BRAZILIAN JOURNAL
OF MEDICAL AND BIOLOGICAL RESEARCH
www.bjournal.com.br

ISSN 0100-879X
Volume 43 (8) 698-811 August 2010

BIOMEDICAL SCIENCES
AND
CLINICAL INVESTIGATION

Braz J Med Biol Res, August 2010, Volume 43(8) 759-766

doi: 10.1590/S0100-879X2010007500059

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Brazilian Journal of Medical and Biological Research (2010) 43: 759-766
ISSN 0100-879X

Estradiol-induced hypophagia is associated with the differential mRNA expression of hypothalamic neuropeptides

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Abstract

Estradiol participates in the control of energy homeostasis, as demonstrated by an increase in food intake and in body weight gain after ovariectomy in rats. In the present study, female Wistar rats (200-230 g, N = 5-15 per group), with free access to chow, were individually housed in metabolic cages. We investigated food intake, body weight, plasma leptin levels, measured by specific radioimmunoassay, and the hypothalamic mRNA expression of orexigenic and anorexigenic neuropeptides, determined by real-time PCR, in ovariectomized rats with (OVX+E) and without (OVX) estradiol cypionate treatment (10 µg/kg body weight, *sc*, for 8 days). Hormonal and mRNA expression were determined at pre-feeding and 4 h after food intake. OVX+E rats showed lower food intake, less body weight gain and lower plasma leptin levels. In the OVX+E group, we also observed a reduction of neuropeptide Y (NPY), agouti-related protein (AgRP) and cocaine- and amphetamine-regulated transcript (CART) mRNA expression in the arcuate nucleus and a decrease in orexin A in the lateral hypothalamic area (LHA). There was an increase in leptin receptor (LepRb), melanocortin-4 receptor (MC4-R), CART, and mainly corticotropin-releasing hormone (CRH) mRNA in the paraventricular nucleus and LepRb and CART mRNA in the LHA. These data show that hypophagia induced by estradiol treatment is associated with reduced hypothalamic expression of orexigenic peptides such as NPY, AgRP and orexin A, and increased expression of the anorexigenic mediators MC4-R, LepRb and CRH. In conclusion, estradiol decreases food intake, and this effect seems to be mediated by peripheral factors such as leptin and the differential mRNA expression of neuropeptides in the hypothalamus.

Key words: Estradiol; Leptin; Orexigenic and anorexigenic neuropeptides; Hypothalamus

Introduction

Gonadal hormones influence food intake and body weight in many mammalian species, including mice, rats, and women (1,2). In female rats, ovariectomy increases food intake and body weight, and these changes can be reversed by treatment with estrogen alone (2,3). Additionally, the energy balance of female rats oscillates as a function of the estrous cycle (3). In humans, abdominal fat increases in postmenopausal women, and estrogen replacement therapy prevents increases in body weight and fat accumulation (4).

The hypothalamus and peripheral signals of the energy store play an important role in the control of energy homeostasis. Several neuropeptides expressed in the hypothalamus possess orexigenic or anorexigenic effects and also affect energy expenditure (5). The paraventricular (PVN) and arcuate (ARC) nuclei and lateral hypothalamic area (LHA) are hypothalamic regions involved in the neuronal circuitry that controls food intake. Neurons in these regions are responsive to leptin, a hormone whose actions are mediated by the long form of the leptin receptor (LepRb) (5).

The ARC contains two distinct neuronal populations that express the cocaine- and amphetamine-regulated transcript (CART) and proopiomelanocortin (POMC), and activation of these neurons is associated with a reduction in food intake (5). The cleavage of POMC in the ARC derives the α-melanocyte-stimulating hormone (α-MSH), which inhibits food intake through hypothalamic melanocortin-3 (MC3-R) and melanocortin-4 (MC4-R) receptors. The other neuronal population co-expresses orexigenic peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP) (5). Both neu-

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Received December 7, 2009. Accepted May 25, 2010. Available online June 18, 2010. Published August 13, 2010.

ronal populations project to second-order neurons located in the PVN, LHA and perifornical area. In the PVN, there is high expression of corticotropin-releasing hormone (CRH) (5), known to affect the energy balance through its effects on food intake and energy expenditure (6). The LHA contains neurons expressing melanin-concentrating hormone (MCH) (7) and orexin A (8), two neuropeptides with potent effects on feeding, as demonstrated by the increase in food intake after intracerebroventricular (*icv*) administration of these peptides (9,10).

