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Estrogen modulates central and peripheral responses to cold in female rats

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Abstract The aim of this study was to determine whether estrogen modulates central and peripheral responses to cold in female rats. In ovariectomized female rats with and without administered estrogen [E_2 (+) and E_2 (−), respectively], the counts of cFos-immunoreactive cells in the medial preoptic nucleus (MPO) and dorsomedial hypothalamic nucleus (DMH) in the hypothalamus were greater in the E_2 (+) rats than in the E_2 (−) rats at 5°C. Examination of the response of normal female rats to exposure to 5°C at different phases of the estrus cycle revealed that counts of cFos-immunoreactive cells in the MPO, DMH, and posterior hypothalamus and the level of uncoupling protein 1 mRNA in the brown adipose tissues were greater in the proestrus phase than on day 1 of the diestrus phase. This result was linked to the level of plasma estrogen. The body temperature during cold exposure was higher in the E_2 (+) rats than in the E_2 (−) rats and was also higher in the proestrus phase than on day 1 of the diestrus phase. We conclude that estrogen may affect central and peripheral responses involved in thermoregulation in the cold.

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Introduction

Female sex hormones (i.e., estrogen and progesterone) are closely associated with sexual characteristics, behavior, and the estrus cycle. Progesterone also has a febrile action [1], which may be associated with a change in body temperature during the estrus cycle. Several studies also indicate a possible involvement of estrogen in thermoregulation: peri- and postmenopausal women suffer from hot flush, i.e., the sudden onset of face flushing, sweating, and hotness [2], a disturbance in circadian body temperature change [3], and a greater sensitivity to cold exposure [4]. Estrogen replacement is sometimes used as a treatment for hot flush [5]. A number of animal studies using ovariectomized rats with and without estrogen replacement have shown that estrogen has an effect on both autonomic and behavioral thermoregulation [6–8]. In contrast, Freedman et al. found that the plasma level of estradiol was not different between postmenopausal women with and without hot flush [9], suggesting a lesser involvement of circulating estrogen in this condition. In another study, estrogen administration was found to have no influence on circadian body temperature rhythm in female rodents [10]. Consequently, it remains unclear if estrogen has an influence on thermoregulatory responses. Further, if it does affect thermoregulatory responses, the mechanism by which it does so is as yet unknown.

The hypothalamus plays an important role in autonomic functions, including thermoregulation. The preoptic area (PO) of the hypothalamus in particular contains many thermosensitive neurons that can show altered neuronal activity in response to the local brain temperature [11]. The PO also contains estrogen-sensitive neurons that are implicated in reproductive behaviors and endocrine responses. In an in vivo slice study, some thermosensitive

neurons in the PO were observed to alter their neural activity in medium supplemented with estrogen [12]. Thus, estrogen may affect thermosensitivity at the level of the hypothalamus. The interscapular brown adipose tissue (iBAT) is a thermoeffector organ involved in non-shivering thermogenesis [13]. The iBAT has been shown to have estrogen-specific macromolecular binding in the cytoplasmic fraction [14], which may also indicate a direct influence of estrogen on iBAT thermogenesis. However, selective assessment of thermogenesis in the iBAT would be difficult in free-moving animals.

The aim of the study reported here was to determine whether estrogen has an influence on central and peripheral responses to cold exposure. These responses are involved in thermoregulation. We first tested the hypothesis that, in ovariectomized rats, systemic administration of estrogen would change the hypothalamic and molecular responses in the iBAT during cold exposure. If the first hypothesis were found to be valid, the second hypothesis was that these responses in normal female rats change in direct correspondence with changes in circulating estrogen level during the estrus cycle.

Methods

Ninety-one adult virgin female Wister rats (body weight 170–270 g, age 9 weeks; Takasugi Experimental Animals Supply, Saitama, Japan) were used in the study. The rats were housed in a plastic cage ($45 \times 25 \times 20$ cm) at an ambient temperature (T_a) of 25°C under a 12/12-h (light/dark) cycle (artificial lighting on at 0700 hours) and had free access to food and water. All procedures were approved by the "Guiding principles for the care and use of animals in the field of physiological sciences" and the Institutional Animal Care and Use Committee, Waseda University.

Experiment 1: effect of 17β-estradiol on thermoregulation in female ovariectomized rats exposed to cold

Rats ($n = 55$) underwent surgery under anesthesia with diethyl ether (Sigma-Aldrich, Tokyo, Japan). A radio transmitter ($15 \times 30 \times 8$ mm; PhysioTel, TA10TA-F40; Data Science, St. Paul, MN) for body core temperature (T_b) measurement was implanted in the peritoneal cavity through a median skin incision. Bilateral ovariectomy was performed through the dorsal skin incision. Penicillin G (1,000 U; Meiji Pharmaceutical, Tokyo, Japan) was injected subcutaneously to prevent post-surgical infection.

At least 7 days after the first surgery, two silastic tubes (inner diameter 1.57 mm, outer diameter 3.18 mm, length 30 mm; Kaneka, Osaka, Japan) containing either 17β-estradiol (E$_2$; Sigma, St. Louis, MO; $n = 26$) or a blank (control; $n = 29$) were placed underneath the right dorsal skin under ether anesthesia. The tubes were prepared, filled with 50–60 mg E$_2$, and sealed with silicone glue as reported previously [15]; this procedure maintained a constant level of E$_2$ in the plasma. At least 7 days after the second surgery, we placed a rat in a plastic cage ($45 \times 25 \times 20$ cm) in a climate chamber (Program Incubator IN602W; Yamato Scientific, Tokyo, Japan) maintained at 25°C, where the T_b was continuously monitored by telemetry. T_b signals from the radio transmitter were obtained through a receiver board (model CTR86; DataScience) at 1-min intervals and stored in a personal computer using a data-logging program (LabView; National Instruments, Austin, TX). The accuracy of the value of T_b was ± 0.1°C. At 1200 hours on the next day, the chamber was set at 5°C for 2 h, or it remained at 25°C as the control. We selected this time of a day because, in a normal environment, T_b is stable (less influence of circadian fluctuation) from 0900 to 1500 hours [16], especially in the middle of the time window (1200–1400 hours; observations from a preliminary study). Following the 2-h cold exposure, the rat was killed with an intraperitoneal injection of overdose pentobarbital Na$^+$ (50 mg/100 g body weight; Somnopentil; Kyoritsu Seiyaku, Tokyo, Japan). The iBAT was removed for the later determination of the level of uncoupling protein 1 (UCP1) mRNA, and a 2-ml blood sample was taken from the left ventricular cavity to determine plasma levels of the sex hormones. The sacrificed rat was then perfused transcardially with 20 ml of normal saline, followed by 300 ml of 4% paraformaldehyde (4°C, cold), and the whole brain was excised for immunohistochemistal analysis.

