 Thermoregulation in a cold environment
—Modulation of estrogen and tail-hiding behavior—

寒冷環境における体温調節
—エストロゲンと尾隠し行動による修飾—

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1. Introduction

The body temperature ($T_b$) of homeothermic animals is regulated by behavioral and autonomic responses (50). As the autonomic responses, human beings induce shivering thermogenesis or activate metabolism in the brown adipose tissue (BAT), but only in the neonates (48). Recent studies have shown that adults can also activate the BAT during cold exposure (67, 86, 87). Rats show shivering, non-shivering thermogenesis in the BAT, and vasoconstriction of the tail (22). Both human beings and rats show behavioral responses. In the cold, both animals try to escape from the place, and seek warmer environment. Human beings put on clothes or turn on air-conditioner, etc. Rats may show a round posture or huddle to decrease their heat loss (22).

The autonomic and behavioral responses are controlled, based on thermal inputs to the body. It is generally accepted that the hypothalamus in the brain plays an important role in autonomic thermoregulation in the cold, although the precise central mechanism involved in thermoregulation remains unclear. Among the areas in the hypothalamus, the medial preoptic area (MPO), the dorsomedial hypothalamus (DMH) and the median preoptic area (MnPO) are key regions in thermoregulation in cold (48). The cold signals activate
GABA neurons in the MnPO via the dorsal horn of the vertebra and the external lateral part of the lateral parabrachial nucleus. The neurons in the MnPO inhibit the GABAergic projection neurons in the MPO to the DMH. Accordingly, the disinhibition of DMH neurons leads to shivering (51). In the contrast to the knowledge about the autonomic thermoregulatory responses, the neural mechanism of behavioral thermoregulation is unknown yet (65).

In the present study, I first evaluated the effect of estrogen on the autonomic thermoregulation during cold exposure, i.e. tail vasoconstriction and thermogenesis in female rats. In the second, I showed that the tail-hiding behavior is one of behavioral thermoregulatory processes in cold.

1.1. Introduction of the systemic administration of estrogen on thermoregulation during a cold exposure in female rats

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 (55), which may be associated with a change in $T_b$ 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 (56), a disturbance in circadian body temperature change (14), and a greater sensitivity to cold exposure (44). Estrogen replacement is sometimes used as a treatment for hot flush (35). 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 (21, 28, 29). In contrast, Freedman et al. found that the plasma level of estradiol was not different between postmenopausal women with and without hot flush (20), 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 (37). 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 (54). 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 (74). 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 (11). The iBAT has been shown to have estrogen-specific macromolecular binding in the cytoplasmic fraction (83), 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.
1.2. Introduction of the effect of hypothalamic application of estrogen on thermoregulation during a cold exposure in female rats

It has been reported that estrogen is involved in body temperature regulation (6, 35, 56) in female animals and women. Numbers of peri- and post-menopause women suffer from hot flush, i.e., a sudden onset of face flushing, sweating, and hotness with a reduction of body temperature. Estrogen replacement is often used as a therapy for hot flush, although estrogen would not be a sole factor involved in hot flush. However, it still remains unclear how estrogen affects body temperature and/or body temperature regulation.

Romanovsky defined that the thermoneutral zone is the ambient temperature \( T_a \) at which animals can maintain \( T_b \) only by changing surface temperature of the dry heat-loss organs such as the tail in rats (63). Several studies have shown that systemic administration of estrogen affects \( T_b \) of ovariectomized rats in slightly lower subneutral (42), subneutral (29), and supraneutral zones (5, 15). In addition, estrogen augments heat production in the subneutral zone (29). In addition, it has been reported that estrogen increases heat loss in the supraneutral zone (5) or decreases (15). We also reported that \( T_b \) in
the subneutral zone differed among estrous phases and was linked with the plasma estrogen level (81). However, we do not know the mechanism behind this. One possible reason may be an abundance of estrogen receptors in the body, which would affect thermoregulation, e.g., in the hypothalamus and blood vessels, etc. Therefore, it is necessary to identify the site at which estrogen works and modulates thermoregulatory responses.

Among the body sites, the hypothalamus may be implicated in the estrogen-induced changes in thermoregulatory responses. In particular, two sub-regions in the hypothalamus, i.e., the MPO in the PO and the DMH, seem to play a key role in thermoregulation. Both the MPO and DMH have estrogen receptors (39). The MPO is a thermosensitive site and sends efferent neurons to other brain sites involved in thermoregulation, such as DMH (50, 52, 54). Disinhibition of the neurons in the DMH increases sympathetic activity to the interscapular brown adipose tissue (iBAT) and skin vasculature, which are related to non-shivering thermogenesis and suppression of dry heat loss in cold (16). Furthermore, Almeida et al. (2006) reported that DMH is involved in the activation of cold-seeking behavior after endotoxin injection.

In the present study, we hypothesized that estrogen affects cold
responses at the level of the MPO and DMH. To test this hypothesis, estrogen was locally administered to the MPO or DMH after ovariectomy in young female rats having no influence of longer-term estrogen imbalance or depletion, and its effect on $T_b$, expression of uncoupling 1 protein (UCP1) mRNA in the iBAT involved in non-shivering thermogenesis, and heat loss from the tail was assessed.

1.3. Introduction of the effect of tail-hiding behavior on thermoregulatory responses during a cold exposure in the fed and fasted rats

In homeothermic animals, body temperature is regulated by autonomic and behavioral processes. For example, in cold environments, rats increase their metabolism by biochemically activating their supplies of brown fat and shivering. The rats also try to escape from the cold by seeking warmer environments. When no such environments are available, they decrease their heat loss by adopting a round posture or by huddling (22). Although several studies have assessed the behavioral responses to thermoregulation using operant systems (1, 27), no study has evaluated how much the behavioral responses contribute to thermoregulation.
Rats use their tail as an effector organ for autonomic thermoregulation (23). Amputation of the tail results in an increase in body temperature during heat exposure (77). In contrast, cold induces the constriction of the tail vessels, which minimizes heat dissipation (22). Romanovsky suggested that skin temperature of the tail could be an indicator in determining whether $T_a$ is in a neutral, subneutral, or supraneutral range (66). Our findings suggest that the tail is also an effector organ for behavioral thermoregulation (82). Ovariectomized rats displayed lower metabolic heat production in cold environments. In addition, they increase the specific behavior of placing their tails underneath the trunk of their body in cold environments. We termed this behavior “tail-hiding behavior” (82). However, it remains unclear whether this behavior contributes to thermoregulation. Therefore, to understand the physiological significance of the tail-hiding behavior, we assessed whether it affected autonomic thermoregulatory responses, such as whether placing the tail under the body reduced metabolic heat demand or attenuated heat dissipation from the tail.

Yoda et al. (85) reported that fasting activates operant warm-seeking/cold-escape behaviors during cold exposure in rats and that fasting attenuates metabolism (49). Therefore, activation of the
operant behavior may result in the maintenance of body temperature without the activation of metabolic heat production. Here, we compared the changes in metabolism and core body temperature between ad-lib fed (high energy availability) and fasting (low energy availability) rats. The responses were also compared between rats where the tail-hiding behavior was prevented and in rats where the behavior was allowed, which was controlled by attaching specific devices to their tails. We hypothesized that the tail-hiding behavior acts as a thermoregulatory process in cold environments and that rats without the ability to perform the tail-hiding behavior would not be able to maintain their core body temperature in the cold during fasting.

2. Effect of systemic administration of estrogen on thermoregulation during a cold exposure in female rats

2.1. Summary

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 E₂ (+) rats than in the E₂ (-) 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 E₂ (+) rats than in the E₂ (-) 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.

2.2. Method

Ninety-one adult virgin female Wistar 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 x 25 x 20 cm) at ambient temperature (Tₐ) 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.

(1) 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 x 30 x 8 mm; PhysioTel, TA10TA-F40; Data Science, St. Paul, MN) for body core temperature (Tb) 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 (E2; 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 E2, and sealed with silicone glue as reported previously (40); this procedure maintained a constant level of E2 in the plasma. At least 7 days after the second surgery, we placed a rat in a plastic cage (45 x 25 x 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; Data-Science) 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(43), 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 immunohistochemical analysis.
(2) **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 (75). 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 (D₁, 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 D₁ phase (75). 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 (75). 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.