Neurons of the PVN and LHA also express MC4-R, which has a significant role in the regulation of appetite. The *icv* injection of an MC4-R-specific antagonist increases food intake (11), and targeted deletion of the MC4-R in mice leads to hyperphagia, maturity onset obesity and hyperinsulinemia (12).

Leptin, the product of the *lep* gene (13), is mainly produced and secreted by white adipocytes in proportion to fat mass, signaling the repletion of body energy stores to the hypothalamus (5). In the ARC, leptin inhibits NPY/AgRP neurons and activates POMC/CART neurons (5). In fact, exogenous administration of leptin results in body weight loss, reduced adiposity, and appetite suppression (14).

Although the effect of estradiol on feeding behavior and body weight is well established, the mechanisms underlying these effects of estradiol on the central nervous system are unknown. To evaluate whether estradiol modulates responses to orexigenic or anorexigenic signals in the hypothalamus, the present study determined food intake, body weight changes and the hypothalamic expression of mediators involved in the control of energy homeostasis before and after food intake in ovariectomized rats with and without estradiol treatment.

Material and Methods

Animals

Female Wistar rats (200-230 g) were housed individually in metabolic cages and maintained in a room with constant temperature (23 ± 2°C) and under a 12/12-h light/dark cycle (dark onset: 18:00 h). Rats were given free access to rat chow and tap water. The estrous cycle was assessed with daily vaginal smears, and only those animals having three consecutive estrous cycles were included in this study.

All procedures performed were approved by Ethics Committee for Animal Use of the School of Medicine of Ribeirão Preto (Protocol #004/2006).

Effect of estradiol on food intake, body weight gain, plasma leptin levels, and hypothalamic mRNA expression

After three consecutive estrous cycles, the animals were subjected to bilateral ovariectomy under anesthesia with 2.5% 2.2.2-tribromoethanol (1 mL/100 g body weight, *ip*; Aldrich, USA). On the next day, rats were treated with estradiol cypionate (OVX+E, 10 µg/kg body weight, *sc*; Pfizer, Brazil) or vehicle (corn oil: 0.1 mL/rat, *sc*, OVX) between 9:00 and 10:00 h for 8 days. Food intake and body weight were determined daily during this period. On the 8th day, food was removed from the metabolic cage at 16:00 h and was returned to the cage at dark onset (18:00 h). One set of OVX and OVX+E rats was decapitated at 17:00 h (pre-feeding) and another subgroup of rats (OVX and OVX+E) was decapitated at 22:00 h (4 h after feeding). Trunk blood and brain tissues from these animals were collected under RNase-free conditions for the determination of plasma leptin levels and mRNA expression, respectively.

Measurement of plasma leptin levels

Plasma leptin concentration was measured by radioimmunoassay using the commercial kit Murine Leptin (Linco Research, USA) according to the manufacturer protocol. The assay sensitivity and intra-assay coefficient of variation were 0.5 ng/mL and 1.6%, respectively.

Microdissection

Coronal sections of 600 (PVN) and 1500 µm (ARC and LHA) thickness were cut from frozen brain in a cryostat. Punches were then immediately obtained based on the rat brain atlas coordinates of Paxinos and Watson (15). With a stainless steel punch needle 1.5 mm in diameter, bilateral PVN punches were taken from sections between -1.7 and -2.4 mm relative to the bregma, and bilateral ARC and LHA punches were taken from sections between -2.4 and 3.8 mm relative to the bregma. Punches containing the selected hypothalamic regions were stored at 4°C in RNAlater reagent (Ambion, USA) for a maximum of 48 h until RNA isolation. All procedures were performed under standard RNase-free conditions to avoid exogenous RNase contamination.

RNA isolation and cDNA synthesis

Total RNA was extracted using Trizol reagent (Invitrogen, USA) according to manufacturer instructions. Total RNA was quantified by spectrophotometric absorption at 260 nm (Eppendorf Biophotometer, USA). The quality and integrity of the isolated RNA were demonstrated using agarose gel (1.2%) stained with ethidium bromide. cDNA was synthesized from 250 ηg of total RNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, USA).

