ELSEVIER

Journal of Nutritional Biochemistry 15 (2004) 328–334

THE JOURNAL OF Nutritional Biochemistry

Physiologic estradiol levels enhance hypothalamic expression of the long form of the leptin receptor in intact rats

Milagros Rocha[a,*], Chen Bing[b], Gareth Williams[b], Marisa Puerta[a]

[a]*Department of Physiology (Animal Physiology II), Faculty of Biological Sciences, Complutense University, 28040 Madrid, Spain*
[b]*Neuroendocrine and Obesity Biology Unit, Department of Medicine, University of Liverpool, University Clinical Departments, Liverpool L69 3GA, United Kingdom*

Received 22 January 2003; received in revised form 12 December 2003; accepted 4 January 2004

Abstract

Estradiol is a potent hypophagic agent that reduces food intake and body weight without a concomitant fall in plasma leptin levels. We investigated whether the hypophagic effect of estradiol is mediated by stimulating POMC and/or inhibiting NPY neuronal pathways in the hypothalamus, which respectively inhibit and stimulate feeding. We examined hypothalamic gene expression of Ob-Rb, NPY, POMC, MC4-R, and AgRP in intact Wistar rats treated with estradiol for 48 hours. Food intake and body weight were reduced in estradiol-treated rats but fat mass was unchanged; plasma leptin and insulin levels were not significantly different from untreated, freely fed controls. In untreated rats that were pair-fed to match the estradiol-treated group, body weight was also reduced without changes in fat mass, although leptin and insulin levels decreased significantly. Ob-Rb expression was increased in both hypophagic groups despite serum leptin were only decreased in pair-fed animals, suggesting an estradiol-stimulating effect on Ob-Rb expression. No significant differences were found in POMC, AgRP, or MC4-R expression among any of the experimental groups. A significant but small decrease in NPY expression was also found in both hypophagic groups; this was explained by the combined effect of both surgery and reduced food intake. These results indicate that estradiol mediated hypophagia in intact rats could be brought about by an enhanced hypothalamic leptin sensitivity but is unlikely to be driven by changes in NPY or melanocortin system.

Keywords: Neuropeptide Y; Long form of leptin receptor; Estradiol; Hypothalamus; Melanocortins; Energy balance; Food intake

1. Introduction

The anorectic effect of estradiol is well documented. Food intake falls just after the estradiol peak in proestrus [1] and also on the last day of pregnancy when estradiol peaks [2]. Administration of estradiol to intact or ovariectomized rats causes a transient decrease in food intake and body weight [3,4]. The central mechanisms of estradiol-induced hypophagia are not fully understood. Estradiol injection into the medial preoptic nucleus (MPN) [5] or in the paraventricular nucleus (PVN) [6] decreases food intake. These sites, especially the PVN, are crucial in coordinating the effects of numerous neural pathways on energy homeostasis, including those containing the appetite-stimulating peptide neuropeptide Y (NPY) and the appetite-inhibiting peptides the melacortins and corticotropin-releasing hormone (CRH).

The arcuate nucleus (ARC) is a key hypothalamic site involved in food intake and body weight. Some ARC neurons express NPY, the injection of which into the third ventricle or PVN potently increases food intake [7–9]. These neurons also express the long form of the leptin receptor (Ob-Rb) [10,11] and are inhibited by leptin [12,13]. A separate population of ARC neurons expresses proopiomelanocortin (POMC), the precursor of α-melanocyte–stimulating hormone (α-MSH), which powerfully inhibits feeding through the hypothalamic melanocortin-3 and melanocortin-4 receptors (MC3-R and MC4-R, respectively) [14,15]. POMC is also colocalized with Ob-Rb [16], and its expression is stimulated by leptin [17,18]. These opposing neuropeptide systems interact at different levels. The NPY neurons co-express agouti-related peptide (AgRP) [19], an antagonist at MC4-R [20], which reinforces the action of NPY by inhibiting the action of α-MSH. In addition, NPY

* Corresponding author. Tel.: 34 91 394 49 90; fax: 34 91 394 49 35.
E-mail address: mpuerta@bio.ucm.es (M. Puerta).

0955-2863/04/$ – see front matter
doi:10.1016/j.jnutbio.2004.01.003

neurons are thought to inhibit POMC neurons via NPY Y_1 receptors [21], whereas the POMC neurons may inhibit NPY expression and release via MC3-R [22].

Previous studies suggest that estradiol may stimulate CRH neurons and/or inhibit NPY neurons. The presence of estradiol receptors and CRH in MPN neurons together with the attenuation of estradiol-mediated hypophagia after the administration of a CRH antagonist supports a role for CRH in the hypophagic effects of estradiol [5,23]. ARC NPY neurons also carry estradiol receptors [24,25] as well as NPY expression and peptide concentration in the PVN decrease after estradiol administration [26,27]. Nonetheless, all of these studies were carried out in ovariectomized rats, which have a higher food intake and are heavier than intact animals [28,29].