(3) Handleings 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-um 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,
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 (QIAGEN).

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: 50-TACCCAGCTGTGCAATGACCA (forward),
50-GCACACAAACATGATGACGTTCC (reverse);
Gapd: 50-GGCACAGTCAAGGCTGAGAATG(forward),
50-ATGGTGTTGAAGACGAGTTTTA (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 (78).

(4) 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 D1 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°C 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 (76). The null hypothesis was rejected at the level of $P<0.05$.

2.3. Results

(1) Body core temperature

Figure 1 shows changes in the $T_b$ during the exposure to 25°C (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].
Tb remained stable in the P and D1 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 Tb in the P group was greater (P<0.05) than that in the D1 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, Tb in the P group was at the level of the baseline (mean 37.4 ± 0.2°C); however, that in the D1 group was lower than the baseline level at 90–120 min (mean P<0.05, 36.8 ± 0.2°C).

(2) 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 E2 (+) group than in the E2 (-) 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 D1 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 D₁ group than in the E₂ (-) group, and lower (P<0.05) in the P group than in the E₂ (+) group. The progesterone level was greater (P<0.05) in the D₁ group than in the E₂ (-) group and also greater (P<0.05) in the P group than in the E₂ (+) group.

(3) 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 E₂ (+) and E₂ (-) 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 E₂ (+) group than in the E₂ (-) 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
Dₑ 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 D₁ 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 E₂ (+) and E₂ (-) groups or the P and D₁ groups.

(4) 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 E₂ (+) and E₂ (-) groups after exposure to 25°C (Fig. 5A). In contrast, the level after the 5°C exposure in the E₂ (+) group was greater (P<0.05) than that in the E₂ (-) 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 D₁ groups at 25°C in Experiment 2, although at 5°C
C, the level was greater in the P group than in the D1 group (Fig. 5B). There was no difference in the level in the D1 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 E2 (+) and P groups or the E2 (-) and D1 groups. The level in the P group was lower than that in the E2 (+) group following the 5°C exposure (P<0.05; Fig. 5A, B).

2.4. 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.

(1) Experiment 1

Tb decreased at 5°C in both ovariectomized rats with and without E2, although the reduction in the E2 (+) group was smaller. However, a similar reduction in Tb was not observed at 5°C in male
rats (31, 68). Hosono et al. (29) reported that the Tb during a 3-h cold exposure at 4 °C was higher in ovariectomized rats with E₂ than in those without E₂. In addition, the Tb in ovariectomized rats with E₂ 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 Tb during the cold exposure because progesterone has a pyrogenic action (55). 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 Tb. However, the progesterone level was not determined in the study of Hosono et al. (29).

As previously reported, we also found that estradiol had no influence on Tb at 25° C (28). However, Fregly et al. found that Tb became lower following ethinyl estradiol treatment (21). The experimental conditions were different between our study and that of Fregly et al. (21) in that in the latter the rats were restrained and Tb was monitored with a thermocouple in the rectum. It has been reported that restraint itself induces a decrease in Tb (72). 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 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 (50) and also receives afferent neurons for thermoregulation (52). The PH is involved in shivering thermogenesis (79), and the cold signal from the skin reaches the MnPO (52). The VMH and DMH involve iBAT thermogenesis (50, 53). The DMH has been reported to be involved in both shivering and non-shivering thermogenesis (79), and it receives neurons from the MPO (53). 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 (45) and PO (8) during a cold exposure. However, most thermosensitive neurons in the PO are warm-sensitive (89), and activation results in the inhibition of thermogenesis (90). 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 (53).
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 raphe pallidus and spinal cord (30). 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 (11). It has been reported that UCP1 knockout mice show a lower cold tolerance (18). Two earlier studies showed that the UCP1 mRNA level in the iBAT increases after a 4- or 24-h cold exposure (12, 84). However, in our study, the UCP1 mRNA level did not change after a 2-h cold exposure in both the E2 (+) and E2 (-) groups, which may have been related to the reduction of Tb. In addition, a significant difference was not observed between the E2 (+) and E2 (-) groups at 25°C. Thus, we conclude that in our experimental model, E2 has little effect on thermogenic response of
the iBAT to the cold. E$_2$ has been reported to increase the level of the α2c adrenoceptor, which partly mediates the cold-induced vasoconstriction of the tail artery (17). 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 (39), and an electrophysiological study (74) revealed that some neurons in the PO area are both thermo- and estrogen-sensitive. In comparison, the DMH has only a few estrogen receptors (39). 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$_b$.

(2) 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 D₁ phase (75). 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 D₁ phases (75). There was no difference in T_b between the phases at 25°C. Marrone et al. (42) 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 (55).

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 (80).

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 (3). 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.

3. Effect of hypothalamic application of estrogen on thermoregulation during a cold exposure in female rats

3.1. Summary

The present study examined the effect of the central administration of estrogen on responses to the cold. Estrogen or cholesterol was applied locally to the medial preoptic nucleus (MPO) or dorsomedial hypothalamic nucleus (DMH) of the hypothalamus in free moving ovariectomized rats. Forty-eight hours after the application, rats had 2-h exposure at 10 or 25 °C. Body temperature ($T_b$) and the tail surface temperature ($T_{tail}$) were continuously measured by telemetry and thermography, respectively. The change of $T_b$ at 10 °C from the 25 °C baseline was higher in the estrogen application in the MPO than that in the cholesterol application; however, such difference was not observed in the DMH application. The uncoupling 1 protein mRNA level in the interscapular brown
adipose tissue involved in non-shivering thermogenesis was not different between the estrogen and cholesterol applications in the MPO and DMH. \( T_{\text{tail}} \) decreased in the cold, which was greater after the estrogen application in the MPO than after the cholesterol application. These results show that estrogen affects the MPO in female rats, changing \( T_b \) in the cold. Moreover, suppression of heat loss from the tail may be involved in the mechanism.

3.2. Method

(1) Animals

Virgin female Wister rats (n=100; bw: 190–290 g; age: 9–11 weeks; Takasugi Experimental Animals Supply, Saitama, Japan) were used in the present study. The rats were housed in a stainless steel cage (45x25x20 cm) at \( T_a \) of 25 °C in a 12:12-h light–dark cycle (lights on at 07:00 h) and were allowed free access to food and water. The Institutional Animal Care and Use Committee of Waseda University approved all experimental procedures in the present study. The rats were divided to two groups and were subjected to two different experimental protocols as follows (Experiments 1 and 2).
(2) Experiment 1: effect of local application of estrogen to the MPO and DMH on cold responses

(2)-1. Surgery

The rats \((n=68)\) underwent surgery under inhalation anesthesia with diethyl ether (Sigma Aldrich, Tokyo, Japan). After a medial skin incision, a radio transmitter (15×30×8 mm; PhysioTel, TA10TA-F40, Data Science, St. Paul, MN) for measuring \(T_b\) and spontaneous activity was implanted into the peritoneal cavity. Bilateral ovariectomy was conducted via a dorsal skin incision, and penicillin G (1,000 U, Meiji Pharmaceutical, Tokyo, Japan) was injected s.c. to prevent post-surgical infection.

At 1 week after surgery, the rats were anesthetized with i.p. administered pentobarbital \(\text{Na}^+\) (10 mg/100 g bw; Somnopentil, Kyoritsu Seiyaku, Tokyo, Japan) and placed on a stereotaxic apparatus for brain surgery (SR-6R; Narishige Scientific Instruments, Tokyo, Japan). A hole was drilled in each rat's skull, and a 21-gauge stainless steel guide cannula (length 2mm) was inserted, the tip of which was located in the right MPO or DMH in accordance with stereotaxic coordinates (58): 5.0mm lateral and 0.8mm caudal to the
bregma and 8.4mm below from the brain surface (the MPO group, n=36), with the same measurements being 5.0, 2.8, and 8.6 mm, respectively, in the DMH group (n=32). The cannula was fixed to the skull with dental cement (Miky Red, Nissin, Kyoto, Japan) and secured with screws. A dummy internal cannula was then put in place to avoid occlusion of the guide cannula (Fig. 6A).

