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Hypothalamic neuronal histamine signaling in the estrogen deficiency-induced obesity

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Abstract

Menopause is one of the triggers that induce obesity. Estradiol (E2), corticotropin-releasing hormone (CRH), and hypothalamic neuronal histamine are anorexigenic substances within the hypothalamus. This study examined the interactions among E2, CRH, and histamine during the regulation of feeding behavior and obesity in rodents. Food intake was measured in rats after the treatment of E2, α-fluoromethyl histidine, a specific suicide inhibitor of histidine decarboxylase that depletes hypothalamic neuronal histamine, or CRH antagonist. We measured food intake and body weight in wild-type mice or mice with targeted disruption of the histamine receptors (H1-R) knockout (H1KO mice). Furthermore, we investigated CRH content and histamine turnover in the hypothalamus after the E2 treatment or ovariectomy (OVX). We used immunohistochemical staining for estrogen receptors (ERs) in the histamine neurons. The E2-induced suppression of feeding was partially attenuated in rats pre-treated with α-fluoromethyl histidine or CRH antagonist and in H1KO mice. E2 treatment increased CRH content and histamine turnover in the hypothalamus. OVX increased food intake and body weight, and decreased CRH content and histamine turnover in the hypothalamus. In addition, E2 replacement reversed the OVX-induced changes in food intake and body weight in wild-type mice but not in H1KO mice. Immunohistochemical analysis revealed ERs were expressed on histamine neurons and western blotting analysis and pre-absorption study confirmed the specificity of ER antiserum we used. These results indicate that CRH and hypothalamic neuronal histamine mediate the suppressive effects of E2 on feeding behavior and body weight.

Keywords: feeding, hypothalamus, histamine, obesity, oestrogen.

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Menopause is one of the triggers that induce a variety of age-related diseases such as osteoporosis, atherosclerosis, and obesity (Genazzani and Gambacciani 2001). The incidence of obesity is higher in post-menopausal women than in age-matched pre-menopausal women; this increase is thought to be associated with the decline of estrogen levels after menopause. In fact, estrogen replacement therapy is an effective treatment to reduce body weight and fat accumulation in post-menopausal women (Svendsen *et al.* 1995). In rats, estrogen treatment reduces energy intake (Donohoe and Stevens 1983), and it has been demonstrated that ovariectomy (OVX) induces an increase in body weight, which can be reversed with estrogen replacement treatment (Wade *et al.* 1985). These studies suggest that estrogen plays an important role in the post-menopausal development of obesity.

The paraventricular nucleus (PVN) within the hypothalamus is a major center of corticotropin-releasing hormone (CRH) neurons (Sawchenko and Swanson 1985). Previous research has demonstrated that estrogen receptors (ERs) and CRH neurons are co-localized within the PVN (Dagnault and

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Abbreviations used: α-hCRH, α-helical CRH; CRH, corticotropin-releasing hormone; DMSO, dimethylsulfoxide; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; ER, estrogen receptor; FMH, α-fluoromethyl histidine; H1KO, histamine receptors knockout; H1-R, histamine receptors; HDC, histidine decarboxylase; i3vt, intra-3rd ventricular; OVX, ovariectomy; PBS, phosphate-buffered saline; PPT, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; t-MH, tele-methylhistamine; TMN, tuberomammillary nucleus.

Richard 1997), and antagonists of CRH have been shown to prevent estradiol (E2)-induced reduction of food intake (Dagnault *et al.* 1993). These results indicate that CRH neurons are a target for the anorectic action of estrogen. On the other hand, it is also the case that E2 still inhibits eating and reduces weight in rats with PVN lesions, indicating that this is not a necessary contribution (Dagnault and Richard 1994).

Neuronal histamine is another brain substance that suppresses appetite. It is mainly produced in the tuberomammillary nucleus (TMN) of the posterior hypothalamus, which has diffuse projections throughout the brain, including the PVN and ventromedial hypothalamic nucleus, also known as the satiety center. Neuronal histamine inhibits feeding behavior via an interaction with H1-receptors (H1-R) in these nuclei (Fukagawa *et al.* 1989). Although histamine is a leading candidate for an anti-obesity drug, peripheral histamine is unable to cross the blood–brain barrier in sufficient quantities. Histidine, a precursor for the synthesis of histamine, is an essential amino acid and abundant in red meat fish such as tuna and bonito (Kasaoka *et al.* 2004). The peripheral injection of histidine suppresses food intake in rats and this histidine-induced suppression diminishes after pre-treatment with an inhibitor of histidine decarboxylase (HDC), α-fluoromethyl histidine (FMH), which indicates that the conversion of histidine to histamine by HDC is necessary to produce the feeding response (Orthen-Gambill 1988; Yoshimatsu *et al.* 2002; Watanabe *et al.* 1990). In fact, histidine treatment induces an elevation of histamine levels within the hypothalamus in rats (Yoshimatsu *et al.* 2002).

It has been reported that the amount of dietary intake was significantly lower for rats fed a histidine-enriched diet than for those fed a control diet and the suppressive effect of histidine on food intake was less in ovariectomized rats than in female rats (Kasaoka *et al.* 2005). Moreover, previous researches have revealed that histamine neurons display immunoreactivity for ER-α in rats and some CRH neurons in the PVN are co-localized with ERα in human (Fekete *et al.* 1999; Bao *et al.* 2005). Furthermore, ERβ mRNA is expressed in various regions of the rat brain, including the TMN (Shughrue *et al.* 1996, 1997). Thus, these results suggest that estrogen may up-regulate histamine and CRH synthesis in the hypothalamus. Recently, we reported that CRH directly activates histaminergic neurons in the TMN via CRH type 1 receptors (CRH1-R) (Gotoh *et al.* 2005). From these behavioral and neuroanatomical studies, we hypothesized that estrogen, CRH, and hypothalamic neuronal histamine may constitute a neuronal network within the hypothalamus that regulates food intake and obesity.