In this study, we examined whether exogenous estradiol alters gene expression of not only NPY but also the other components of the system (namely, hypothalamic Ob-Rb, POMC, MC4-R, and AgRP), as their balance clearly controls food intake under normal condition and other physiological adjustments. We studied rats treated with estradiol to reach the physiological levels known to reduce both food intake and body weight [3,30]. Intact rats were used because ovariectomy increases hypothalamic NPY expression [27] and protein content in PVN [29], whereas it decreases hypothalamic CRH inmunoreactivity [31], with these effects being reverted by estradiol administration [26,32]. Thermoneutrality was selected to avoid thermoregulatory requirements influencing food intake [33]. To allow for any nonspecific effects of underfeeding and weight loss, mRNA levels of the hypothalamic neuropeptides and receptors were also examined in freely fed, untreated controls and in untreated rats that were pair-fed to match the intake of the estradiol-treated rats.

2. Methods and materials

2.1. Animals

Female Wistar rats (180–200 g) were housed in individual cages and maintained at 28°C (thermoneutrality) with a dark:light cycle of 12-hour light, 12-hour dark (lights on at 8 AM). Each rat's estrous cycle was assessed for 4 days before the experiment with the estrous phase defined as day 0 of the experiment. Animals were divided into three groups: 1) rats freely fed and estradiol-treated; 2) those untreated; and 3) those freely fed, untreated, and pair-fed to match the intake of the estradiol-treated group. All untreated animals received an empty Silastic capsule (0.5 cm long; 3.2 mm OD; 1.5 mm ID; Dow Corning, Midland, MI), implanted subcutaneously between the scapulae under halothane anesthesia. In the estradiol-treated group the capsule was filled with 6.1 ± 0.2 mg of 17β-estradiol (Sigma Diagnostics, St. Louis, MO) and released 0.15–0.20 mg daily. The pair-fed rats received an empty Silastic capsule and were given the average food intake of the estradiol-treated group on the appropriate day after implantation. All rats were given a commercial diet (Panlab, Barcelona, Spain) containing 66.7% carbohydrate, 19.3% protein, 3.4% fat, 4.9% cellulose, and 5.7% mineral (w/w). Food intake, body weight and estrous cycle phase were monitored daily, and the animals were maintained in accordance with the principles of The Council of European Communities (86/609 EEC).

After 2 days of treatment, animals were killed by decapitation between 10 AM and 12 noon, and blood trunk was collected and allowed to clot on ice. Serum was obtained and stored at −80°C until analysis. Left-sided white adipose tissue (WAT) pads (inguinal, parametrial, retroperitoneal, perirenal, and periovarian) were dissected and weighed. The hypothalamus was dissected *en bloc* under a binocular microscope, from a frontal slice between the optic chiasm and the mammillary bodies, cutting immediately below the anterior commissure and vertically through each perihypothalamic sulcus [34].

2.2. Serum assays

Serum leptin and insulin concentrations were determined with commercial RIA kits from Linco Research (St. Louis, MO). The intra- and interassay coefficients of variation were 4.6% and 5.7% for leptin and 4.6% and 10.8% for insulin. Serum estradiol was determined using a commercial RIA kit (DiaSorin, Saluggia, Italy), with intra- and interassay coefficients of variation being 8.0% and 9.7%, respectively.

2.3. Measurement of hypothalamic gene expression using reverse transcriptase–polymerase chain reaction

Total RNA was extracted using TriReagent (Sigma Diagnostics, St. Louis, MO), according to the manufacturer's instructions. To avoid DNA contamination, samples were treated with DNase I (Roche Molecular Biochemicals, Mannheim, Germany) for 15 minutes at 37°C followed by incubation at 75°C for 5 minutes to inactivate the enzyme. First-strand complementary DNA (cDNA) was prepared from total RNA by using a Reverse-iT first strand synthesis kit (ABgene, Surrey, UK) in a final volume of 20 μL. Using 0.2 mL Thermo-tube (ABgene), 0.5–1.0 μg RNA sample was added with 0.5 μg of anchored oligo-dT and free DNase, RNase water (Sigma Diagnostics) in a final volume of 13 μL. Samples were heated to 70°C for 5 minutes to remove any secondary structures and then placed on ice. A quantity of 4 μL of 5xfirst-strand synthesis buffer, 2 μL of dNTP mix (5 mmol/L each), and 1 μL of reverse transcriptase (Reverse-iT RTase Blend) was added to each tube. Samples were incubated at 47°C for 30 minutes followed by exposure at 75°C for 10 minutes to inactivate the RTase. Polymerase chain reaction (PCR) was performed in a final volume of 25 μL: 2 μL c-DNA, 0.5 μL of sense and

Table 1
Oligonucleotide primers used for RT-PCR amplification of Ob-Rb, NPY, POMC, MC-4 R, AgRP, and HPRT

