oestrogen acts on the liver, cardiovascular system, bones, and the brain<sup>12,43,44</sup>. Therefore, ovarian dysfunction can elicit many symptoms. In this study, we mimicked ovarian dysfunction/menopause through ovariectomies. We observed that OVX reduced mRFP1 fluorescence in the SON and PVN (ap, dp, m). Thus, OXT production may have been decreased in all hypothalamic areas. Decreased OXT production suggests that broad-ranging central OXT actions may be attenuated by OVX.

There are three known variants of oestrogen – oestrone (E1), oestradiol (E2), and oestriol (E3), and three known subtypes of ERs – ER $\alpha$ , ER $\beta$ , and G protein-coupled receptor 30 (GPR30)<sup>45</sup>. ERs are expressed systemically, and ER $\alpha$  and ER $\beta$  are localized in the brain. However, only ER $\beta$  has been reported to be

expressed in the PVN and SON in the hypothalamus<sup>11</sup>. E2, which elicits the strongest effects, is often used as an experimental or therapeutic drug. In this experiment, the type of oestrogen used was E2. Oestrogen replacement has various effects on the liver, cardiovascular system, bones, and the brain. It is further used to treat menopausal symptoms<sup>46-48</sup>. The subcutaneous administration of oestrogen has fewer side effects compared to intravenous and oral administration and can reproduce the systemic effects of oestrogen<sup>49</sup>. In our work, the subcutaneous administration of E2 to OVX female rats restored mRFP1 fluorescence intensity in the SON and PVN (ap, dp, m) in a dose-dependent manner. Thus, the subcutaneous administration of E2 may increase OXT production in all hypothalamic regions.

Oestrogen has further been reported to suppress feeding. Mechanistically, oestrogen has been shown to increase pro-opiomelanocortin (POMC) gene expression, which induces appetite suppression through signal transducers and activates transcription 3 (STAT-3) in the hypothalamus<sup>50</sup>. Oestrogen also regulates hypothalamic OXT activity in the NTS of the solitary tract and thereby suppresses food intake<sup>18</sup>. Similar to the previous reports, we demonstrated that oestrogen administration suppressed feeding and promoted weight loss. Furthermore, we confirmed that hypothalamic OXT production increased depending on oestrogen levels. This suggests that food consumption may differ depending upon oestrogen levels.

Oestrogen and OXT share a common antifeedant activity<sup>20</sup>. Food consumption was measured following the administration of CCK-8, which is known to selectively activate OXT neurons<sup>21</sup>. We demonstrated the link between OXT and oestrogen by counting Fos<sup>+</sup>/OXT<sup>+</sup> neurons. Fos is an early expression pro-oncogene expressed at low levels in most cell types<sup>51</sup> and can be activated by various second messenger signals. However, Fos is an indicator of cell activation. Accordingly, the evaluation of Fos expression cannot be used to determine any direct effects related to feeding versus other secondary effects, which may likewise induce cellular activation. The gastrointestinal hormone CCK-8 acts on OXT in the hypothalamus through the NTS to increase blood OXT concentrations and suppress appetite<sup>21</sup>. We demonstrated that food consumption was lowest when CCK-8 was administered alongside oestrogen supplementation. Thus, we demonstrated that oestrogen could further induce OXT production when CCK-8 was administered. Likewise, the strongest levels of appetite suppression corresponded with the highest proportions of OXT<sup>+</sup>/Fos<sup>+</sup> neurons. These results suggest that oestrogen administration may enhance hypothalamic OXT production clinically.

Many studies have demonstrated the efficiency of oestrogen and OXT as anti-obesity peptides<sup>52</sup>. Intracerebroventricular or peripheral (i.p. and subcutaneous) injection of OXT decreases food intake, body weight, and fat mass in rats and mice<sup>37,53</sup>. Fat was divided into visceral fat and subcutaneous fat, with differences between sexes. The percentage of visceral fat was higher in males, and subcutaneous fat was altered in females. However, in this experiment, it is unclear whether OXT was directly involved in fat composition.

To the best of our knowledge, this is the first report demonstrating a difference in the dynamics of the hypothalamic OXT based on the sex of rats. Additionally, we demonstrated that hypothalamic OXT expression is specifically dependent upon oestrogen, as E2 administration increased central OXT production in OVX rats. However, our study has several limitations. We did not assess any association of hypothalamic OXT with sex hormones (e.g. progesterone) other than oestrogen. All hypothalamic examinations were conducted in rats and not clinically in humans. No adverse effects of oestrogen replacement have been considered.

## Methods

### Rats Models

All rats were treated after 10 weeks of age once the oestrus cycle was established. Adult male and female OXT-mRFP1 Wistar transgenic rats (aged 10-16 weeks and weighing 223–474 g) were bred and maintained as described previously<sup>19,20</sup>. The OXT-mRFP1 transgenic rat was created by inserting the *mRFP1* gene into the *OXT* gene. This reporter strain facilitates the visualization of OXT dynamics in the hypothalamus through the quantification of OXT fluorescence intensity changes under various stimulation loads<sup>28,29</sup>. All rats were genotypically screened through PCR analysis of their genomic DNA extracted via ear biopsies<sup>19</sup>. Adult female Wistar rats (aged 15-16 weeks and weighing 271–337g) were used in experiment 4 (below). All rats were housed as three per plastic cage (transparent polymethylpentene, TR-TPX-200A, TOKIWA KAGAKU KIKAI, Tokyo Japan) in an air-conditioned room (22–25 °C) with a 12-h light cycle (7:00 A.M. to 7:00 P.M.) and ad libitum access to food (CLEA Rodent Diet CE-2, CLEA Japan, Tokyo Japan) and water. All transgenic rats and Wistar rats delivered at the same time were used for each experiment. All experiments were performed in strict accordance with guidelines on the use and care of laboratory animals as set forth by the Physiological Society of Japan and approved (No.AE10-012) by the Ethics Committee of Animal Care and Experimentation of University of Occupational and Environmental Health, Japan.

### Surgical procedures

Bilateral ovariectomies were conducted on rats to induce an oestrogen-deficient state. Ovariectomies were conducted by opening the flanks of the rat by ~1 cm and removing the ovaries attached to the end of the double-horned uterus. In sham operations, the abdominal cavity was closed without treatment. Rats undergoing OVX and sham operations were anesthetized with an i.p. injection of a cocktail of three different anaesthetic agents (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol).

For hormone replacement, hormone-containing tubes were subcutaneously implanted into the mid-back region of the rats. Rats were anesthetized with isoflurane (3% isoflurane with a flow rate of 5.0 L/min). Silastic tubing (1.57 mm inner diameter; 3.18 mm outer diameter; 37.0 mm in length; Dow

Corning, Midland, MI, USA) was filled with fat-soluble E2 (17 $\beta$ -oestradiol  $\geq$  98%, Sigma-Aldrich, Tokyo, Japan) dissolved in sesame oil (Sigma-Aldrich) <sup>54,55</sup>.

For i.c.v. administration, animals were implanted with stainless steel canulae targeting the lateral ventricle. They were anaesthetized (i.p. injection of a cocktail of three different anaesthetic agents (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol)) and placed in a stereotaxic frame. Stainless steel guide canulae (550  $\mu$ m outer diameter and 10 mm length) were stereotaxically implanted at the following coordinates: 0.8 mm posterior to the Bregma, 1.4 mm lateral to the midline, and 2.0 mm below the surface of the left cortex, such that canula tips were 1.0 mm above the left cerebral ventricle <sup>56</sup>. Two stainless steel anchoring screws and acrylic dental cement were used to secure cannulae in place. After the surgical procedure, animals were handled daily, individually housed in a plastic cage, and allowed to recover for at least 10 days.