Experiment 2: changes in thermoregulation in normal female rats exposed to cold during the estrus cycle

For this experiment, we used normal female rats with a regular estrus cycle ($n = 36$) for this experiment. These rats had surgery to implant the T_b radio transmitter, as carried out in Experiment 1. To determine the estrus cycle for each rat, a vaginal smear was taken every morning for at least 10 days [17]. Briefly, vaginal epithelium was obtained with a thin moist cotton swab, and a smear was made on a slide glass and stained by Giemsa's method. Based on the smear image, the estrus phase (i.e., the first day of diestrus, the second day of diestrus, proestrus, or estrus phases) was determined. A rat either in the estrus phase or at the second day of diestrus was selected and moved into the climate chamber as in Experiment 1. At 0730 hours on the following day, a vaginal smear was again made to verify that the phase was either proestrus

(P, $n = 17$) or the first day of diestrus (D$_1$, $n = 19$). These two phases were selected because the circulating level of estrogen is the highest in the P phase and the lowest in the D$_1$ phase [17]. The chamber was set at 5°C or remained at 25°C from 0900 to 1100 hours. In the P phase, plasma progesterone starts increasing around 1300 hours due to a surge in luteinizing hormone, with a peak around 1900 hours [17]. We therefore selected a different time window in Experiment 2 (0900–1100 hours) to minimize the influence of plasma progesterone on thermoregulation. In ovariectomized rats in Experiment 1, the difference in T_b between 0900–1100 and 1200–1400 hours was less than 0.2°C. Thus, we assumed that the influence of the difference in the time window on T_b was minimal. Following the cold or control exposure, the rat was killed in the same manner as Experiment 1.

Handlings and analyses of the blood, brain, and iBAT samples

The blood was centrifuged at 4°C and the plasma stored at −80°C until assay. The plasma levels of estradiol and progesterone were determined by an enzyme-linked immumosorbent assay (ELISA) in duplicate (Estradiol EIA kit and Progesterone EIA kit; Cayman Chemical, Ann Arbor, MI).

The brain was first soaked in 4% paraformaldehyde at 4°C overnight and then in 25% sucrose in phosphate buffered saline (PBS) for another 48 h. The brain was frozen in crushed dry ice, and coronal sections (40-μm thickness) were prepared on a cryostat (CM1510S; Leica, Wilzlar, Germany). The sections were rinsed with PBS and incubated sequentially in: (1) 0.3% hydrogen peroxide in PBS with 0.3% Triton X-100 for 30 min; (2) rabbit primary anti-cFos polyclonal IgG (1:15,000 dilution; Calbiochem, Merck, Tokyo, Japan) for 12 h; (3) biotinylated donkey anti-rabbit IgG (1:1,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA) for 90 min; (4) avidin–biotin complex (1:1,000 dilution; Vectastain Elite ABC standard kit; Vector Laboratories, Burlingame, CA) for another 90 min. After rinsing, the sections were reacted with 5% diaminobenzidinetetrahydrochloride (Sigma) in PBS and mounted on gelatin-coated glass slides. Each slide was covered with a glass microcoverslip. Digital images of the dorsomedial hypothalamic nucleus (DMH), medial preoptic nucleus (MPO), median preoptic nucleus (MnPO), ventromedial hypothalamus (VMH), and posterior hypothalamus (PH) were captured with a CCD camera system (Digital Camera HC 2500 3CCD; FUJIFILM, Tokyo, Japan) mounted on a microscope (ECLIPSE E600; Nikon, Tokyo, Japan), which was controlled by imaging software (Image-Pro Plus; MediaCybernetics, Bethesda, MD). The images were saved in TIFF format, and cFos-immunoreactive cells

(cFos-IR cells) in three consecutive sections were manually dotted with image software (Adobe Photoshop; Adobe System, San Jose, CA). The counts were determined using image analyzing software (Image J; NIH, Bethesda, MD).

The iBAT was immersed in RNA-stabilization reagent (RNA Later; QIAGEN, Tokyo, Japan) at 4°C for 12 h and stored at −80°C until assay. Total RNA was extracted from the iBAT using the RNeasy Lipid Tissues Mini kit (QIA-GEN). Briefly, the frozen tissue samples were thawed at room temperature and homogenized in lysis reagent (a monophasic solution of phenol and guanidine thiocyanate). After adding chloroform, the homogenate was separated into aqueous and organic phases by centrifugation. The RNA-containing aqueous phase was extracted, and ethanol was added to provide appropriate binding conditions. The sample was then applied onto a silica membrane. After several washes, RNA was eluted in RNase-free water (Ultra Pure Water; Invitrogen, Tokyo, Japan). The purity and concentration of the total RNA were determined by spectrophotometry at an absorbance of 260 and 280 nm (NanoDrop ND-1000 spectrophotometer; Thermo Scientific, Wilmington, DE). The extracted RNA was then subjected to first-stand cDNA synthesis using the PrimeScript RT reagent kit (TAKARABIO, Otsu, Japan). The final volume was adjusted to 50 μl with EASY Dilution (TAKARABIO, Otsu, Japan) in accordance with the manufacturer's instructions. In quantitative reverse transcription (RT)-PCR, diluted cDNA was added to a SYBER Premix Ex Taq reaction mixture (TAKARABIO, Otsu, Japan) containing 200 nM PCR (forward and reverse) primers. The oligonucleotide sequences for the primers were:

UCP1: 5′-TACCCAGCTGTGCAATGACCA (forward), 5′-GCACACAAACATGATGACGTTCC (reverse); Gapd: 5′-GGCACAGTCAAGGCTGAGAATG (forward), 5′-ATGGTGGTGAAGACGCCAGTA (reverse).

Amplification was performed using an ABI-Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The PCR amplification program consisted of an initial denaturation step for 10 s at 95°C, followed by the Shuttle PCR standard protocol and dissociation protocol. UCP1 mRNA levels were determined using the Threshold Cycle method in accordance with the manufacturer's protocol. To standardize the level of UCP1 mRNA, glyceraldehyde-3-phosphate dehydrogenase (Gapd) mRNA, one of the housekeeping genes, was used for the reference in the same way as in a previous study in which the UCP1 mRNA level during cold exposure was assessed [18].