Target	Primer Sequence	Concentration (μg RNA/μL)	Cycles (no.)	Temperature (°C)	Size (bp)
Ob-Rb					
Sense	5′-TGAAACATTTGAGCATCTTT-3′	1.0	34	54.5	370
Antisense	5′-CGATGCACTGGCTGACAGAA-3′				
NPY					
Sense	5′-CGCCATGATGCTAGGTAAC-3′	0.5	30	54.0	379
Antisense	5′-CAGACTGGTTTCACAGGATG-3′				
POMC					
Sense	5′-CCTGTGAAGGTGTACCCCAATGTC-3′	0.5	30	58.0	266
Antisense	5′-CACGTTCTTGATGATGGCGTTC-3′				
MC-4 R					
Sense	5′-TATGGTACTGGAGCGCGTAA-3′	0.5	35	60.5	370
Antisense	5′-TCAGACGGAGGATGCTATGA-3′				
AgRP					
Sense	5′-AGAGTTCTCAGGTCTAAGTCT-3′	1.0	32	58.0	210
Antisense	5′-CTTGAAGAAGCGGCAGTAGCACGT-3′				
HPRT					
Sense	5′-CAGTCCCAGCGTCGTGATTA-3′	0.5	30	58.0	138
Antisense	5′-AGCAAGTCTTTCAGTCCTGTC-3′				

Concentrations of RNA used, number of cycles, and annealing temperature selected together with the size of generated cDNA products are also indicated.

antisense primers at 0.02 mmol/L and 22 μL of 1.1 × Reddy Mix PCR Master Mix (ABgene), which contains 1.5 mmol/L Mg Cl_2, 1.25 units Taq polymerase, 75 mmol/L Tris HCl (pH 8.8 at 25 °C), 20 mmol/L $(NH_4)_2$ SO_4, 0.01% Tween 20 (v/v), 0.2 mmol/L each of dATP, dCTP, dGTP, and dTTP, and precipitant and red dye for electrophoresis.

PCR primers used to amplify Ob-Rb, NPY, POMC, MC4-R, AgRP, and house-keeping gene hypoxanthine-phosphoribosyl transferase (HPRT) are listed in Table 1. PCR amplification was performed on a PCR express thermal cycler (Hybaid, Ashford, UK). After initial denaturation at 94°C for 5 minutes, samples were subjected to amplification cycles (Table 1) that consisted of denaturation at 94°C for 25 seconds, annealing for 30 seconds at an optimal temperature for each gene (Table 1), and extension at 72°C for 30 seconds. After the last cycle, samples underwent final elongation at 72°C for 10 minutes.

PCR products were visualized on a 1.0 % agarose gel containing 0.4% (v/v) ethidium bromide (Sigma Diagnostics) and the band density was analyzed with Kodak 1D Image Analysis System (Kodak Digital Science, Eastman Kodak Company, Rochester, NY).

2.4. Statistical analyses

One-way analysis of variance (ANOVA) was performed to compare food intake, body weight, WAT deposit weight, concentrations of serum estradiol, leptin, and insulin, and specific PCR products (namely, Ob-Rb, NPY, POMC, MC4-R, and AgRP). The Student-Newman-Keuls test was used for *post hoc* comparisons. Data are shown as mean ± SEM. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Metabolic and weight changes

Estradiol treatment increased serum estradiol concentration to values similar to those in proestrus (Table 2) and also induced estrous-like changes in vaginal epithelium (data not shown). Food intake was reduced in all three groups 24 hours after capsule implantation, but freely fed controls recovered their initial food intake by 48 hours. By contrast, estradiol-treated rats showed a continuing reduction in food intake that was 32% below controls at the moment of sacrifice (Fig. 1A). At 48 hours, body weight was increased by 1% in controls whereas a significant decline was observed in both estradiol-treated (−1.5%, $P < 0.05$) and pair-fed (−0.5%, $P < 0.05$) groups (Fig. 1B). There were no significant differences in fat masses among any of the groups (Table 2). A positive correlation was found between total fat mass and leptin levels in untreated groups ($r = 0.786$, $P = 0.021$), whereas estradiol-treated did not show any correlation. Serum leptin and insulin levels were reduced in pair-fed rats but unaffected with estradiol administration (Table 2).

3.2. Hypothalamic gene expression changes

The RT-PCR generated products for hypothalamic genes (Ob-Rb, NPY, POMC, MC4-R, AgRP, and HPRT) were detectable in the hypothalamus of all the rats (left panels of figures). Ob-Rb mRNA levels were significantly increased above controls in both estradiol-treated (24%, $P < 0.05$) and pair-fed (39%, $P < 0.01$) groups, with no significant difference between these two groups (Fig. 2).