We examined this hypothesis from several different perspectives. First, we investigated whether the anorectic effect of estrogen is modulated in rats pre-treated with FMH, a suicide inhibitor of histamine synthesizing HDC, or α-helical $CRH_{9–41}$ (α-hCRH), a competitive CRH receptor antagonist. We also examined the effect of estrogen treatment on food intake in H1-R knockout (H1KO) mice. Second, we examined whether the administration of estrogen or the surgical ablation of estrogen production (OVX) affects CRH content and histamine turnover in the hypothalamus. We also examined whether E2 replacement after OVX modulates the suppressive effect of E2 on food intake and body weight, in both rats and H1KO mice. Finally, we determined whether ERα and ERβ are expressed in the histaminergic neurons of the TMN.

Materials and methods

Animals

Female Sprague–Dawley rats (250–280 g; Seac Yoshitomi, Fukuoka, Japan) and female H1KO mice (25–30 g; Kyushu University, Fukuoka, Japan) were housed in a room with daily illumination from 7 AM to 7 PM (12-h light–dark cycle) and maintained at 21 ± 1°C with 55 ± 5% humidity. H1KO mice for backcrossing were maintained at Oita University (Yufu, Japan). Backcrossing H1-R−/− homozygous mice with the strain C57BL/6N (Kyudo, Fukuoka, Japan) for six generations resulted in incipient congenital N5 mice of two genotypes (H1-R−/− and H1-R+/+), which were used in this study. All genotypes were confirmed by Southern blot analysis, as described in a previous report (Inoue *et al.* 1996). Animals were permitted *ad libitum* access to standard chow (Clea chow, Clea, Japan) and tap water. All rats and mice were handled for 5 min each on four successive days to equilibrate their arousal levels before the experiment (Sakata 1982). On the testing day, the animals had recovered to at least their pre-treatment body weight. All studies were conducted in accordance with the Oita University Guidelines, which are based on the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

Surgical procedures

Ten days before beginning treatment, the rats were anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) and placed in a stereotaxic apparatus (Narishige, Tokyo, Japan). A stainless steel cannula (23 gauge, 15 mm long) was inserted into the third ventricle at the midline (6.0 mm anterior to ear bar zero, 7.8 mm below the cortical surface) according to the atlas developed by Paxinos and Watson (1986). Details of the surgical procedure have been described previously (Sakata *et al.* 1981). A stainless steel wire stylet (30 gauge) was inserted into the guide cannula to prevent leakage of CSF and obstruction of the cannula.

Rats were all treated at diestrus stage of the estrus cycle to avoid the influence of endogenous E2 on feeding, other hypothalamic hormones, and ER expression at 9 AM. Stage of estrus cycle was determined by examining the appearance and abundance of cells within vaginal cytology samples. Diestrus stage was characterized by leukocytes and clusters of cornified cells and estrus was characterized by abundance of cornified cells.

On the other hand, using mice, bilateral OVX was performed through an abdominal incision down the midline, under sterile conditions. The sham operation was performed using the same procedure as OVX, except that the ovaries were not excised. All mice received either an OVX or sham operation 3 weeks before the beginning of the experiment.

Reagents

On the day of the experiment, α-hCRH (1 μg/μL), pargyline hydrochloride (1 mmol/mL; Sigma, St. Louis, MO, USA), and FMH (100 mg/mL; Research Biochemical International, Natick, MA, USA) were freshly dissolved in phosphate-buffered saline (PBS). Estradiol benzoate (E2; Sigma), 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; 100 μg/0.1 mL), a specific ERα agonist (Tocris, Ellisville, MO, USA) and 2,3-bis(4-hydroxy-phenyl)-propionitrile (DPN; 500 μg/0.1 mL), a specific ERβ agonist (Tocris), were dissolved in dimethylsulfoxide (DMSO; 0.1 mL; Wako, Osaka, Japan). The pH of each solution was adjusted to 6.5–7.5.

Measuring food intake

Estradiol-regulated changes in food intake in histamine-depleted rats

Food intake was measured for 1- and 24-h periods after the i.p. administration of DMSO, E2, FMH, or FMH followed by E2 with gonadally intact rats. Each treatment group consisted of six rats matched by body weight. Food was withheld for 24 h before the experiment. On the day of the experiment, FMH (50 mg/kg/0.1 mL, i.p.: a dose previously determined to deplete most neuronal histamine in the hypothalamus) was administered 2 h before treatment with E2 (25 μg/kg/0.1 mL, i.p.), which is a physiological dose, or DMSO (0.1 mL, i.p.) (Flanagan-Cato *et al.* 2001; Santollo *et al.* 2007). In groups not pre-treated with FMH, an equal volume of PBS was administered 2 h before i.p. infusion with E2 or DMSO.

Estradiol-regulated changes in food intake in gonadally intact rats after pre-treatment with α-hCRH

Food intake was measured for 1- and 24-h periods after the administration of PBS, E2, α-hCRH, or α-hCRH followed by E2 with intact-female rats. Each treatment group consisted of six rats matched by body weight. Food was removed 24 h before the experiment. On the day of the experiment, α-hCRH (10 μg/10 μL/10 min) was centrally administered 2 h before i.p. administration with E2 (25 μg/kg/0.1 mL) or DMSO (0.1 mL). In groups not pre-treated with α-hCRH, an equal volume of PBS was administered 2 h before i.p. administration with E2 or DMSO.

Estradiol-regulated changes in food intake in H1-R knockout mice

Food was withheld for 24 h before the experiment. On the day of the experiment, H1KO (n = 6) or wild-type mice (n = 6) were given an i.p. administration of E2 (25 μg/kg/10 μL) or DMSO (10 μL). Food intake was then measured over 1- and 24-h periods following the treatment.