Quantitative real-time PCR

Quantitative real-time PCR was performed using Applied Biosystems 7500 real-time PCR system under the following conditions: initial denaturation at 95°C for 10 min during the first stage and a denaturation (95°C for 15 s) and annealing/extension (60°C for 1 min) steps for 40 amplification cycles in the second stage. The following Taqman® Gene Expression Assays (Applied Biosystems, USA) were used in this study: Rn 01431703_g1 (AgRP), Rn 00567382_m1

(CART), Rn 01462137 (CRH), Rn 01491866_s1 (MC4-R), Rn 01410145_m1 (NPY), Rn 00561465_m1 (LepRb), Rn 00565995_m1 (orexin A), Rn 00595020_m1 (POMC), Rn 00561766_g1 (pro-MCH). Each PCR assay was performed in triplicate. Water instead of cDNA was used as a negative control. β-actin, glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA were used as housekeeping genes to normalize the reactions. The relative quantitation was determined by the ΔΔCT method. For each sample, the threshold cycle (Ct: the cycle at which emission intensity rises above baseline values) was determined and normalized to the average of housekeeping genes ($\Delta CT = Ct_{unknown} - Ct_{housekeeping\ genes}$). The fold change of mRNA in the unknown sample (OVX+E at 17:00 or 22:00 h) relative to the respective control group (OVX at 17:00 or 22:00 h) was determined by $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT_{unknown} - \Delta CT_{control\ group}$). Data are reported as relative mRNA expression to that of the control group.

Data analysis

Data are reported as means ± SEM. Statistical analysis was performed using the SigmaStat 3.1 software. Data concerning 24-h food intake, body weight gain and mRNA expression were analyzed by the unpaired *t*-test. Plasma leptin levels were analyzed by two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post hoc* test. Differences between means were considered to be significant when $P < 0.05$.

Results

Food intake, body weight and plasma leptin levels

Estradiol treatment (N = 15) induced lower food intake ($P < 0.01$; Figure 1) and body weight gain compared to the untreated OVX group (N = 13; $P < 0.01$).

The OVX+E group showed higher plasma leptin levels compared to the OVX group at 17:00 h (pre-feeding: 2.4 ± 0.3 *vs* 1.6 ± 0.3 ηg/mL) and at 22:00 h (post-feeding: 3.7 ± 0.3 *vs* 2.5 ± 0.3 ηg/mL; Figure 1). Plasma leptin levels were higher ($P < 0.05$) at 22:00 h than at 17:00 h in both OVX and OVX+E groups.

Hypothalamic mRNA expression

Figure 2 shows a significant reduction ($P < 0.05$) in NPY and AgRP mRNA expression in the ARC at 17:00 h in estradiol-treated animals. In addition, estradiol treatment decreased CART mRNA expression in this nucleus both at 17:00 and 22:00 h. There was no difference in POMC or LepRb mRNA expression in the ARC at 17:00 and 22:00 h in either the OVX or OVX+E group.

LepRb, MC4-R, CRH, and CART mRNA expression in the PVN is shown in Figure 3. At 17:00 h, LepRb, MC4-R, CART, and mainly CRH mRNA expression levels were significantly higher in the OVX+E group compared to the OVX group. However, no significant difference in mRNA

