Oestrogen-dependent hypothalamic oxytocin expression with changes in feeding and body weight in female rats

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Oxytocin (OXT) is produced in the hypothalamic nuclei and secreted into systemic circulation from the posterior pituitary gland. In the central nervous system, OXT regulates behaviours including maternal and feeding behaviours. Our aim is 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 and expression of the OXT and mRFP1 genes in the hypothalamic nuclei is highest during the oestrus stage in female rats and decreased significantly in ovariectomised rats. Oestrogen replacement caused significant increases in fluorescence intensity and gene expression in a dose-related manner. This is also demonstrated in the rats' feeding behaviour and hypothalamic Fos neurons using cholecystokinin-8 and immunohistochemistry. Hypothalamic OXT expression is oestrogen-dependent and can be enhanced centrally by the administration of oestrogen.
Oxytocin (OXT) is produced in the paraventricular (PVN) and supraoptic nuclei (SON) of the hypothalamus. Peripheral OXT is a neurohypophysial hormone that is originally synthesised in the magnocellular PVN (mPVN) and SON, and is secreted from the posterior pituitary gland (PP) into the systemic circulation. In the periphery, OXT regulates parturition and lactation. Recent studies have suggested that in addition to its peripheral effects, hypothalamic OXT produced in the mPVN and SON acts within the central nervous system to regulate many functions, including social recognition and trust-building. In addition, OXT is produced in the dorsal parvocellular PVN (dpPVN) and in this context, is involved in the modulation of stress and pain. Interestingly, the OXT pathway from the PVN is involved in the control of feeding. In particular, hypothalamic OXT has an anorectic action; therefore, modulating this pathway is anticipated to reduce obesity and high blood glucose levels. However, while the peripheral actions of OXT in pregnant and lactating females are well known, the sex differences in hypothalamic OXT dynamics remain unclear.

Oestrogen is produced in the ovaries and placenta, and binds to systemic oestrogen receptors (ER) via blood to produce oestrogenic activity. Oestrogen plays an important role in maintaining the physiological functions of systemic organs. Additionally, through the oestrous 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. Furthermore, the effects of oestrogen on food intake are thought to be mediated through ERα and ERβ within the central nervous system. ERβ are located on OXT neurons and are the predominant ER subtype in the PVN, a hypothalamic area involved in eating. However, details regarding the correlation between oestrogen and hypothalamic OXT dynamics are unknown.

In the present study, we first assessed the effects of sex and oestrogen on body weight and body fat mass in female rats using micro-computed tomography (micro-CT). We used reporter OXT-monomeric red fluorescent protein 1 (mRFP1) transgenic rats to visualise OXT expression and clarify the relationship between hypothalamic OXT and the oestrous cycle. To confirm the relationship between oestrogen and OXT, we assessed the expression of both OXT-mRFP1 and the OXT-mRFP1 gene in bilaterally ovariectomised (OVX) rats with or without exogenous oestrogen replacement. Then, we assessed whether oestrogen replacement and OVX impact the regulation of OXT produced by hypothalamic neurons, thereby regulating central nervous system functions. We evaluated differences in the expression of the hypothalamic OXT and mRFP1 genes in OVX rats with or without exogenous oestrogen replacement. We then assessed whether oestrogen replacement and OVX impact the regulation of OXT produced by hypothalamic neurons, thereby regulating central nervous system functions. We evaluated differences in the expression of the hypothalamic OXT and mRFP1 genes in OVX rats with or without exogenous oestrogen replacement using in situ hybridisation histochemistry. 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. 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). Thus, we aimed to investigate whether oestrogen could regulate and control hypothalamic OXT dynamics.