(2)-2. Local application of estrogen or cholesterol to the MPO and DMH

At 1 week after the second operation, an internal cannula filled with 0.1 mg 17-β estradiol or cholesterol crystalline (Sigma, St. Louis, MO) was used to replace the dummy cannula for 4 h (the estrogen-treated and control rats, respectively) as previously reported (70). Neither estradiol nor cholesterol was left in the cannula after the 4-h administration. One week after the first trial, the procedure was repeated using the other drug; i.e., when estrogen was used in the first trial, a cannula filled with cholesterol was applied. The effect of estrogen in hypothalamus was estimated by the immunohistological analysis of ERα expression. In addition, we verified that the effect of estrogen administration on the ERα expression disappeared 1 week after the administration.
(2)-3. Exposure protocol and measurements

Forty-six hours after the drug administration in the brain, each rat was moved from its cage to a climatic chamber (Program Incubator IN602W, Yamato Scientific, Tokyo, Japan) maintained at 25 °C for 2 h, in which $T_b$ and spontaneous activity were continuously monitored by telemetry. Spontaneous activity was estimated by the changes in the intensity of the telemetry signals. Signals from the radio transmitter were obtained through a receiver board placed underneath the cage (model CTR86, Data Science, St. Paul, MN) at 1-min intervals and stored on a personal computer using a data-logger (LabView, National Instruments, Austin). Before the experiments, we calibrated the transmitter by immersing water at three different temperatures at 35, 37, and 39 °C. $T_a$ was monitored with a thermocouple placed 10 cm above the bottom of in the climatic chamber. Accuracy of the $T_b$ measurement was ±0.1 °C. The chamber was then set at 10 °C for 2 h (12:00–14:00 h, the subneutral exposure) or 25 °C. Set at 10 °C, $T_a$ reached at the level within 30 min. $T_a$ was maintained within ±0.5 °C of each set temperature. After the temperature exposure, the rats were killed via i.p. injection of an overdose of pentobarbital Na+ (50 mg/100 g bw; Somnopentil, Kyoritsu Seiyaku, Tokyo, Japan).
The iBAT was then removed, immersed in RNA-stabilization reagent (RNA Later, QIAGEN, Tokyo, Japan) at 4 °C for 12 h, and stored at −80 °C. A 2-ml blood sample was taken from the left ventricular cavity and centrifuged at 4 °C, and the plasma was stored at −80 °C until the assay. Each rat was perfused transcardially with normal saline, followed by 4% paraformaldehyde, and then had their brain removed.

(3) Experiment 2: effect of local application of estrogen to the MPO on heat loss from the tail

(3)-1. Surgery

Virgin female Wister rats (n=32) underwent surgery for ovariectomy and the implantation of a radio transmitter, and a stainless steel guide cannula was placed into the MPO (Fig. 6B). 17-β estradiol (the estrogen-treated rats, n=16) or cholesterol (the control rats, n=16) was administered through the cannula. All these procedures were similar to those performed in Experiment 1.

(3)-2. Exposure protocol and measurement

Each rat was placed in a chamber: a polyethylene pail (20×32×14
cm) placed in a larger pail (diameter, 38 cm; height, 42 cm). Water was then added to the space between the two pails and was circulated to and from a temperature-controlled reservoir. $T_a$ was monitored with a thermocouple placed 10 cm above the bottom of the chamber, and was set to 25 °C for the first 2 h (10:00 to 12:00 h) and 10 or 25 °C for the following 2 h (12:00 to 14:00 h). Set at 10 °C, $T_a$ reached at the level within 30 min. $T_a$ was maintained within ± 1.0 °C of each set temperature. $T_b$ and tail surface temperatures were monitored by telemetry and infrared thermography (Neo Thermo TVS-700, Nihon Avionics, Tokyo, Japan), respectively, at 1-min intervals. Based on the thermograms, tail surface temperature was analyzed at two points (one-third of the tail length from the tail tip and root) and then averaged ($T_{\text{tail}}$). When the tail could not be seen, the missing $T_{\text{tail}}$ value was replaced with that of the previous time point. During cold exposure, the rats occasionally hid their tails underneath their bodies. We named this response “tail-hiding behavior” and calculated the total duration that the behavior was observed.

After the measurements, the rats were killed and perfused transcardially as in Experiment 1. The whole brain was excised to verify the site of the cannula, and the iBAT was then weighed.
(3)-3. Handling and analyses of the blood, brain, and iBAT

Determination of plasma estradiol level

The plasma levels of estradiol was determined by ELISA in duplicate (Estradiol EIA Kit and Progesterone EIA Kit, Cayman Chemical, Ann Arbor, MI).

(3)-4. Immunohistochemistry of cFos and estrogen receptor α in the hypothalamic areas

The brain was soaked in 4% paraformaldehyde at 4 °C overnight and 25% sucrose in PBS for another 48 h while being frozen in crushed dry ice. Coronal sections were prepared with a cryostat with a 40-μm thickness (CM1510S, Leica, Wilzlar, Germany), and the sections were then rinsed with PBS and incubated overnight in 0.3% hydrogen peroxide in PBS mixed with 0.3% Triton X-100 for 30min and rabbit anti-cFos polyclonal IgG (1:15,000 dilution; PC38, Calbiochem, Merck, Tokyo, Japan) or rabbit anti-estrogen receptor α polyclonal IgG (1:10,000 dilution, Cat No. sc-542, Santa Cruz Biotechnology, Santa Cruz, CA). The anti-cFos antibody was raised against residues 4–17 of the human peptide gene product, and the specificity of the labeling was tested in previous study (Gelez and Fabre-Nys, 2006). The anti-estrogen receptor α antagonist specifically binds to
the estrogen receptors α and does not show a cross-reaction with the 
estrogen receptors β (Hou et al., 2003). On the next day, the 
sections were incubated with biotinylated donkey anti-rabbit IgG 
(1:1,000 dilution; Jackson ImmunoResearch Laboratories, West Grove, 
PA) for 90 min and avidin-biotin complex (1:1,000 dilution; Vectastain 
Elite ABC standard kit, Vector Laboratories, Burlingame, CA) for 
another 90 min, before being reacted with 5% 
diaminobenzidinetetrahydrochloride (Sigma, St. Louis, MO) in PBS 
and mounted on gelatin-coated glass slides, each of which were 
coverslipped. Digital images of the DMH, MPO, MnPO, VMH, and PH 
were captured (Digital Camera HC 2500 3CCD, FUJIFILM, Tokyo, 
Japan; ECLIPSE E600, Nikon, Tokyo, Japan; Image-Pro Plus, Media 
Cybernetics, Bethesda, MD) and saved in TIFF format. cFos 
immunoreactive (cFos-IR) cells in three consecutive sections in the 
right side of the brain were manually dotted with image analyzing 
software (Adobe Photoshop, Adobe System, San Jose, CA). The 
counts were determined using image analyzing software (Image J, NIH, 
Bethesda, MD) and the averaged were calculated. The atlas of (58) 
was used to define the position of labeled cells.