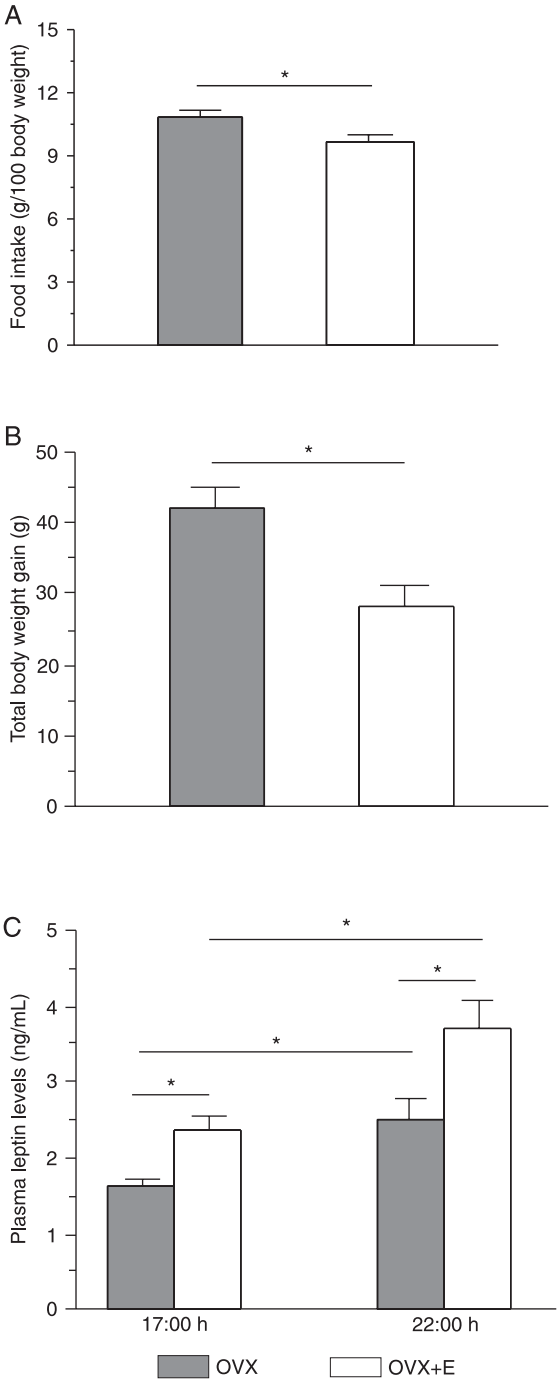

Figure 1. Mean daily food intake (Panel A), total body weight gain (Panel B) after 8 days of treatment with oil (OVX, N = 13) or estradiol (OVX+E, N = 15) in ovariectomized rats. Plasma leptin levels (Panel C) in the pre-feeding (17:00 h) and post-feeding (22:00 h) periods of OVX (N = 7-8) and OVX+E (N = 7-8) groups. Data are reported as means ± SEM. *$P < 0.05$ (unpaired *t*-test or two-way ANOVA followed by the Student-Newman-Keuls *post hoc* test).

levels of these neuropeptides between the OVX and OVX+E groups was found at 22:00 h. Figure 4 shows LepRb, MC4-R, orexin A, pro-MCH, and CART mRNA expression in the LHA of OVX and OVX+E groups. There was no difference in the MC4-R and pro-MCH mRNA expression between the OVX and OVX+E groups, either at 17:00 or 22:00 h. It was also observed that LepRb and CART mRNA expression at 22:00 h were higher in the OVX+E group compared to the OVX group, with no difference at 17:00 h. Orexin A mRNA expression was lower at 17:00 h in the OVX+E rats, with no difference between groups at 22:00 h.

Discussion

The present study demonstrated that estradiol treatment of OVX rats induced lower food intake, less body weight gain and lower plasma leptin levels both before and after feeding. Furthermore, the mRNA expression of several

