Published in final edited form as:

J Neuroendocrinol. 2013 June ; 25(6): 570–579. doi:10.1111/jne.12032.

Oestradiol decreases melanin-concentrating hormone (MCH) and MCH receptor expression in the hypothalamus of female rats

Jessica Santollo* and Lisa A. Eckel

Program in Neuroscience, Florida State University, Tallahassee, FL 32306 USA

Abstract

Previous studies have shown that oestradiol (E2) decreases the orexigenic effect of melanin-concentrating hormone (MCH). Here, we examined whether this action of E2 is mediated by its ability to decrease the expression of MCH or its receptor (MCHR1). Using immunocytochemistry and Western blotting, we examined whether E2 decreases MCH-immunoreactive neurones or MCHR1 protein content in the hypothalamus of female rats. We found that both MCH and MCHR1 protein expression was decreased by acute E2 treatment in OVX rats, and by the peri-ovulatory increase in circulating E2 in pro-oestrous rats, relative to rats at other cycle stages. To determine whether these changes in MCH/MCHR1 protein expression may be mediated by E2's ability to directly regulate the transcription of MCH and MCHR1 genes, the effect of E2 treatment on MCH and MCHR1 mRNA expression in a neuronal hypothalamic cell line was examined using real time RT-PCR. We also determined whether MCH and oestrogen receptor alpha (ER α) are co-expressed in the hypothalamus of female rats. E2 treatment did not decrease MCH or MCHR1 mRNA expression *in vitro*, and no hypothalamic neurones were identified that co-expressed MCH and ER α . We conclude that E2-dependent decreases in hypothalamic MCH/MCHR1 protein expression mediate E2's ability to decrease MCH-induced feeding. The current findings suggest, however, that E2 exerts these actions indirectly, likely through interactions with other neuronal systems that provide afferent input to MCH and MCHR1 neurones.

Keywords

Food Intake; Oestrogen; Lateral Hypothalamus; Oestrogen Receptor α ; PPT

Introduction

Oestradiol (E2) is an important endocrine signal involved in the control of food intake. Specifically, female rats display cyclic changes in feeding that are associated with fluctuations in circulating E2 (1–3), increases in food intake following ovariectomy (4), and decreases in food intake following peripheral or central administration of E2 (5, 6). E2 exerts this inhibitory effect on feeding by modulating the activity of peptide, endocrine, and neurotransmitter systems implicated in the physiological control of meal size. For example, E2 increases the anorexigenic effect of cholecystokinin and serotonin, both of which suppress feeding by a selective decrease in meal size (7–10).

E2's inhibitory effect on food intake may also be mediated by its ability to decrease the activity of orexigenic peptides, including melanin-concentrating hormone (MCH). MCH

*Correspondence to: Jessica Santollo, Department of Psychology, University at Buffalo, Buffalo, NY 14260, United States. Tel.: 716-645-0271; Fax: 716-645-3801; jsantoll@buffalo.edu.

increases short-term (1–4 h) food intake in male and female rodents (11–14) by increasing meal size (15, 16). MCH exerts this behavioural effect through binding with the MCH type 1 receptor (MCHR1), a G-protein coupled receptor that is expressed throughout the rodent central nervous system with particularly high concentrations in hypothalamic nuclei involved in the control of food intake (17). Our lab has demonstrated that the orexigenic effect of MCH is attenuated in female rats, relative to male rats (16). This sex difference is likely the result of the higher circulating levels of E2 in females, relative to males, since MCH's orexigenic effect is decreased by the pro-oestrous rise in plasma E2 in cycling rats and E2 treatment in OVX rats (16).

The classic oestrogen receptors (ERs), ER α and ER β , mediate many of E2's diverse effects. Following ligand binding, these ERs form homo- and hetero-dimeric complexes that can regulate gene transcription, resulting in functional changes in protein expression (18, 19). Available pharmacological data suggest that E2's anorexigenic effect is mediated, at least in part, by its ability to regulate the expression of feeding-related peptides via activation of ER α , with minimal or no involvement of ER β (20–23). The expression of ER α in brain regions that contain both MCH and MCHR1 neurones (17, 24–26) raises the possibility that E2 may decrease MCH-induced feeding through changes in MCH and/or MCHR1 gene expression that promote decreased MCH signalling.

Few studies have examined the effect of E2 on MCH gene expression. In one study, acute administration of E2 decreased prepro-MCH mRNA expression in the ZI of OVX rats (27). Because prepro-MCH encodes multiple peptides, not just MCH (17), additional research is necessary to determine whether this change in gene expression results in a change in MCH protein expression. It is also unknown whether endogenous E2 decreases MCH or MCHR1 gene/protein expression in cycling rats, or if E2-dependent changes in MCH or MCHR1 activity are a direct result of ER α -mediated decreases in MCH/MCHR1 gene transcription or an indirect result of ER α -mediated changes in the transcription of genes that provide afferent input to MCH/MCHR1 neurones.