## **Test substances**

Cholecystokinin-8 (CCK-8) (Peptide Institute, Osaka, Japan) was dissolved in 0.9% sterile physiological saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to a concentration of 10  $\mu$ g/ml.

OXT receptor antagonist (OXTR-A) (L-368899, Tocris Bioscience, Bristol, UK) was dissolved in 0.9% sterile physiological saline (150 ng/ $\mu$ l).

## **Experimental procedure**

### **1. Assessing differences in OXT-mRFP1 fluorescence in male and female transgenic rats**

We first aimed to ascertain differences in OXT-mRFP1 fluorescence between 10-week-old female rats undergoing a normal oestrus cycle and 10-week-old male rats [first experiment (Exp. A), n = 30]. Female OXT-mRFP1 transgenic rats were further divided based on the four oestrus stages (prooestrus, oestrus, metoestrus, and dioestrus stages). In total, we assessed five groups (1 male, 4 females; n = 5-9 per group). The oestrus cycle was confirmed by examination of the vaginal smear from the rats collected every morning by two researchers. In brief, the prooestrus stage is identified mainly by nucleated cells, oestrus by all keratinocyte cells, metoestrus by the presence of many white blood cells, and dioestrus by the presence of few white blood cell and various cells. Rats with irregular oestrus cycles were excluded from the experiment.

### **2. Assessing the effects of OVX on OXT-mRFP1 fluorescence**

In the second experiment (Exp. B, n = 33), all OXT-mRFP1 transgenic rats were divided into six groups, including five sham-operated groups (consisting of both males and females at all oestrus cycle stages) and one OVX group (n = 5-6 per group). We confirmed the oestrogen-deficient state (like dioestrus stage) according to the vaginal smear of OVX rats.

### **3. Assessing OXT-mRFP1 fluorescence after oestrogen replacement**

For the third experiment (Exp. C, n = 24), we conducted hormone replacement experiments involving subcutaneous implantation of oestrogen-containing tubes in the mid-back region of the rat. Female OXT-mRFP1 transgenic rats with OVX were divided into four groups (n = 5-6 in each group): control (sham back operation), vehicle (subcutaneous sesame oil only), low E2 replacement (20 µg β-oestradiol/ml sesame oil), and high E2 replacement (400 µg β-oestradiol/ml sesame oil)<sup>54,55</sup>. Low doses of E2 have previously been shown to negatively affect luteinizing hormone (LH) pulses but did not induce LH surges in OVX rats. High E2 levels were shown to induce an LH surge and resulted in an oestrus-like state in OVX rats<sup>54</sup>. We confirmed the effect of low E2 and high E2 in the rats through vaginal smears examinations.

#### **4. Measurement of body weight and food intake**

For the fourth experiment (Exp. D, n = 30), in male and female Wistar rats we assessed body weight and food intake. Wistar rats were divided into 5 groups (n=6 in each group): sham-operated male, sham-operated female, only OVX, OVX plus low E2 replacement, and OVX plus high E2 replacement groups. We performed sham operations and OVX in 10-week-old male and female rats, conducted hormone replacement (tube implantation) with OVX rats at week 14, and assessed cumulative food intake for one day at 9, 13, and 16 weeks.

#### **5. Assessing the effects on administration of CCK-8**

For the fifth experiment (Exp. E, n = 24), in female Wistar rats, we assessed food intake. Ovariectomised Wistar rats were divided into 4 groups: vehicle (subcutaneous sesame oil only) with i.p. administration of saline, vehicle (subcutaneous sesame oil only) with i.p. administration of CCK-8 (50 µg/kg body weight), high E2 (400 µg β-oestradiol/ml sesame oil) with saline injection, and high E2 with CCK-8 injection. Ovariectomies were performed at 10 weeks and tube implantation was performed at 14 weeks of age; saline and CCK-8 were injected at 16 weeks. On week 16, cumulative food intake was measured at 0.5 hours (h), 1 h, 1.5 h, 3h and 6 h after i.p. administration of saline and CCK-8. Prior to injections, all rats were fasted for 24 h (n = 6 in each sub-group). CCK-8 was administered, and the effect on food intake was assessed.

For the sixth experiment (Exp. F, n = 24), in female Wistar rats, we conducted immunohistochemistry to assess the levels of Fos and OXT in the hypothalamus. Ovariectomised Wistar rats were divided into four groups: vehicle (subcutaneous sesame oil only) with i.p. administration of saline, vehicle (subcutaneous sesame oil only) with i.p. administration of CCK-8 (50 µg/kg body weight), high E2 (400 µg β-oestradiol/ml sesame oil) with saline injection, and high E2 with CCK-8 injection. Ovariectomies were performed at 10 weeks and tube implantation was performed at 14 weeks of age; saline and CCK-8 were injected at 16 weeks. All rats were anesthetized and sacrificed at 1.5 h after i.p. administration of saline and CCK-8. Slices of brain tissues were harvested and evaluated for the expression of Fos and OXT through double-FIHC.

#### **6. Assessing the effects on administration of CCK-8 and OXTR-A**

For the seventh experiment (Exp. G, n = 24), in female Wistar rats, we assessed food intake. Ovariectomised Wistar rats were divided into 4 groups: vehicle (subcutaneous sesame oil only) with i.p. administration of CCK-8 (50 µg/kg body weight) and administration of i.c.v. saline, vehicle (subcutaneous sesame oil only) with i.p. administration of CCK-8 and administration of i.c.v. OXTR-A (150 ng/µl), high E2 (400 µg β-oestradiol/ml sesame oil) with CCK-8 i.p. and saline (sterile 0.9% saline) i.c.v., and high E2 with CCK-8 i.p. and OXTR-A i.c.v.. Ovariectomies were performed at 10 weeks and tube implantation and i.c.v. treatments were performed at 14 weeks of age. On week 16, cumulative food intake was measured at 3 h after i.p. administration of CCK-8 and i.c.v. administration of saline or OXTR-A.

### **Extraction of the hypothalamus and pituitary gland of OXT-mRFP1 transgenic rat**

Anesthetized rats were perfused transcardially with 0.1 M phosphate buffer (PB) (pH 7.4) containing heparin (1,000 U/L), followed by 4% paraformaldehyde in 0.1 M PB. Rat brains and pituitaries were carefully extracted, and a small block encapsulating the hypothalamus was isolated. Blocks were post-fixed with 4% paraformaldehyde in 0.1 M PB for 48 h at 4 °C as described previously<sup>57</sup>. Tissue was cryoprotected in 20% (w/v) sucrose in 0.1 M PB for 48 h at 4 °C. Fixed tissue was cut coronally to a thickness of 30 µm using a microtome (REM-700; Yamato Kohki Industrial Co. Ltd, Saitama, Japan). Sections were rinsed with 0.1 M PB and placed on glass slides. Pituitary glands were not treated.

### **Evaluation of mRFP1 fluorescence in the hypothalamus and pituitary gland of OXT-mRFP1 transgenic rat**

The locations of the SON and PVN were determined according to the coordinates in the atlas of Paxinos and Watson<sup>56</sup>. The ap, dp, and m divisions of the PVN were divided and quantified. Sections containing the SON, PVN, and the intact pituitary gland tissues were also examined using a fluorescence microscope (ECLIPSE E 600; Nikon Corp., Tokyo, Japan) equipped with an mRFP1 filter (Nikon Corp.) to visualise OXT-mRFP1 expression. The images were captured with a digital camera (DS-Qi1Mc; Nikon Corp.). For each animal, we averaged the mRFP1 fluorescence intensities across both sections for each region. The average mRFP1 fluorescence intensity per unit area in the SON, apPVN, dpPVN, mPVN, and PP was quantified with an imaging analysis system (NIS-Elements; Nikon Corp.).