**Results**

**Relationship between body weight, fat mass, and feeding.** We observed a significant change in body weight depending on sex, OVX, and oestrogen replacement. Wistar rats were divided into five groups: sham-operated male, sham-operated female, only OVX, OVX + low β-oestradiol (E2) replacement, and OVX + high E2 replacement groups (Exp. A). We observed a significant change in body weight depending on 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 in a dose-dependent manner (Fig. 1a). The difference in body weight was determined by assessing the amount of visceral and subcutaneous fat using micro-CT (Fig. 1b, e, h). There was a difference in fat mass between the 9th (Fig. 1b, c), 14th (Fig. 1e, f), and 16th (Fig. 1h, i) week. We observed a significant change in feeding depending on sex, OVX, and oestrogen replacement (Fig. 1d, g, j).

**OXT-mRFP1 fluorescence differences between female and male rats.** We used adult male and female OXT-mRFP1 Wistar transgenic rats that were maintained under normal laboratory conditions (12-h light, 12-h dark cycle) with free access to food and drinking water. 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 (Exp. B). Female OXT-mRFP1 transgenic rats were further divided based on the four oestrous stages (pro-oestrus, oestrus, metoestrus, and dioestrus stages). Similar to a previous study, we observed the entire hypothalamus [SON, anterior parvocellular PVN (apPVN), dpPVN, and mPVN] in OVX and mPVN transgenic rats using high-power fluorescence microscopy. (Fig. 2a). 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 (Fig. 2b). 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. To further investigate the influence of sex on OXT expression, OVX (Exp. C) was performed 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. B, 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 (Fig. 3a). 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 (Fig. 3b). 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. Considering that oestrogen levels were expected to be affected by OVX, oestrogen supplementation experiments were performed (Exp. D). Oestrogen was supplemented in female OVX-OXT-mRFP1 rats. Among the OVX groups, the rats in the groups with supplementation of low E2 and high E2 elicited significant changes in OXT-mRFP1 levels in the hypothalamus (SON, apPVN, dpPVN, and mPVN) and...
Interestingly, the high-dose E2 group demonstrated higher levels of OXT-mRFP1 fluorescence in the hypothalamus (SON, apPVN, dpPVN, and mPVN) compared to the low-dose group (Fig. 4b). This represents the first evidence that oestrogen regulates OXT expression in hypothalamic OXT neurons in a dose-related manner.

**Correlation between plasma oestrogen and OXT-mRFP1 fluorescence.** Among the OVX groups, the rats who received high and low E2 supplementation demonstrated significant changes in plasma E2 concentration (Fig. 4c). We investigated the correlation between plasma E2 and OXT-mRFP1 fluorescence in the hypothalamus (SON, apPVN, dpPVN, and mPVN) and PP. The results demonstrated that plasma E2 concentration significantly correlated with OXT-mRFP1 fluorescence in the SON, mPVN, and PP. Plasma E2 concentration was positively associated with OXT-mRFP1 fluorescence in the hypothalamus (SON, apPVN, dpPVN, and mPVN) and PP, and it had no significant association with apPVN and dpPVN (Fig. 4c).

**Effects of oestrogen replacement on OXT gene expression.** We performed OVX in 10-week-old female OXT-mRFP1 transgenic rats, conducted hormone replacement (oil only, low E2, and high E2) on week 14, and performed experiments on week 15. We investigated whether oestrogen affects OXT and mRFP1 gene expression in decapitated rats (Exp. E). Among the OVX groups, the rats in the groups with low and high E2 supplementation demonstrated significant changes in plasma E2 and OXT concentration (Fig. 5a). In addition, plasma E2 concentration was significantly and positively correlated with plasma OXT concentration (Fig. 5b). The expression of OXT mRNA in the SON,
dpPVN and mPVN was significantly increased in the rats with supplementation of high E2 compared with rats in the oil only group (Fig. 5c). Regarding OXT mRNA probe binding affinity in the SON, dpPVN, and mPVN, we found a statistical difference in the OVX + oil only and OVX + high E2 groups (Fig. 5d). The high-dose E2 group demonstrated higher levels of OXT mRNA in the dpPVN than the low-dose group (Fig. 5d). We investigated the correlation between plasma E2, plasma OXT, and OXT mRNA levels in the hypothalamus (SON, dpPVN, and mPVN). The results demonstrated that plasma E2 concentration significantly and positively related to OXT mRNA in the dpPVN and mPVN (Fig. 5f). This indicates that oestrogen regulates the hypothalamic OXT gene in a dose-related manner.

