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Neuropeptide Y regulates the leptin receptors in rat hypothalamic and pituitary explant cultures

María Paula Di Yorio, María Guillermina Bilbao, Alicia Graciela Faletti *

Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Facultad de Medicina, Universidad de Buenos Aires, Argentina

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ABSTRACT

The aim of this work was to investigate whether the expression of leptin receptors (OBR) in the hypothalamic–pituitary (HP) axis is regulated by the orexigenic neuropeptide Y (NPY) during ovulation. To this end, we performed in vitro assays, using cultures of both hypothalamic and anterior pituitary explants from immature rats primed with gonadotropins to induce ovulation. In hypothalamic explants, protein expression of both the long and short OBR isoforms was increased by the presence of NPY at 100–500 ng/ml and at 300–500 ng/ml, respectively. Similarly, in pituitary explants, protein expression of the long isoform was increased between 30 and 300 ng/ml while that of the short isoform was increased only at 300 ng/ml. When both tissues were incubated with NPY and BIBP3226, a specific antagonist of the NPY Y1 receptor subtype, the NPY-induced protein expression was totally reversed by the antagonist at almost every concentration assayed. However, this antagonist was not always capable of blocking the increase caused by the presence of NPY at transcript level. In conclusion, our results indicate that NPY is able to regulate the expression of both the long and the short isoforms of OBR in the HP axis, at least in part, through the NPY Y1 receptor. These results reinforce the fact that NPY and its NPY Y1 receptor play a critical role in reproduction by modulating leptin sensitivity.

1. Introduction

Leptin is a protein hormone synthesized and secreted mainly by adipocytes and transported through the blood to act in specific central and peripheral systems. This protein plays an important role in neuroendocrine signaling and reproduction. Leptin is recognized by the leptin receptor (OBR) [1], which is encoded by the *Lepr* gene [2,3]. Six leptin receptor isoforms generated by alternative splicing have been identified to date [4,5]: i) the long form (OBRb), which is the primary isoform expressed in the hypothalamus and which contains all the intracellular motifs required for effective signaling; ii) four truncated forms (OBRa, OBRc, OBRd and OBRf), which may act as transporters of leptin through physiological barriers like the blood–brain barrier, and of which OBRa is the most physiologically significant; and iii) the soluble form (OBRe), which lacks both the intracellular and transmembrane domains, and serves as a plasma leptin-binding protein [4,6].

The reproductive function is regulated by the interaction of the hypothalamus, the pituitary and the gonads, which form the reproductive axis. In humans and rodents, different OBR isoforms have been found to be widely distributed in many organs, including the hypothalamus [7–9], the pituitary [10–13] and the ovary [10,14–17]. Leptin is able to cause both stimulatory [18–21] and inhibitory [17,22–24] effects on the ovarian function. We have previously found that an acute treatment with leptin significantly inhibits ovulation [25] but that a daily treatment with a low dose of leptin enhances ovulation in comparison with control animals [26]. Furthermore, leptin is able to modulate the expression of its own receptors in the reproductive axis in a differential way [27].

Neuropeptide Y (NPY), a potent stimulator of food intake, has been shown to affect the reproductive axis in several species including rats, although discrepant stimulatory and inhibitory effects have been observed [28–31]. In the hypothalamus, NPY is synthesized in abundance by neurons of the arcuate nucleus (ARC) [32] and transported to other hypothalamic nuclei involved in the regulation of appetite [33,34]. NPY exerts its effects mainly at the hypothalamic level, stimulating the release of the gonadotropin-releasing hormone (GnRH) [35], although direct effects, such as regulation of both the luteinizing hormone (LH) [36–38] and prolactin secretion [39–41], have also been reported.

OBR is present in NPY neurons in the ARC [42] and the pituitary [43]. NPY has been proposed to mediate the hypothalamic effect of leptin, since the administration of leptin attenuates the overexpression of NPY mRNA in the ARC of *ob*/*ob* mice [44] and reduces both NPY mRNA expression [45] and NPY levels [46] in ARC of fasted rats. NPY and leptin have been proposed as key neuroendocrine integrators between energy balance and reproduction [47,48]. These and other results support the possibility that leptin and NPY are regulated in one direction. Therefore, the aim of this work was to investigate whether the expression of OBR in the hypothalamic–pituitary axis is regulated by NPY during ovulation in rats.

* Corresponding author at: CEFYBO-CONICET, Facultad de Medicina-UBA, Paraguay 2155, 16° P, C1121ABG Buenos Aires, Argentina. Tel./fax: +54 11 4508 3680.
E-mail address: agfaletti@yahoo.com.ar (A.G. Faletti).