Measuring CRH and tele-methylhistamine contents in the hypothalamus

Estradiol-regulated changes in CRH and tele-methylhistamine contents

Sixty-six rats were divided into 11 groups of six rats each, matched by body weight. All rats received either an OVX or sham operation at the same time and were allowed to recover for 3 weeks. On the day of the experiment, all groups were administered pargyline hydrochloride (0.33 mmol/kg, i.p.) 2 h prior to the start of the experiment. Pargyline hydrochloride inhibits monoamine oxidase B, which induces the extraneuronal accumulation of tele-methylhistamine (t-MH), a major metabolite of released neuronal histamine.

At the start of the experiment, the drugs were administered as follows: Group 1 – Sham-OVX, PBS [10 μL, intra-3rd ventricular (i3vt)] followed by E2 (25 μg/kg/0.1 mL, i.p.); Groups 2 and 3 – OVX, PBS (10 μL, i3vt) followed by DMSO (0.1 mL, i.p.); Groups 4 and 5 – Sham-OVX, PBS (10 μL, i3vt) followed by DMSO (0.1 mL, i.p.); Groups 6 and 7 – Sham-OVX, α-hCRH (10 μg/10 μL, i3vt) followed by E2 (25 μg/kg/0.1 mL, i.p.); Groups 8 and 9 – Sham-OVX, PPT, a specific ERα agonist (100 μg/0.1 mL, i.p.); and Groups 10 and 11 – Sham-OVX, DPN, a specific ERβ agonist (500 μg/0.1 mL, i.p.). Treatments groups were duplicated to allow for the measurement of CRH and t-MH. A 2-h gap was left between the pre-treatment and the E2, PPT, DPN, or DMSO treatment.

All rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then exsanguinated following transcardiac perfusion with 100 mL of saline containing 200 U of heparin. After decapitation, sections containing the hypothalamus were cut based on the rat brain map developed by Paxinos and Watson (1986) for measuring t-MH contents. The tissue blocks were collected, placed in 400 μL of 0.5 M acetic acid, and homogenized. The homogenate was boiled for 10 min, and a 50-μL aliquot was removed for protein assay (Bio-Rad, Hercules, CA, USA). CRH contents were measured in punches from PVN specific sections in hypothalamus, using a CRH radioimmuno assay kit (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). t-MH contents were assayed via HPLC, using the deproteinized supernatants containing the amine extracts. The details of amine assays have been described elsewhere (Sakata *et al.* 1981).

Measuring food intake and body weight in H1KO or wild-type mice after OVX and E2 treatment and plasma estrogen levels

H1-R knockout or wild-type mice were divided into the following groups: (i) wild-type mice after the sham operation (wild-sham, n = 6), (ii) wild-type mice after OVX (wild-OVX, n = 6), (iii) H1KO mice after the sham operation (H1KO-sham, n = 6), and (iv) H1KO mice after OVX (H1KO-OVX, n = 6). E2 (25 μg/kg/10 μL, i.p.) was administered daily to the wild-OVX and H1KO-OVX groups and DMSO (10 μL, i.p.) was administered daily to the wild-sham and H1KO-sham for 1 week after the post-operative 3-week recovery. Feeding rhythm was measured during both the dark and light phases 3 weeks after the operation. Moreover, the daily food intake and body weight were measured for all mice throughout the 4-week period. Finally, blood of all mice was obtained by cardiac puncture and centrifuged at 400 g for 10 min at 4°C. Plasma was immediately frozen and stored at −80°C until analyzed using oestraiol enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI, USA).

Immunohistochemistry

Diestrus female rats (n = 24) were anesthetized with 50 mg/kg sodium pentobarbital i.p. and then transcardiacally perfused with 50 mL of PBS containing 50 U of heparin, followed by 50 mL of 4% p-formaldehyde in ice-cold PBS. The brains were removed and divided into three segments, i.e. the forebrain, the diencephalons, and the brainstem. Next, the specimens were frozen rapidly at −80°C and stained for ERα, ERβ (n = 6, in each), and negative control of ERα or ERβ (n = 6, in each). Frozen sections were cut from hypothalami at a thickness of 40 μm using a cryostat at −20°C. The sections were incubated overnight at 4°C with polyclonal rabbit

antiserum against HDC (specificity for rat, 1 : 2000; Chemicon Inc., Temecula, CA, USA), followed by detection with biotin-conjugated goat anti-rabbit IgG and FITC-conjugated streptavidin (ABC reagent; Vector Laboratories, Burlingame, CA, USA). The sections were then incubated overnight at 4°C with polyclonal rabbit antiserum against the last 15 amino acids ERα (specificity for rat, 1 : 50 000; Upstate, Lake Placid, NY, USA) and against a peptide corresponding to amino acids 467–485 of the carboxy terminus of ERβ (specificity for rat, 1 : 2000; Affinity BioReagents Inc., Golden, CO, USA) in a buffer of 0.3% Triton X-100 and 1% normal goat serum (NGS) (Cardona-Gomez *et al.* 2000; Somponpun *et al.* 2004). Next, the sections were exposed to biotin-conjugated goat anti-rabbit IgG (ABC reagent; Vector Laboratories) and rhodamine-conjugated streptavidin (ABC reagent; Vector Laboratories). For each experiment, a negative control (single staining for HDC) was performed in which one primary antibody (ERα or ERβ) was replaced with normal serum, followed by incubation with both secondary antibodies. Moreover, to determine the specificity of ERα and ERβ antiserum, each antiserum was pre-incubated with recombinant ERα or ERβ protein. Increasing amounts of protein were added to a fixed concentration of ERα and ERβ antiserum at a 1 : 1, 1 : 5, and 1 : 10 molar concentration of each antiserum to molar concentration of each recombinant protein. The stained sections were examined under a confocal immunofluorescence microscope (Olympus, Tokyo, Japan) and analyzed with imaging software (Lumina Vision; Mitsutani Corp., Tokyo, Japan). The FITC-stained and rhodamine-stained areas were visualized as green and red signals, respectively. The resultant digital images of the same section could be manipulated using reconstruction techniques. The images were not adjusted or altered in any way, except an occasional adjustment for brightness. In addition the ratio of signal co-expression was also assessed by counting single- and double-labeled HDC positive neurons. This analysis included every fifth section from serial sample taken through the posterior hypothalamus of six rats (seven sections from each animal). ERα and ERβ immunoreactivity was determined by drawing an area of TMN in the hypothalamus and applying an optical density scale using automated counting tools from imaging software (Lumina Vision; Mitsutani Corp.).