Table 2
Body weight, visceral white adipose tissue (WAT) deposits, and serum estradiol, leptin, and insulin concentrations in control, estradiol, and pair-fed rats

	Control	Estradiol	Pair-fed
Body weight (g)			
Initial	200.3 ± 2.0	197.9 ± 1.9	197.9 ± 2.3
Final	202.3 ± 2.1[a]	195.0 ± 1.6[b]	197.0 ± 1.9[a,b]
Gain	2.0 ± 0.9[a]	−2.9 ± 0.7[b]	−0.9 ± 1.0[b]
Visceral WAT deposits (g)*	3.93 ± 0.30	3.29 ± 0.23	3.46 ± 0.22
Estradiol (nmol/L)	0.076 ± 0.034[a]	0.636 ± 0.146[b]	0.060 ± 0.020[a]
Leptin (nmol/mL)	0.42 ± 0.08[a]	0.52 ± 0.06[a]	0.17 ± 0.03[b]
Insulin (nmol/mL)	0.26 ± 0.03[a]	0.28 ± 0.03[a]	0.14 ± 0.03[b]

Data are expressed as mean ± SEM of eight animals each group.
* Sum of inguinal, parametrial, retroperitoneal, periovaric, and perirenal left-sided depots.
Different superscript letters indicate differences of $P < 0.05$ between the experimental groups (by one-way analysis of variance).

NPY mRNA levels were slightly but significantly lowered in both estradiol-treated groups (6%, $P < 0.05$) and pair-fed groups (9%, $P < 0.01$) compared with controls, with no significant difference between these two groups (Fig. 3). A negative correlation was found between NPY and Ob-Rb levels in untreated groups ($r = 0.525$, $P = 0.037$), whereas estradiol-treated did not show any correlation.

No significant differences in mRNA levels of POMC, MC4-R, and AgRP were found among any of the experimental groups (Fig. 4).

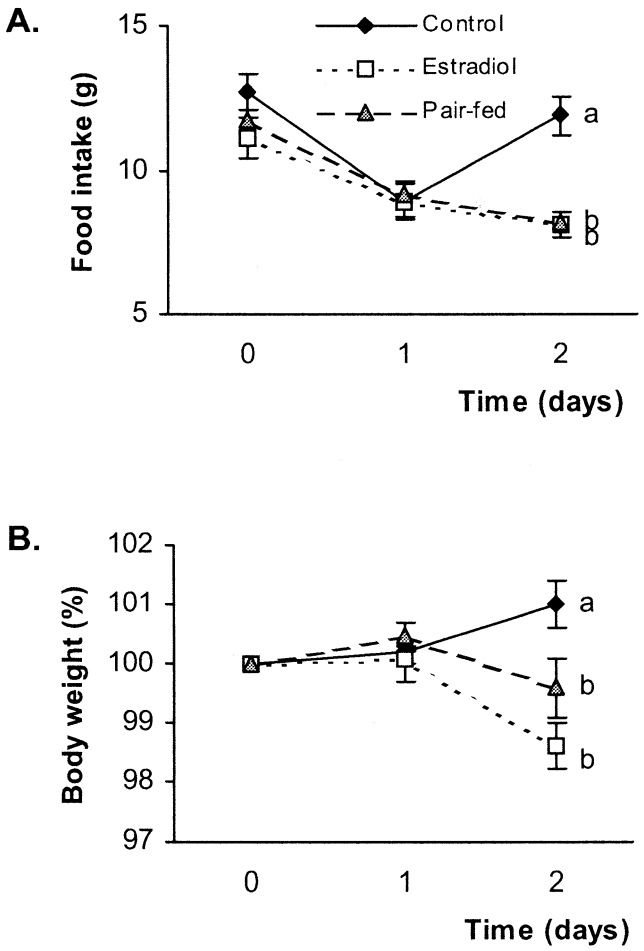

Fig. 1. Food intake (A) and body weight (B) in rats receiving estradiol during 2 days of treatment or pair-fed to the estradiol group. Each point represents mean ± SEM of eight animals. Different superscript letters indicate differences of $P < 0.05$ between the experimental groups by one-way analysis of variance.

4. Discussion

The hypophagic effect of estradiol was again confirmed in this study. Although capsule implantation transiently decreased food intake, estradiol treatment continued to reduce food intake, consistent with previous reports [3]. Body weight was comparably reduced in both estradiol-treated and pair-fed animals, suggesting that hypophagia was primarily responsible. The length of our experiment was determined by the transient effect of estradiol, which peaks in 2–3 days after capsule implantation and disappears in 1 week in intact rats [3], although it last much longer in ovariectomized rats [4]. On the other hand, the length of this experiment mimics the short-term physiological changes that occur during the estrous cycle, when plasma estradiol is only enhanced for less than 24 hours [35].

Serum leptin and insulin levels fell in the pair-fed group, consistent with the effects of food deprivation [36] but were

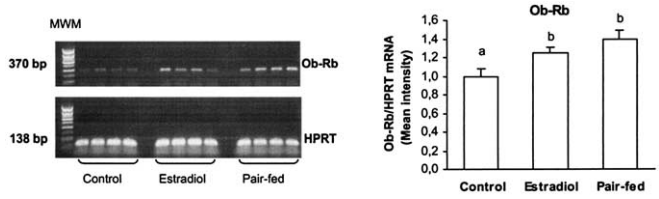

Fig. 2. mRNA levels of Ob-Rb in the hypothalamus of control, estradiol-treated, and pair-fed groups. Left panel shows a representative gel electrophoresis of the PCR products for Ob-Rb and HPRT mRNA. A 100–base pair ladder was used as molecular weight marker (MWM). Right panels show the relative abundance of Ob-Rb mRNA levels, expressed as a ratio to HPRT mRNA. Each bar represents mean ± SEM of eight animals. Different superscript letters indicate differences of $P < 0.05$ between the experimental groups by one-way analysis of variance.