The goal of this study was to investigate the neural mechanism underlying E2's ability to decrease the orexigenic effect of MCH in female rats. Based on the available data, we hypothesized that E2 decreases hypothalamic expression of MCH and/or MCHR1 protein. Because activation of ER α is both sufficient and necessary for E2's anorexigenic effect (e.g., (21, 23), we further hypothesized that any E2-dependent decreases in hypothalamic MCH/MCHR1 protein expression would be mediated by ER α .

Materials and Methods

Subjects, housing and surgery

Female Long-Evans rats (Charles River; 200–225 g body weight) were singly housed in a colony room that was maintained at $20 \pm 2^\circ\text{C}$ with a 12:12 light cycle. In all experiments, rats were given free access to food (Purina 5001) and tap water. Rats utilized in Experiment 1 were anesthetized with intraperitoneal (i.p.) injections of a mixture of ketamine (50 mg/kg, Ketaset, Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (4.5 mg/kg, Rompun, Mobay Corporation, Shawnee, KS) and then bilaterally OVX using an intra-abdominal approach (28). Following surgery, rats received i.p. injections of butorphanol (0.5 mg/kg, Fort Dodge Animal Health, Fort Dodge, IA) and gentamicin (10 mg/ml, Butler, Dublin, OH) to minimize post-surgical pain and the risk of infection, respectively. Animal usage and all procedures used in these experiments were approved by the Florida State University Institutional Animal Care and Use Committee.

Cell culture and reagents

N-42 neurones (CELLutions Biosystems, Burlington, NC), a clonal, murine (harvested from a mixture of male and female embryos) hypothalamic cell model that expresses a number of neuronal cell markers (29), were cultured in monolayer in DMEM (Sigma, St. Louis MO) and supplemented with 5% fetal bovine serum (Sigma, St. Louis MO), 4.5 mg/ml glucose and penicillin/streptomycin and maintained at 37°C in 5% CO₂. This hypothalamic cell line was chosen because it expresses ER α , MCH, and MCHR1 gene products, and it has been demonstrated that E2 treatment decreases neuropeptide Y (NPY) and agouti-related protein (AgRP) expression within these cells (30).

Experiment 1: Does E2 treatment decrease MCH or MCHR1 protein expression in OVX rats?

Protocol—OVX rats (n = 7–8/group) received single, subcutaneous (s.c.) injections (0.1 ml) of 2 μ g oestradiol benzoate (EB, Sigma, St. Louis, MO), 75 μ g of a selective ER α agonist (4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; PPT, Tocris, Ellisville, MO), or sesame oil vehicle 4 h prior to dark onset. The dose of EB was chosen because it produces circulating levels of E2 that model those observed in cycling rats (6, 31). The dose of PPT was chosen because its anorexigenic effect is similar to a physiological dose of EB (21). Rats were anesthetized 9 h after vehicle/EB treatment and 6 h after PPT treatment. The former time point was chosen because serum E2 levels rise rapidly (within 6–9 h) following an acute injection of EB (6). The latter time point was chosen because PPT exerts a more rapid anorexigenic effect than EB (21). Following transcardial perfusion, brains were dissected out of the skull and a coronal cut at the most rostral level of the cerebellum was made to obtain forebrain blocks of tissue containing the hypothalamus. This forebrain tissue was exposed to 4% PF overnight and then stored in a 30% sucrose solution in PB for five days at 4°C. Tissue was then sectioned on a freezing, sliding microtome (Zeiss, HM 440E) at 40 μ m coronal sections from the level of the MPOA (+ 0.48 bregma) throughout the ZI (–5.16 bregma). Brain sections containing the LH and ZI were examined for expression of MCH immunoreactivity (as described below) because the synthesis of MCH is primarily limited to these two brain regions (17, 24, 32).

Western blots were used to examine whether E2 decreases hypothalamic MCHR1 protein content. Western blot analysis was used instead of immunocytochemistry (ICC) because of the technical concerns associated with ICC antibody specificity for G-protein coupled receptors. OVX rats (n = 7–8 per group) received acute, s.c. injections of 2 μ g EB, 10 μ g EB, 75 μ g PPT, or vehicle. A larger dose of EB was used in this experiment to avoid the possibility of results being masked by analyzing the entire hypothalamus, as opposed to individual nuclei. Rats were anesthetized at the same time points described above. Once unresponsive, rats were decapitated and the hypothalamus was dissected out of the brain. Coronal cuts were made immediately rostral and 5 mm caudal to the optic chiasm. Next, sagittal cuts were made 4 mm bilateral to the midline. Finally, the hypothalamus was isolated by a horizontal cut below the anterior commissure. Hypothalamic blocks were stored at –80°C until protein purification and were processed for MCHR1 protein content via Western Blot (as described below).