### **Fos and OXT double-FIHC of Wistar rat**

Serial 40-µm thick sections were rinsed twice with 0.1 M phosphate-buffered saline (PBS) and washed in 0.1 M Tris buffer (pH, 7.6) containing 0.3% Triton X-100. Sections were incubated for 72 h at 4 °C in primary antibody solution (goat c-Fos, Santa Cruz Biotechnology, TX, USA; 1:500 or rabbit anti-OXT, Sigma-Aldrich, MO, USA; 1:5000)<sup>58</sup>. After washing twice in 0.3% Triton X-100 in PBS, floating sections were incubated for 24 h at 4 °C with a secondary antibody (Alexa Fluor 546 donkey anti-goat IgG or Alexa Fluor 488 donkey anti-rabbit IgG; Molecular Probes, OR, USA; 1:2,000 in PBS containing 0.3% Triton X-100)<sup>58</sup>. Sections were washed twice in PBS and then mounted on the slides and coverslipped using vectashield (Vector Laboratories Co. Ltd., CA, USA)<sup>59</sup>. Images of Fos<sup>+</sup>, OXT<sup>+</sup>, and Fos<sup>+</sup>/OXT<sup>+</sup> double-

labelled cells were counted manually by two researchers who were blinded to avoid bias. The number and percentage of Fos<sup>+</sup>, OXT<sup>+</sup>, and Fos<sup>+</sup>/OXT<sup>+</sup> cells in the SON and PVN were estimated.

## Statistical analysis

All data points are presented as the mean ± standard error of the mean. Statistical significances were calculated based on one-way analysis of variance (ANOVA) and repeated-measures ANOVA, using a Tukey-Kramer-type adjustment for multiple comparisons. A P-value <0.05 was considered statistically significant.

## Declarations

### Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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### Author Contributions

Study was designed by K.N.1, and Y.U.1; The paper was written by K.N.1; Experiments were performed by K.N.1, N.I.1, K.B.1, K.S.1, Y.A.1, H.N.1, K.T.1, S.S.1, H.U.1, M.Y.1 T.M.1, and K.Y.2; Data analysis and Interpretation of data were performed by K.N.1, K.Y.2, T.H.3 and Y.U.1; Draft and Figures are prepared by K.N.1; Final Approval was made by Y.U.1 All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy. All authors designated as authors qualify for authorship, and all those who qualify for authorship are listed.

### Competing interests

The authors declare that they have no competing interests.

## References

- 1 Carmichael, M. S. *et al.* Plasma oxytocin increases in the human sexual response. *J Clin Endocrinol Metab* **64**, 27-31, doi:10.1210/jcem-64-1-27 (1987).
- 2 Kiss, A. & Mikkelson, J. D. Oxytocin–anatomy and functional assignments: a minireview. *Endocr Regul* **39**, 97-105 (2005).

- 3 Ludwig, M. & Leng, G. Dendritic peptide release and peptide-dependent behaviours. *Nat Rev Neurosci***7**, 126-136, doi:10.1038/nrn1845 (2006).
- 4 Higashida, H. *et al.* Oxytocin signal and social behaviour: comparison among adult and infant oxytocin, oxytocin receptor and CD38 gene knockout mice. *J Neuroendocrinol***22**, 373-379, doi:10.1111/j.1365-2826.2010.01976.x (2010).
- 5 Pedersen, C. A., Vadlamudi, S. V., Boccia, M. L. & Amico, J. A. Maternal behavior deficits in nulliparous oxytocin knockout mice. *Genes Brain Behav***5**, 274-281, doi:10.1111/j.1601-183X.2005.00162.x (2006).
- 6 Lund, I. *et al.* Repeated massage-like stimulation induces long-term effects on nociception: contribution of oxytocinergic mechanisms. *Eur J Neurosci***16**, 330-338 (2002).
- 7 Petersson, M., Wiberg, U., Lundeberg, T. & Uvnas-Moberg, K. Oxytocin decreases carrageenan induced inflammation in rats. *Peptides***22**, 1479-1484 (2001).
- 8 Flanagan, L. M., Olson, B. R., Sved, A. F., Verbalis, J. G. & Stricker, E. M. Gastric motility in conscious rats given oxytocin and an oxytocin antagonist centrally. *Brain Res***578**, 256-260 (1992).
- 9 Yosten, G. L. & Samson, W. K. The anorexigenic and hypertensive effects of nesfatin-1 are reversed by pretreatment with an oxytocin receptor antagonist. *Am J Physiol Regul Integr Comp Physiol***298**, R1642-1647, doi:10.1152/ajpregu.00804.2009 (2010).
- 10 Sabatier, N. alpha-Melanocyte-stimulating hormone and oxytocin: a peptide signalling cascade in the hypothalamus. *J Neuroendocrinol***18**, 703-710, doi:10.1111/j.1365-2826.2006.01464.x (2006).
- 11 Isgor, C., Cecchi, M., Kabbaj, M., Akil, H. & Watson, S. J. Estrogen receptor beta in the paraventricular nucleus of hypothalamus regulates the neuroendocrine response to stress and is regulated by corticosterone. *Neuroscience***121**, 837-845 (2003).
- 12 Grodstein, F. & Stampfer, M. The epidemiology of coronary heart disease and estrogen replacement in postmenopausal women. *Prog Cardiovasc Dis***38**, 199-210 (1995).
- 13 Hulley, S. *et al.* Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group. *JAMA***280**, 605-613 (1998).
- 14 Rossouw, J. E. *et al.* Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. *JAMA***288**, 321-333 (2002).
- 15 Geary, N., Asarian, L., Korach, K. S., Pfaff, D. W. & Ogawa, S. Deficits in E2-dependent control of feeding, weight gain, and cholecystokinin satiation in ER-alpha null mice. *Endocrinology***142**, 4751-4757, doi:10.1210/endo.142.11.8504 (2001).

- 16 Wade, G. N. & Gray, J. M. Gonadal effects on food intake and adiposity: a metabolic hypothesis. *Physiol Behav***22**, 583-593 (1979).
- 17 Heine, P. A., Taylor, J. A., Iwamoto, G. A., Lubahn, D. B. & Cooke, P. S. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A***97**, 12729-12734, doi:10.1073/pnas.97.23.12729 (2000).
- 18 Sloan, D. K., Spencer, D. S. & Curtis, K. S. Estrogen effects on oxytocinergic pathways that regulate food intake. *Horm Behav***105**, 128-137, doi:10.1016/j.yhbeh.2018.08.007 (2018).
- 19 Katoh, A. *et al.* Highly visible expression of an oxytocin-monomeric red fluorescent protein 1 fusion gene in the hypothalamus and posterior pituitary of transgenic rats. *Endocrinology***152**, 2768-2774, doi:10.1210/en.2011-0006 (2011).
- 20 Katoh, A. *et al.* Fluorescent visualisation of the hypothalamic oxytocin neurones activated by cholecystokinin-8 in rats expressing c-fos-enhanced green fluorescent protein and oxytocin-monomeric red fluorescent protein 1 fusion transgenes. *J Neuroendocrinol***26**, 341-347, doi:10.1111/jne.12150 (2014).
- 21 Ebenezer, I. S. Systemic administration of cholecystokinin (CCK) inhibits operant water intake in rats: implications for the CCK-satiety hypothesis. *Proc Biol Sci***263**, 491-496, doi:10.1098/rspb.1996.0074 (1996).
- 22 Olson, B. R. *et al.* Oxytocin and an oxytocin agonist administered centrally decrease food intake in rats. *Peptides***12**, 113-118, doi:10.1016/0196-9781(91)90176-p (1991).
- 23 Verbalis, J. G., Blackburn, R. E., Olson, B. R. & Stricker, E. M. Central oxytocin inhibition of food and salt ingestion: a mechanism for intake regulation of solute homeostasis. *Regul Pept***45**, 149-154, doi:10.1016/0167-0115(93)90198-h (1993).
- 24 Arase, K. *et al.* Possible involvement of central oxytocin in cisplatin-induced anorexia in rats. *J Physiol Sci***68**, 471-482, doi:10.1007/s12576-017-0550-z (2018).
- 25 Matsuura, T. *et al.* Fluorescent Visualisation of Oxytocin in the Hypothalamo-neurohypophysial/-spinal Pathways After Chronic Inflammation in Oxytocin-Monomeric Red Fluorescent Protein 1 Transgenic Rats. *J Neuroendocrinol***27**, 636-646, doi:10.1111/jne.12290 (2015).
- 26 Ohkubo, J. *et al.* Electrophysiological effects of kainic acid on vasopressin-enhanced green fluorescent protein and oxytocin-monomeric red fluorescent protein 1 neurones isolated from the supraoptic nucleus in transgenic rats. *J Neuroendocrinol***26**, 43-51, doi:10.1111/jne.12128 (2014).
- 27 Ohno, S. *et al.* Increased oxytocin-monomeric red fluorescent protein 1 fluorescent intensity with urocortin-like immunoreactivity in the hypothalamo-neurohypophysial system of aged transgenic rats. *Neurosci Res***128**, 40-49, doi:10.1016/j.neures.2017.08.001 (2018).