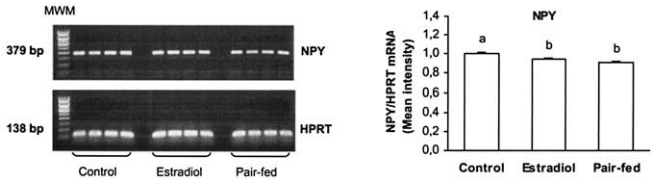

Fig. 3. Depiction of mRNA levels of NPY in the hypothalamus of control, estradiol-treated, and pair-fed groups. Left panel shows representative gel electrophoresis of the PCR products for Ob-Rb and HPRT mRNA. A 100–base pair ladder was used as molecular weight marker (MWM). Right panels show the relative abundance of Ob-Rb mRNA levels, expressed as a ratio to HPRT mRNA. Each bar represents mean ± SEM of eight animals. Different superscript letters indicate differences of $P < 0.05$ between the experimental groups by one-way analysis of variance.

unaltered in the estradiol-treated group. This agrees with the failure of leptin levels to fall in hypophagic, underweight, estradiol-treated rats, which was observed previously [3], and gives support to the suggestion that estradiol has an independent stimulatory effect on leptin; indeed, estradiol enhances leptin synthesis and release *in vitro* [37].

Hypothalamic Ob-Rb mRNA was increased in the pair-fed untreated group that lost weight, consistent with previous reports showing enhanced expression of Ob-Rb mRNA associated with decreased plasma leptin [38]. Ob-Rb mRNA levels were similarly increased in the estradiol-treated group, although plasma leptin did not fall. This suggests that estradiol may modulate *per se* the hypothalamic expression of the Ob-Rb receptor. If functional Ob-Rb protein levels were also increased, it is possible that leptin sensitivity could be enhanced. Therefore, the increased Ob-Rb mRNA levels, together with the unchanged serum leptin in the estradiol-treated group suggest that estradiol may inhibit feeding by reinforcing leptin action at hypothalamic level.

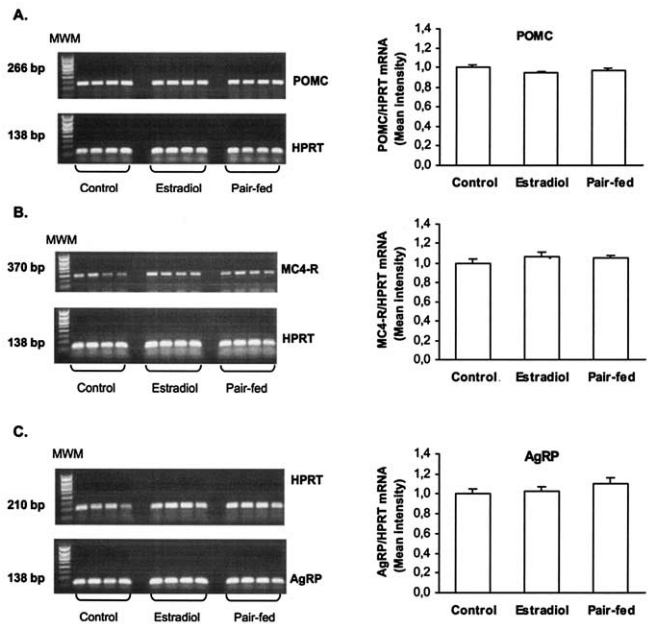

Fig. 4. Depiction of mRNA levels of POMC (A), MC4-R (B), and AgRP (C) in the hypothalamus of control, estradiol-treated, and pair-fed groups. Left panel shows representative gel electrophoresis of the PCR products for Ob-Rb and HPRT mRNA. A 100–base pair ladder was used as molecular weight marker (MWM). Right panels show the relative abundance of Ob-Rb mRNA levels, expressed as a ratio to HPRT mRNA. Each bar represents mean ± SEM of eight animals.

Because Ob-Rb receptors are colocalized with NPYergic and POMC neurons [10,11,16], the relatively enhanced leptin action induced by estradiol could bring about hypophagia by stimulating POMC and/or by inhibiting NPY neurons. Both estradiol-treated and pair-fed rats showed a slight but significant decrease in NPY mRNA. Previous results have showed an enhancement in NPY after long-term fasting [39,40], but an actual reduction after short-term (24 hours) food restriction similar to that used in the pair-fed rats of this study [41]. Moreover, systemic enhanced concentration of glucocorticoids is known to decrease NPY levels [42] and indeed surgery for capsule implantation increase plasma corticosterone concentration [43]. These factors were working in both the estradiol-treated and the pair-fed group so that our results do not support a role for NPY in the hypophagia mediated by estradiol in intact rats.