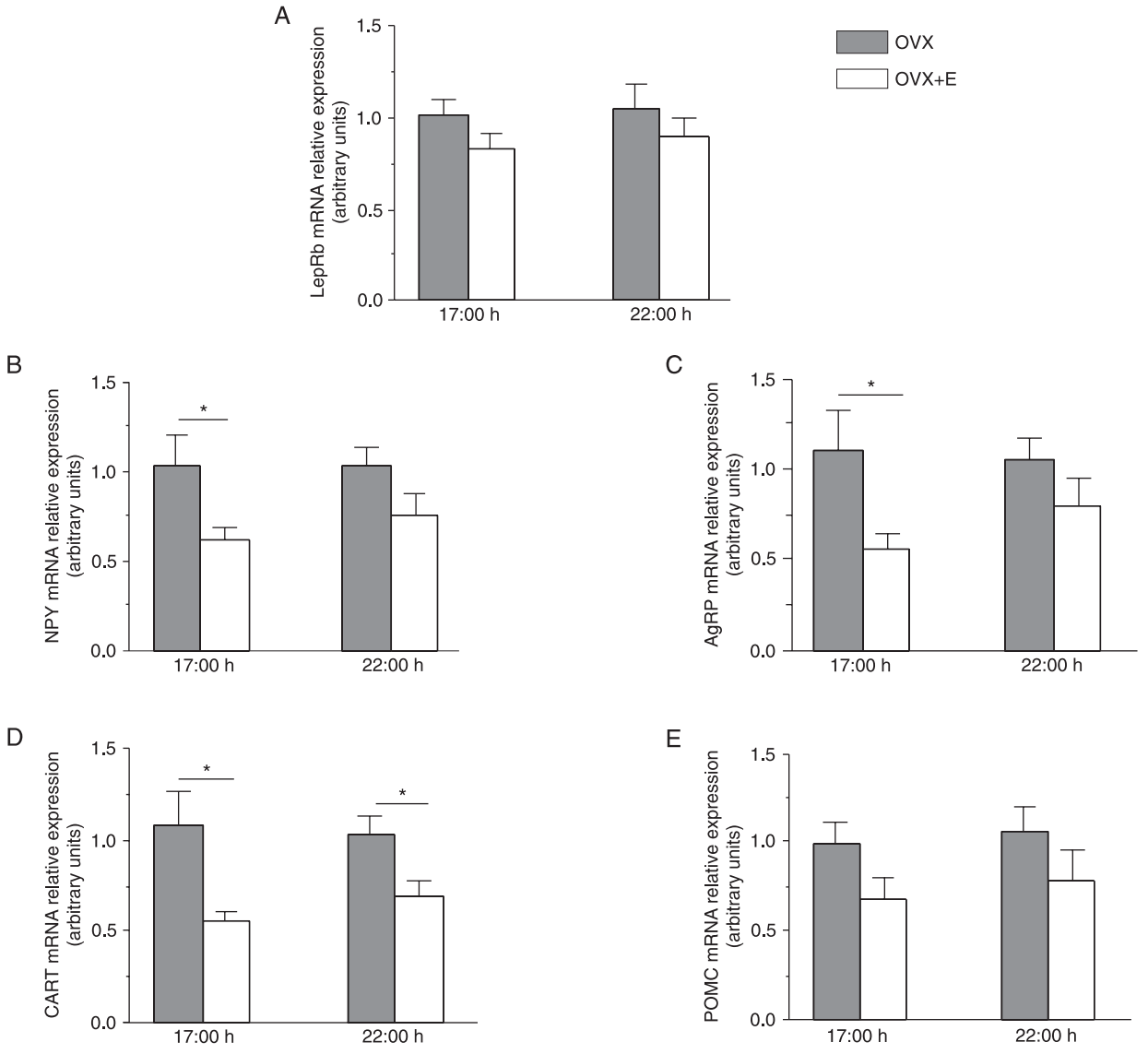

Figure 2. Relative NPY, AgRP, CART, LepRb, and POMC mRNA expression in the arcuate nucleus at 17:00 h (pre-feeding) and 22:00 h (post-feeding) of ovariectomized rats with (OVX+E) and without estradiol treatment (OVX), N = 4-7 per group. Values are reported as means ± SEM. LepRb = leptin receptor (Panel A); NPY = neuropeptide Y (Panel B); AgRP = agouti-related protein (Panel C); CART = cocaine- and amphetamine-regulated transcript (Panel D); POMC = proopiomelanocortin (Panel E). *P < 0.05 (unpaired *t*-test).

neuropetides in specific hypothalamic nuclei showed that estradiol can modify the mRNA expression of neuropeptides and receptors localized in the ARC, PVN and LHA.

The lower food intake and reduced body weight gain of the OVX+E group is in agreement with the well-established role of estradiol in modulating energy homeostasis. Indeed, variation of food intake and body weight of adult female rats can be correlated with the estrous cycle, with lower food intake during the peri-ovulatory phase (2).

We observed increased plasma leptin levels at 17:00 and 22:00 h in estradiol-treated animals. These data agree with a previous report (16), but differ from data presented in another study, which reports that estradiol treatment does not modify plasma leptin levels (17). The reason for this discrepancy may be related to the experimental protocols, duration of estradiol treatment and feeding status. To reinforce the notion that estrogens affect leptin secretion, it has been shown that estradiol has a direct effect on white adipocytes, increasing the synthesis and secretion of leptin (16), as well as its mRNA expression (18). The presence of estrogen receptors in adipocytes (19) provides a possible mechanism by which estrogen may modulate food intake, increasing leptin release.

We also observed increased plasma leptin levels in the post-feeding period (22:00 h), when compared to the pre-feeding period (17:00 h) in both groups (OVX and OVX+E). Leptin secretion is related to adiposity; however, leptin gene expression in white adipose tissue and circulating leptin are also regulated by acute or short-term stimulation (20,21). These data suggest that food intake may modulate leptin secretion and reinforce the notion that it may play a role in the process of satiety, modulating meal size (22). The increase in plasma leptin levels after feeding may be mediated, in part, by insulin, which directly stimulates the synthesis and secretion of leptin by adipocytes (23).