- 28 Nishimura, H. *et al.* Neuropathic Pain Up-Regulates Hypothalamo-Neurohypophysial and Hypothalamo-Spinal Oxytocinergic Pathways in Oxytocin-Monomeric Red Fluorescent Protein 1 Transgenic Rat. *Neuroscience***406**, 50-61, doi:10.1016/j.neuroscience.2019.02.027 (2019).
- 29 Matsuura, T. *et al.* Possible Involvement of the Rat Hypothalamo-Neurohypophysial/-Spinal Oxytocinergic Pathways in Acute Nociceptive Responses. *J Neuroendocrinol***28**, doi:10.1111/jne.12396 (2016).
- 30 Brownstein, M. J., Russell, J. T. & Gainer, H. Synthesis, transport, and release of posterior pituitary hormones. *Science***207**, 373-378 (1980).
- 31 Veening, J. G., de Jong, T. R., Waldinger, M. D., Korte, S. M. & Olivier, B. The role of oxytocin in male and female reproductive behavior. *Eur J Pharmacol***753**, 209-228, doi:10.1016/j.ejphar.2014.07.045 (2015).
- 32 Ludwig, M. & Pittman, Q. J. Talking back: dendritic neurotransmitter release. *Trends Neurosci***26**, 255-261, doi:10.1016/S0166-2236(03)00072-9 (2003).
- 33 Rilling, J. K. & Young, L. J. The biology of mammalian parenting and its effect on offspring social development. *Science***345**, 771-776, doi:10.1126/science.1252723 (2014).
- 34 Young, L. J. & Barrett, C. E. Neuroscience. Can oxytocin treat autism? *Science***347**, 825-826, doi:10.1126/science.aaa8120 (2015).
- 35 Meyer-Lindenberg, A., Domes, G., Kirsch, P. & Heinrichs, M. Oxytocin and vasopressin in the human brain: social neuropeptides for translational medicine. *Nat Rev Neurosci***12**, 524-538, doi:10.1038/nrn3044 (2011).
- 36 Condes-Lara, M., Martinez-Lorenzana, G., Rodriguez-Jimenez, J. & Rojas-Piloni, G. Paraventricular hypothalamic nucleus stimulation modulates nociceptive responses in dorsal horn wide dynamic range neurons. *Neurosci Lett***444**, 199-202, doi:10.1016/j.neulet.2008.08.027 (2008).
- 37 Maejima, Y. *et al.* Nesfatin-1-regulated oxytocinergic signaling in the paraventricular nucleus causes anorexia through a leptin-independent melanocortin pathway. *Cell Metab***10**, 355-365, doi:10.1016/j.cmet.2009.09.002 (2009).
- 38 Geenen, V. *et al.* The neuroendocrine thymus: coexistence of oxytocin and neurophysin in the human thymus. *Science***232**, 508-511 (1986).
- 39 Brandsborg, B. Pain following hysterectomy: epidemiological and clinical aspects. *Dan Med J***59**, B4374 (2012).
- 40 Montes, G. S. & Luque, E. H. Effects of ovarian steroids on vaginal smears in the rat. *Acta Anat (Basel)***133**, 192-199 (1988).

- 41 Ill, J. F. S., Barbieri, R. L. & Gargiulo, A. R. *Yen & Jaffe's Reproductive Endocrinology: Physiology, Pathophysiology, and Clinical Management, 8e.* (Elsevier, 2018).
- 42 Yue, C., Ponzio, T. A., Fields, R. L. & Gainer, H. Oxytocin and vasopressin gene expression and RNA splicing patterns in the rat supraoptic nucleus. *Physiol Genomics***35**, 231-242, doi:10.1152/physiolgenomics.90218.2008 (2008).
- 43 Ohmichi, M. *et al.* Rapid changes of flow-mediated dilatation after surgical menopause. *Maturitas***44**, 125-131 (2003).
- 44 Melby, M. K., Lock, M. & Kaufert, P. Culture and symptom reporting at menopause. *Hum Reprod Update***11**, 495-512, doi:10.1093/humupd/dmi018 (2005).
- 45 Prossnitz, E. R. *et al.* Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol***70**, 165-190, doi:10.1146/annurev.physiol.70.113006.100518 (2008).
- 46 Scarabin, P. Y. *et al.* Haemostatic variables and menopausal status: influence of hormone replacement therapy. *Thromb Haemost***70**, 584-587 (1993).
- 47 Maclennan, A. H., Broadbent, J. L., Lester, S. & Moore, V. Oral oestrogen and combined oestrogen/progestogen therapy versus placebo for hot flushes. *Cochrane Database Syst Rev*, CD002978, doi:10.1002/14651858.CD002978.pub2 (2004).
- 48 Rossouw, J. E. *et al.* Postmenopausal hormone therapy and risk of cardiovascular disease by age and years since menopause. *JAMA***297**, 1465-1477, doi:10.1001/jama.297.13.1465 (2007).
- 49 Nelson, H. D. Commonly used types of postmenopausal estrogen for treatment of hot flashes: scientific review. *JAMA***291**, 1610-1620, doi:10.1001/jama.291.13.1610 (2004).
- 50 Gao, Q. & Horvath, T. L. Cross-talk between estrogen and leptin signaling in the hypothalamus. *Am J Physiol Endocrinol Metab***294**, E817-826, doi:10.1152/ajpendo.00733.2007 (2008).
- 51 Morgan, J. I., Cohen, D. R., Hempstead, J. L. & Curran, T. Mapping patterns of c-fos expression in the central nervous system after seizure. *Science***237**, 192-197 (1987).
- 52 Maejima, Y. *et al.* Impact of sex, fat distribution and initial body weight on oxytocin's body weight regulation. *Sci Rep***7**, 8599, doi:10.1038/s41598-017-09318-7 (2017).
- 53 Maejima, Y. *et al.* Peripheral oxytocin treatment ameliorates obesity by reducing food intake and visceral fat mass. *Aging (Albany NY)***3**, 1169-1177, doi:10.18632/aging.100408 (2011).
- 54 Cagampang, F. R., Maeda, K. I., Tsukamura, H., Ohkura, S. & Ota, K. Involvement of ovarian steroids and endogenous opioids in the fasting-induced suppression of pulsatile LH release in ovariectomized rats. *J Endocrinol***129**, 321-328 (1991).