α-MSH elicits its effects on energy balance by acting on MC4-R whose density is inversely related to the synaptic availability of the agonist [44,45]. Neither POMC mRNA – the precursor of α-MSH – nor MC4-R mRNA were altered by estradiol; indicating that the POMC system is unlikely to be involved in mediating the hypophagic effects of estradiol either in intact rats (as we have shown here) or in ovariectomized rats after short-term estradiol treatment [46]. Neither there were changes in the expression of AgRP, the endogenous antagonist of MC4-R that modulates MC4-R activity under some conditions of altered nutritional state such as dietary obesity and food restriction [47].

Neither POMC nor AgRP mRNA levels were altered by food restriction in pair-fed rats, which is at variance with previous studies showing reduced POMC mRNA [39,48] or increased AgRP mRNA [30]; however, food restriction in these cases lasted much longer (2–3 weeks) than in our study. Thus, those neuropeptides may be more important in responding to longer and more severe food restriction.

Because AgRP is coexpressed by the NPY neurons [19] and both peptides appear to be regulated similarly in conditions of altered nutritional status [49,50], a decrease in AgRP expression could be expected in hypophagic groups. However, recent studies have shown differential gene expression of NPY and AgRP not only in obese rats after fasting [51] but also in late pregnancy—a physiological situation associated with the highest endogenous progesterone levels [52]. Consequently, it may be that NPY and AgRP are not always regulated in the same manner.

The negative results found with the main neuropeptide systems involved in the control of food intake (namely, NPY and POMC) must probably be viewed on the light of the dual model of neural circuit proposed to explain hypophagia as a result of either food unavailability—which in-

volves NPY and POMC systems—or as a result of voluntary behavior [53]. This last circuit includes CRH and melanin concentrating hormone (MCH) in the lateral hypothalamus (LHA), two neuropeptides that have been shown to be involved in the hypophagic effect of estradiol [23,30] and that we did not study in this work. Even more, Ob-Rb receptors have been found to be expressed in the LHA [54] and in neurons that express CRH and MCH [55], suggesting that the changes in Ob-Rb we have observed here could involve these specific subsets of hypothalamic neurons.

In conclusion, short-term administration to intact rats of elevated estradiol to values similar to those found in normal proestrus increases Ob-Rb mRNA, maintaining plasma leptin under conditions when it would normally fall, pointing to this receptor as target for estradiol induced hypophagia. We have demonstrated that a pair-feeding schedule mirroring the amount of food eaten during estradiol treatment has the same effects on mRNA levels of NPY, POMC, MC-4 R, and AgRP as estradiol treatment, suggesting that these genes are unlikely involved in mediating hypophagia induced by estradiol.