MCH ICC—Free-floating sections were washed in PB, incubated for 30 min in 0.3% H₂O₂, and rinsed in PB. Sections were then incubated for 1 h in PB containing 10% normal goat serum (NGS) and 0.4% Triton X-100 and then incubated overnight at 4°C with MCH antibody (H-070-47; Phoenix Pharmaceuticals, Burlingame,) diluted 1:20,000 (MCH) in PB/2% NGS/0.4% Triton X-100. Sections were then rinsed in PB, incubated for 1 h with biotinylated goat anti-rabbit antibody (BA-100; Vector Labs, Burlingame, CA) diluted 1:1000 or 1:400 in PB/2% NGS/0.4% Triton X-100 (MCH or MCHR1, respectively).

Sections were then washed in PB, incubated for 1 h with avidin-biotin complex (ABC; Vector Labs, Burlingame, CA) diluted 1:500, washed in PB, and incubated in PB containing 0.04% 3,3'-diaminobenzide tetra hydrochloride (DAB; Vector Labs, Burlingame, CA) and 0.01% H₂O₂ for 5 min. Sections were then mounted on glass slides and coverslipped. As a negative control, an additional set of sections were processed without incubation in the primary antibody.

Quantification of immunoreactivity—Approximately 25 sections per brain (every fourth section ranging from −1.20 to −5.16 bregma) were digitized at 4 and 20x magnification (Olympus AX70). Examination of these sections revealed that the majority of MCH staining was limited to ~ 10 serial sections (−1.84 to −3.4 bregma). MCH staining within these 10 sections (containing the LH and ZI) was quantified. Because the abundant MCH staining on the border of the LH and ZI made it difficult to delineate the two nuclei, the numbers of MCH-immunoreactive neurones within these two nuclei were summed to create a composite LH/ZI score.

The number of neurones (i.e., cell bodies) that expressed MCH immunoreactivity within brain regions of interest was quantified with the aid of automated counting software (ImagePro, Media Cybernetics) and a constant set of threshold detection criteria based on object density, shape and size. Although density of staining was used to provide an objective way to identify MCH-immunoreactive neurones, it is possible that some MCH neurones may not have been counted if they expressed MCH at levels below our threshold of detection. For individual rats, the numbers of MCH-immunoreactive cell bodies were counted unilaterally within hemisections obtained from target nuclei, using the fornix, third ventricle and optic tract as landmarks (33).

Western blot analysis and quantification—Hypothalamic tissue blocks were processed for MCHR1 protein content via Western blots using established protocols from our laboratory (34). Membranes were incubated in a primary polyclonal goat antibody (SC-5534; Santa Cruz, Santa Cruz, CA; 1:500 dilution) directed against MCHR1. Previously, this antibody has been used to identify MCHR1 protein via Western blot analysis in the rodent brain, including the hypothalamus (e.g., (35, 36)). Immunoreactive bands were visualized by incubation with horseradish peroxidase-labelled secondary donkey anti-goat antisera (SC-2020; Santa Cruz, Santa Cruz, CA; 1:20,000) followed by chemiluminescence (ECL Plus kit). Following x-ray visualization, membranes were stripped and then reprocessed for the control protein (β -actin; SC-69879; Santa Cruz, Santa Cruz, CA) at a 1:10,000 dilution. MCHR1 and β -actin labeling was visualized from x-ray film and quantified using a computerized imaging program (Image J, NIH) to measure optical density. All MCHR1 bands were normalized to β -actin controls.

Experiment 2: Are changes in endogenous E2 levels associated with changes in MCH or MCHR-1 immunoreactive neurones in cycling rats?

Protocol—To assess the effects of endogenous E2 on MCH protein expression, cycle stage was monitored daily, as described previously (31), in a group (n = 24) of female rats. On test days, rats in varying cycle stages (i.e., diestrus, pro-oestrus, and oestrus) were anesthetized with i.p. injections of sodium pentobarbital (50 mg/kg, Henry Schein, Melville, NY) during the mid-light phase. Once anesthetized, rats were transcardially perfused following the collection of 2 ml of blood via cardiac puncture. Perfused brains were sectioned and processed for MCH immunoreactivity as described above. Blood was analyzed for serum E2 concentrations via radioimmunoassay (RIA; as described below).