**Effects of oestrogen replacement on mRFP1 gene expression.**

The expression of mRFP1 mRNA in the SON, dpPVN and mPVN was significantly increased in the rats with high E2 supplementation than in the rats in the oil-only group (Fig. 6a). There was also a statistically significant difference in mRFP1 mRNA probe binding affinity in the SON, dpPVN, and mPVN between the OVX + oil-only and OVX + high E2 groups (Fig. 6b). The high-dose E2 group demonstrated higher levels of OXT mRNA in the dpPVN than the low-dose group (Fig. 6b). We investigated the correlation between plasma E2, plasma OXT, and changes in mRFP1 mRNA levels in the hypothalamus (SON, dpPVN, and mPVN). The results demonstrated that plasma E2 concentrations were significantly and positively correlated with mRFP1 mRNA in the dpPVN and mPVN. Plasma OXT concentration was also significantly positively related to mRFP1 mRNA in the dpPVN and mPVN (Fig. 6d). These results
indicates that oestrogen regulates the hypothalamic OXT-\textit{mRFP1} gene in a dose-dependent manner.

**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. F). Rats receiving high doses of oestrogen experienced significant weight loss (Fig. 7a). 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 (Fig. 7b). CCK-8 was administered to examine the amount of food consumed. Cumulative food intake was significantly decreased at 0.5 h, 1 h, and 1.5 h after i.p. administration of CCK-8. There was a significant difference between the OVX + oil only and OVX + high E2 groups at 1.5 h after i.p. administration of saline and at 3 h after i.p. administration of CCK-8. After 6 h, there was no significant difference in cumulative food intake among all groups (Fig. 7c).

**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. G). 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 via double-fluorescence immunohistochemistry (FIHC). We quantified immunofluorescently-labelled OXT\textsuperscript{+}/Fos\textsuperscript{+} double-labelled cells in the SON and PVN at 1.5 h after i.p. administration of CCK-8. The number and percentage of OXT\textsuperscript{+}/Fos\textsuperscript{+} cells were significantly higher when CCK-8 was administered than when saline was administered. Among these rats, the number and percentage of OXT\textsuperscript{+}/Fos\textsuperscript{+} cells were significantly higher in the OVX + high E2 group than in the OVX + oil only group (Fig. 7e).

**Effect of pre-treatment with OXTR-A on food intake.** In the previous experiment (Exp. F), there was a significant difference in food intake between the OVX + oil-only and OVX + high E2 groups at 3 h after i.p. administration of CCK-8. Therefore, we assessed food intake for 3 h 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
The present study provides the first evidence that hypothalamic-neurohypophyseal OXT is oestrogen-dependent and shows dynamic changes during the oestrus cycle. The novelty of this study was the success in identifying oestrogen-dependent hypothalamic-pituitary OXT changes by observing the fluorescent intensities of mRFP1 in OXT neurons and their axon terminals in the PP. 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 and OXT-mRFP1 mRNA expression levels in the SON and PVN in OVX rats in a dose-related manner. Thus, the dynamics of hypothalamic OXT expression is regulated by oestrogen. The advantage of this study was the quantitative demonstration of the hypothalamic-pituitary OXT system by examining OXT blood concentrations, correlation coefficient and OXT mRNA expression levels in the SON and PVN. We confirmed that feeding suppression induced by the peripheral administration of CCK-8 resulted in a difference in the activation of OXT neurons, and this was enhanced among oestrogen-replaced OVX female rats. Therefore, we investigated changes in food intake when CCK-8, oestrogen, and OXTR-A administration are combined and proved the relationship between oestroge and OXT.