55 Nishimura, K. *et al.* Effect of oestrogen-dependent vasopressin on HPA axis in the median eminence of female rats. *Sci Rep***9**, 5153, doi:10.1038/s41598-019-41714-z (2019).

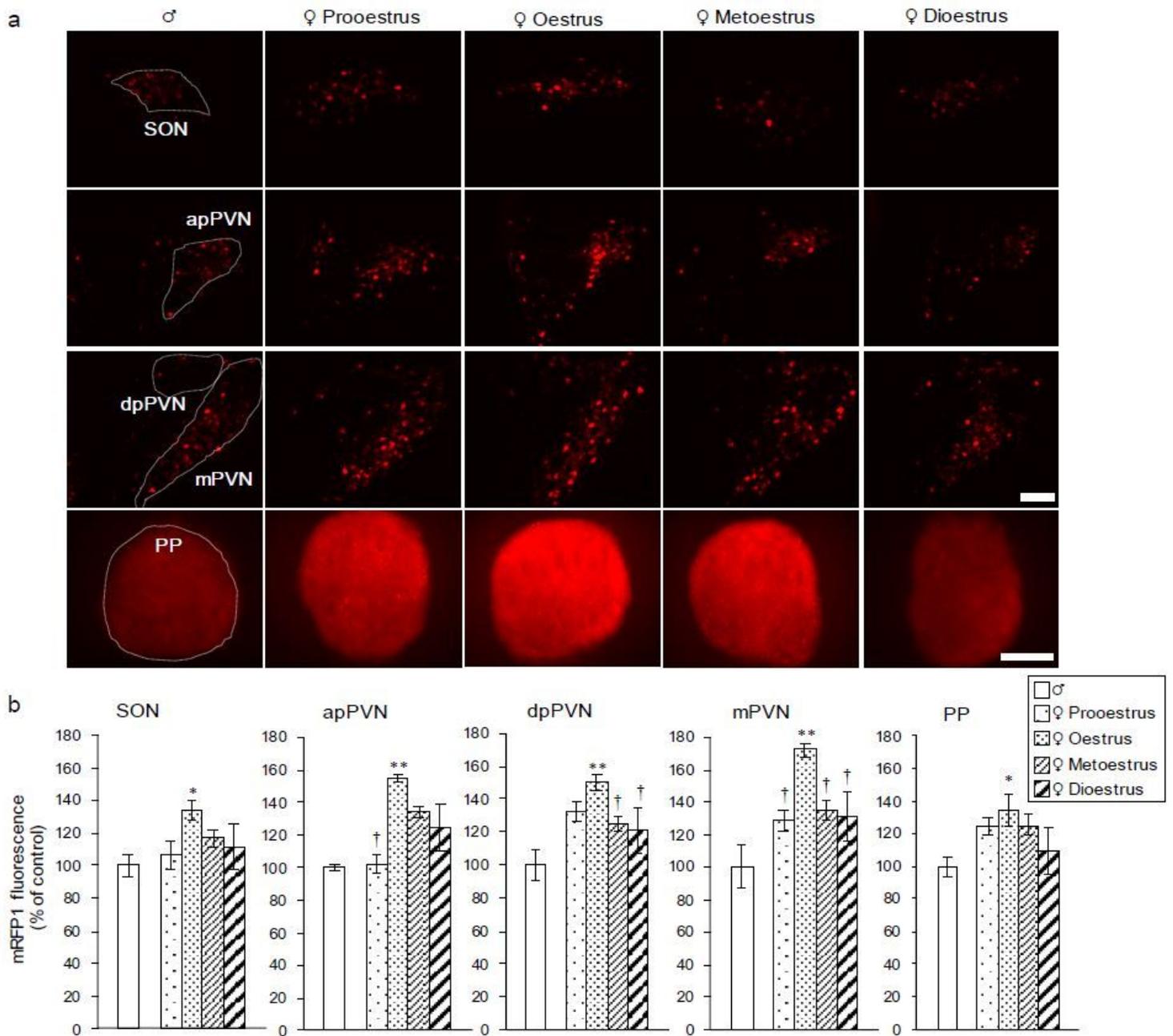
56 Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates, Sixth Edition.* (Academic Press, 2006).

57 Yoshimura, M. *et al.* A c-fos-monomeric red fluorescent protein 1 fusion transgene is differentially expressed in rat forebrain and brainstem after chronic dehydration and rehydration. *J Neuroendocrinol***25**, 478-487, doi:10.1111/jne.12022 (2013).

58 Yoshimura, M. *et al.* Activation of endogenous arginine vasopressin neurons inhibit food intake: by using a novel transgenic rat line with DREADDs system. *Sci Rep***7**, 15728, doi:10.1038/s41598-017-16049-2 (2017).

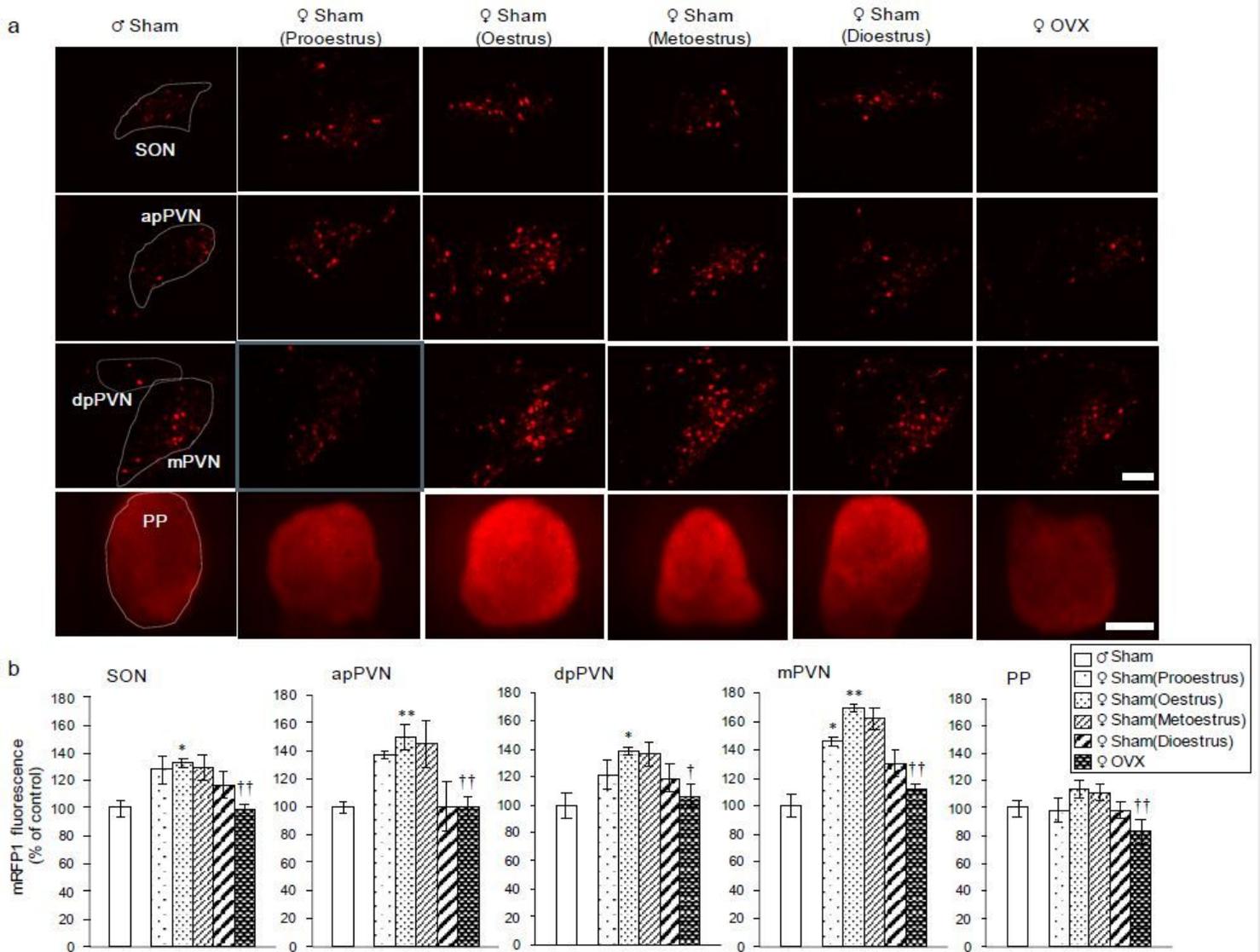
59 Saito, R. *et al.* Centrally administered kisspeptin suppresses feeding via nesfatin-1 and oxytocin in male rats. *Peptides***112**, 114-124, doi:10.1016/j.peptides.2018.12.003 (2019).