To assess the effects of endogenous E2 on MCHR1 protein expression, cycle stage was monitored daily in a second group of rats ($n = 20$). On test days, rats were anesthetized (at each cycle stage) during the mid-light phase and fresh hypothalamic tissue was collected and processed for MCHR1 protein content via Western Blot (as described above).

E2 RIA—Clotted blood samples, collected in uncoated tubes, were centrifuged at 10,000 $\times$ g for 15 min. Serum was collected and stored at -20°C until assayed for E2 using a non-extraction, solid phase RIA kit (TKE21; Siemens Diagnostic, Los Angeles, CA). An automated gamma counter (Apex 41600, Titertek Instruments Inc.) and related software were used to quantify serum E2 concentration. The analytical sensitivity for this assay is 1 pg of E2 per ml of serum. All samples were within the linear detection range and analyzed in duplicate.

Experiment 3: Does E2 treatment decrease MCH or MCHR1 mRNA expression in neuronal cultures?

Protocol—Prior to hormone treatment, cells were serum starved for 12 h in phenol red-free DMEM. A total of 24 plates containing 85% confluent N-42 neurones in phenol red-free medium supplemented with charcoal-stripped serum were treated with either 10 nM β -estradiol (β -E2, Sigma E2758) or 0.0001% ethanol vehicle. Previously, this dose of β -E2 decreased NPY and AgRP gene expression in N-42 cells (30). Cells were incubated in β -E2 or vehicle for 2, 4, 6 or 24 h. This was repeated 3 times, providing 24 samples (8 groups, $n = 3/\text{group}$). Following hormone treatment, cDNA samples from the cell cultures were processed for gene expression of MCH, MCHR1 and the housekeeping gene γ -actin. In addition, cDNA samples from the 2-h vehicle- and β -E2-treated groups were processed for AgRP gene expression as a positive control.

cDNA synthesis—Total RNA from sample plates was isolated using TRI Reagent (Sigma, St. Louis, MO) with modifications to remove DNA using the Qiagen RNeasy columns and DNase I Kit (Qiagen, Valencia, CA). RNA was stored at -80°C in RNase-free H_2O supplemented with the RNase inhibitor Supersasin (Ambion, Austin, TX) according to the manufacturer's directions. Quality of RNA was determined using UV spectrophotometry and agarose gel visualization of intact RNA. First-strand cDNA was synthesized from 500 ng total RNA using Superscript III reverse transcriptase (RT) and a mixture of oligo dT and random hexamer primers according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Real Time RT-PCR—Real-time PCR was performed using an iCycler instrument (Bio-Rad, Hercules, CA) with SYBR green PCR master mix (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. All primers were designed to span introns and synthesized by IDT (Integrated DNA Technologies, IA). Control reactions were performed where amplification was carried out on samples in which the reverse transcriptase was omitted (RT-). γ -actin was used as an internal control for quantification of individual mRNA. The primer sequences were: MCH sense, 5' TCC CAG CTG AGA ATG GAG TTC AGA; antisense, 5' TCT TCC CAG CAT ACA CCT GAG CAT, MCHR1 sense, 5' TTG CCG TGG TGA AGA AAT CCA AGC, antisense, 5' AGT GCC AGA CAC CAT TAC CCA TGA, γ -Actin sense, 5' AGA TCT GGC ACC ACA CCT TCT ACA; antisense, 5' ATA CAA GGA CAG CAC CGC CTG AAT, AgRP sense, 5' CGG AGG TGC TAG ATC CAC AGA, antisense, 5' AGG ACT CGT GCA GCC TTA CAC.

Experiment 4: Is ER α and MCH co-localized in hypothalamic neurones in female rats?

Protocol—To further investigate whether E2 may affect MCH signalling via a direct action on MCH neurones, we examined whether MCH- and ER α -immunoreactive neurones are co-

localized in the LH/ZI of female rats. This experiment was designed to extend our *in vitro* study involving a murine-derived cell line (Experiment 3). We deemed this necessary in light of the fact that our behavioural studies demonstrating E2's ability to decrease the orexigenic effect of MCH were conducted in rats and it is not known whether a similar effect is observed in mice. Eight free-floating brain sections (back-up tissue from Experiment 2), spanning the LH/ZI were dually processed for ER α /MCH immunoreactivity. Representative sections from the dorsal hippocampus and PVN were also processed as negative controls since MCH and ER α are not expressed in these nuclei, respectively. Due to the lack of highly specific MCHR1 antibodies validated for ICC, we could not perform double-label ICC for ER α and MCHR1. Thus, this experiment was limited to examining the potential co-localization of ER α and MCH.