It has been demonstrated that estrogen-deficient rats show insensitivity to central leptin administration, which may contribute to their increased body weight (24). We observed an increase of LepRb mRNA expression in the PVN in the pre-feeding period (17:00 h) and in the LHA in the post-feeding period (22:00 h) in the OVX+E group. Using the whole hypothalamus, Rocha et al. (17) demonstrated an increase in LepRb mRNA expression in intact rats treated with estradiol. The abundance of leptin receptors is likely

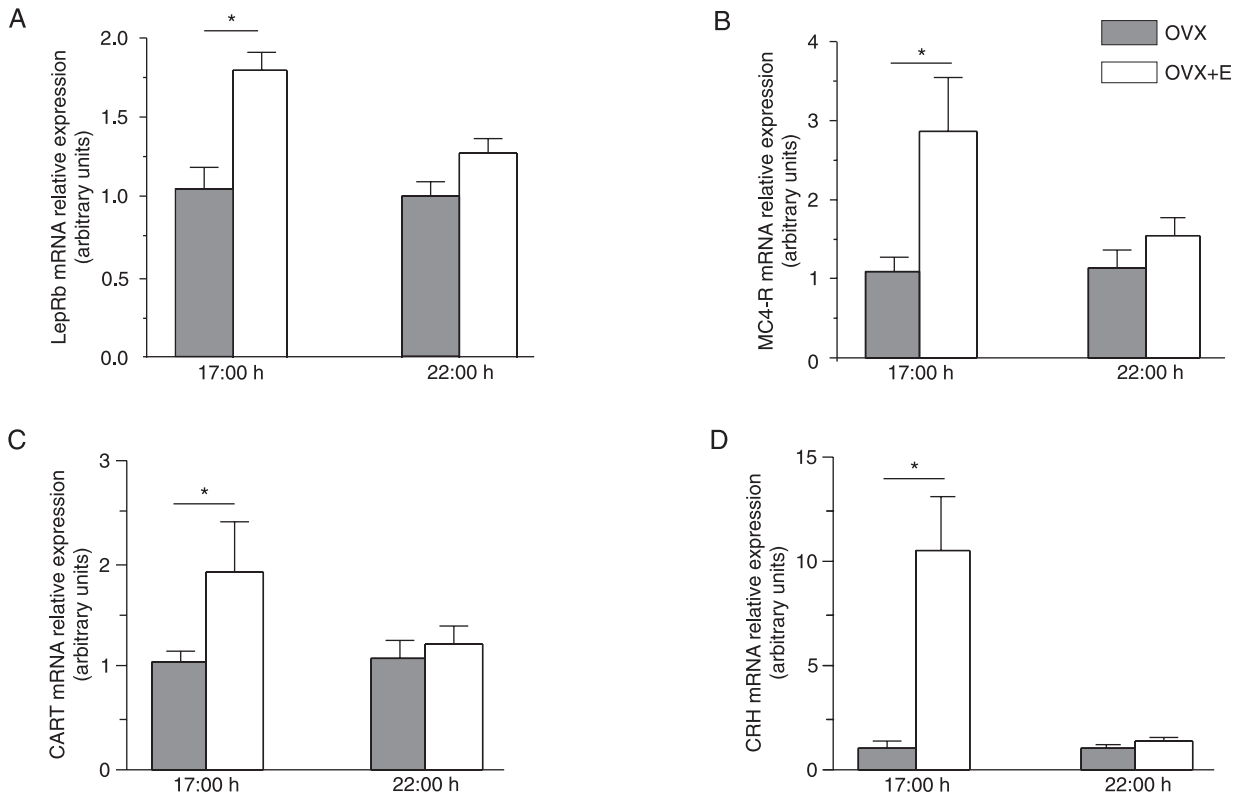

Figure 3. Relative LepRb, MC4-R, CART, and CRH mRNA expression in the paraventricular nucleus at 17:00 h (pre-feeding) and 22:00 h (post-feeding) of ovariectomized rats with (OVX+E) and without estradiol treatment (OVX), N = 4-8 per group. Data are reported as means ± SEM. LepRb = leptin receptor (Panel A); MC4-R = melanocortin-4 receptor (Panel B); CART = cocaine- and amphetamine-regulated transcript (Panel C); CRH = corticotropin-releasing hormone (Panel D). *P < 0.05 (unpaired *t*-test).

to be an important determinant of leptin sensitivity (25). Therefore, though no change of LepRb mRNA expression was observed in the ARC in the present study, increased expression of this receptor in the PVN and LHA might contribute to the lower food intake by OVX+E rats.