Statistical analysis

All values are shown as the mean ± standard error (SE). Values for T_b were averaged every 5 min. Differences

between E_2 (+) and E_2 (−) groups or those between the P and D_1 groups were assessed by two-way analysis of variance (ANOVA; StatView; SAS, Cary, NC). A change in T_b from the 30-min averaged value before the 5° or 25°C exposure (baseline value) was also evaluated. A post hoc test to identify any significant difference at a specific time point was performed using the Tukey–Kramer method [19]. The null hypothesis was rejected at the level of $P < 0.05$.

Results

Body core temperature

Figure 1 shows changes in the T_b during the exposure to 25° (Fig. 1A, C) and 5°C (Fig. 1B, D) in Experiments 1 and 2. In Experiment 1, the T_b during the 25°C exposure remained unchanged in the E_2 (+) and E_2 (−) groups, and there was no difference between the two groups (Fig. 1A; mean temperature 37.4 ± 0.1 and 37.5 ± 0.2°C, respectively). The T_b in the E_2 (−) group was lower ($P < 0.05$) than that in the E_2 (+) group following 35–120 min of exposure to 5°C (Fig. 1B; 37.1 ± 0.1 and 36.7 ± 0.1°C at 115 min, respectively). In addition, the T_b in both groups decreased ($P < 0.05$) from the baseline value [at 40–120 and 10–120 min in the E_2 (+) and E_2 (−) groups, respectively]. T_b remained stable in the P and D_1 groups during the 25°C exposure and was not different between the two groups in Experiment 2 (Fig. 1C; mean temperature 37.2 ± 0.1 and 37.3 ± 0.1°C, respectively). However, during the 5°C exposure, the T_b in the P group was greater ($P < 0.05$) than that in the D_1 group at 80–95 min (Fig. 1D; 37.3 ± 0.1 and 36.9 ± 0.1°C at 90 min, respectively). During the cold exposure, T_b in the P group was at the level of the baseline (mean 37.4 ± 0.2°C); however, that in the D_1 group was lower than the baseline level at 90–120 min (mean $P < 0.05$, 36.8 ± 0.2°C).

Plasma levels of estradiol and progesterone

Figure 2 shows the plasma levels of estradiol (Fig. 2A, C) and progesterone (Fig. 2B, D) in Experiments 1 and 2. In Experiment 1, the plasma estradiol level was higher in the E_2 (+) group than in the E_2 (−) group ($P < 0.05$; 250 ± 30 and 30 ± 3 pg/ml, respectively; Fig. 2A) without any significant difference in the plasma progesterone level (2.5 ± 0.1 and 2.3 ± 0.2 ng/ml, respectively; Fig. 2B). The estradiol level was higher in the P group than in the D_1 group in Experiment 2 ($P < 0.05$; 100 ± 13 and 55 ± 5 pg/ml, respectively; Fig. 2C), but the progesterone level was not different between the two groups (4.6 ± 0.1 and 4.5 ± 0.1 ng/ml, respectively; Fig. 2D). Compared

with the data in Experiment 1, the estradiol level was greater ($P < 0.05$) in the D_1 group than in the E_2 (−) group, and lower ($P < 0.05$) in the P group than in the E_2 (+) group. The progesterone level was greater ($P < 0.05$) in the D_1 group than in the E_2 (−) group and also greater ($P < 0.05$) in the P group than in the E_2 (+) group.

cFos-IR cells in the hypothalamic areas

Figure 3 shows the immunohistochemical images of cFos during exposure to 5°C in Experiments 1 and 2 (Fig. 3A, B, respectively). The images are obtained from the sites showing a difference in cFos expression in each experiment. The counts of cFos-IR cells in the hypothalamic areas are summarized in Fig. 4. In both experiments, the number of cFos-IR cells increased with cold exposure at 5°C in the DMH, MPO, MnPO, VMH, and PH. In Experiment 1, the counts of cFos IR cells following exposure to 25°C were low throughout the hypothalamic areas in both the E_2 (+) and E_2 (−) groups (Fig. 4A). After the 5°C exposure, the counts in the MPO (Fig. 3A: a, b) and DMH (Fig. 3A: c, d) were higher in the E_2 (+) group than in the E_2 (−) group; however, there were no differences in counts between the MnPO, VMH, and PH (Fig. 4B). The counts of cFos-IR cells in the hypothalamic areas were not different between the P and D_1 groups following exposure to 25°C in Experiment 2 (Fig. 4C). Although the counts in the MnPO and VMH were not different between the two groups after exposure to 5°C, those in the MPO (Fig. 3B: a, b), DMH (Fig. 3B: c, d), and PH (Fig. 3B: e, f) were greater in the P group than in the D_1 group ($P < 0.05$, Fig. 4D). We also assessed the localization of cFos-IR cells in the two hypothalamic areas. The cells were mainly located in the middle and caudal parts of the MPO and all the areas in the DMH. The pattern of the cell location in each area was not different between the E_2 (+) and E_2 (−) groups or the P and D_1 groups.

The UCP1 mRNA level in the interscapular brown adipose tissue

Figure 5 shows the UCP1 mRNA level in the iBAT in Experiments 1 and 2. In Experiment 1, the UCP1 mRNA level was not different between the E_2 (+) and E_2 (−) groups after exposure to 25°C (Fig. 5A). In contrast, the level after the 5°C exposure in the E_2 (+) group was greater ($P < 0.05$) than that in the E_2 (−) group; however, there were no statistical differences in the UCP1 mRNA level between the 5 and 25°C exposures in both groups. The UCP1 mRNA level was not different between the P and D_1 groups at 25°C in Experiment 2, although at 5°C, the level was greater in the P group than in the D_1 group (Fig. 5B). There was no difference in the level in the D_1