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 enabled the visualisation of OXT expression and previous studies on OXT-mRFP1 transgenic rats reported different findings. OXT+ neurons in the SON and PVN project their axons to the PP, where OXT is thereby secreted into the systemic circulation and elicits activity peripherally. 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. With respect to the central functions, OXT is also somatodendrical and neurohypophyseal released from neurons in the SON and PVN and acts directly on the brain. Neurons expressing OXT receptors are ubiquitous in the brain and have a wide range of functions. It has been reported that OXT is not only associated with confidence and bond formation but is also strongly associated with autism. 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. This pathway...
Regression analysis for plasma E2 and OXT. The regression line and probability value for the slope are shown. The statistical significance of female OXT-mRFP1 transgenic rats in the OVX surgeries. We speculate that these observations could be reported to be a tenth as severe as that after other open surgeries.

Chronic pain after caesarean section is associated with decreased pain thresholds. Humans have an oestrus cycle of approximately 28 days while females have a shorter cycle of 4–5 days. The blood levels of hormones fluctuate periodically, and these dynamics are common between humans and rats. Oestrogen acts on the mucous membrane epithelium and changes its histology. Notably, it is used to treat menopausal symptoms.

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. Therefore, ovarian dysfunction can elicit various 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 effects may be attenuated by OVX.

There are three known variants of oestrogen—oestrone (E1), oestradiol (E2), and oestril (E3), and three known subtypes of ERs—ERα, ERβ, and G protein-coupled receptor 30. ERs are expressed systemically, and ERα and ERβ are localised in the brain. However, only ERβ has been reported to be expressed in the PVN and SON in the hypothalamus. 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.

Oestrus occurs several hours later. In OXT-mRFP1 transgenic rats, the fluorescence intensity of mRFP1 has been shown to be delayed by several hours after stimulation. It is thought that the fluorescence intensity of mRFP1 increases several hours after the point of the highest pro-oestrous production of oestrogen by the ovary. This, therefore, indicates that fluorescence intensity peaks during the oestrous stage.

Spontaneous oovulator 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 smear examination. Based on this examination, the oestrus cycle in rats can be classified into the pro-oestrus, oestrus, metoestrus, and dioestrus cycles. The highest increase in blood oestrogen levels occurs when the luteinizing hormone (LH) surge coincides with the pro-oestrous stage. Ovulation subsequently occurs several hours later. In OXT-mRFP1 transgenic rats, the fluorescence intensity of mRFP1 has been shown to be delayed by several hours after stimulation. It is thought that the fluorescence intensity of mRFP1 increases several hours after the point of the highest pro-oestrous production of oestrogen by the ovary. This, therefore, indicates that fluorescence intensity peaks during the oestrous stage.

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Oestrogen has further been reported to suppress feeding. Mechanistically, oestrogen has been shown to increase pro-opiomelanocortin gene expression, which induces appetite suppression via the signal transducer and activator of transcription 3 (STAT3). Oestrogen has further been reported to suppress feeding. In OVX female rats, restoration of mRFP1 fluorescence intensity in the SON and PVN (ap, dp, m) in a dose-related manner. Thus, the subcutaneous administration of E2 may increase OXT production in all hypothalamic regions.

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Fig. 6 Effects of oestrogen replacement on mRFP1 gene expression. a Representative images hybridised with a 35S-labelled oligodeoxynucleotide probe complementary to mRFP1 in the SON, dpPVN, and mPVN of female OXT-mRFP1 transgenic rats in the OVX + oil-only (n = 6), OVX + low E2 (n = 6), and OVX + high E2 (n = 6) groups, respectively. The scale bar indicates 100 μm. b mRFP1 mRNA levels in the SON, dpPVN, and mPVN of the rats (OVX + oil-only, OVX + low E2, and OVX + high E2 groups). The data are presented as the mean ± SEM (one-way ANOVA) (**P < 0.01, *P < 0.05, compared with OVX + oil only group). c Regression analysis for plasma E2 and changes in mRFP1 mRNA levels in the SON, dpPVN, and mPVN. The regression line and probability value for the slope are shown. The statistical significance of the slope was set at P < 0.05. d Regression analysis for plasma OXT and changes in mRFP1 mRNA levels in the SON, dpPVN, and mPVN. The regression line and probability value for the slope are shown. The statistical significance of the slope was set at P < 0.05.