(4) UCP1 mRNA analysis
Total RNA was extracted from the iBAT with the RNeasy Lipid Tissues Mini Kit (QIAGEN, Tokyo, Japan). Briefly, the frozen tissue was thawed at room temperature and homogenized with QIAzol Lysis Reagent® (a monophasic solution of phenol and guanidine thiocyanate). After the addition of chloroform (Sigma Aldrich, Tokyo, Japan), the liquid centrifuged at 12,000×g for 15 min at 4 °C after vigorous shaking. Seventy-percent ethanol (Sigma Aldrich, Tokyo, Japan) was added to the supernatant, which was centrifuged using a flow-through column. Rnase free water (Ultra PureWater, Invitrogen, Tokyo, Japan) was put on a membrane of the flow-through column, which was centrifuged to elute. The total RNA concentration in the eluent was determined based on the ratio of the absorbance at 260 and 280 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Scientific, Wilmington, DE). Reverse transcription for the total iBAT RNA of 1000 ng was conducted using a reverse transcription kit (PrimeScript RT reagent Kit, TAKARABIO, Otsu, Japan). Denaturation was performed at 37 °C for 15 min, followed by 85 °C for 5 s in a thermal cycler (I Cycler, Bio-Rad Laboratories, Hercules, CA). To quantify the mRNA level of UCP1, RT-PCR was conducted using an RT-PCR kit (SYBER Premix Ex Taq Real Time, TAKARABIO, Otsu, Japan). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH)
gene was used as the reference as in a previous report that determined the UCP1 mRNA level of the iBAT in the cold (Takayanagi et al., 2008). The oligonucleotide sequences of the primers used were as follows: UCP1: 5′-TACCCAGCTGTGCAATGACCA (forward) and 5′-GCACACAAACATGATGACGTTCC (reverse); and GAPDH: 5′-GGCACAGTCAAGGCTGAGATG (forward) and 5′-ATGGTGGTGAAGACGCCAGTA (reverse). The amplification was performed with an ABI-Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The denaturation protocol was 95 °C for 10 s, 95 °C for 5 s by 40 cycles, 60 °C for 32 min, 95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s.

(5) Statistical analysis

All values are shown as means±SE. Values for Tb, Ttail, and spontaneous activity were averaged every 5 min. The baseline value (the mean for the 30 min before 10 or 25 °C exposure), changes in Tb and Ttail, and changes in spontaneous activity from the baseline levels were calculated. Differences between the estrogen-treated and control rats were assessed by two-way ANOVA with repeated measures (SPSS, Chicago, IL). Differences between the MPO and DMH groups in each trial were assessed by two-way ANOVA. A post
hoc t-test was performed to identify significant differences at specific time points. Differences in the counts of cFos-IR cells and the UCP1 mRNA level in the iBAT between the estrogen-treated and control rats, the MPO and DMH groups, or the cold and control exposures were assessed by three-way ANOVA with SPSS. Differences in the weights of the iBAT between the estrogen-treated and control rats or the 10 and 25 °C exposures were assessed by the Kruskal–Wallis test. The null hypothesis was rejected at the level of P<0.05.

3.3. Results

(1) Effect of local application of estrogen to the MPO and DMH on cold responses (Experiment 1)

(1)-1. The number of estrogen receptor α immunoreactive cells in the MPO and plasma level of estradiol

Fig. 6A illustrates application sites of estrogen and cholesterol in the MPO or DMH with 2-h 25 °C control or 10 °C exposure. Fig. 7 shows an immunohistochemical analysis of estrogen receptor α immunoreactive (ERα-IR) cells (Figs. 7A, B, and C) in the MPO. In ovariectomized rats that did not receive a local estrogen administration, many ERα-IR cells were seen in both the central and
rostral parts of the MPO (Fig. 7A-a and b). In the estrogen treated rats in the MPO group, ERα-IR cells were abundantly observed in the rostral MPO (Fig. 7B-b); however, few were present in the central part (Fig. 7B-a). In the estrogen-treated rats of the DMH group, there were many ERα-IR cells in both the central and rostral parts (Fig. 7C-a and b). The plasma estradiol level was not different between the control and estrogen-treated rats in Experiment 1 (57.5±11.0 and 56.3±13.0 pg/ml, respectively).

(1)-2. Change in body temperature

Fig. 8 shows the change in Tb from the 25°C baseline during exposure to T_a of 25 and 10 °C in Experiment 1 (A and B, and C and D, respectively). The baseline Tb was not different between the control and estrogen-treated rats in the MPO group (37.5±0.0 and 37.4±0.0 °C, respectively) or the DMH group (37.3±0.0 and 37.5±0.0 °C, respectively). The change in Tb was not different between the control and estrogen-treated rats in the MPO (Fig. 8A) or DMH groups (Fig. 8B) at 25 °C or the DMH group (Fig. 8D) at 10 °C. In the MPO group, the change in Tb in the estrogen-treated rats at 10 °C was higher (P<0.05) than that in the control rats at 30–40, 50–70, and 95–100min (Fig. 8C), although no statistical change from the baseline was
observed in the two treatments.

(1)-3. Change in spontaneous activity (movement in a cage)

The change in spontaneous activity from the baseline during exposure to $T_a$ of 25 and 10 °C in Experiment 1 are shown in Fig. 8 (E and F, and G and H, respectively). The change in spontaneous activity was not different between the control and estrogen-treated rats in the MPO (Fig. 8E) or DMH groups (Fig. 8F) during exposure to $T_a$ of 25 °C, or in the DMH group during exposure to $T_a$ of 10 °C (Fig. 8H). In contrast, during exposure to $T_a$ of 10 °C, the change in spontaneous activity in the MPO group was greater ($P<0.05$) in the estrogen-treated rats than in the control rats at 5–20, 100, and 120 min (Fig. 8G).

(1)-4. Counts of cFos immunoreactive (cFos-IR) cells in the hypothalamic areas

Fig. 9 shows the immunohistochemical images of cFos in the MPO and DMH after exposure to $T_a$ of 10 °C in Experiment 1 (A and B, respectively). The counts of cFos-IR cells in the hypothalamus after exposure to $T_a$ of 25 or 10 °C in Experiment 1 are summarized in Figs. 10 (A and B, and C and D, respectively).
5 hypothalamic areas, i.e., the median preoptic nucleus (MnPO), MPO, DMH, ventromedial hypothalamus (VMH), and posterior hypothalamus (PH), there were no differences in the counts between the 25 and 10 °C treatments in the control or estrogen-treated rats in either the MPO or DMH group. In addition, there was no difference between the control and estrogen-treated rats under any environmental condition in any group.

(1)-5. Uncoupling 1 protein (UCP1) mRNA level and the weight of the interscapular brown adipose tissue (iBAT)

Fig. 11 illustrates the UCP1 mRNA level in the iBAT in Experiment 1 (A and B, respectively). In the MPO and DMH groups, the UCP1 mRNA level was not different between the estrogen-treated and control rats after either 25 or 10 °C exposure. On the other hand, in the control rats, the UCP1 mRNA level after exposure to $T_a$ of 10 °C was higher ($P<0.05$) than that after exposure to $T_a$ of 25 °C in both the MPO and DMH groups. The weight of the iBAT was not significantly different between the control and estrogen-treated rats in the MPO group (85±20 and 85±1 mg at 25 °C and 89±18 and 50±12mg at 10 °C, respectively).
(2) Effect of local application of estrogen to the MPO on heat loss from the tail (Experiment 2)

(2)-1. Change in tail surface temperature

Application sites of estrogen and cholesterol in the MPO or DMH with 2-h 25 °C control or 10 °C exposure were shown in Fig. 6B. The changes in tail surface temperature (T_{tail}) from the baseline at 25 and 10 °C are summarized in Figs. 7A and B, respectively. The baseline T_{tail} at 25 and 10 °C was not significantly different between the control and estrogen treated rats (28.3±0.7 and 28.0±0.5 °C, and 26.4±0.7 and 28.2±0.3 °C, respectively). T_{tail} decreased during exposure to 10 °C in both the control and estrogen-treated rats. Although the change in T_{tail} during exposure to 25 °C was not different between the control and estrogen-treated rats (Fig. 12A), that during exposure to 10 °C was greater (P<0.05) in the estrogen-treated rats than in the control rats at 55–95 and 105–110min (Fig. 12B).

(2)-2. Tail-hiding behavior

Fig. 12C shows the length of time that the rats hid their tails underneath their bodies during exposure to 25 and 10 °C. In both the control and estrogen-treated rats, the duration of hiding at 10 °C was
greater (P<0.05) than that at 25 °C. However, the tail-hiding duration was not different between the control and estrogen-treated rats at either 25 or 10 °C (4±3 and 5±1 min and 43±5 and 31±9 min, respectively).