## Figures



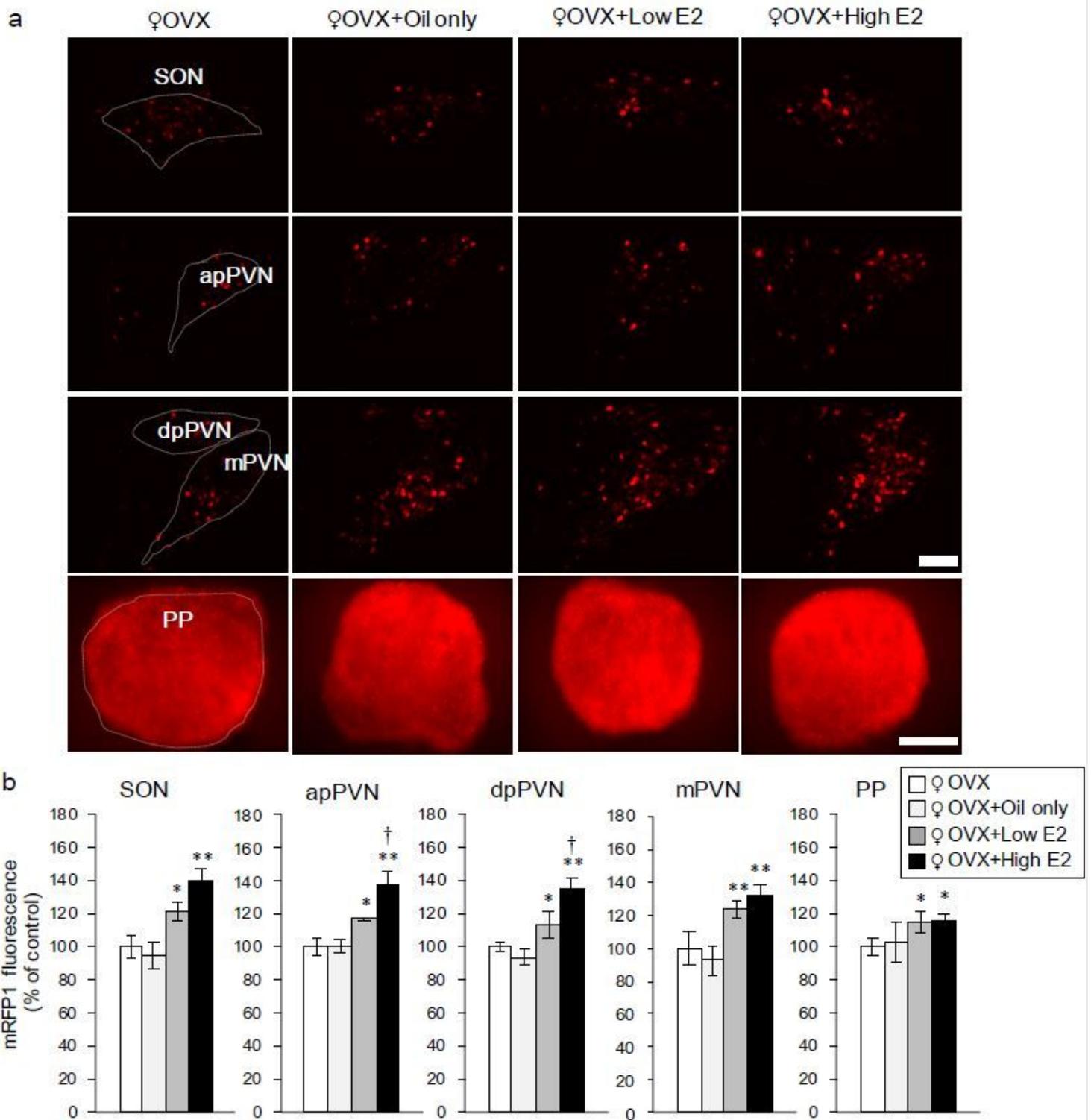
**Figure 1**

OXT-mRFP1 fluorescence in females and male rats a. OXT-mRFP1-positive neurons fluoresce in the SON, apPVN, dpPVN, and mPVN of male, prooestrus female, oestrus female, metroestrus female, and dioestrus female OXT-mRFP1 transgenic rats (first 3 rows). Scale bar indicates 100  $\mu$ m. OXT-mRFP1 fluorescence in the PP of male, prooestrus, oestrus, metroestrus, and dioestrus female OXT-mRFP1 transgenic rats (last row). Scale bar indicates 500  $\mu$ m. b. OXT-mRFP1 expression in the SON, apPVN, dpPVN, mPVN, and PP of the rats [male (n = 5), prooestrus female (n = 5), oestrus female (n = 9), metroestrus female (n = 6), dioestrus female (n = 6)]. The data are presented as the mean  $\pm$  SEM (one-way ANOVA) (\*\*P<0.01, \*P<0.05 compared with male rats; †P<0.05 compared with oestrus female rats).