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2. Materials and methods

2.1. Animals

Immature female Sprague Dawley rats (21 days old) were purchased from the School of Veterinarian Sciences (University of Buenos Aires, Argentina) and maintained under controlled conditions of light (14-h light, 10-h dark), temperature (22 °C) and humidity, with free access to food and water. Animals were handled according to the Guiding Principles for the Care and Use of Research Animals approved by the Animal Care and Use Committee of the Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET) — School of Medicine (University of Buenos Aires). At 26–28 days of age, rats were injected with 15 IU of equine chorionic gonadotropin (eCG) i.p. (in 0.10 ml saline) to induce the growth of the first generation of preovulatory ollicles. Forty-eight hours later, the animals were injected with 15 IU of human chorionic gonadotropin (hCG) i.p. (in 0.10 ml saline) to induce ovulation, which, in this rat strain, usually occurs within 12 h after hCG administration.

2.2. Incubation of tissue explants

Animals were killed by decapitation 4 h after gonadotropin treatment to allow hCG to reach its target, but before the sequential transcription of the genes involved in ovulation is in their maximal expression, as previously described [49]. The medial basal hypothalamus and the anterior pituitary were immediately removed and placed in different plates containing DMEM/F12 (1:1) medium (Bio Rad Laboratories, CA, USA) with 25 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone and 2 mM L-glutamine, as described previously [27]. Each hypothalamus or anterior pituitary was placed individually in a final volume of 300 μl/well of culture medium at 37 °C under a humidified atmosphere (5% CO_2:95% O_2). After 30-min preincubation, the medium was replaced by fresh medium in the presence of either vehicle (dimethyl sulfoxide) or a combination of NPY (0.3–500 ng/ml) and BIBP3226, an NPY Y1 receptor subtype antagonist (10^{-7}–10^{-6} M), and incubated for 2 or 3 h for the hypothalamus [50] and anterior pituitary [51], respectively. The concentrations of both NPY and NPY Y1 receptor antagonist used in these studies were based on previous reports [50,52]. After the respective incubation periods, all tissues were recovered and frozen on dry ice and stored at −72 °C to measure OBR mRNA expression by RT-PCR and/or OBR protein expression by Western blot analysis. Six independent experiments were run for each culture condition by using different tissue preparations, with two replicates per experiment. As control of stimulation, we used only the tissues from those rats that exhibited ovaries with adequate size and weight, characteristic of gonadotropin-induced simulation.

2.3. RNA isolation and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from all frozen tissues using TRI Reagent® (Molecular Research Center, Cincinnati, USA) according to the manufacturer's instructions. The organic phase of each sample was saved for protein extraction. Total RNA was quantitated and purity was determined by spectral absorption (A260/280) prior to reverse transcription reactions. Complementary DNA was synthesized from 4 μg RNA in 25 μl of reaction mixture containing 200 U Moloney murine leukemia virus (MMLV, Promega, Madison, WI, USA), oligonucleotides (Random Primers, Invitrogen, Carlsbad, CA, USA) and deoxyribonucleotides (dNTPs, Promega). Complementary DNA was amplified by PCR in a total volume of 25 μl using the primers and the cycles detailed in Table 1. Each reaction also contained 1 U *Taq*-DNA polymerase (Invitrogen), 0.2 mM of each primer (Invitrogen), 0.2 mM of each dNTP, and 1.5 mM $MgCl_2$. The PCR prolife consisted of an initial denaturing step at 94 °C for 5 min and an appropriate number of denaturing cycles at 94 °C for 40 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The primer sequences used to amplify the mRNA of both the long and short OBR isoforms were those described by Ryan et al. [53], whereas those for β-actin mRNA were those described by Bilbao et al. [54] (Table 1). In preliminary experiments, the optimum cycle number was determined for each target, so that signals were always in the exponential portion of the amplification curve (Table 1). An aliquot of each sample of the PCR reaction was electrophoresed in 2% (m/v) agarose gel with subsequent ethidium bromide (10 mg/ml) staining. The mRNA bands were visualized and quantified using Image Quant RT ECL (General Electric, Amersham Bioscience Argentina SA, CABA, Argentina) and ImageJ, respectively. Data were normalized to β-actin mRNA in each sample. Negative controls were performed without reverse transcriptase or RNA.