Fig. 1 Changes in body core temperature (T_b) at 25°C (**A, C**), and 5°C (**B, D**) in Experiments 1 and 2, respectively. T_b is shown as the 5-min average during a 2-h exposure at 5 or 25°C after a 2-h baseline at 25°C. Values are given as the mean ± standard error (SE) [**A** $n = 25$ (11 in the E_2 (+) group and 14 in the E_2 (−) group), **B** $n = 30$ (15 in the E_2 (+) group and 15 in the E_2 (−) group), **C** $n = 16$ (7 in the P group and 9 in the D_1 group), **D**, $n = 20$ (10 in the P group and 10 in the D_1 group)]. *Significant difference between the E_2 (+) and E_2 (−) groups or between the P and D_1 groups at $P < 0.05$. #Significant difference from the baseline value, $P < 0.05$. E_2 17β-estradiol, P proestrus, D_1 first day of diestrus
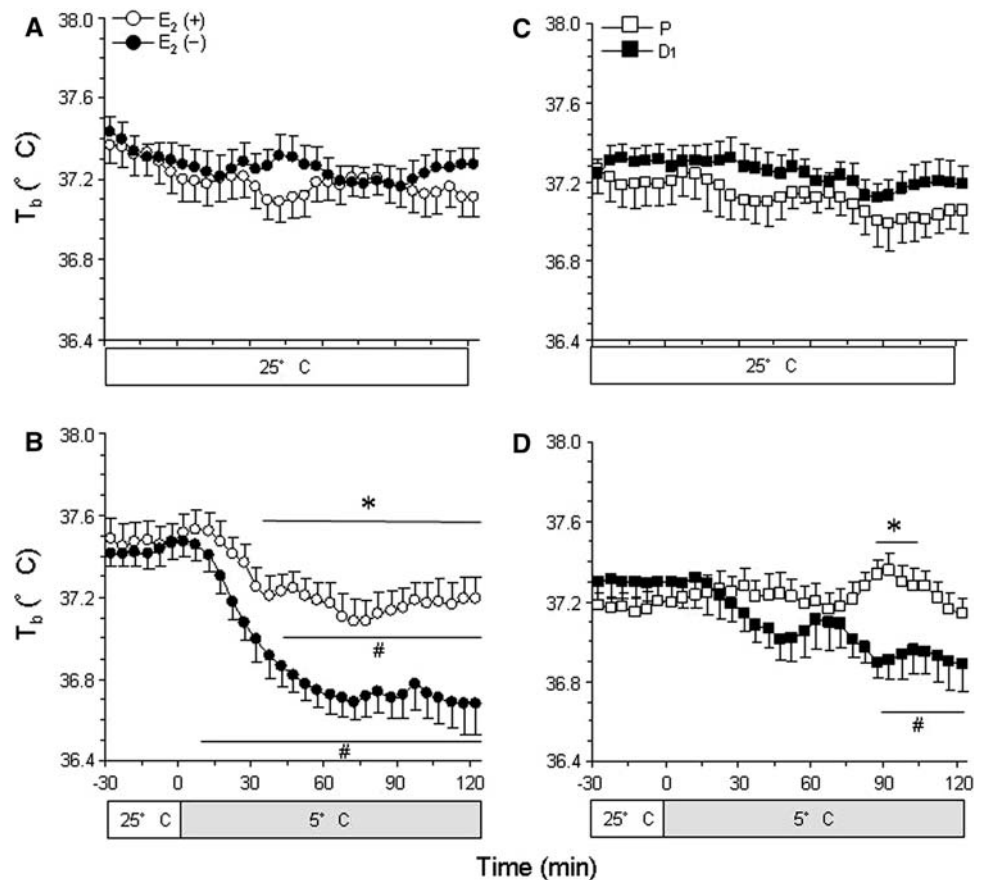
group between the 5 and 25°C exposures; however, the level was greater ($P < 0.05$) after 5°C exposure in the P group. After 25°C exposure, the UCP1 mRNA level was not different between the E_2 (+) and P groups or the E_2 (−) and D_1 groups. The level in the P group was lower than that in the E_2 (+) group following the 5°C exposure ($P < 0.05$; Fig. 5A, B).

Discussion

The aim of our study was to evaluate the effect of estrogen on the central and peripheral responses which are known to be involved in metabolic thermoregulation during cold exposure. Analyses of cFos expression in the hypothalamus and UCP1 mRNA level in the iBAT indicate that the central and peripheral tissues or organs may show different responses to the cold and that these may be linked with circulating estrogen level.
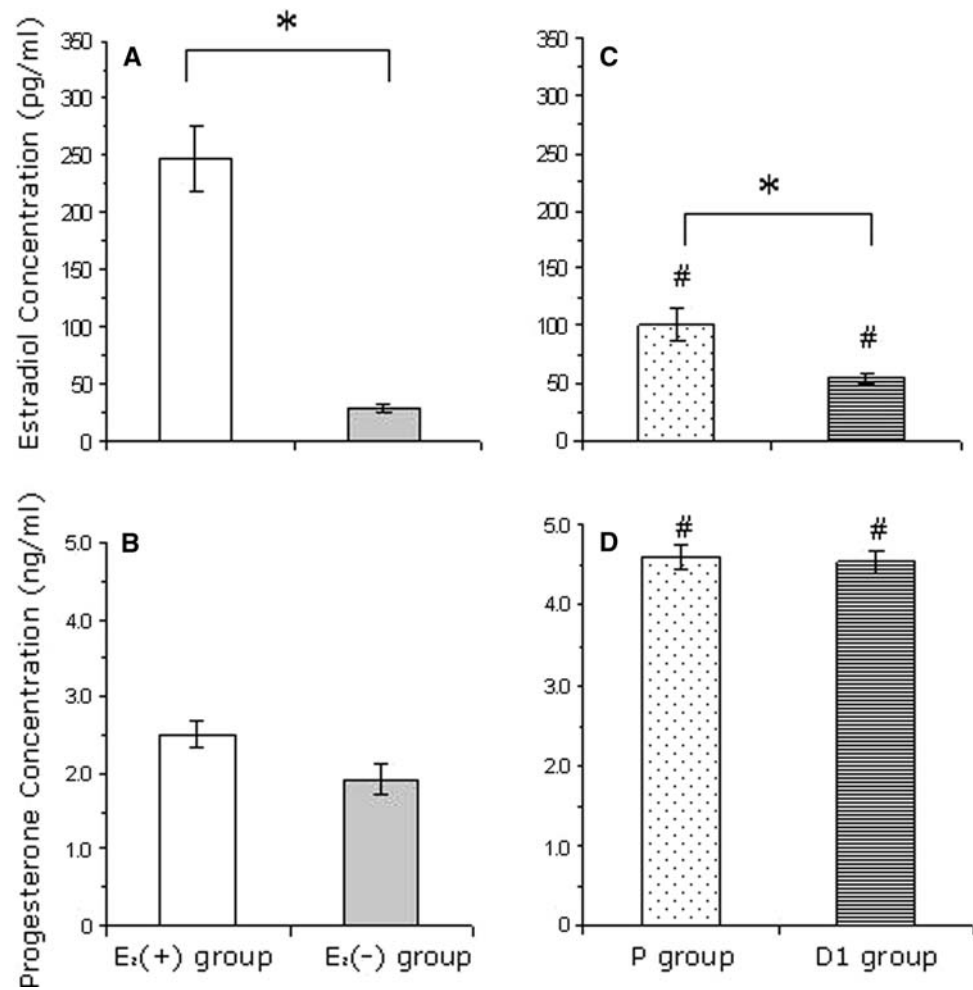
Experiment 1