ER α /MCH double-label ICC—Tissue sections were processed for ER α immunoreactivity using established methods (37). Sections were washed in PB and incubated for 1h in 1% H₂O₂. After a PB rinse, tissue was incubated for 1h in PB containing 2% NGS/0.5% Triton X-100 and then incubated at 4°C for 2 days in anti-ER α rabbit IgG (MC-20, Santa Cruz, Santa Cruz, CA; diluted 1:20,000). After a PB rinse, tissue was incubated for 2h in biotinylated anti-rabbit goat IgG (Vector Labs; Burlingame, CA) diluted 1:500 in PB. After a PB rinse, tissue was incubated for 1.5h with ABC diluted 1:500, washed with PB, and incubated in PB containing 0.02% nickel intensified DAB and 0.01% H₂O₂ for 5 min. Following a 1 h PB rinse, sections were processed for MCH immunoreactivity as described above and then mounted on glass slides and coverslipped.

Quantification of immunoreactivity—Dually-processed tissue sections were viewed at 20x under a research microscope (Olympus AX70). The presence of single- and double-labelled cells was visualized using image analysis software (Image Pro) capable of discriminating nuclear and cytoplasmic staining within individual cell bodies. Double-labelled neurones (ER α /MCH) were identified as cell bodies that expressed punctate, black nuclear staining (indicating the presence of ER α) surrounded by brown cytoplasmic staining (evidence of MCH). Because our goal was to determine whether MCH and ER α are expressed in the same cells, we only counted double-labelled cells.

Statistical analysis—Data are presented as means + SEM throughout. The effect of hormone treatment on the number of MCH-immunoreactive neurones in OVX rats (Experiment 1) was analyzed using a one-way ANOVA. Tukey's HSD multiple-comparison, post-hoc test was used to assess group differences following significant ($P < 0.05$) ANOVA effects. To quantify MCHR1 expression in Experiment 1, the optical density measures obtained from western blots were normalized to β -actin control and then each hormone-treated group was compared to the vehicle-treated control group via independent t-tests (each hormone group was run on one membrane with the vehicle group, yielding three membranes and in turn three analyses). Data from hormone-treated rats were then expressed as a percentage of the data obtained in the vehicle-treated group in order to present all of the data in a single figure. Regression analyses were used to examine the relationship between serum E2 level and the number of MCH-immunoreactive neurones and MCHR1 protein content within hypothalamic nuclei in cycling rats (Experiment 2). The RT-PCR values from Experiment 3 were calculated using the delta-delta CT quantification method. The effect of hormone treatment on MCH and MCHR1 expression were analyzed using a two-factor ANOVA (drug by time) and AgRP mRNA expression was analyzed using an independent t-test.

Results

MCH and MCHR1 protein expression in OVX rats

Expression of MCH-immunoreactive neurones was limited to the LH and ZI (Fig. 1). Quantification revealed that MCH expression in the LH/ZI was influenced by hormone treatment in OVX rats, $F(2,20) = 4.61$, $P < 0.05$ (Fig. 2A). The number of MCH-immunoreactive neurones was decreased in both EB- and PPT-treated rats, relative to oil-treated rats, $P_s < 0.05$. MCHR1 protein content was also influenced by hormone treatment in OVX rats. While hypothalamic MCHR1 protein content was similar in rats treated with oil and 2 μg E2, $t(13) = -1.19$, n.s., MCHR1 protein content was decreased in rats treated with 10 μg E2 and 75 μg PPT, relative to rats treated with oil, $t(13) = 2.45$ and 4.29, $P < 0.05$ and 0.0001, respectively (Fig. 2B,C).

MCH and MCHR1 protein expression in cycling rats

A negative association between MCH expression and serum E2 level was detected in cycling rats, $R^2 = 0.30$, $P < 0.01$, with the number of MCH-immunoreactive neurones decreasing as a function of increasing serum E2 (Fig. 3A). Additionally, when classified by stage of the oestrous cycle, pro-oestrus rats had fewer MCH-immunoreactive neurones compared to rats in dioestrus and oestrus, $F(2,21) = 5.17$, $P < 0.05$ (Fig. 3B). A negative association was also observed between MCHR1 expression and serum E2, $R^2 = 0.21$, $P < 0.05$, with hypothalamic MCHR1 protein content decreasing as a function of increasing serum E2 (Fig. 3C). Re-analysis of these data by cycle stage revealed less hypothalamic MCHR1 protein content in pro-oestrous rats, relative to dioestrus and oestrus rats, $F(2,17) = 5.69$, $P < 0.01$ (Fig. 3D).