Acknowledgments

M. Rocha was the recipient of a Scholarship from Complutense University.

References

[1] Ter Haar MB. Circadian and estrual rhythms in food intake in the rat. Horm Behav 1972;3:213–9.
[2] Lu CC, Tsai SC, Wang SW, Tsai CL, Lau CP, Shih HC, Chen YH, Chiao YC, Liaw C, Wang PS. Effects of ovarian steroid hormones and thyroxine on calcitonin secretion in pregnant rats. Am J Physiol 1998;274:E246–52.
[3] Rocha M, Grueso E, Puerta M. The anorectic effect of oestradiol does not involve changes in plasma and cerebrospinal fluid leptin concentration in the rat. J Endocrinol 2001;171:349–54.
[4] Wade GN. Some effects of ovarian hormones on food intake and body weight in female rats. J Comp Physiol Psychol 1975;88:183–93.
[5] Dagnault A, Richard D. Involvement of the medial preoptic area in the anorectic action of estrogens. Am J Physiol 1997;272:R311–7.
[6] Butera PC, Beikirch RJ, Willard DM. Changes in ingestive behaviors and body weight following intracranial application of 17alpha-estradiol. Physiol Behav 1990;47:1291–3.
[7] Flynn MC, Plata-Salaman CR, Ffrench-Mullen JM. Neuropeptide Y-related compounds and feeding. Physiol Behav 1999;65:901–5.
[8] Kalra SP, Dube MG, Fournier A, Kalra PS. Structure-function analysis of stimulation of food intake by neuropeptide Y: effects of receptor agonists. Physiol Behav 1991;50:5–9.
[9] Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. Proc Natl Acad Sci USA 1985;82:3940–3.
[10] Baskin DG, Schwartz MW, Seeley RJ, Woods SC, Porte D, Breininger JF, Jonak Z, Schaefer J, Krouse M, Burghdat C, Campfield LA, Burn P, Kochan JP. Leptin receptor long-form splice-variant protein expression Y mRNA in the arcuate nucleus. J Histochem Cytochem 1999;47:353–62.
[11] Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT, Morgan PJ, Trayhurn P. Coexpression of leptin receptor and preproneuropeptide Y mRNA in ARC of mouse hypothalamus. J Neuroendocrinol 1996;8:733–5.
[12] Stephens TW, Basinski M, Bristow PK, Bue-Valleskey JM, Burgett SG, Craft L, Hales J, Hoffmann J, Hsiung HM, Kriauciunas A, MacKeller W, Rosteck P, Schoner B, Smith D, Tinsley FC, Zhang XY, Heiman M. The role of neuropeptide Y in the antiobesity actions of the gene product. Nature 1995;377:530–2.
[13] Wang Q, Bing C, Al-Baranzanji K, Mooskowaska DE, Wang X-M, McBay DL, Neville WA, Taddayon M, Pickavance L, Dryden S, Thomas MEA, McHale MT, Gloyer IS, Wilson S, Buckingham R, Arch JRS, Trayhurn P, Williams G. Interactions between leptin and hypothalamic neuropeptide Y neurons in the control of food intake and energy homeostasis in the rat. Diabetes 1997;46:335–41.
[14] Fan W, Boston B, Kesterson R, Hruby V, Cone R. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. Nature 1997;385:165–8.
[15] Grill HJ, Ginsberg AB, Seeley RJ, Kaplan JM. Brainstem application of melanocortin receptor ligands produces long-lasting effects on feeding and body weight. J Neurosci 1998;18:10128–35.
[16] Cheung CC, Clifton DK, Steiner RA. Proopiomelanocortin neurons are direct targets for leptin in the hypothalamus. Endocrinology 1997;138:4489–92.
[17] Cowley MA, Smart JL, Rubinstein M, Cerdán MG, Diano S, Horvath TL, Cone RD, Low MJ. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. Nature 2001;411:480–4.
[18] Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG. Leptin increases hypothalamic proopiomelanocortin mRNA expression in the rostral arcuate nucleus. Diabetes 1997;46:2119–23.
[19] Broberger C, Johansen J, Johansson C, Schalling M, Hokfelt T. The neuropeptide Y/agouti gene-related protein (AGRP) brain circuitry in normal, anorectic, and monosodium glutamate-treated mice. Proc Natl Acad Sci USA 1998;95:15043–8.
[20] Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. Science 1997;278:135–8 (published erratum appears in Science 1998;281:1615).
[21] Fuxe K, Tinner B, Caberlotto L, Bunnemann B, Agnati LF. NPY Y_1 receptor like immunoreactivy exists in a sub population of beta-endorphin inmunoreactive nerve cells in the arcuate nucleus: a double immunolabelling analysis in the rat. Neurosci Lett 1997;225:49–52.
[22] Bagnol D, Lu X-Y, Kaelin CB, Day HEW, Ollman M, Gantz I, Akil H, Barsh GS, Watson SJ. Anatomy of an endogenous antagonist: relationship between agouti-related protein and proopiomelanocortin in brain. J Neurosci 1999;19 RC26(1–7).
[23] Dagnault A, Ouerghi D, Richard D. Treatment with alpha-helical-CRF(9-41) prevents the anorectic effect of 17-beta-estradiol. Brain Res Bull 1993;32:689–92.
[24] Shughrue PJ, Lane MV, Merchenthaler I. Comparative distribution of estrogen receptor-alpha and -beta mRNA in the rat central nervous system. J Comp Neurol 1997;15:507–25.
[25] Sun F, Yu J. The effects of a special herbal tea on obesity and anovulation in androgen-sterilized rats. Proc Soc Exp Biol Med 2000;223:295–301.
[26] Bonavera JJ, Dube MG, Kalra PS, Kalra SP. Anorectic effects of estrogen may be mediated by decreased neuropeptide-Y release in the hypothalamic paraventricular nucleus. Endocrinology 1994;134:2367–70.
[27] Shimizu H, Ohtani K, Kato Y, Tanaka Y, Mori M. Withdrawal of estrogen increases hypothalamic neuropeptide Y (NPY) mRNA expression in ovariectomized obese rats. Neurosci Lett 1996;204:81–4.
[28] Richard D. Effects of ovarian hormones on energy balance and brown adipose tissue thermogenesis. Am J Physiol 1986;250:R245–9.