T_b decreased at 5°C in both ovariectomized rats with and without E_2, although the reduction in the E_2 (+) group was smaller. However, a similar reduction in T_b was not

observed at 5°C in male rats [20, 21]. Hosono et al. [8] reported that the T_b during a 3-h cold exposure at 4°C was higher in ovariectomized rats with E_2 than in those without E_2. In addition, the T_b in ovariectomized rats with E_2 became higher than the baseline level in association with metabolic heat production. In addition to differences in sex and/or the length of cold exposure, differences in progesterone level may have also affected T_b during the cold exposure because progesterone has a pyrogenic action [1]. In our study, the progesterone level in the ovariectomized rats was half that found in the normal rats, which may have resulted in the decreased T_b. However, the progesterone level was not determined in the study of Hosono et al. [8].

As previously reported, we also found that estradiol had no influence on T_b at 25°C [7]. However, Fregly et al. found that T_b became lower following ethinyl estradiol treatment [6]. The experimental conditions were different between our study and that of Fregly et al. [6] in that in the latter the rats were restrained and T_b was monitored with a thermocouple in the rectum. It has been reported that restraint itself induces a decrease in T_b [22]. It is possible that estrogen may have an effect on the restraint-induced hypothermia, but it is not known whether estrogen affects factors associated with thermoregulation during restraint, such as stress and autonomic responses. Another possible

Fig. 2 Plasma levels of estradiol (**A**, **C**) and progesterone (**B**, **D**) in Experiments 1 and 2, respectively. Values are given as the mean ± SE [**A** $n = 17$ (9 in the E_2 (+) group and 8 in the E_2 (−) group), **B** $n = 13$ (6 in the E_2 (+) group and 7 in the E_2 (−) group), **C** $n = 36$ (17 in the P group and 19 in the D_1 group), **D** $n = 36$ (17 in the P group and 19 in the D_1 group)]. *Significant difference between the E_2 (+) and E_2 (−) groups, or the P and D_1 groups, $P < 0.05$. #Significant difference between the E_2 (+) and P groups or between the E_2 (−) and D_1 groups for circulating levels of estradiol or progesterone, $P < 0.05$