Effect of β -E2 treatment on MCH and MCHR1 mRNA expression in cultured hypothalamic neurones

Prior to hormone treatment we verified that N-42 cells express MCH and MCHR1 mRNA and ER α protein (data not shown). As shown in Fig. 4A, neither MCH nor MCHR1 mRNA expression was influenced by hormone treatment (main effect: $F(1,16) = 0.01$ and 0.2, respectively, n.s.; interaction: $F(3,16) = 0.13$ and 0.38, respectively, n.s.). In addition, MCH mRNA was not influenced by time ($F(3,16) = 1.00$, n.s.). We did, however, detect that MCHR1 mRNA was influenced by a main effect of time ($F(3,16) = 3.73$, $P < 0.05$), with greater MCHR1 mRNA expression at 24 h, relative to 2 and 6 h (data not shown). As shown in Fig. 4B, AgRP mRNA was decreased in cells treated for 2-h with β -E2, relative to cells treated with vehicle ($t(4) = 2.40$, $P < 0.05$).

Localization of ER α and MCH-immunoreactive neurones in the hypothalamus of cycling rats

MCH and ER α immunoreactive neurones were present in the hypothalamus of female rats (Fig. 5A). MCH staining was limited to the LH, particularly around the fornix, and the ZI. ER α staining was moderately expressed in the LH, ZI, and DMH, heavily expressed in the VMH and ARC, and absent in the paraventricular nucleus of the hypothalamus (PVN). MCH and ER α neurones were both present in the LH and ZI and neurones expressing each protein often lay in close proximity to one another (Fig. 5B). However, no neurones that expressed both MCH and ER α were observed throughout the rostral-caudal extent of the LH and ZI (Fig. 5C).

Discussion

Both exogenous and endogenous E2 decreased hypothalamic MCH immunoreactivity and MCHR1 protein content in female rats. These findings suggest that E2's ability to decrease

the orexigenic effect of MCH is mediated via a decrease in hypothalamic MCH signalling. Our finding that MCH and MCHR1 protein expression was lowest in pro-oestrous rats (i.e., during the late light phase prior to behavioural oestrous) suggests that a decrease in MCH signalling may contribute to oestrous-related decreases in food intake in cycling rats. Although we hypothesized that E2 may decrease MCH signalling via direct transcriptional regulation of MCH and MCHR1 genes, our findings suggested otherwise. Neither short-term (2 h) nor sustained (24 h) E2 treatment decreased MCH or MCHR1 mRNA expression in cultured hypothalamic neurones, and ER α - and MCH-immunoreactive neurones were not co-localized in the LH/ZI of female rats. Collectively, these findings suggest that E2's inhibitory effect on hypothalamic MCH and MCHR1 protein expression is mediated indirectly via E2-mediated changes in the afferent inputs to MCH and MCHR1 neurones.

MCH/MCHR1 expression in OVX rats

Acute E2 treatment decreased the number of MCH-immunoreactive neurones and MCHR1 protein content within the hypothalamus of OVX rats. That the latter required a larger dose of E2 suggests that MCH neurones may be more sensitive than MCHR1 neurones to E2 treatment. This differential sensitivity may also be the result of methodological differences because the number of MCH-immunoreactive neurones was quantified in individual hypothalamic areas, whereas MCHR1 protein content was quantified in whole hypothalamic tissue blocks. Accordingly, a higher dose of E2 may have been necessary to reveal its inhibitory effect on MCHR1 protein expression when examining the whole hypothalamus, which would have included MCHR1 neurones within individual hypothalamic nuclei that may not be responsive to E2 treatment.

Previous studies have shown that E2 influences MCH expression. For example, acute administration of E2 (5 – 200 μ g) decreased prepro-MCH mRNA in the ZI of OVX rats (27) and the LH of obese male mice (38). It has also been shown that chronic, pharmacological doses of E2 (22 day treatment with a pellet containing 7.5 mg of E2) blocked the increase in lateral hypothalamic MCH mRNA expression that is associated with negative energy balance in male rats (39). The current findings extend these studies by confirming that E2-dependent changes in hypothalamic MCH mRNA expression likely translate to changes in the functional protein product.

Acute administration of the ER α agonist PPT decreased hypothalamic MCH immunoreactivity and MCHR1 protein content to a similar degree as that observed following E2 treatment. This suggests that selective activation of ER α is sufficient to mediate E2-dependent changes in MCH and MCHR1 protein expression. To our knowledge, this provides the first evidence that ER α can regulate the expression of feeding-related proteins *in vivo*. Thus, our finding provides new insight into the mechanism by which activation of ER α promotes decreased feeding in female rats, and it adds to a growing literature that activation of ER α is sufficient to mediate E2's anorexigenic effect in OVX rats (20–22).