3.4. Discussion

The goal of the present study was to understand whether the hypothalamic application of estrogen affects cold responses. Our main finding was that, in ovariectomized rats, local administration of estrogen to the MPO affected the change in $T_b$ in the cold, which correlates with the heat loss response of the tail.

(1) Evaluation of the effective area of estrogen in the hypothalamus after the local application

It would be difficult to know the local concentration of estrogen in the brain areas. Instead, we analyzed ERα-IR cells around the administration site in the MPO because it is well known that estrogen with higher concentration suppresses ER expression in the PO (73, 91). The present procedure used for the local application of estrogen enabled us to influence the estrogen level within a 0.7-mm radius from the tip of cannula. Thus, we speculated that the direct effect of
estrogen was limited to a small area around the tip of the cannula. We also assessed whether locally administering estrogen to the MPO or DMH affects the plasma estrogen level. Estrogen application in the MPO or DMH did not affect the plasma estrogen level and the level was below than the lowest level in normal rats (10, 75). Therefore, the administered estrogen remained within a small area in the brain.

(2) Estrogen application in the MPO changed the response of Tb in the cold

In Experiment 1, we assessed the effect of the local administration of estrogen to the MPO and DMH on the response of Tb under cold conditions. Estrogen application in the MPO affected the change in Tb in the cold. In contrast, the influence of estrogen application in the DMH was small. Although the application sites of estrogen may not to be equally distributed in the MPO, we could not see any difference in Tb among the sites such as the rostral vs. caudal during the 25 or 10 °C exposure. The MPO and DMH are reported to be involved in thermoregulation in the cold (50). Electrophysiological analysis (74) has indicated that estrogen directly affects thermosensitive neurons in the PO including the MPO. As shown in a previous report (39), estrogen α and β receptors are abundant in the
On the other hand, the DMH has only a few estrogen receptors (39). Our results indicate that estrogen directly affects the neurons in the MPO, which facilitates cold responses and changed the change in $T_b$ in ovariectomized rats.

(3) Estrogen application in the MPO affected the change in spontaneous activity in the cold

Spontaneous activity may have contributed to the difference in the change in $T_b$ because both values increased at the same time in the estrogen-treated rats. However, it is still disputed whether activity affects $T_b$ in the cold (19, 60). Gordon (22) suggested that activity affects heat production and loss simultaneously in small animals. Estrogen application in the MPO affects the change in activity in the cold, which may be involved in the increase of the change in $T_b$ in the cold.

(4) The number of cFos-IR cells in hypothalamic areas was not affected by estrogen application in the MPO

We counted the number of cFos-IR cells in the hypothalamus, in which the number of cFos-IR cells was reported to increase in the cold (88). Our previous study showed that the number of cFos-IR
cells during exposure to a temperature of 5 °C was augmented in the MPO and DMH in ovariectomized rats when a pharmacological level of estrogen was systemically administered (81). In addition, the reduction of $T_b$ in the cold was also attenuated in rats that received estrogen administration, as resemble to the result in the present study. However, in the present study, estrogen application in the MPO or DMH did not affect the number of cFos-IR cells in any hypothalamic areas despite the difference in the change in $T_b$ in the cold. Therefore, exposure to 10 °C may not have been strong enough to increase the number of cFos-IR cells in the hypothalamus. In addition, the augmented cFos-IR cells observed at 5 °C in the MPO and DMH might not have been related to $T_b$ changes. It was reported that exposure to $T_a$ of 4 °C increased the circulating corticosterone level as a stress response (71). Some stresses such as restraint and immobilization increase the number of cFos-IR cells in the MPO and DMH (9). Estrogen was also reported to increase the stress response in ovariectomized mice (46). Thus, estrogen may modulate the number of cFos-IR cells in the hypothalamus under severe cold stress; however, such responses are not likely to be associated with $T_b$ differences in the cold.
(5) UCP1 mRNA level in the iBAT was not influenced by
estrogen application in the MPO

We selected UCP1 in the iBAT as a molecular response to the
cold because it closely involved in non-shivering thermogenesis in
rodents (11). It has been reported that the level of UCP1 mRNA in
the iBAT increases after cold exposure: 3-h exposure at 4 °C in mice
(12) and 3-h exposure at 4 °C (84) and 1-, 5-, 48-h exposures at 5 °C
(63) in rats. In contrast, we previously reported that ovariectomized
rats showed smaller UCP1 mRNA level even after 2-h cold exposure at
5 °C (81). Various effects of the systemic administration of estrogen
on UCP1 synthesis and its mRNA level have been reported (i.e., it
increases them (59), suppresses them (61), or has no effect (41, 64).
Estrogen application in the MPO did not change total UCP1 mRNA
level in sampled tissue of the iBAT. It was reported that exposure to
the cold (4 °C for 2 h) decreased iBAT weight (47); however, the iBAT
weight did not change after the cold in either estrogen or cholesterol
application in the MPO. Although the cold stimulus was milder than
those in previous reports, the 2-h exposure would be long enough to
induce the molecular response in the iBAT in normal rats. Thus, the
influence of estrogen in the MPO on non-shivering thermogenesis in
the iBAT may have been small.
(6) Estrogen application in the MPO suppressed heat loss from tail in the cold

We also assessed the effect of local estrogen administration to the MPO on heat loss response by estimating tail surface temperature in Experiment 2 because the tail is the main effector organ for heat dissipation in rats (23). Previous studies show that systemic administration of estrogen decreased $T_{\text{tail}}$ in rats (7, 28) and mice (57) at a $T_a$ of 24–25 °C. However, in the present study, no such response was observed. $T_{\text{tail}}$ decreased in the cold, probably due to strong vasoconstriction. In addition, the estrogen application in the MPO augmented the response in ovariectomized rats. Thus, the estrogen present in the MPO may specifically increase the vasoconstriction response of the tail in the cold, decreasing $T_{\text{tail}}$ and minimizing heat loss from the tail.

(7) Tail-hiding behavior was not changed by estrogen application in the MPO

In the cold, behavioral thermoregulatory responses such as huddling and augmented food intake are seen (Gordon, 1993). In Experiment 2, we assessed tail-hiding behavior, which might be a
thermoregulatory response designed to suppress heat loss, because it is well known that the tail is involved in heat dissipation in rats (Gordon, 1990). The tail-hiding behavior increased with the cold exposure; however, the estrogen application in the MPO did not affect the tail-hiding behavior, suggesting no contribution of the behavioral response to the difference of the change in $T_b$.

(8) Conclusion

The present study demonstrated that the local administration of estrogen to the MPO affected the change in body temperature in the cold. Two possible mechanisms could be involved in this response: increased the change in spontaneous activity and the suppression of heat loss from the tail. Estrogen may affect thermoregulatory responses in the cold via estrogen receptors in the hypothalamus.

4. Effect of tail-hiding behavior on thermoregulatory responses during a cold exposure in the fed and fasted rats

4.1. Summary

Rats place their tails underneath their body trunks when cold (tail-hiding behavior). The aim of the present study was to determine
whether this behavior is necessary to maintain body temperature.

Male Wistar rats were divided into ‘fed’ and ‘42-h fasting’ groups. A one-piece tail holder (8.4 cm in length) that prevented the tail-hiding behavior or a three-piece tail holder (2.8 cm in length) that allowed for the tail-hiding behavior was attached to the tails of the rats. The rats were exposed to 27°C for 180 minutes or to 20°C for 90 minutes followed by 15°C for 90 minutes with continuous body temperature and oxygen consumption measurements. Body temperature decreased by -1.0 ± 0.1°C at 15°C only in the rats that prevented tail-hiding behavior of the 42-h fasting group, and oxygen consumption increased at 15°C in all animals. Oxygen consumption was not different between the rats that prevented tail-hiding behavior and the rats that allowed the behavior in the fed and 42-h fasting groups under ambient conditions. These results show that the tail-hiding behavior is involved in thermoregulation in the cold in fasting rats.