Western blotting analysis

To confirm the molecular weight of proteins recognized by the ERα and ERβ antibodies used in the immunohistochemistry experiments, we performed western blotting analysis with solutions containing baculovirus system derived, human recombinant full-length ERα (~66.4 kDa; Sigma) and human recombinant full-length ERβ (~53.4 kDa; Sigma). The recombinant proteins were diluted to their final protein concentrations in sample buffer (50 mM Tris–HCl, pH 8.0, 10% glycerol, 1 mM EDTA, 2 mM dithiothreitol, and 1 mM *o*-vanadate). All samples were heated at 94°C for 4 min and then centrifuged (12 000 *g*). The samples were separated on 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred electrophoretically onto a polyvinylidene difluoride membrane. After blocking with 0.5% non-fat milk, the membrane was incubated with the same antiserum against ERα and ERβ used in the immunohistochemistry experiments overnight at 4°C. After repeated washing, the membrane was incubated with goat anti-rabbit IgG. Immunopositive bands were visualized using enhanced chemiluminescence after exposure to Hyperfilm (GE Healthcare Bioscience, Piscataway, NJ, USA).

Statistics

The data are expressed as mean ± SEM. Statistical significance was evaluated using two-way ANOVA followed by Scheffe's test for *post hoc* comparison. For all tests, the level of significance was set at $p < 0.05$.

Results

Effect of pre-treatment with FMH or α-hCRH on the E2-induced suppression of food intake

Estradiol treatment significantly decreased the cumulative food intake compared with the control level in the 1- and 24-h periods, and pre-treatment with FMH partially attenuated the E2-induced suppression of food intake. The administration of FMH alone did not affect the cumulative food intake compared with the control level (Fig. 1a and b). The E2-induced suppression of food intake was also attenuated by pre-treatment with α-hCRH, and treatment with α-hCRH alone did not alter feeding compared with the control group (Fig. 1c and d).

Attenuation of the anorexic effects of E2 in H1KO mice

Estradiol treatment caused a significant reduction in food intake in the wild-type mice (40% in 1 h and 25% in 24 h), whereas the same treatment had a significantly lesser effect in the H1KO mice (20% in 1 h and 13% in 24 h; Fig. 1e and f).

Effect of E2 infusion on CRH and t-MH contents in the hypothalamus

Estradiol treatment significantly increased the CRH content in the PVN compared with the control level (Fig. 2a). E2 treatment also increased the pargyline-induced accumulation of t-MH, and pre-treatment with α-hCRH attenuated this effect (Fig. 2a and b).

Effect of selective estrogen receptor agonists infusions on CRH and t-MH contents in the hypothalamus

The ERα agonist PPT treatment significantly increased the CRH content in the PVN and the pargyline-induced accumulation of t-MH compared with the control level, but not the ERβ agonist DPN (Fig. 2c and d).

Effect of E2 treatment on food intake, body weight and plasma estrogen concentration in wild-OVX or H1KO-OVX mice

There was no difference in daily food intake between wild-sham and H1KO-sham mice. Daily food intake and body weight increased significantly in both wild-OVX and H1KO-OVX mice when compared with the respective control values