[29] Ainslie DA, Morris MJ, Wittert G, Turnbull H, Proietto J, Thorburn AW. Estrogen deficiency causes central leptin insensitivity and increased hypothalamic neuropeptide Y. Int J Obes 2001;25:1680–8.
[30] Mystkowski P, Seely RJ, Hahn TM, Baskin DG, Havel PJ, Matsumoto AM, Wilkinson CW, Peacock-Kinzig K, Blake KA, Schwartz MW. Hypothalamic melanin-concentrating hormone and estrogen-induced weight loss. J Neurosci 2000;20:8637–42.
[31] Haas DA, Geirge SR. Gonadal regulation of corticotropin-releasing factor immunoreactivity in hypothalamus. Brain Res Bull 1988;20: 361–7.
[32] Roy BN, Reid RL, Van Vugt DA. The effects of estrogen and progesterone on corticotropin-releasing hormone and arginine vasopressin messenger ribonucleic acid levels in the paraventricular nucleus and supraoptic nucleus of the rhesus monkey. Endocrinology 1999;140:2191–8.
[33] Puerta M, Nava MP, Abelenda M, Fernández A. Inactivation of brown adipose tissue thermogenesis by oestradiol treatment in cold-acclimated rats. Pflügers Arch 1990;416:659–62.
[34] Williams G, Gill JS, Lee YC, Cardoso HM, Okpere BE, Bloom SR. Increased neuropeptide Y concentrations in specific hypothalamic regions of streptozotocin-induced diabetic rats. Diabetes 1989;38: 321–7.
[35] Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. Endocrinology 1974;94:1704–8.
[36] Ahima RS, Prabakaran D, Mantzoros CS, Qu D, Lowell BB, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. Nature 1996;382:250–2.
[37] Machinal F, Dieudonne MN, Leneveu MC, Pecquery R, Giudicelli Y. *In vivo* and *in vitro* ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroids hormones. Endocrinology 1999;140:1567–74.
[38] Bing C, Taylor S, Tisdale MJ, Williams G. Cachexia in MAC16 adenocarcinoma: suppression of hunger despite normal regulation of leptin, insulin and hypothalamic neuropeptide Y. J Neurochem 2001; 79:1004–12.
[39] Brady LS, Smith MA, Gold PW, Herkenham M. Altered expression of hypothalamic neuropeptide RNAs in food-restricted and food-deprived rats. Neuroendocrinology 1990;52:441–7.
[40] Schwartz MW, Sipols AJ, Grubin CE, Baskin DG. Differential effect of fasting on hypothalamic expression of genes encoding neuropeptide Y, galanin, and glutamic acid decarboxylase. Brain Res Bull 1993;31:361–7.
[41] Frankish HM, Dryden S, Wang Q, Bing C, MacFarlane IA, Williams G. Nicotine administration reduces neuropeptide Y and neuropeptide Y mRNA concentrations in the rat hypothalamus: NPY may mediate nicotine's effects on energy balance. Brain Res 1995;694:139–46.
[42] Zakrzewska KE, Cusin I, Stricker-Krongrad A, Boss O, Ricquier D, Jeanrenaud B, Rohner-Jeanrenaud F. Induction of obesity and hyperleptinemia by central glucocorticoid infusion in the rat. Diabetes 1999;48:365–70.
[43] Grueso E, Rocha M, Puerta M. Plasma and cerebrospinal fluid leptin levels are maintained despite enhanced food intake in progesterone-treated rats. Eur J Endocrinol 2001;144:659–65.
[44] Blake AD, Bot G, Li S, Freeman JC, Reisine T. Differential agonist regulation of the human kappa-opioid receptor. J Neurochem 1997; 68:1846–52.
[45] Shockley MS, Burford NT, Sadee W, Lameh J. Residues specifically involved in down-regulation but not internalization of the m1 muscarinic acetylcholine receptor. J Neurochem 1997;68:601–9.
[46] Treiser SL, Wardlaw SL. Estradiol regulation of proopiomelanocortin gene expression and peptide content in the hypothalamus. Neuroendocrinology 1992;55:167–73.
[47] Harrold JA, Williams G, Widdowson PS. Changes in hypothalamic agouti-related protein (AGRP) but not a alpha-MSH or pro-opiomelanocortin concentrations in dietary-obese and food-restricted rats. Biochem Biophys Res Commun 1999;258:574–7.
[48] Kim EM, Wejch CC, Grace MK, Billington CJ, Levine AS. Chronic food restriction and acute food deprivation decrease mRNA levels of opioid peptides in arcuate nucleus. Am J Physiol 1996;270:R1019–24.
[49] Wang H, Storlien LH, Huang XF. Effects of dietary fat types on body fatness, leptin, and ARC leptin receptor, NPY, and AgRP mRNA expression. Am J Physiol 2002;282:E1352–9.
[50] Chen P, Li C, Haskell-Luevano C, Cone RD, Smith MS. Altered expression of agouti-related protein and its colocalization with neuropeptide Y in the arcuate nucleus of the hypothalamus during lactation. Endocrinology 1999;140:2645–50.
[51] Korner J, Savontaus E, Chua SC, Leibel RL, Wardlaw SL. Leptin regulation of *Agrp* and *NPY* mRNA in the rat hypothalmus. J Neuroendocrinol 2001;13:959–66.
[52] Rocha M, Bing C, Williams G, Puerta M. Pregnancy-induced hyperphagia is associated with increased gene expression of hypothalamic agouti-related peptide in rats. Regul Peptides 2003;114:159–65.
[53] Watts AG, Sanchez-Watts G, Kelly AB. Distinct patterns of neuropeptide gene expression in the lateral hypothalamic area and arcuate nucleus are associated with dehydration-induced anorexia. J Neurosci 1999;19:6111–21.
[54] Funahashi H, Ryushi T, Mizushima H, Katoh S, Shioda S. Ultrastructural localization of the receptor for leptin in the rat hypothalamus. Horm Behav 2000;37:327–34.
[55] Håkansson ML, Brown H, Ghilardi N, Skoda RC, Meister B. Leptin receptor immunoreactivity in chemically defined target neurons of the hypothalamus. J Neurosci 1998;18:559–72.