4.2. Method

(1) Animals

Male Wistar rats (n=80; body mass, 200–340 g; age, 7–9 week; Takasugi Experimental Animals Supply; Saitama, Japan) were used in
the study. The rats were individually housed in stainless steel cages (22500 cm$^3$) at a $T_a$ of 25°C under a 12:12 h light-dark cycle (lights on at 07:00) and were allowed free access to food and water.

(2) Surgery

The rats underwent surgery using inhalation anesthesia with diethyl ether (Sigma Aldrich; Tokyo, Japan). After making a medial skin incision, a radio transmitter device (15 × 30 × 8 mm; PhysioTel, TA10TA-F40; Data Science; St. Paul, USA), which measures body temperature ($T_b$), was implanted in the peritoneal cavity, and penicillin G (1,000 units; Meiji Pharmaceutical; Tokyo, Japan) was injected subcutaneously to prevent post-surgical infection. The rats were not used in the experiments until at least 1 week after surgery.

(3) Assessment of the tail-hiding behavior in the cold, and the influence of a tail-holder attachment on behavior (Experiment 1).

We first assessed that male rats displayed the same tail-hiding behavior in cold environments as ovariectomized rats. Rats (n=8) were individually placed in an experimental chamber that consisted of a plastic box (8960 cm$^3$) inside a cylindrical box (47608 cm$^3$). All rats
were familiarized with being in the chamber three times before the experiment was performed. Water was filled in the space between the two boxes, which was circulated with a pump connected to a temperature-controlled water reservoir. $T_a$ in the chamber was maintained at 27°C for 2 h (from 10:00 to 12:00 h) with the circulating water and was followed by varying the temperature to 15°C or 27°C for 2 h (12:00 to 14:00 h). $T_a$ reached a stable level within 30 min, which was monitored with a thermocouple located 10 cm from the bottom of the chamber. The cooling rate was maintained at a constant level. The accuracy of the set temperatures was ± 1.0°C. $T_a$ was monitored every 1 min. Images of the rat in the chamber were collected at 1-min intervals from the top of the chamber.

We assessed the tail-hiding behavior in rats (n=12), and the behavior was modulated using a tail holder attachment. Figure 1 shows the two different types of tail holders used in the study. One holder was a three-piece holder (28 mm in length; Fig. 13a), which allowed for the tail-hiding behavior (TA), and the other holder was a one-piece holder (84 mm in length; Fig. 13b), which prevented the tail-hiding behavior (TP). The holders were made of a thin copper sheet (0.5 mm thickness) and were secured with thin fabric tape. One day before the experiment, a tail holder was attached to a rat’s
tail while the rat was under anesthesia with diethyl ether. The next day, the rats were placed in the experimental chamber in the same manner as described. We evaluated the tail-hiding behavior by counting the intervals where the tail images were missing in the last 90 min of the experiment.

(4) Evaluation of the role of the tail-hiding behavior in the cold (Experiment 2).

Rats (n=64) were divided into two groups: the ad-lib fed and 42-h fasting groups. Fasting began 42 h before the experiment (at 18:00 2 days before the experiment). All rats had free access to water. Each group was further divided into two subgroups depending on the type of tail holder that was attached (TA or TP). The rats were placed in a semi-closed Plexiglas metabolic box (11655 cm³) with paper flakes at the bottom (Crecia; Tokyo, Japan) at 06:00, and the rats remained there overnight. The box was in a climate-controlled chamber (Program Incubator IN602W; Yamato Scientific; Tokyo, Japan) that was maintained at 27°C. On the next day, the $T_a$ in the chamber was set to 20°C for 90 min (12:00 - 13:30 h) followed by 15°C for 90 min (13:30 - 15:00 h) or was maintained at 27°C as a control. $T_a$ reached the set temperature within 30 min.
(5) Measurements

$T_b$ measurements were collected through a receiver board (model CTR86, DataScience; St. Paul, USA) underneath the chambers in both experimental protocols and stored in a personal computer using a data-logging program (LabView, National Instruments; Austin, USA) at 1-min intervals. $T_a$ was monitored with a thermocouple. The accuracy of the $T_b$ and $T_a$ measurements was $\pm 0.1^\circ C$. The metabolic box in Experiment 2 had an air inlet and outlet attached to an airflow system with a constant flow rate of 2100 ml/min. The difference in the oxygen tension between the air in the inlet and outlet of the box was continuously monitored with an electrochemical oxygen analyzer (model LC-700E, Toray; Tokyo, Japan), and the data were stored every minute. Oxygen consumption ($\dot{V}O_2$) was calculated as the product of the difference between the oxygen tension and flow rate divided by the post-experimental body mass (fed and fasting groups, 330 $\pm$ 16 and 232 $\pm$ 6 g, respectively) to the power of 0.75 (Brody-Kleiber formula, (33)). The data were corrected to standard temperature ($0^\circ C$) and pressure (760 mm Hg) under dry conditions.

In Experiment 2, we estimated the thermal resistance in each ambient condition as an index of the overall heat dissipation using the
following calculation: \((T_b - T_a)/\text{heat production}\) (4). Heat production was assessed using the \(\dot{V}O_2\) values and respiratory quotients at 27°C and 15°C during ad-lib feeding (0.89 and 0.80, respectively) and fasting (0.80 and 0.65, respectively; (26, 34, 38, 62).

(6) Statistical analysis

All values are shown as the mean ± SE. The values of \(T_b\) and \(\dot{V}O_2\) were averaged every 30 min during the experiments, and the average in the second 30 min while the rat was at 27°C was defined as the baseline. Differences in \(T_b\) and \(\dot{V}O_2\) among the four groups (the TA and TP subgroups in the fed and fasting groups, respectively) were assessed by a three-way ANOVA with repeated measurements (SPSS, IBM; Chicago, USA). A post-hoc analysis was conducted using Fisher’s t-test to identify significant differences at specific time points. The null hypothesis was rejected at the level of \(P<0.05\).

4.3. Results

(1) Assessment of the tail-hiding behavior in the cold, and the influence of the tail-holder attachment on behavior

(Experiment 1)
Figure 14a shows the duration that the tail hiding-behavior was observed. The duration at 27°C was 0 min in rats without the tail holder attached (holder-free group); however, the duration increased at 15°C (60.0 ± 12.3 min). The duration in the TA group also increased at 15°C (44.0 ± 4.0 min duration) without any differences in the holder-free group. Figures 13b and 13c show $T_b$ during exposure to 27°C and 15°C, respectively. $T_b$ was not different among the three groups.

(2) Evaluation of the role of the tail-hiding behavior in the cold (Experiment 2)

Figure 15 illustrates $T_b$ in the fed and fasting groups in Experiment 2. The value at time 0 denotes the baseline at 27°C in each group. The baseline was not different between the TA and TP subgroups in any exposure protocol. $T_b$ remained unchanged during a 180-min exposure at 27°C in the TA and TP subgroups of each group (Figs. 15a and 3b). At a $T_a$ of 20°C and 15°C, $T_b$ in the two subgroups of the fed group did not change from baseline (Fig. 15c). However, in the fasting group, $T_b$ at 15°C decreased from baseline in the TP subgroup, which was lower than the TA subgroup (120–180 min, Fig. 15d). $T_b$ in the TA subgroup of the fed group was not
different from the values in Experiment 1 at a given $T_a$. Body mass
decreased by $23.4 \pm 0.8 \text{ g}$ after 48 h of fasting (body mass in the fed
group was $250 \pm 3 \text{ g}$, and body mass in the fasting group was $253.8 \pm
7.0 \text{ g}$ before fasting and $230 \pm 6.5 \text{ g}$ after fasting).

Figure 16 shows $\dot{V}O_2$ in the fed and fasting groups in Experiment
2. The baseline was not different between the TA and TP subgroups
in each condition. $\dot{V}O_2$ at $15^\circ\text{C}$ increased from the baseline in the TA
and TP subgroups in the fed and fasting groups, although the
differences were not significant among the groups (Figs. 16c and 16d).