Fig. 3 Photo images of brain sections. Coronal sections, including the MPO (**A** a, b) and DMH (**A** c, d) in Experiment 1, and the MPO (**B** a, b), DMH (**B** c, d), and PH (**B** e, f) in Experiment 2. The rats were exposed to 5°C for 2 h. Cells stained in *dark* denote cFos-immunoreactive cells. *MPO* Medial preoptic nucleus, *DMH* dorsomedial hypothalamic nucleus, *PH* posterior hypothalamus. The drawings are modified from the stereotaxic atlas [40]
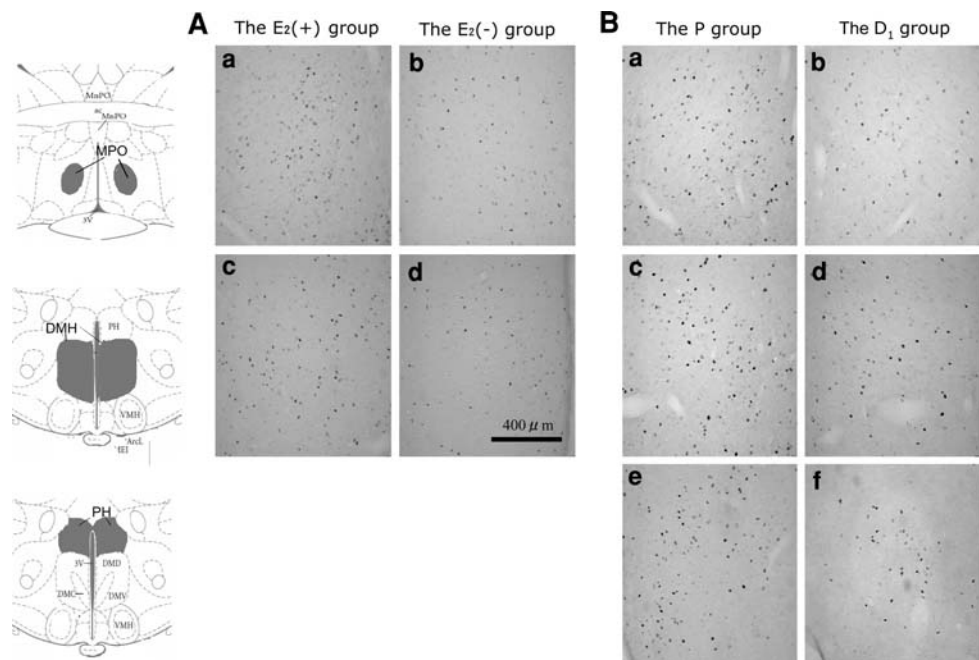
Fig. 4 Counts of c-Fos-immunoreactive cells in the hypothalamic areas. Counts of cFos-IR cells in the hypothalamic areas after 25°C exposure in Experiment 1 (**A**), 5°C exposure in Experiment 1 (**B**), 25°C exposure in Experiment 2 (**C**),and 5°C exposure in Experiment 2 (**D**). Values are given as the mean $\pm$ SE [**A** $n = 7$ (4 in the E_2 (+) group and 3 in the E_2 (−) group), **B** $n = 25$ (15 in the E_2 (+) group and 10 in the E_2 (−) group), **C** $n = 16$ (7 in the P group and 9 in the D_1 group), **D** $n = 20$ (10 in the P group and 10 in the D_1 group)]. *Significant difference between the E_2 (+) and E_2 (−) groups or the P and D_1 groups, $P < 0.05$. *MnPO* Median preoptic nucleus, *VMH* ventromedial hypothalamus, T_a ambient temperature
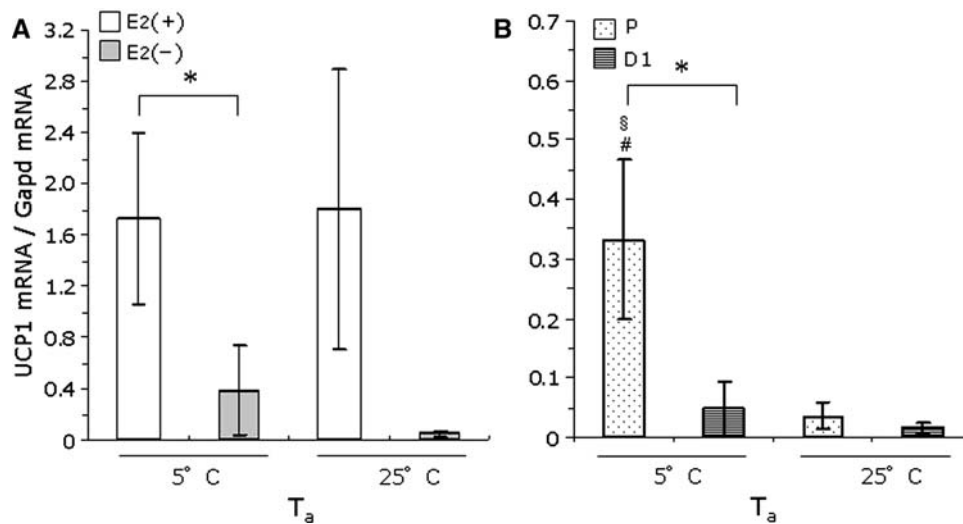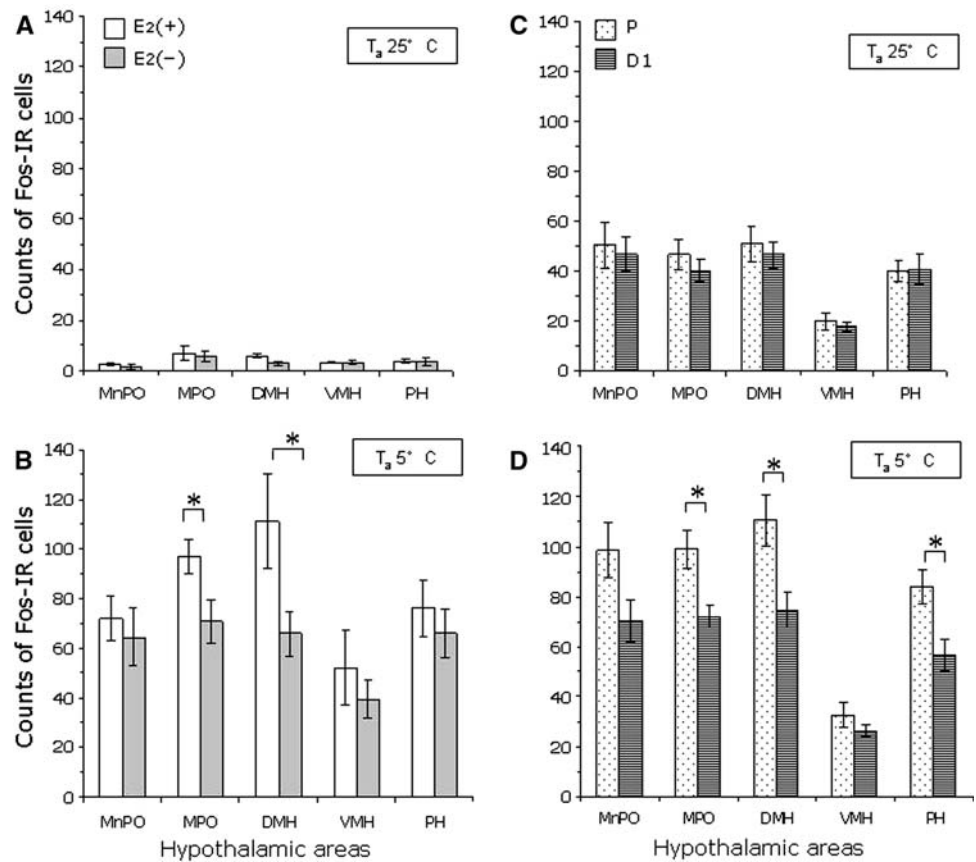
Fig. 5 Uncoupling protein 1 (*UCP1*) mRNA level with respect to the glyceraldehyde-3-phosphate dehydrogenase (*Gapd*) level in the interscapular brown adipose tissues in Experiments 1 and 2 (**A**, **B**, respectively). Values are given as the mean $\pm$ SE [**A** $n = 34$ (10 in the E_2 (+) group and 9 in the E_2 (−) group after 5°C exposure, and 7 in the E_2 (+) group and 8 in the E_2 (−) group after 25°C exposure; **B** $n = 34$ (9 in the P group and 10 in the D_1 group after 5°C exposure, and 7 in the P group and 8 in the D_1 group after 25°C exposure]. *Significant difference between the E_2 (+) and E_2 (−) groups or between the P and D_1 groups, $P < 0.05$. #Significant difference between the P and E_2 (+) groups after the 5°C exposure, $P < 0.05$. §Significant difference between the 5 and 25°C exposure in the P group, $P < 0.05$

reason for the difference may be that estrogen facilitates behavioral responses for thermoregulation in ovariectomized rats, resulting in the unchanged level of T_b in the free-moving animals at 25°C.

We assessed the central responses to the cold by counting cFos-IR cells in the hypothalamic areas, an approach widely reported in the literature. The PO area is the site which sends many efferent neurons for thermoregulation [23] and also receives afferent neurons for thermoregulation [24]. The PH is involved in shivering thermogenesis [25], and the cold signal from the skin reaches the MnPO [24]. The VMH and DMH involve iBAT thermogenesis [23, 26]. The DMH has been reported to be involved in both shivering and non-shivering thermogenesis [25], and it receives neurons from the MPO [26]. In our study, exposure to cold increased the number of cFos-IR cells in all these areas.