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Oestrogen and OXT share a common antifeedant activity. Food consumption was measured following the administration of CCK-8, which is known to selectively activate OXT neurons. We demonstrated the link between OXT and oestrogen by counting Fos+ neurons. Fos is an early expression progenitor gene expressed at low levels in most cell types and can be activated by various second messenger signals. However, Fos is an indicator of cell activation. 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 neurons in the hypothalamus through the solitary tract nucleus to increase blood OXT concentrations and suppress appetite. 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+/Fos+ neurons. These results suggest that oestrogen administration may enhance hypothalamic OXT production clinically.

Various studies have demonstrated the efficiency of oestrogen and OXT as anti-obesity peptides. Intracerebroventricular or peripheral (i.p. and subcutaneous) injection of OXT decreases food intake, body weight, and fat mass in rats and mice. In the present study, 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. In this experiment, oestrogen was associated with fat composition. However, it is unclear whether OXT was directly involved.

Oestrogen, OXT and CCK-8 decreases food intake. CCK-8 acts on OXT neurons in the hypothalamus to decreases appetite. Oestrogen, OXT, and CCK-8 have been known to suppress feeding, but no studies combining them were conducted. In this study, the addition of oestrogen to CCK-8 resulted in a more marked decrease in food intake and activation of OXT neurons. Oestrogen was also found to regulate OXT neurons in transgenic rats via in situ experiments. The oestrogen receptor ERβ is localised in the PVN and SON of the hypothalamus. Therefore, oestrogen may act on these hypothalamic areas via ERβ to activate OXT neurons.

To the best of our knowledge, this is the first report demonstrating a difference in the dynamics of hypothalamic OXT in rats based on the sex. 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. First, we did not assess any associations among hypothalamic OXT, the OXT
receptor, and sex hormones other than oestrogen (e.g., progesterone). Second, all hypothalamic examinations were conducted in rats and not clinically in humans. Finally, no adverse effects of oestrogen replacement were considered.

Methods

Animals. All rats were treated after 10 weeks of age once the oestrous 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 under normal laboratory conditions (12-h light, 12-h dark cycle) with free access to food and drinking water. The OXT-mRFP1 transgenic rats were created by inserting the mRFP1 gene into the OXT gene. This reporter strain facilitates the visualisation of OXT dynamics in the hypothalamus through the quantification of OXT fluorescence intensity changes under various stimulation loads. All rats were genotypically screened through polymerase chain reaction analysis of their genomic DNA extracted via ear biopsies. Adult female Wistar rats (aged 15–16 weeks and weighing 271–337 g) were used in experiment 4 (below). All rats were housed as one or three per plastic cage (transparent polymethylpentene; TR-TPX-200A, Tokiwa Kagaku Kikai, Tokyo, Japan) in an air-conditioned room (22°C, 50% relative humidity; 12-h light cycle (7:00 A.M. to 7:00 P.M.) and ad libitum access to food (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 the 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 anaesthetised 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 anesthetised (i.p. injection of a cocktail of three different anaesthetic agents). For i.c.v. administration, animals were implanted with stainless steel canulae targeting the lateral ventricle. They were anaesthetised (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 μm 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. Two stainless steel anchoring screws and acrylic dental cement were used to secure the
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.** 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 μg/ml (OXTR-A (L-368899, Tocris Bioscience, Bristol, UK) was dissolved in 0.9% sterile physiological saline (150 ng/μl).