MCH/MCHR1 expression in cycling rats

As would be predicted on the basis of our findings in OVX rats, hypothalamic MCH and MCHR1 protein expression was influenced by endogenous E2 in cycling rats. Specifically, the number of MCH-immunoreactive neurones and MCHR1 protein content decreased in the hypothalamus as a function of increasing serum E2 concentration. This resulted in decreased MCH and MCHR1 expression in pro-oestrous rats, relative to rats at other cycle stages. In cycling rats, serum E2 peaks 4–6 h prior to the onset of behavioural oestrus, a time in which feeding is maximally suppressed (4). Thus, the current pattern of results suggests that an E2-dependent decrease in hypothalamic MCH and MCHR1 protein expression contributes not

only to E2's ability to decrease MCH-induced feeding (35, 51), but also to the transient decrease in feeding that is observed during oestrus in cycling rats (1–3). Additionally, our findings extend previous reports that the expression of other orexigenic peptides, including NPY and orexin, are influenced by stage of the oestrous cycle (40). They also provide the first demonstration that cyclic changes in MCH and MCHR1 are linked to changes in endogenous E2 in cycling rats and thus place our findings obtained in OVX rats treated with E2 in a more physiologically-relevant context.

MCH/MCHR1 expression *in vitro*

As a steroid hormone, E2 could decrease MCH and MCHR1 protein expression by directly affecting the transcription of MCH and MCHR1 genes. To investigate this hypothesis, we examined the effects of E2 treatment on MCH and MCHR1 mRNA expression in cultured hypothalamic (N-42) neurones. This particular cell line was chosen because it has been used previously to investigate E2's ability to directly regulate the expression of other orexigenic peptides (30). We found that E2 failed to influence either MCH or MCHR1 mRNA expression at all of the time points (2, 4, 6 and 24 h) tested here. Although we cannot exclude the possibility that a sub-threshold dose of E2 was used in this experiment or that the chosen time points were not adequate to observe a change in MCH/MCHR1 mRNA expression, we believe this is unlikely because the same dose of E2 was sufficient to decrease AgRP gene expression in our study (at 2 h), and to decrease both AgRP and NPY mRNA expression in N-42 neurones in a previous study (30). Thus, a more parsimonious explanation is that E2 regulates the expression of MCH and MCHR1 indirectly through interactions with E2-responsive neurones that provide afferent input to neuronal populations expressing MCH and MCHR1. Alternatively a decrease in MCHR1 protein could occur in the absence of changes in MCHR1 mRNA through increased receptor internalization following E2 exposure. Future studies will be needed to test this hypothesis.

Hypothalamic expression of MCH and ER α neurones

Although our cell culture study suggested that the E2-dependent decreases in MCH/MCHR1 protein expression observed here were mediated indirectly, we conducted an additional experiment to examine whether MCH and ER α are co-localized in hypothalamic neurones in female rats. It was of particular importance to provide further support for the results from our *in vitro* studies because the cultured hypothalamic neurones utilized here were derived from a mixture of male and female mice embryos (29), whereas our previous demonstration that E2 decreases MCH-induced feeding was obtained in female rats (14, 16). Although MCH- and ER α -immunoreactive neurones were often observed in close proximity to each other, we found no evidence that the two proteins were co-localized within the same neurones (Fig. 5C). This demonstrates that E2, acting via ER α , cannot directly regulate MCH gene expression *in vivo* because these two proteins are not present in the same neurones. Our findings in female rats support and extend a previously study in which MCH and ER α did not appear to be co-expressed in the hypothalamus of male rats (41). The results from this experiment, together with the results from our *in vitro* study, provide strong evidence that E2, acting via ER α , cannot directly influence MCH or MCHR1 expression.

Possible mechanisms by which E2 decreases MCH/MCHR1 protein expression

Because E2 had no effect on MCH or MCHR1 gene expression *in vitro* and MCH and ER α were not co-expressed in the female rat hypothalamus, it is likely that E2-mediated decreases in MCH and MCHR1 expression are mediated indirectly through changes in the afferent signals to MCH and MCHR1 neurones. For example, ARC POMC and AGRP neurones, as well as lateral hypothalamic GABA and glutamate neurones, all of which innervate and influence the activity of MCH neurones, are regulated by E2 (30, 42–47). Additionally, the activity of peripheral signals that influence MCH neurones, such as the

anorexigenic hormone leptin, is decreased by E2 (48–52). Thus, E2 may function to inhibit one or more afferent signals that converge on MCH neurones. This in turn could promote a decrease in MCH gene and subsequent protein expression.