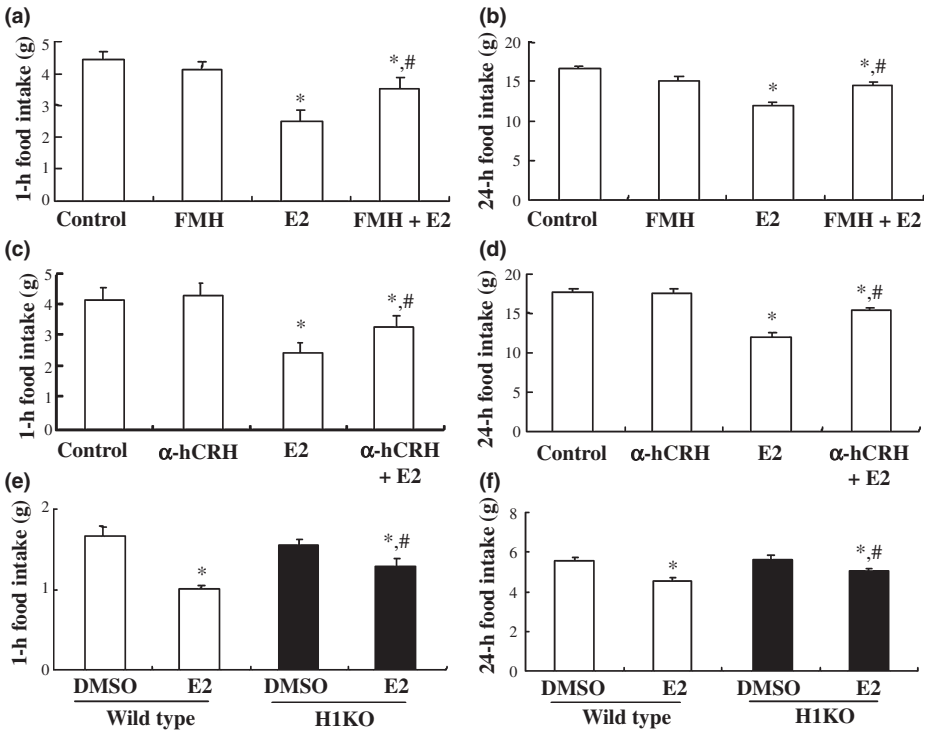

Fig. 1 Food intake for the 1-h (a) and 24-h (b) periods after administration of FMH, E2, or FMH followed by E2. Control, PBS followed by DMSO; FMH, FMH followed by DMSO; E2, PBS followed by E2; FMH + E2, FMH followed by E2; $^{*}p < 0.01$ versus control and FMH and $^{\#}p < 0.05$ versus E2. Food intake for the 1-h (c) and 24-h (d) periods after administration of α-hCRH, E2, or α-hCRH followed by E2. α-hCRH, α-hCRH followed by DMSO; α-hCRH + E2, α-hCRH followed by E2; $^{*}p < 0.05$ versus control and α-hCRH and $^{\#}p < 0.05$ versus E2. Food intake for the 1-h (e) and 24-h (f) periods after E2 administration in wild-type and H1KO mice. DMSO, i.p. administration of DMSO; E2, i.p. administration of E2; $^{*}p < 0.05$ versus DMSO (wild-type and H1KO) and $^{\#}p < 0.05$ versus E2 (wild-type).

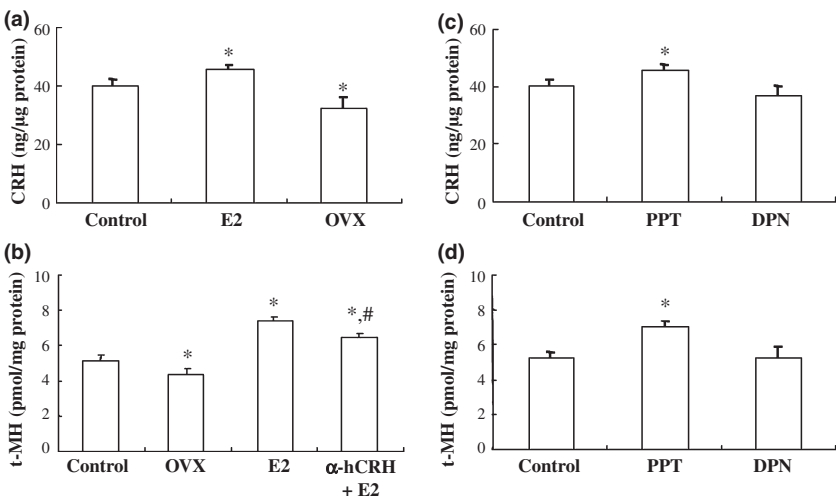

Fig. 2 The CRH (a) contents in the PVN of the hypothalamus after i.p. infusion with DMSO or E2 in sham-operated and OVX rats. t-MH (b) contents in the hypothalamus after i.p. infusion with either E2 or α-hCRH followed by E2, in sham-operated and OVX rats. Rats underwent the designated surgical procedures and were allowed to recover for 3 weeks. Control, PBS followed by DMSO; E2, PBS followed by E2; OVX, PBS followed by DMSO after 3 weeks of post-operative recovery, α-hCRH + E2, α-hCRH followed by E2; $^{*}p < 0.05$ versus control and $^{\#}p < 0.05$ versus E2. The CRH (c) and t-MH (d) contents in the PVN of the hypothalamus after i.p. infusion with DMSO or PPT or DPN in intact-female rats. Control, DMSO; PPT, a specific ERα agonist PPT; DPN, a specific ERβ agonist DPN; $^{*}p < 0.05$ versus Control.