Our data showed that estradiol treatment reduced NPY and AgRP mRNA expression in the ARC that could be due to higher plasma leptin levels in the OVX+E group. This result is consistent with a report from Pelletier et al. (26), which showed that estradiol administration to OVX female mice reduced NPY mRNA expression in the ARC. The present results reinforce the idea that the interaction

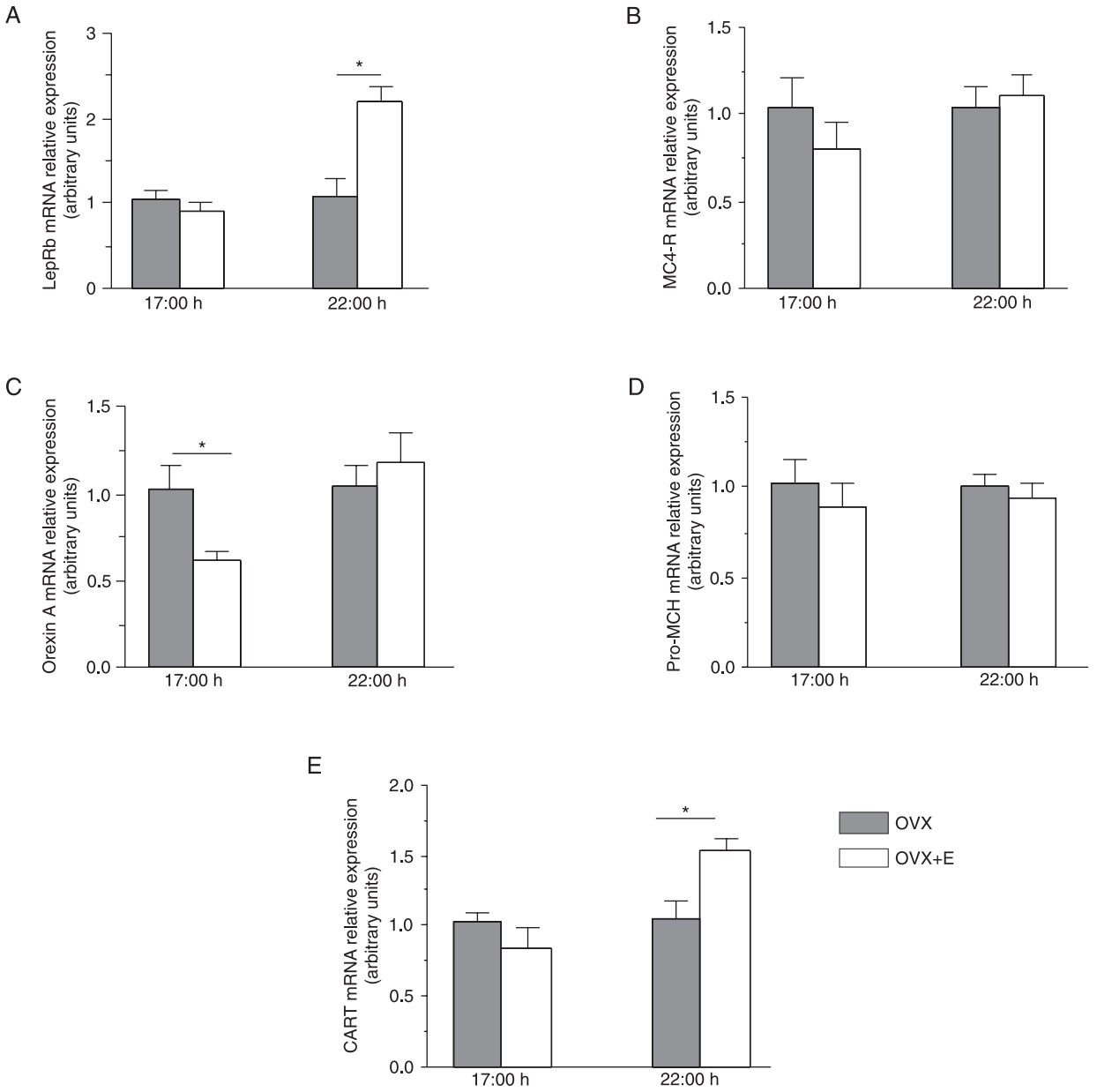

Figure 4. Relative LepRb, MC4-R, orexin A, pro-MCH, and CART mRNA expression in the lateral hypothalamic area at 17:00 h (pre-feeding) and 22:00 h (post-feeding) of ovariectomized rats with (OVX+E) and without estradiol treatment (OVX), N = 5-8 per group. Data are reported as means ± SEM. LepRb = leptin receptor (Panel A); MC4-R = melanocortin-4 receptor (Panel B); orexin A (Panel C); pro-MCH = pro-melanin-concentrating hormone (Panel D); CART = cocaine- and amphetamine-regulated transcript (Panel E). *P < 0.05 (unpaired *t*-test).