Previous studies have also found that cFos-IR cells increase in number in the MPO [27] and PO [28] during a cold exposure. However, most thermosensitive neurons in the PO are warm-sensitive [29], and activation results in the inhibition of thermogenesis [30]. There is a specific region in the MPO that sends inhibitory signals to the DMH, and it is this region that is involved in the iBAT thermogenesis [26]. This region in the MPO is located more rostral to where we found a high number of cFos-IR cells following cold exposure (Fig. 3), and we also found fewer cFos-IR cells in this specific region. The physiological meaning of MPO neural activation remains unclear; however, we speculate that the activation may reflect cold signals from the skin, which results in the suppression of the warm-sensitive neurons in the MPO. An earlier study also suggested that the subregion in the DMH (i.e., the dorsolateral and ventromedial parts) separately innervates the nucleus raphé pallidus and spinal cord [31]. However, we did find any different location of cFos-IR cells between the estrogen levels.

UCP1 in the iBAT specifically converts energy to heat in the mitochondria [13]. It has been reported that UCP1 knockout mice show a lower cold tolerance [32]. Two earlier studies showed that the UCP1 mRNA level in the iBAT increases after a 4- or 24-h cold exposure [33, 34]. However, in our study, the UCP1 mRNA level did not change after a 2-h cold exposure in both the E_2 (+) and E_2 (−) groups, which may have been related to the reduction of T_b. In addition, a significant difference was not observed between the E_2 (+) and E_2 (−) groups at 25°C. Thus, we conclude that in our experimental model, E_2 has little effect on thermogenic response of the iBAT to the cold. E_2 has been reported to increase the level of the α_{2c} adrenoceptor, which partly mediates the cold-induced vasoconstriction of the tail artery [35]. It is possible that E_2 is involved in the heat loss suppression in the cold, thereby affecting T_b.

Estrogen administration increased the number of cFos-IR cells in the MPO and DMH after the cold exposure to a level that was more than 30% higher than the control level. This increase in cFos-IR cells was not linked with the UCP1 mRNA level in the iBAT. Both the α- and β-estrogen receptors are abundant in the PO area [36], and an electrophysiological study [12] revealed that some neurons in the PO area are both thermo- and estrogen-sensitive. In comparison, the DMH has only a few estrogen receptors [36]. Thus, we speculate that systemic 17β-estradiol augments cold-sensitivity at the PO area, and modulates cold responses in the DMH. Moreover, because the cold sensitivity in the MPO was weakened in the ovariectomized rats, they were unable to activate their thermoregulatory response to the cold, thereby decreasing T_b.

Experiment 2

In Experiment 2, we examined whether the estrus cycle (i.e., fluctuation of sex hormones) affects body temperature and assessed central and peripheral responses to cold exposure. We especially looked at the involvement of estrogen. To this end, we selected two parameters: estrus phase and time of day. During the 4- or 5-day estrus cycle of rats, plasma estradiol level is the highest in the P phase and the lowest in the D_1 phase [17]. In addition, to minimize the effect of plasma progesterone, the 2-h cold exposure was conducted at 0900–1100 hours, during which time window the plasma progesterone remains lower and there is no difference between the P and D_1 phases [17].

There was no difference in T_b between the phases at 25°C. Marrone et al. [37] reported that the difference in T_b during the estrus cycle became apparent 10 h after light-onset. However, as these researchers did not estimate the plasma progesterone level, T_b may have been influenced by the pyrogenic action of progesterone [1].

In the cold, T_b was maintained and UCP1 mRNA level became greater in the P phase; these results differ from those of Experiment 1. Therefore, we speculate that estrogen also plays a key role in the cold response, although via a different metabolic mechanism. Endogenous estrogen with progesterone may be important in maintaining T_b with an increase in the iBAT activity in the cold. A previous study showed that progesterone did not affect thermosensitive neurons in the PO in ovariectomized rats and that 38% of the thermosensitive neurons in the PO in ovariectomized rats were excited when estradiol was also added to the model system [38].

In Experiment 2, counts of cFos-IR cells at 25°C seemed to be higher than those in Experiment 1. This result may reflect the difference in progesterone level between the two experimental systems. However, we did not assess the statistical difference because the immunohistochemistry

was separately conducted with different lots of antibodies. Counts of cFos after the cold exposure became greater in the P phase; different from Experiment 1, the counts were also greater in the PH. It has been reported that a chemical stimulation of the PH induced an increase in the iBAT thermogenesis [39]. Thus, the maintenance of T_b in the cold in the P phase is possibly associated with the response in the PH, although the UCP1 mRNA level was lower than that in the ovariectomized rats with E_2.

The results of our study show that plasma estrogen affects cFos expression in the hypothalamus during cold exposure when a pharmacological level of estrogen was systemically given to ovariectomized rats. Endogenous estrogen may also affect the responses to the cold in normal female rats in a different manner from that of systemic-administrated estrogen reaching the pharmacological level. Estrogen seems to affect thermoregulation via both the hypothalamus and iBAT; however, the mechanism remains unclear and needs to be clarified.