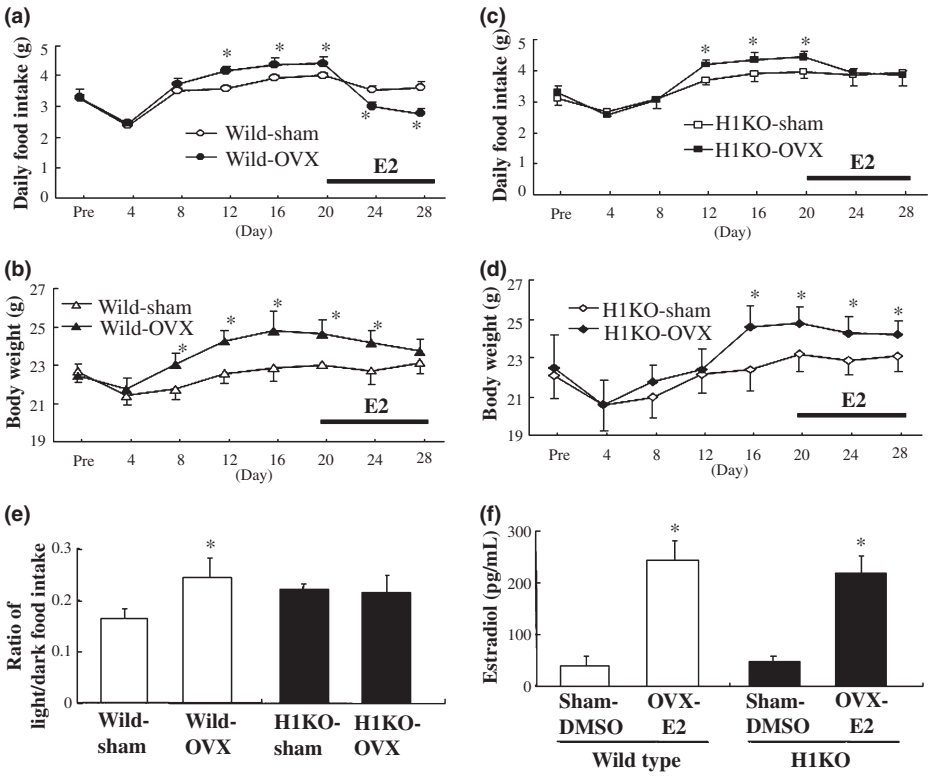

Fig. 3 Effect of E2 treatment on food intake and body weight in wild-OVX (a and b) or H1KO-OVX mice (c and d). Mice underwent the designated surgical procedures and were allowed to recover for 3 weeks, at which time they received an i.p. infusion of E2 daily for 1 week. Food intake and body weight were monitored throughout the 4-week period. Wild-sham, wild-type mice after the sham operation; Wild-OVX, wild-type mice after OVX; H1KO-sham, H1KO mice after the sham operation; H1KO-OVX, H1KO mice after OVX; $^*p < 0.05$ versus Wild-sham or H1KO-sham. The ratio of light- to dark-phase food intake in H1KO and wild-type mice after 3 weeks of post-operative recovery (e). Plasma estradiol concentration in wild-sham mice treated with DMSO, wild-OVX treated with E2, H1KO-sham treated with DMSO, and H1KO-OVX treated with E2 (f). Sham-DMSO, DMSO administration after sham operation; OVX-E2, E2 administration after OVX; $^*p < 0.05$ versus Wild-sham or sham-DMSO.

for wild-sham and H1KO-sham. E2 replacement over 1 week suppressed the increases in food intake and body weight in the wild-OVX group compared with the wild-sham group (Fig. 3a and b). In contrast, the administration of E2 failed to suppress food intake and body weight gain in the H1KO-OVX group compared with the H1KO-sham group (Fig. 3c and d). In addition, the ratio of food consumption in the light versus dark phases (light/dark) was greater in not only the wild-OVX group but also H1KO-sham group than in the wild-sham group (Wild-sham, 0.16 ± 0.02; Wild-OVX, 0.24 ± 0.04; H1KO-sham, 0.22 ± 0.01; Fig. 3e). No difference was observed between the H1KO-OVX and H1KO-sham groups. Moreover, Plasma estrogen levels were significantly higher by E2 treatment in both wild-OVX and H1KO-OVX groups (Fig. 3f).

Immunohistochemical staining for ERα and ERβ in histaminergic neurons

Immunohistochemical analysis with double labeling demonstrated that the majority of histaminergic neurons expressed ERα in the cell nucleus (78 ± 4.3%; Fig. 4a). In contrast, very few histaminergic neurons expressed ERβ in the nucleus (5.3 ± 0.7%; Fig. 4d) though ERβ were detected in the TMN of the hypothalamus. To further confirm the specificity of ERα and ERβ antibodies, we performed immunohistochemistry after pre-absorbing the antibodies with purified ERα and ERβ protein. When ERα and ERβ antibody was pre-absorbed with ERα and ERβ protein, respectively, both of antibody labeling was greatly diminished (Fig 4b and e). Moreover, the specificity of the labeling was controlled by omitting the ERα or ERβ antiserum. (Fig 4c and f). The results from the semi-quantitative analysis of the ERα immunolabeling following pre-absorption with ERα protein are displayed in Fig. 4g. With increasing concentrations of the full-length ERα recombinant protein relative to primary antibody (1 : 1, 1 : 5, and 1 : 10 molar ratio of ERα antibody concentration to recombinant protein concentration), the ERα immunolabeling was significantly decreased compared with the immunolabeling with the primary antibody alone. The diminution of the anti-ERα antibody signal with full-length ERα showed a linear reduction in staining up to five times the molar ratio of

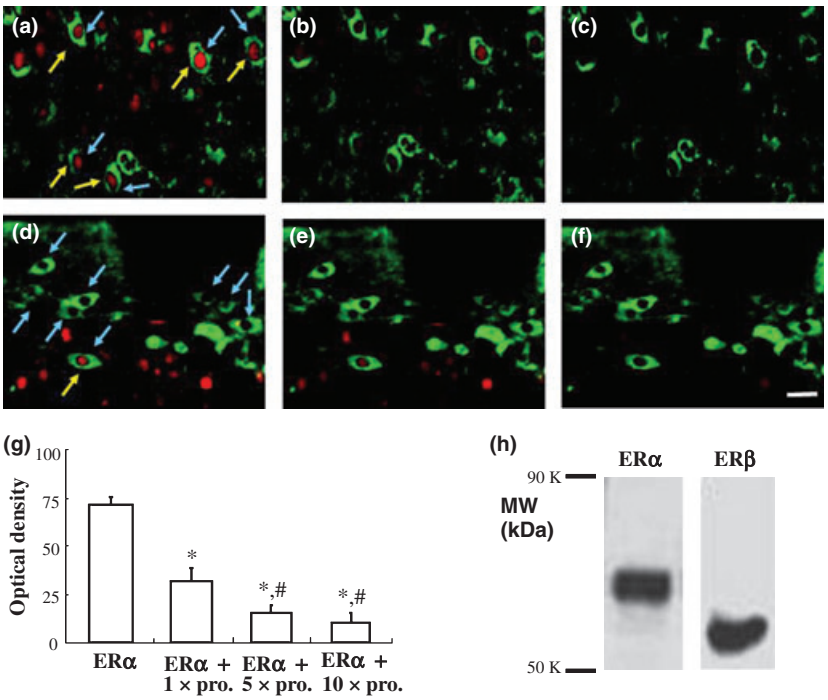

Fig. 4 Immunohistochemical staining for ERα and ERβ in the histaminergic neurons (yellow arrows) of the TMN. (a) ERα (red) is expressed in the nucleus of histamine neurons (green, blue arrows). (d) Very little ERβ (red) is expressed in the nucleus of histamine neurons (green). (b and e) ERα or ERβ antibody pre-absorbed with 1× ERα or ERβ protein. (c and f) Substitution of ERα or ERβ antibodies with normal serum and incubation with secondary antibody as for double staining results in no detectable signal. Scale bar, 10 μm. (g) Bar graph showing the intensity of ERα labeling in the presence and absence of ERα recombinant protein at 1 : 1 (×1 pro.), 1 : 5 (×5 pro.), and 1 : 10 (×10 pro.) molar concentrations of ERα antibody. The values in this graph represent the mean total immunolabeling; $^{*}p < 0.05$ versus ERα and $^{\#}p < 0.05$ versus ERα + 1 × 1 pro. (h) Western blotting analysis reveals ERα and ERβ antibodies detect the full-length ERα (~66 kDa) and ERβ (~54 kDa) protein, respectively. Molecular weight size markers in kDa are shown to the left.

antigen to antibody. Pre-absorption with ERβ protein yielded similar results (data not shown).