**Figure 2**

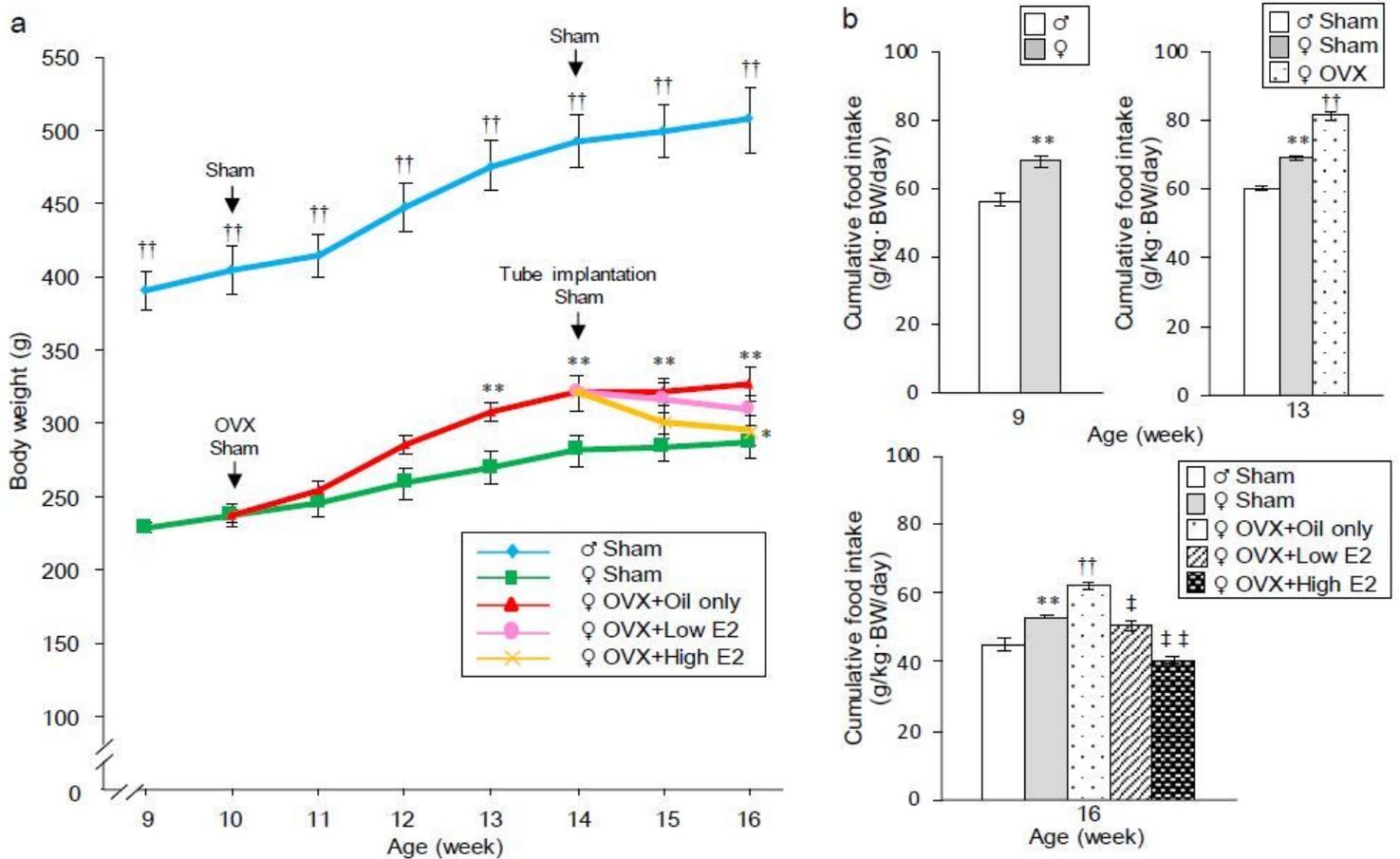
Effects of OVX on OXT-mRFP1 fluorescence a. OXT-mRFP1-positive neurons fluoresce in the SON, apPVN, dpPVN, and mPVN of sham-operated male, sham-operated female in the prooestrus, oestrus, metoestrus, and dioestrus stages, and OVX female OXT-mRFP1 transgenic rats (first 3 rows). Scale bar indicates 100  $\mu$ m. OXT-mRFP1 fluorescence in the PP of sham-operated male, sham-operated female in the prooestrus, oestrus, metoestrus, and dioestrus stages, and OVX female OXT-mRFP1 transgenic rats (last row). Scale bar indicates 500  $\mu$ m. b. OXT-mRFP1 expression in the SON, apPVN, dpPVN, mPVN, and PP of the rats [male (n = 5), prooestrus female (n = 5), oestrus female (n = 5), metoestrus female (n = 5), dioestrus female (n = 5), and OVX female (n = 6)] The data are presented as the mean  $\pm$  SEM (one-way ANOVA) (\*\*P < 0.01, \*P < 0.05, compared with male rats; ††P < 0.01, †P < 0.05, compared with oestrus and OVX female rats).



**Figure 3**

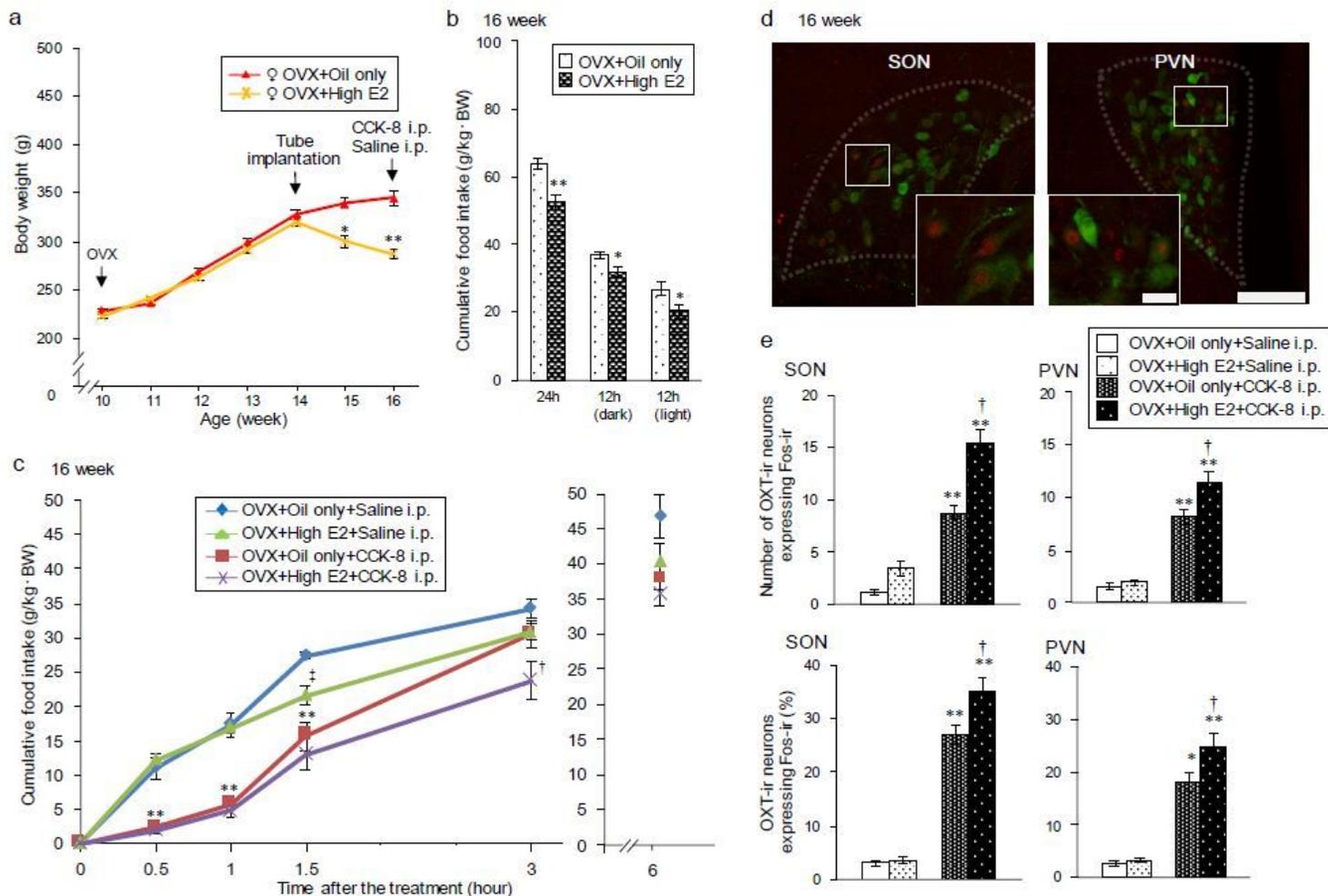
Effects of oestrogen replacement on OXT-mRFP1 fluorescence a. OXT-mRFP1-positive neurons fluoresce in the SON, apPVM, mPVN, and dpPVN of female OXT-mRFP1 transgenic rats in the OVX group, OVX + oil only group, OVX + low E2 (20 µg β-oestradiol/ml sesame oil) group, and OVX + high E2 (400 µg β-oestradiol/ml sesame oil) group, respectively (first 3 rows). Scale bar indicates 100 µm. OXT-mRFP1 fluorescence in the PP of female OXT-mRFP1 transgenic rats in the OVX group, OVX + oil only group, OVX

+ low E2 group, and OVX + high E2 group (last row). Scale bar indicates 500  $\mu\text{m}$ . b. OXT-mRFP1 expression of the SON, apPVN, dpPVN, mPVN, and PP of the rats [OVX (n = 6), OVX + oil only (n = 5), OVX + low E2 (n = 6) and OVX + high E2 (n = 6)]. The data are presented as the mean $\pm$ SEM (one-way ANOVA) (\*\*P<0.01, \*P<0.05, compared with OVX group; †P<0.05, compared with low E2 and high E2 groups).