Figure 17 illustrates the relationship between $T_b$ and $\dot{V}O_2$ during
exposure at $20^\circ\text{C}$ and $15^\circ\text{C}$ in the two subgroups of the fed and fasting
groups (Figs. 17a and 17b, respectively). In the fed group, the
relationship was similar between the TA and TP subgroups (Fig. 17a).
During exposure at $20^\circ\text{C}$, $T_b$ in both subgroups reached $36.6^\circ\text{C}$ and
displayed an increased $\dot{V}O_2$, although no significant increase was
detected (Fig. 17c). $\dot{V}O_2$ increased during exposure at $15^\circ\text{C}$ without
a decrease in $T_b$. In the fasting group, the value of $T_b$ at a given $\dot{V}O_2$
was lower in the TP group compared with the TA subgroup (Fig. 17b).

Figure 18 shows the estimated thermal resistance in Experiment 2.
Thermal resistance at $27^\circ\text{C}$ was not different between the TA and TP
subgroups in the fed and fasting groups. The resistance at $20^\circ\text{C}$ and
15°C increased from the level observed at 27°C in the two subgroups of the fed and fasting groups. However, there were no differences among the four subgroups in the two groups.

4.4. Discussion

The goal of the study was to determine the role of the tail-hiding behavior in rats, which is observed during cold exposure. We determined that fasting rats with the tail-hiding behavior prevented could not maintain their T_b in the cold. This result indicates that the behavior serves a thermoregulatory process.

(1) Tail-hiding behavior in male rats in the cold

We assessed the tail-hiding behavior during cold exposure in male rats. The behavior was not observed at 27°C in fed or fasting rats in Experiment 1; however, the duration of the tail-hiding behavior increased when the T_a was 15°C. The surface area of the tail is approximately 7% of the total body surface area of a rat (Gordon 1993). Even in thermoneutral conditions, where autonomic thermoregulatory responses are small, the amount of heat dissipated from the tail surface is 20% greater than from the total body surface (Gordon 1993). Therefore, we assumed that the tail-hiding behavior decreased heat
dissipation in cold environments.

(2) Thermoregulatory responses in rats with the tail holders

The tail holders used in the study modulated the rats’ tail-hiding behavior, as shown in Fig. 13. In addition, baseline $T_b$ at 27°C was not different among rats with different types of tail holders or without the tail holder. Therefore, the tail holders had no influence on heat loss from the tail at a $T_a$ of 27°C.

The tail-hiding behavior was less important for the fed animals because $T_b$ was controlled during exposure to 20°C and 15°C (Fig. 15c) with increases in $\dot{V}O_2$, which was observed in the TA and TP subgroups (Fig. 16c). The relationships between $T_b$ and $\dot{V}O_2$ in the cold (Fig. 17a) and thermal resistance (Fig. 18c) indicated that the effect of the tail-hiding behavior on thermoregulation was small during ad-lib feeding. These results suggest that heat dissipation was suppressed by vasoconstriction of the tail skin in the TP subgroup and by the tail-hiding behavior in the TA subgroup, although we did not assess the skin temperature of the tail.

The tail-hiding behavior was important for the fasting animals. At 15°C, the rats were able to maintain their $T_b$ by employing the tail-hiding behavior (the TA subgroup; Fig. 15d); however, the $T_b$ in the
rats that were unable to perform the tail-hiding behavior (TP subgroup) decreased. These results indicate that the tail-hiding behavior is involved in thermoregulation in the cold during fasting.

In contrast to the findings of a previous report (49), the baseline $\dot{V}O_2$ at 27°C was not attenuated in the fasting group (Fig 15b). One possible reason for this is the difference in $T_a$ between the two study conditions. $\dot{V}O_2$ was previously assessed at a $T_a$ of 23°C (49). In addition, 65-h fasting decreased the threshold $T_b$ for metabolic heat production in rats (69). A $T_a$ of 20°C may be cold enough to activate $\dot{V}O_2$ in fed rats; however, it is not enough in fasting rats, which may have caused the observed differences in $\dot{V}O_2$.

We also expected that 42-h fasting would attenuate $\dot{V}O_2$ in the cold. Fasting decreased shivering thermogenesis at 10°C in anesthetized rats (32) and decreased the weight of brown adipose tissue that is related to non-shivering thermogenesis (11, 24, 25). However, in this study, $\dot{V}O_2$ increased in the cold in the fed and fasting rats without any differences between the two feeding conditions and two types of tail holders (Figs. 16c and 16d). It is believed that a reduction in $T_b$ and $T_a$ are factors that induce metabolic heat production. The analysis of the relationship between $T_b$ and $\dot{V}O_2$ in the cold (Fig. 17b) suggests that a reduction in $T_b$ has little effect on $\dot{V}O_2$; however, a reduction in
$T_a$ increased $\dot{V}O_2$. These phenomena may be explained by a reduction of the threshold of body temperature for metabolic heat production, as previously reported (69). Feeding conditions affect thermoregulation in the cold. In particular, fasting may blunt the response of metabolic heat production to a reduction in $T_b$. However, fasting may not abolish the tail-hiding behavior, which prevents $T_b$ reduction.

Fasting is also a strong stimulus that suppresses heat loss from the tail, probably through vasoconstriction (49). Because we did not assess the surface temperature of the tail skin, it is unknown if fasting rats could produce further vasoconstriction in the cold. Thermal resistance during cold exposure with fasting was not different between the TA and TP groups (Fig. 18d). In addition, the level was similar to the fed group. These results may indicate that the level of tail vasoconstriction during fasting was not affected by the cold. Moreover, the tail-hiding behavior is necessary to prevent heat dissipation from the tail.

Despite similar levels of thermal resistance and $\dot{V}O_2$ between the fed and fasting groups, $T_b$ only decreased in the TP subgroup in the fasting group. This result may indicate that we overestimated the value of thermal resistance for the TP subgroup during fasting. $T_b$
was not stable during cold exposure at 20°C and 15°C in the fasting group (Fig. 17b). Therefore, heat flux was not stable, and the estimated resistance values were not accurate. Although we did not assess behavioral responses to the cold during fasting, fasting may have increased the duration of the tail-hiding behavior, which prevents heat dissipation in the cold. Therefore, the TP subgroup resulted in hypothermia because of a lack of the tail-hiding behavior. When the metabolic responses are impaired, such as during fasting, the tail-hiding behavior may be an important mechanism of thermoregulation and directly affect $T_b$.

The present study demonstrated that rats change their tail position based on the ambient temperature and their energy state to achieve thermoregulation. The behavior is preserved during fasting and is important in maintaining body temperature, although the mechanism is currently unknown.

(3) Limitations and future directions

Behavioral thermoregulation is crucial for energy savings in humans and animals. To assess behavioral thermoregulation in animals, thermal gradients (2) and operant systems (1, 13, 27) have been used. However, these systems have limited utility for
estimating behavioral thermoregulation. First, in the thermal gradient system, we cannot assess the effectiveness and/or behavioral response because animals stay still in their preferred temperature. Second, operant behavior is not a natural behavior for thermoregulation. In addition, animals need training to obtain the behavior and some animals fail, and there have been few studies that have assessed natural behaviors such as huddling in the cold (36). The tail-hiding behavior, which is a natural behavior, may be a simple indicator in assessing behavioral thermoregulation in rats.

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### 7. Figure legends

Figure 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

Figure 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
Figure 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 (58).

Figure 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 ± SE [A n = 7 (4 in the E₂ (+) group and 3 in the E₂ (-) group), B n = 25 (15 in the E₂ (+) group and 10 in the E₂ (-) group), C n = 16 (7 in the P group and 9 in the D₁ group), D n = 20 (10 in the P group and 10 in the D₁ group)]. *Significant difference between the E₂ (+) and E₂ (-) groups or the P and D₁ groups, P<0.05. MnPO Median preoptic nucleus, VMH ventromedial hypothalamus, Tₐ ambient temperature.

Figure 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 ± SE [A n = 34 (10 in the E\textsubscript{2} (+) group and 9 in the E\textsubscript{2} (-) group after 5°C exposure, and 7 in the E\textsubscript{2} (+) group and 8 in the E\textsubscript{2} (-) group after 25°C exposure; B n = 34 (9 in the P group and 10 in the D\textsubscript{1} group after 5°C exposure, and 7 in the P group and 8 in the D\textsubscript{1} group after 25°C exposure)]. *Significant difference between the E\textsubscript{2} (+) and E\textsubscript{2} (-) groups or between the P and D\textsubscript{1} groups, P<0.05. # Significant difference between the P and E\textsubscript{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.