While central regulators of MCHR1 gene expression are unknown, the locations of MCHR1 neurones suggest mechanisms by which this protein could be indirectly decreased by E2. MCHR1 neurones are located in many hypothalamic and hindbrain nuclei, such as the MPOA, ARC and NTS – sites where E2 acts to decrease food intake (5, 53, 54). Additional studies will be necessary to determine whether MCHR1 neurones in these specific nuclei are influenced by E2.

Conclusions

We have shown that MCH and MCHR1 protein expression is decreased by E2 in OVX rats and negatively associated with serum E2 in cycling rats. These findings support the notion that an E2-dependent decrease in MCH signalling underlies E2's ability to decrease the orexigenic effect of MCH, and furthers our understanding of how E2 modulates the overall tone of the neuroendocrine circuitry controlling food intake. Studies examining the neural mechanism underlying the anorexigenic effect of E2, like the one reported here, have the potential to help us understand the role of ovarian hormones in mediating sex differences in obesity and eating disorders.

Acknowledgments

We thank Drs. Larissa Nikonova, Yan Liu, Xixi Jia, Missy Cavallin and Kristal Tucker for technical training and assistance. We thank Dr. Anne Etgen for helpful comments on the manuscript. This work was supported by grants from the National Institutes of Health, DK073936 (L. A. Eckel) and NS062667 (J. Santollo).

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Figure 1.

Representative photomicrographs depicting the rostral-caudal distribution of MCH-immunoreactive neurones in female rats. MCH neurones were observed in the ZI at -1.84 mm from Bregma (A), and in the LH and ZI at -2.04 mm from Bregma (B) and, to a greater extent, at -2.64 mm from Bregma (C). Abbreviations: f, fornix; opt, optic tract; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; ic, internal capsule; mt, mammillothalamic tract; DMH, dorsal medial hypothalamus.

Figure 2.

Effect of hormone treatment on hypothalamic MCH and MCHR1 protein expression in OVX rats. (A) Acute administration of both EB and the selective $ER\alpha$ agonist PPT decreased the number of MCH-immunoreactive neurones in the LH and ZI. (B) Representative immunoblots of MCHR1 and β -actin from oil- and EB-treated rats. (C) Quantification of the optical density of the protein bands revealed that 10 µg EB and 75 µg PPT decreased whole hypothalamic MCHR1 protein content, relative to that observed following oil vehicle treatment. Note that MCHR1 protein content in hormone-treated rats is expressed as a percentage of that observed in oil-treated rats. *Less than oil vehicle, $P < 0.05$.

Figure 3.

A negative association between endogenous E2 levels and MCH and MCHR1 protein was detected in cycling rats. (A) The number of neurones that expressed MCH in the LH and ZI decreased as serum E2 levels increased, $P < 0.01$. (B) When the same data were expressed in relation to cycle stage, fewer MCH-expressing neurones were detected in pro-oestrous rats, in comparison to dioestrous and oestrous rats. (C) MCHR1 protein content in the hypothalamus decreased as serum E2 levels increased, $P < 0.05$. (D) When the same data were expressed in relation to cycle stage, hypothalamic MCHR1 protein content was decreased in pro-oestrous rats, in comparison to dioestrous and oestrous rats. Abbreviations: D; dioestrus, P; pro-oestrus, E; oestrus. *Less than D and E, $P_s < 0.05$.

Figure 4.

β -E2 treatment did not influence MCH or MCHR1 mRNA in hypothalamic cell cultures. A) At 2 h following hormone treatment, MCH and MCHR1 mRNA expression was similar in β -E2- and vehicle-treated cells. B) At this same time point, AgRP mRNA was decreased in β -E2-treated cells, relative to vehicle-treated cells. *Less than vehicle, $P < 0.05$.

Figure 5.

Representative photomicrographs depicting the distribution of MCH- and ERα-immunoreactive neurones in multiple hypothalamic brain areas in female rats at -2.64 mm from Bregma. (A) MCH- and ERα-immunoreactive neurones were both expressed in the LH and ZI. In addition, ERα-immunoreactive neurones were expressed in the VMH and ARC. (B) Higher magnification of the area denoted in (A) revealed that MCH- and ERα-immunoreactive neurones were expressed in close proximity within the LH. (C) Higher magnification of the area denoted in (B) revealed that MCH- and ERα-immunoreactive neurones in the LH were not co-localized within the same cells. Abbreviations: LH, lateral hypothalamus; ZI, zona incerta; f, fornix; opt, optic tract; ic, internal capsule; mt, mammillothalamic tract; VMH, ventromedial nucleus of the hypothalamus; ARC, arcuate nucleus; 3V, third ventricle.