**Micro-CT.** Rats were anesthetised 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) before undergoing micro-CT scanning. Body fat images were acquired by a micro-CT system (CosmoScan GX; Rigaku, Tokyo, Japan) with a resolution of 148 × 148 × 148 μm^3 (90 kVp, 88 μA, 555.33 ms integration time). The CT images of body fat were visualised using Analyze 12.0 software (AnalyzeDirect, Inc., KS, USA). The total fat volume in the body was measured from the base of the eniform cartilage to the pelvic floor, and the fat volume was further distinguished into visceral and subcutaneous fat.

**Experimental procedure**

Measurement of body weight, fat mass, and feeding. We first aimed to ascertain the effects of sex and oestrogen on body weight, fat mass, and feeding. In the first experiment (Exp. A, n = 30), we assessed body weight in male and female Wistar rats. Wistar rats were divided into five groups (n = 6 in each group): sham-operated male, sham-operated female, only OVX, OVX + low E2 (20 μg β-oestradiol/ml sesame oil) replacement groups. We performed sham operations and OVX in 10-week-old male and female rats, and conducted hormone replacement (tube implantation) with OVX rats at week 14. Micro-CT was performed at 9, 14, and 16 weeks of age to measure the ratio of visceral and subcutaneous fat to internal organs.

Assessing differences in OXT-mRFP1 fluorescence in male and female transgenic rats. We 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 [second experiment (Exp. B), n = 30]. Female OXT-mRFP1 transgenic rats were further divided based on the four oestrus stages (pro-oestrus, oestrus, metoestrus, and dioestrus stages). In total, we assessed five groups (one male, four females; n = 5 9 per group). The stage of the oestrus cycle was confirmed by examining vaginal smears collected every morning from the rats by two researchers. Briefly, the pro-oestrus stage is identified mainly by nucleated cells, oestrus stage by all keratinocyte cells, metoestrus stage by the presence of many white blood cells, and dioestrus stage by the presence of few white blood cells and other cells. Rats with irregular oestrous cycles were excluded from the experiment.

Assessing the effects of OXV on OXT-mRFP1 fluorescence. For the third experiment (Exp. C, n = 33), all OXT-mRFP1 transgenic rats were divided into six groups, including five sham-operated groups (comprising both males and females at all oestrous cycle stages) and one OVX group (n = 5 6 per group). We confirmed the oestrogen-deficient state (e.g., dioestrus stage) according to the vaginal smears of OVX rats.
administration of saline (sterile 0.9% saline), and high E2 with i.p. administration of CCK-8 and i.c.v. administration of OXTR-A. Ovariectomies were performed at 10 weeks of age, and the rats were treated with either saline or CCK-8 for 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. Anesthetised rats were perfused transcardially with 0.1 M phosphate buffer (pH 7.4) containing heparin (1000 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 prepared by adding 4% paraformaldehyde to 0.1 M PB (pH 7.4) containing heparin (1000 U/L). Tissue was cryoprotected in 20% (w/v) sucrose in 0.1 M PB for 48 h at 4 °C. Fixed tissue was cut coronally, and sections were thawed on a stage at 30 mm using a rotator (REM-700, Yamato Kohki Industrial Co. Ltd., Saitama, Japan). The sections were divided into three groups, so that approximately the same brain region was included. The first group of 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 Watson65. The ap, dp, and m divisions of the PVN were divided and quantified. Sections containing the SON, PVN, and intact pituitary gland tissues were 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.). Using a light source of μ paraformaldehyde in 0.1 M PB for 48 h at 4 °C prepared by adding 4% paraformaldehyde to 0.1 M PB (pH 7.4) containing heparin (1000 U/L). Tissue was cryoprotected in 20% (w/v) sucrose in 0.1 M PB for 48 h at 4 °C. Fixed tissue was cut coronally, and sections were thawed on a stage at 30 mm using a rotator (REM-700, Yamato Kohki Industrial Co. Ltd., Saitama, Japan). The sections were divided into three groups, so that approximately the same brain region was included. The first group of sections were rinsed with 0.1 M PB and placed on glass slides. Pituitary glands were not treated.