References

1. Nakayama T, Suzuki M, Ishizuka N (1975) Action of progesterone on preoptic thermosensitive neurons. Nature 258:80
2. Nelson HD (2008) Menopause. Lancet 371:760–770
3. Copinschi G, Van Cauter E (1995) Effects of ageing on modulation of hormonal secretions by sleep and circadian rhythmicity. Horm Res 43:20–24
4. Melby MK (2007) Chilliness: a vasomotor symptom in japan. Menopause 14:752–759
5. Kobayashi T, Tamura M, Hayashi M, Katsuura Y, Tanabe H, Ohta T, Komoriya K (2000) Elevation of tail skin temperature in ovariectomized rats in relation to menopausal hot flushes. Am J Physiol Regul Integr Comp Physiol 278:R863–R869
6. Fregly MJ, Kelleher DL, Black DJ (1979) Tolerance of estrogen-treated rats to acute cold exposure. J Appl Physiol 47:59–66
7. Hosono T, Chen XM, Miyatsuji A, Yoda T, Yoshida K, Yanase-Fujiwara M, Kanosue K (2001) Effects of estrogen on thermoregulatory tail vasomotion and heat-escape behavior in freely moving female rats. Am J Physiol Regul Integr Comp Physiol 280:R1341–R1347
8. Hosono T, Chen XM, Zhang YH, Kanosue K (1997) Effects of estrogen on thermoregulatory responses in freely moving female rats. Ann N Y Acad Sci 813:207–210
9. Freedman RR, Norton D, Woodward S, Cornelissen G (1995) Core body temperature and circadian rhythm of hot flashes in menopausal women. J Clin Endocrinol Metab 80:2354–2358
10. Labyak SE, Lee TM (1995) Estrus- and steroid-induced changes in circadian rhythms in a diurnal rodent, octodon degus. Physiol Behav 58:573–585
11. Nakayama T, Eisenman JS, Hardy JD (1961) Single unit activity of anterior hypothalamus during local heating. Science 134:560–561
12. Silva NL, Boulant JA (1986) Effects of testosterone, estradiol, and temperature on neurons in preoptic tissue slices. Am J Physiol 250:R625–R632
13. Cannon B, Nedergaard J (2004) Brown adipose tissue: function and physiological significance. Physiol Rev 84:277–359
14. Wade GN, Gray JM (1978) Cytoplasmic 17 beta-[3H]estradiol binding in rat adipose tissues. Endocrinology 103:1695–1701
15. Legan SJ, Coon GA, Karsch FJ (1975) Role of estrogen as initiator of daily LH surges in the ovariectomized rat. Endocrinology 96:50–56
16. Matthew CB, Sils IV, Tartarini KA (1999) The effect of estrogen on thermoregulation, heart rate, and activity in the female rat with comparisons to males. J Therm Biol 24:167–174
17. Smith MS, Freeman ME, Neill JD (1975) The control of progesterone secretion during the estrous cycle and early pseudo-pregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology 96:219–226
18. Takayanagi Y, Matsumoto H, Nakata M, Mera T, Fukusumi S, Hinuma S, Ueta Y, Yada T, Leng G, Onaka T (2008) Endogenous prolactin-releasing peptide regulates food intake in rodents. J Clin Invest 118:4014–4024
19. Sokal RR, Rohlf FJ (1994) Biometry, 3rd edn. WH Freeman, New York
20. Ishiwata T, Saito T, Hasegawa H, Yazawa T, Kotani Y, Otokawa M, Aihara Y (2005) Changes of body temperature and thermoregulatory responses of freely moving rats during GABAergic pharmacological stimulation to the preoptic area and anterior hypothalamus in several ambient temperatures. Brain Res 1048:32–40
21. Saito T, Ishiwata T, Hasegawa H, Nomoto S, Otokawa M, Aihara Y (2005) Changes in monoamines in rat hypothalamus during cold acclimation. J Therm Biol 30:229–235
22. Shimada SG, Stitt JT (1983) Inhibition of shivering during restraint hypothermia. Can J Physiol Pharmacol 61:977–982
23. Nagashima K, Nakai S, Tanaka M, Kanosue K (2000) Neuronal circuitries involved in thermoregulation. Auton Neurosci 85:18–25
24. Nakamura K, Morrison SF (2008) Preoptic mechanism for cold-defensive responses to skin cooling. J Physiol 586:2611–2620
25. Tanaka M, Tonouchi M, Hosono T, Nagashima K, Yanase-Fujiwara M, Kanosue K (2001) Hypothalamic region facilitating shivering in rats. Jpn J Physiol 51:625–629
26. Nakamura Y, Nakamura K, Matsumura K, Kobayashi S, Kaneko T, Morrison SF (2005) Direct pyrogenic input from prostaglandin EP3 receptor-expressing preoptic neurons to the dorsomedial hypothalamus. Eur J Neurosci 22:3137–3146
27. Miyata S, Ishiyama M, Shido O, Nakashima T, Shibata M, Kiyohara T (1995) Central mechanism of neural activation with cold acclimation of rats using fos immunohistochemistry. Neurosci Res 22:209–218
28. Bratincsak A, Palkovits M (2004) Activation of brain areas in rat following warm and cold ambient exposure. Neuroscience 127:385–397
29. Yoshida K, Maruyama M, Hosono T, Nagashima K, Fukuda Y, Gerstberger R, Kanosue K (2002) Fos expression induced by warming the preoptic area in rats. Brain Res 933:109–117
30. Zhang YH, Yanase-Fujiwara M, Hosono T, Kanosue K (1995) Warm and cold signals from the preoptic area: which contribute more to the control of shivering in rats? J Physiol 485[Pt 1]:195–202
31. Hosoya Y, Ito R, Kohno K (1987) The topographical organization of neurons in the dorsal hypothalamic area that project to the spinal cord or to the nucleus raphe pallidus in the rat. Exp Brain Res 66:500–506

32. Enerback S, Jacobsson A, Simpson EM, Guerra C, Yamashita H, Harper ME, Kozak LP (1997) Mice lacking mitochondrial uncoupling protein are cold-sensitive but not obese. Nature 387:90–94

33. Carmona MC, Valmaseda A, Brun S, Vinas O, Mampel T, Iglesias R, Giralt M, Villarroya F (1998) Differential regulation of uncoupling protein-2 and uncoupling protein-3 gene expression in brown adipose tissue during development and cold exposure. Biochem Biophys Res Commun 243:224–228

34. Yamashita H, Sato Y, Mori N (1999) Difference in induction of uncoupling protein genes in adipose tissues between young and old rats during cold exposure. FEBS Lett 458:157–161

35. Eid AH, Maiti K, Mitra S, Chotani MA, Flavahan S, Bailey SR, Thompson-Torgerson CS, Flavahan NA (2007) Estrogen increases smooth muscle expression of alpha2C-adrenoceptors and cold-induced constriction of cutaneous arteries. Am J Physiol Heart Circ Physiol 293:H1955–H1961

36. Laflamme N, Nappi RE, Drolet G, Labrie C, Rivest S (1998) Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. J Neurobiol 36:357–378

37. Marrone BL, Gentry RT, Wade GN (1976) Gonadal hormones and body temperature in rats: effects of estrous cycles, castration and steroid replacement. Physiol Behav 17:419–425

38. Tsai CL, Kanosue K, Matsumura K (1992) Effects of estradiol treatment on responses of rat preoptic warm sensitive neurons to progesterone in vitro. Neurosci Lett 136:23–26

39. Amir S (1990) Activation of brown adipose tissue thermogenesis by chemical stimulation of the posterior hypothalamus. Brain Res 534:303–308

40. Paxinos G, Watson C (2005) The rat brain in stereotaxic coordinates, 5th edn. Elsevier, Amsterdam