Western blotting analysis

To confirm the specificity of the ERα and ERβ antibodies used in the immunohistochemistry experiments, western blotting analysis was performed. As seen in Fig. 4h, we detected an immunopositive band at the predicted molecular weight of full-length ERα (~66.4 kDa) and full-length ERβ (~53.4 kDa) protein, suggesting that the two primary antibodies were indeed capable of recognizing ERα and ERβ.

Discussion

Estradiol, CRH, and hypothalamic neuronal histamine act as anorexigenic substances in the hypothalamus (Wade *et al.* 1985; Sawchenko and Swanson 1985; Fukagawa *et al.* 1989). However, their functional relationships, especially that between E2 and histamine, are still unclear. Our results demonstrate that the depletion of neuronal histamine by pre-treatment with FMH partially attenuated the E2-induced suppression of food intake. In addition, this attenuation of the E2-induced response was mimicked in H1KO mice. These findings indicate that endogenous neuronal histamine and the associated H1-R partially mediate the anorexigenic effects of E2. Therefore, it is important to determine how E2 affects the level of hypothalamic neuronal histamine. After its release from the nerve terminals, histamine is rapidly converted into its metabolite t-MH, which is then deaminated. Consequently, it is more informative to analyze histamine release by measuring its metabolite than by measuring total brain histamine. Pre-treatment with pargyline, an inhibitor of monoamine oxidase B, induces the accumulation of t-MH in the extraneuronal spaces (Oishi *et al.* 1987).

We showed that E2 treatment increased the hypothalamic levels of t-MH. Moreover, this study indicates that ERα, but very little ERβ, was expressed in the nucleus of histaminergic neurons in the TMN and confirms the specificity of ERα and ERβ we used, considering the results that both of antibody labeling was diminished with pre-absorption of ERα and ERβ protein, and the immunopositive band at the predicted molecular weight of ERα and ERβ protein was detected in western blotting analysis. Our results are in agreement with previous findings demonstrating that ERα, but not ERβ, has a major influence on energy homeostasis and that the central infusion of histamine accelerates energy

expenditure through H1-R in the hypothalamus (Heine *et al.* 2000; Masaki *et al.* 2001; Roesch 2006; Santollo *et al.* 2007). In this study, ERα agonist but not ERβ increased CRH and t-MH contents in the hypothalamus. This result that ERα mediates the ability of estrogens to decrease food intake is supported by previous observations that ERα and ERα/β knockout mice accumulate more adipose tissue than wild-type mice, suggesting that ERα mediates the attenuating effects of estrogen on weight gain (Heine *et al.* 2000; Ohlsson *et al.* 2000). Therefore, we speculate that the i.p. infusion of E2 increases histamine turnover, synthesis, and release in the hypothalamus via ERα.

Ovariectomy-induced obesity in rats mimics obesity induced by estrogen deficiency in humans, making it a useful model for studying the mechanisms through which hypoestrogenism increases body fat and modifies the leptin level. Previous research has shown that E2 supplementation in OVX rats caused a decline in the serum leptin level, reducing total fat mass and restoring the body to a composition similar to that in euoestrogenism. E2 supplementation also elevated leptin receptors in the PVN and ventromedial hypothalamic nucleus. In addition, leptin treatment activated histaminergic neurons in the hypothalamus, even though these neurons do not express leptin receptors (Yoshimatsu *et al.* 1999). We show here that OVX-induced hyperphagia and weight gain disappeared after E2 supplementation in wild-type mice but that these effects were attenuated in H1KO mice. Hence, we suggest that E2 suppresses feeding directly through the activation of neuronal histamine synthesis, or indirectly via the modulation of leptin sensitivity in histaminergic neurons. Furthermore, the supplementation of histidine, a precursor for the synthesis of histamine, may contribute to the decrease in obesity in postmenopausal women without a requirement for concomitant E2 therapy and its accompanying untoward effects (Kasaoka *et al.* 2005).

In addition, we demonstrate that the i.p. administration of E2 increased the CRH content in the PVN of the hypothalamus. The central administration of α-hCRH prevented the reduction in food intake induced by a single injection of E2 although a single infusion of α-hCRH did not affect food consumption, suggesting that CRH mediates the anorectic effect of E2 in the hypothalamus (Dagnault *et al.* 1993; Lu *et al.* 2003). Furthermore, research in gonadectomized rodents has indicated that E2 regulates the expression of CRH, which results in a decrease in hypothalamic CRH immunoreactivity (Haas and George 1988; Vamvakopoulos and Chrousos 1993). This effect may be mediated through estrogen-responsive elements that are present in the promoter region of the rodent CRH gene (Haas and George 1988). These results are consistent with our findings, in which OVX mice displayed a reduced CRH level in the PVN; furthermore, pre-treatment with α-hCRH, a CRH antagonist, partially attenuated the E2-induced suppression of food intake. This study describes that E2 caused a rapid effect on food intake and CRH expression in the hypothalamus. Previous studies demonstrated that subcutaneous administration of 1 μg dose of E2 also rapidly increase the number of phosphorylated cAMP-response element binding protein-immunoreactive cells in the mouse brain and a single i.p. injection of E2 (15 μg/kg) rapidly increased extracellular signal-regulated protein kinase 2 phosphorylation in the PVN of rat hypothalamus (Abraham *et al.* 2004; Bryant *et al.* 2005). These data indicate that systemic administration of E2 activates neuronal cells including hypothalamus rapidly.