In situ hybridisation histochemistry. To measure changes in the expression of the mRFP1 and OXT genes in the PVN and SON via in situ hybridisation histochemistry, transgenic rats were sacrificed by decapitation after hormone replacement in the state of O VX. We performed in situ hybridisation based on previous reports61,62. Brain tissue was frozen immediately and carefully using crushed dry ice after decapitation. The tissues were cut with a microtome to a thickness of 12 μm to create sections for observation via in situ hybridisation. As mentioned above, the nucleus of interest was identified by referring to the rat atlas60. To select the sections that corresponded to those in the atlas, they were checked with dark-field microscopy. Two sections containing the SON and PVN were used from each rat to determine the autoradiography density of the autoradiographs. 35S-labelled oligo-odeoxynucleotide probe and protocol for in situ hybridisation histochemistry (ISH) in this experiment have been used many times before with good reliability. The details of the probe sequence and protocol for in situ hybridisation have been completed. The digoxigenin-labelled OXT and mRFP1 (OXT probe sequence, 5′-CTC GGA GAA GGC AGC TTA AGG TGC GCA GGA-3′; mRFP1 probe sequence, 5′-GGG CTG TAC TGT TGC ATG GTG TAC TGC TGG TCT-3′). The probe was 3′-end labelled by terminal deoxynucleotidyl transferase and [35S] deoxy-ATP61,64. The hybridised sections were exposed to autoradiography films (Amersham Hyperfilm, Buckinghamshire, UK) for 3 days (mRFP1) and 6 h (OXT), respectively. The gene expression in the obtained image was analysed semiquantitatively using ImageJ software (National Institutes of Health, Baltimore, MD, USA). Using a cryostat (OFT5000, Bright Instrument Co Ltd., England), brains were sliced into 12-μm-thick coronal sections at −20 °C. The sections were thawed after mounting them on gelatin/chrome alum-coated slides. The PVN and arcuate nuclei regions were determined by referring to the coordinates provided in the rat brain atlas.

Fos and OXT double-FIHC. 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 anti-c-Fos, Santa Cruz Biotechnology, TX, USA; 1:500 or rabbit anti-OXT, Sigma-Aldrich, MO, USA; 1:5000)65. 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 488 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)60,61. Sections were washed twice in PBS and then mounted on the slides and overspalled using vetchesheet (Vector Laboratories Co. Ltd., CA, USA)65. Images of Fos+, OXT+, and Fos+/OXT+ double-labelled cells were counted manually by two researchers who were blinded to avoid bias. The number and percentage of Fos+, OXT+, and Fos+/OXT+ cells in the SON and PVN were estimated.

Measurement of plasma OXT and oestrogen concentration. Plasma OXT levels were measured in rat blood samples taken upon decapitation by a radio-immunoassay (RIA) method using specific anti-OXT antisera66. The intra- and inter-assay coefficients of variation for measuring plasma OXT levels were 4 and 10%, respectively.

Statistical and reproducibility. All data points are presented as the mean ± standard error of the mean. P values were calculated by using two-tailed Student’s t test for pair wise comparisons. An unpaired t-test was used to detect differences between male and female rats. Statistical significances were calculated based on one-way and two-way analysis of variance (ANOVA) as well as repeated-measures ANOVA, using a Tukey-Kramer-type adjustment for multiple comparisons. Correlation analyses were performed using GraphPad Prism 9. A P-value < 0.05 was considered statistically significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The source data are provided with this paper (Supplementary Data 1). Any further requests can be directed to the corresponding author.

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Competing interests
The authors declare no competing interests.

Additional information
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