Figure 6. Estrogen and cholesterol application sites in Experiments 1 (A) and 2 (B). The diagrams were modified from the stereotaxic atlas (58) and show coronal sections including the MPO and DMH. MPO, Medial preoptic nucleus; DMH, dorsomedial hypothalamic nucleus.

Figure 7. Images of brain sections including the central (a) and rostral (b) parts of the MPO in Experiment 1 (A, B, and C) are shown. The dark dots indicate estrogen receptor α immunoreactive cells in
ovariectomized rats (A) and estrogen-treated rats in the MPO (B) and DMH (C) groups. MPO, medial preoptic nucleus; DMH; dorsomedial hypothalamic nucleus; central MPO, the central part of the MPO (bregma −0.8 mm); rostral MPO, the rostral part of the MPO (bregma −0.1 mm); OVX, ovariectomy; 3V, the third ventricle. The diagrams are modified from the stereotaxic atlas (58). The squares in the diagrams denote the area in which the image was taken.

Figure 8. Changes in body temperature ($T_b$) from the baseline at 25 (A and B) and 10 °C (C and D) and changes in spontaneous activity from the baseline at 25 (E and F) and 10 °C (G and H) in Experiment 1. Values are means±SE (A, B, and D, n=16 rats each/ estrogen=8 and control=8; C, n=20 rats/estrogen=10 and control=10; E, F, and H, n=16 rats each/estrogen=8 and control=8; G, n=20 rats/estrogen=10 and control=10). The baseline $T_b$ was not different between the control and estrogen-treated rats in the MPO group (37.5±0.0 and 37.4±0.0 °C, respectively) or the DMH group (37.3±0.0 and 37.5±0.0 °C, respectively). *Significant difference between the estrogen-treated and control rats, P<0.05. The arrows in C and G indicate the time point at which both the change in body temperature and spontaneous activity increased simultaneously.
Figure 9. Photoimages of brain sections. Coronal sections, including the MPO (A-a, b) and DMH (A-c, d) in the MPO group, and the MPO (B-a, b) and DMH (B-c, d) in the DMH group. The rats were exposed to 10 °C for 2 h. Cells stained in dark denote cFos immunoreactive cells. The drawings are modified from the stereotaxic atlas (58).

Figure 10. Counts of cFos immunoreactive cells in the hypothalamus after exposure to 25 (A and B) and 10 °C (C and D) in Experiment 1. The counts were the average for 3 consequent sections. Values are means±SE (A and B, n=12 rats each/ estrogen=6 and control=6; C, n=24 rats/estrogen=12 and control=12; D, n=12 rats/estrogen=6 and control=6). MnPO, median preoptic nucleus; VMH, ventromedial hypothalamus; PH, posterior hypothalamus.

Figure 11. UCP1 mRNA level with respect to GAPDH mRNA level in the iBAT in Experiment 1. Values are means±SE (A, n=32 rats/ estrogen=10 and control=10 after exposure to 10 °C, and estrogen=6 and control=6 after exposure to 25 °C; B, n=27 rats/ estrogen=6 and control=10 after exposure to 10 °C, and estrogen=5 and control=6 after exposure to 25 °C). #Significantly different between the 10 and
25 °C exposures, P<0.05.

Figure 12. Changes in tail surface temperature (T\text{\textsubscript{Tail}}) from the baseline at 25 (A) and 10 °C (B) and the time for which tail-hiding behavior was observed (C) in Experiment 2. Values are means±SE (A and B, n=16 rats each/estrogen=8 and control=8; C, n=16 rats/estrogen=8 and control=8 after exposure to 10 °C, and n=16 rats/estrogen=8 and control=8 after exposure to 25 °C). #Significantly different between the 10 and 25 °C exposures, P<0.05. *Significantly different between the estrogen-treated and control rats, P<0.05.

Figure 13. Tail-holders that allowed (TA, a) and prevented the tail-hiding behavior (TP, b). The holders were constructed out of a thin copper sheet, which consisted of three pieces in the TA group and one piece in the TP group.

Figure 14. The duration that the tail-hiding behavior was observed (a) and core body temperature (T\text{\textsubscript{b}}) in the last 90 min of the exposure at 27°C (b) and 15°C (c) in Experiment 1. Values are the means ± SE (n=16; 4 in holder-free group, n=8 in the TA group, and n=8 in TP group). *Significant difference between the groups, P<0.05.
Figure 15. Core body temperature ($T_b$) during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2. Values are the means ± SE (n=8 in the TA and TP subgroups in each group, respectively). *Significant difference between the TA and TP subgroups, P<0.05. #Significant difference from the baseline value at 0 min, P<0.05.

Figure 16. Oxygen consumption ($\dot{V}O_2$) during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2. Values are the means ± SE (n=8 in the TA and TP subgroups in each group, respectively). #Significant differences from the baseline shown at 0 min, P<0.05.

Figure 17. Relationship between the core body temperature ($T_b$) and oxygen consumption ($\dot{V}O_2$) during exposure at 27°C, 20°C and 15°C in the fed (a) and fasting groups (b) in Experiment 2. Each point represents the means ± SE for 8 rats during a 30-min recording period. The arrows indicate the time order (time of 0, 30, 60, 90, 120, 150 and
180 min). Numbers next to symbols indicate the ambient temperature that the data were obtained.

Figure 18. Thermal resistance during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2. Values are the means ± SE (n=8 in the TA and TP subgroups in each group, respectively). #Significant differences from the baseline shown at 0 min, P<0.05.
Figure 1. Changes in body core temperature ($T_b$) at $25^\circ$C (A, C), and $5^\circ$C (B, D) in Experiments 1 and 2.
Figure 2. Plasma levels of estradiol (A, C) and progesterone (B, D) in Experiments 1 and 2
Figure 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.
Figure 4. Counts of c-Fos immunoreactive cells in the hypothalamic areas
Figure 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.
Figure 6. Estrogen and cholesterol application sites in Experiments 1 (A) and 2 (B)
Figure 7. Images of brain sections including the central (a) and rostral (b) parts of the MPO in Experiment 1 (A, B, and C)
Figure 8. Changes in body temperature (Tb) from the baseline at 25 (A and B) and 10 °C (C and D) and changes in spontaneous activity from the baseline at 25 (E and F) and 10 °C (G and H) in Experiment 1.
Figure 9. Photoimages of brain sections. Coronal sections, including the MPO (A-a, b) and DMH (A-c, d) in the MPO group, and the MPO (B-a, b) and DMH (B-c, d) in the DMH group.

Figure 10. Counts of cFos immunoreactive cells in the hypothalamus after exposure to 25 °C (A and B) and 10 °C (C and D) in Experiment 1.
Figure 11. UCP1 mRNA level with respect to GAPDH mRNA level in the iBAT in Experiment 1.
Figure 12. Changes in tail surface temperature (T_{tail}) from the baseline at 25 °C (A) and 10 °C (B) and the time for which tail-hiding behavior was observed (C) in Experiment 2.
Figure 13. Tail-holders that allowed (TA, a) and prevented the tail-hiding behavior (TP, b)

Figure 14. The duration that the tail-hiding behavior was observed (a) and core body temperature ($T_b$) in the last 90 min of the exposure at 27°C (b) and 15°C (c) in Experiment 1
Figure 15. Core body temperature (Tb) during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2.
Figure 16. Oxygen consumption (VO2) during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2.
Figure 17. Relationship between the core body temperature (Tb) and oxygen consumption (VO2) during exposure at 27°C, 20°C and 15°C in the fed (a) and fasting groups (b) in Experiment 2.
Figure 18. Thermal resistance during exposure at 27°C in the fed and fasting groups (a and b, respectively) and at 20°C and 15°C in the fed and fasting groups (c and d, respectively) in Experiment 2.