**Figure 4**

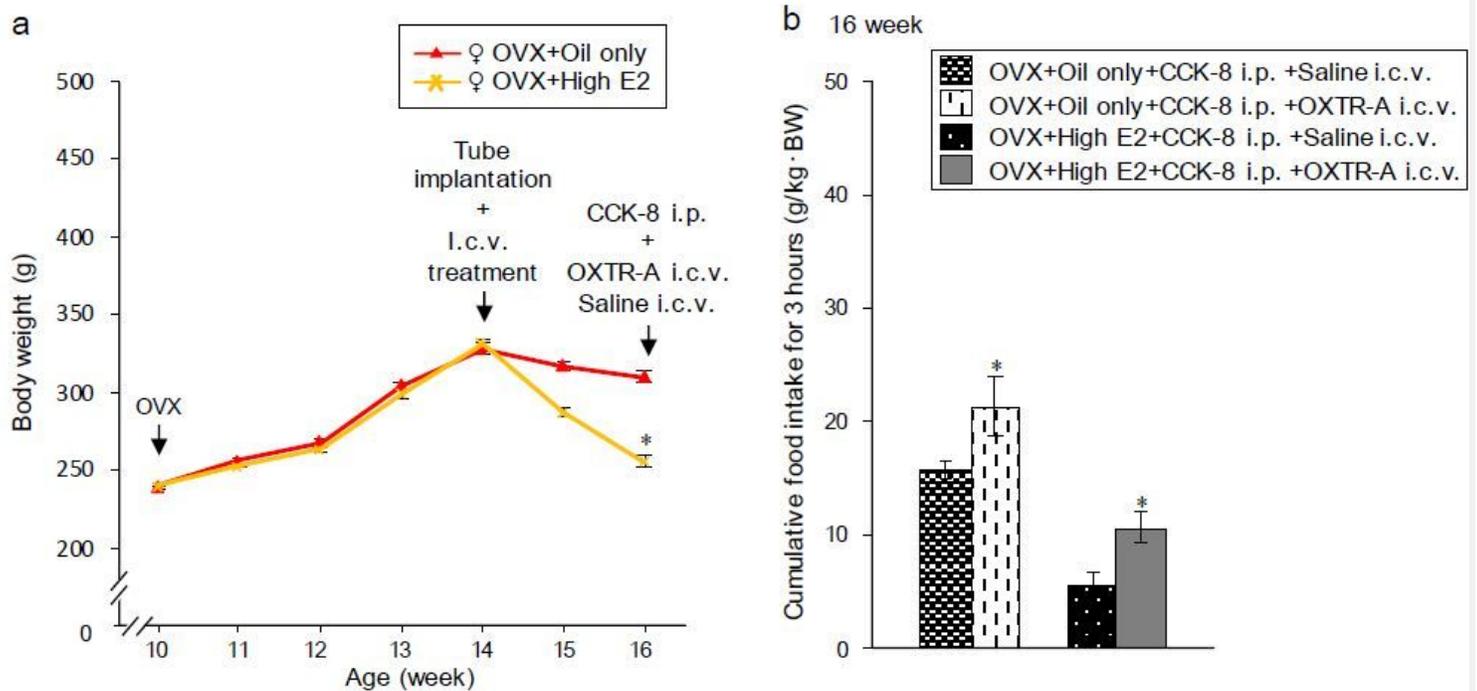
Relationship between rat body weight and feeding a. Changes in body weight after treatment. Sham-operated male, sham-operated female, and OVX female rats at 10 weeks of age. Tube implantation (oil only, low E2 and high E2) was performed in OVX female rats at 14 weeks of age. The data are presented as the mean $\pm$ SEM (repeated-measures ANOVA) (\*\*P<0.01, compared with the treated rats; ††P<0.01, compared with all female rats). b. Cumulative food intake for one day at 9, 13, and 16 weeks of age. The data are presented as the mean $\pm$ SEM (one-way ANOVA) (†, ‡, \*\*P<0.01, \*P<0.05, compared with oil only and high E2 groups).



**Figure 5**

Effect of peripheral administration of CCK-8 on food intake with oestrogen replacement a. Changes in body weight after treatment. Tube implantation (oil only and high E2) was performed in OVX female rats at 14 weeks of age. The data are presented as the mean±SEM (repeated-measures ANOVA) (\*\*P<0.01, \*P<0.05, compared with oil only and high E2 groups). b. Cumulative food intake for one day, dark-time, and light-time. The data are presented as the mean±SEM (one-way ANOVA) (\*\*P<0.01, \*P<0.05, compared with oil only and high E2 groups). c. Cumulative food intake 0.5 h, 1 h, 1.5 h, 3 h and 6 h after i.p. administration of saline or CCK-8 (50 µg/kg body weight). The data are presented as the mean±SEM (one-way ANOVA) (\*\*P<0.01 compared with saline and CCK-8 i.p. groups; †P<0.05, compared with oil only and high E2 groups; ‡P<0.05, compared with oil only and high E2 groups). d. Photomicrographs of the SON and PVN obtained during double-FIHC of Fos and OXT 1.5 h after i.p. administration of CCK-8 to the rats in the OVX + high E2 group. Representative images of Fos-immunoreactive (ir) expression are shown as red-coloured cells, and OXT-ir expression is shown as green cytoplasmic cells in the panel. Merged images of Fos-ir and OXT-ir neurons in the SON and PVN. Scale bars in the low-power and high-power photomicrographs indicate 100 µm and 20 µm, respectively. e. The number of OXT-ir neurons expressing Fos-ir in the SON and PVN 1.5 h after i.p. administration of saline or CCK-8 to the rats in the OVX+ oil only group or OVX+ high E2 group. The average percentage of OXT-ir neurons expressing Fos-ir in the SON and

PVN 1.5 h after i.p. administration of saline or CCK-8 to the rats in the OVX+ oil only group or OVX+ high E2 group. The data are presented as the mean±SEM (one-way ANOVA) (\*\*P<0.01, compared with saline i.p. group; †P<0.05, compared with oil only and high E2 group).



**Figure 6**

Effect of pre-treatment with OXT receptor antagonist (OXTR-A) on food intake a. Changes in body weight after treatment. Tube implantation (oil only and high E2) and i.c.v. treatment was performed in OVX female rats at 14 weeks of age. The data are presented as the mean±SEM (repeated-measures ANOVA) (\*P<0.05, compared with oil only and high E2 groups). b. Cumulative food intake for 3 h after i.p. administration of CCK-8 (50 µg/kg body weight) and i.c.v. administration of saline or OXTR-A (150 ng/µl) to the rats in the OVX+ oil only group or OVX+ high E2 group. The data are presented as the mean±SEM (one-way ANOVA) (\*P<0.05, compared with saline i.c.v. and OXTR-A i.c.v. group).

## Supplementary Files

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