of leptin and estrogen signaling in hypothalamic regions is related to energy homeostasis (27,28). It is also important to point out that estradiol may also interact with the action of ghrelin on NPY neurons by attenuating the orexigenic effects of this peptide produced by the stomach (2).

In the ARC, we found a decrease of CART expression in OVX+E rats. CART neurons in the ARC have been shown to project to areas containing neurons that show immunoreactivity to gonadotropin-releasing hormone (GnRH) (29). Therefore, CART neurons from the ARC, under the influence of estradiol, might be involved in the control of the hypothalamic-pituitary-ovarian axis.

It is interesting to note that, in spite of the co-localization of CART and POMC in the ARC, CART was reduced in OVX+E rats, but there was no change in POMC mRNA expression in these rats. This result agrees with previous reports using different experimental models (30,31) that also showed a differential expression of CART and POMC mRNAs, suggesting a distinct intracellular regulation of these neuropeptides.

We observed a marked increase of CRH mRNA in the PVN of OVX+E rats during the pre-feeding period. CRH has also been shown to mediate the anorexigenic effects of leptin (32). Besides having anorexigenic effects, CRH has been shown to increase sympathetic nerve activity in brown adipose tissue involved in the control of thermogenesis (6) and also to mediate leptin actions on the expression of uncoupling protein mRNA in this tissue (33). Parvocellular PVN neurons sensitive to estrogen have been shown to possess reciprocal projections with neurons in the nucleus of the solitary tract, which participate in the PVN-hindbrain pathway involved in the control of energy homeostasis (2). Thus, our result suggests that estradiol, by increasing CRH mRNA expression in the PVN, may contribute to diminished food intake and to enhanced energy expenditure.

We also observed that estradiol increased CART gene expression in the PVN during the pre-feeding period. Wang et al. (34) showed that the injection of this neuropeptide into the PVN decreased NPY-induced feeding and body weight. Thus, our data indicate that the increased CART in the PVN could mediate the inhibitory effect of estradiol on food intake. In addition, estradiol treatment induced an increase in MC4-R mRNA expression in the PVN during the pre-feeding period. The activation of this receptor decreases food intake and also stimulates sympathetic outflow to brown adipose tissue and, consequently, thermogenesis (35). A decrease in MC4-R expression after food intake was also observed in the group treated with estradiol. This result is consistent with previous studies that demonstrated a down-regulation of this receptor after feeding (36,37). On the basis of the present study, we suggest that increased downstream MC4-R signaling may also contribute to the inhibition of food intake and the enhancement of energy expenditure induced by estradiol.

The hypophagic effect of estradiol was also associated with a reduction of orexin A mRNA expression in the LHA at 17:00 h, indicating that change in this neuropeptide expression also contributes to the decrease in food intake observed in OVX+E rats. There is substantial evidence demonstrating that orexin has other physiological effects, being involved in the control of sexual behavior and GnRH and LH release (38). The significance of the differential expression of orexin mRNA found in the present study and its participation in the hypothalamic-pituitary-ovarian axis remains to be established. We observed that in the LHA there was no difference in MCH mRNA expression, but there was higher CART expression at 22:00 h in the OVX+E group. It was shown that CART colocalizes extensively with MCH in the LHA (39). Though the functions of CART in this area are not well established, it could counteract the orexigenic effect of MCH (39).

The results of the present study provide evidence that estradiol affects energy homeostasis through its effects on food intake, which are mediated by peripheral factors, such as leptin released by white adipose tissue and by differential expression of hypothalamic neuropeptides.

Acknowledgments

The authors thank Maria Valci Aparecida dos Santos Silva, Marina Holanda, and Rubens Fernando de Melo for excellent technical assistance. Research supported by FAPESP, CAPES and CNPq.

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