Finally, our results provide insight into the relationship between CRH and neuronal histamine. We have previously demonstrated that the CRH-induced increase in histamine turnover is suppressed by pre-treatment with α-hCRH. In addition, CRH1-Rs are expressed on the cell bodies of histaminergic neurons in the TMN (Gotoh *et al.* 2005). Therefore, we hypothesized that E2 signaling regulates neuronal histamine via CRH. Our results support this hypothesis, in that the E2-induced increase in hypothalamic histamine turnover was attenuated by pre-treatment with α-hCRH.

However, there are conflicting data that E2s action is not sufficient to decrease daily food intake when E2 is implanted in the PVN (Hrupka *et al.* 2002). This result is not necessarily contradictory to our study. The centrally injected E2 spreads to distant brain sites including the PVN and the TMN of the hypothalamus. We have demonstrated that E2 induces the reduction of feeding by activating histamine neurons through ERα in the TMN. Considering the previous report that histamine also activates CRH neurons in the PVN, it is possible that some CRH neurons may be stimulated with not E2 but activated histamine neurons by E2 (Kjaer *et al.* 1998).

Various behavioral processes, including the sleep–wake cycle, feeding behavior, and certain neuropeptides, are affected by the neuronal circadian clock located within the hypothalamic suprachiasmatic nucleus (SCN) (Turek *et al.* 2005; Shimba *et al.* 2005). Recent studies have demonstrated that the circadian rhythm of feeding behavior is crucial for regulating body weight (Turek *et al.* 2005; Shimba *et al.* 2005). Disruption of this feeding rhythm is often observed in obese patients (Andersen *et al.* 2004). It was also recently demonstrated that *Clock* genes regulate circadian nutrient homeostasis in mice (Turek *et al.* 2005). *Clock* mutant mice exhibited an attenuated diurnal feeding rhythm, hyperphagia, obesity, and the development of metabolic syndromes such as hyperleptinemia, hyperinsulinemia, and hyperglycemia (Ando *et al.* 2005). These observations indicate that the circadian *Clock* gene network in the SCN has an important role in feeding behavior and metabolism. In a previous study, we found that histamine promotes c-fos expression in the SCN, which expresses H1-R. Furthermore, H1KO mice exhibited abnormal circadian rhythms of food intake relative

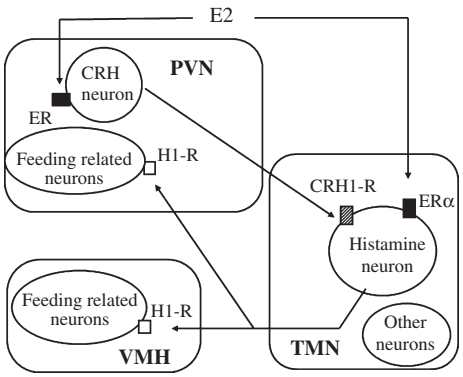

Fig. 5 Feeding proposed model for the activation of histaminergic neurons by E2. E2 regulates histamine neurons in the TMN through ERα, which is expressed in the nucleus of histaminergic neurons. Histamine suppresses food intake via H1-R, which are expressed on feeding-related neurons in the PVN and the ventromedial hypothalamic nucleus (VMH). In addition, E2 stimulates histaminergic neurons via estrogen receptors expressed on CRH neurons, which directly activate histaminergic neurons through CRH-R1. 'feeding-related neurons' mean the neurons which produce peptides such as brain-derived neurotrophic factor and neuropeptide-Y or co-localize with receptors such as H1-R and CRH type 2 receptors, influencing feeding behavior.

to wild-type mice. In addition, the ratio of food intake in the light versus dark phases is greater in OVX wild-type mice than in sham-operated animals, although this difference was not observed in H1KO mice (Masaki *et al.* 2004).

Another study reported that the plasma leptin level increased in OVX rats compared with the level in sham-operated rats and that the effect of centrally administered leptin on food intake was significantly less in OVX rats than in sham-operated rats. Given that there was no difference in the expression level of hypothalamic leptin receptor mRNA between OVX and sham-operated animals, these findings suggest that estrogen-deficient rats may be insensitive to leptin (Ainslie *et al.* 2001). Both CRH and neuronal histamine are under the control of leptin. Previous studies have shown that the central administration of leptin increases CRH neuron activation and histamine turnover in the hypothalamus (Yoshimatsu *et al.* 1999; Huang *et al.* 1998). Leptin-induced suppression of feeding was attenuated in rats pre-treated with FMH, which depletes neuronal histamine, as well as in H1KO mice that lack H1-R (Yoshimatsu *et al.* 1999). Moreover, we have previously demonstrated that CRH mediates the effect of leptin signaling on neuronal histamine turnover (Gotoh *et al.* 2005). In estrogen-deficient rats, increased body weight is associated with central leptin insensitivity. In addition, the administration of E2 activates CRH and histaminergic neurons, both of which are targets for leptin regulation in the hypothalamus (Gotoh *et al.* 2005; Clegg *et al.* 2006). Therefore, we speculate that E2 and leptin signaling cooperate in the regulation of histaminergic neurons.

We have developed a working model of the neuronal network that regulates feeding and satiety via a signaling cascade from E2 to neuronal histamine (Fig. 5). E2 may act histaminergic neurons through ERα directly and CRH neurons directly via ER or indirectly through activated histamine neurons. Furthermore, activated CRH neurons also may regulate histaminergic neurons through CRH1-R. This study provides novel insight into the action of E2 in the hypothalamic regulation of energy metabolism.

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