2.4. Protein isolation and Western blot analysis

Total protein content was isolated from all frozen tissues with TRI Reagent® (Molecular Research Center) according to the manufacturer's instructions. Equal amounts of protein (100 μg) were separated by SDS-PAGE (7.5%). Proteins were transferred to polyvinylidene difluoride membranes (Bio Rad Laboratories) for 60 min in a cold chamber using a Bio Rad transblot apparatus. Membranes were first blocked at 4 °C overnight in Tris-HCl: saline (50 mM Tris-HCl: 150 mM NaCl, pH 7.5) containing 5% (w/v) of non-fat milk powder, and then incubated at 4 °C overnight with an antibody against OBR raised in rabbit (H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody is recommended for detection of the long isoforms (OBRb) and all the short isoforms (OBRs) of OBR of mouse, rat and human origin by Western blot, as indicated by the manufacturers since it is a rabbit polyclonal antibody raised against amino acids 541–840 mapping within an internal domain of OBR of human origin. The final dilution of antibody was 1:200. After washing, membranes were treated for 1 h at room temperature with goat anti-rabbit IgG (1:2500) as the secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized using chemiluminescence detection reagents (Sigma-Aldrich, St. Louis, MO, USA) and Image Quant RT ECL (General Electric), and quantified using ImageJ software. Before reuse, membranes were stripped, blocked, and reprobed according to the manufacturer's instructions. The membranes were reprobed with anti-actin antibody (A2066, Sigma-Aldrich of Argentina SA). Negative controls were carried out by omitting the incubation with the primary antibody and no bands were detected. Molecular weight standards (Kaleidoscope, Bio Rad Laboratories) were run under the same conditions to identify the protein bands. Protein bands at 150 and 110 kDa were consistent with the predicted size of

Table 1
Oligonucleotide sequences used in RT-PCR.

Gen	Oligonucleotide sequences	Annealing temperature	Cycles	Product
Rat OBRb	Sense 5′-GTTCCTGGGCACAAGGACTTAAT-3′ Antisense 5′-GGTTCCCTGGGTGCTCTGA-3′	57 °C	35	281 pb
Rat OBRa	Sense 5′-GTTCCTGGGCACAAGGACTTAAT-3′ Antisense 5′-ACTGTTGGGAGGTTGGTAGATTG-3′	57 °C	40	101 pb
Rat β-actin	Sense 5′-AGCCATGTACGTAGCCATCC-3′ Antisense 5′-CTCTCAGCTGTGGTGGTGAA-3′	57 °C	35	228 pb

OBRb and OBRs, respectively, based on amino acid composition. Thus, these two bands were considered to be leptin receptors. The data were normalized to β-actin protein levels in each sample to avoid procedural variability.

2.5. Gonadotropin assays

GnRH was measured by radioimmunoassay (RIA) in the hypothalamic culture medium, as described previously [55]. The highly specific GnRH antiserum was kindly provided by Ayala Barnea (University of Texas Southwestern Medical Center, Dallas, TX, USA). The sensitivity of the assay was 0.2 pg per tube and the curve was linear up to 100 pg GnRH. The intra-assay coefficient of variation of the GnRH RIA ranged from 4 to 7.3%, and the inter-assay coefficient of variation was 8.9%. LH was measured by RIA in the pituitary culture medium as described previously [27] and using a double antibody and reagents kindly provided by the NIAMDD Rat Pituitary Program. The sensitivity of the assay was 4 pg per tube and the curve was linear up to 1000 pg LH. The intra- and interassay coefficients of variation were 8 and 10% respectively. GnRH and LH for iodination were purchased from Peninsula Laboratories and iodine-125 from New England Nuclear. All samples were measured in duplicate. The results are expressed as pg per hypothalamus or pituitary.

2.6. Statistical analysis

All data are expressed as means ± SEM. Groups were compared using one-way ANOVA with Dunnett's multiple comparison test between each concentration and controls for NPY assays and two-way ANOVA with Bonferroni post-tests for the NPY Y1 receptor antagonist. Levene's test and a modified Shapiro–Wilk test were used to assess homogeneity of variances and normal distribution, respectively. If these assumptions were not met, a logarithmic transformation was applied to the data before ANOVA. Differences between groups were considered significant when $P < 0.05$.

3. Results

3.1. Effect of NPY on gonadotropin secretion

As NPY has both stimulatory and inhibitory effects on gonadotropin levels, the effect of NPY on GnRH and LH secretion was measured in the hypothalamic and pituitary incubation media, respectively. The levels of both GnRH and LH were significantly increased at 300 ng/ml and 100 ng/ml, respectively, without differences at the other concentrations of NPY as compared with controls (Table 2).

3.2. Effect of NPY on OBR in hypothalamic and pituitary explants

Figs. 1 and 2 show the protein expression of both OBR isoforms in hypothalamic and pituitary explants after incubation for 2 and 3 h, respectively, in the presence or absence of different concentrations of NPY (0.3–500 ng/ml). In hypothalamic explants, NPY significantly increased the expression of both isoforms: between 44 and 118% at 100–500 ng/ml for OBRb, and between 54 and 72% at 300–500 ng/ml for OBRs, as compared with controls (Fig. 1). In pituitary explants, NPY also caused significant increases in the expression of both isoforms at different concentrations: at 10–300 ng/ml for OBRb and only at 300 ng/ml for OBRs. These increases ranged between 44 and 67% when compared with controls (Fig. 2).

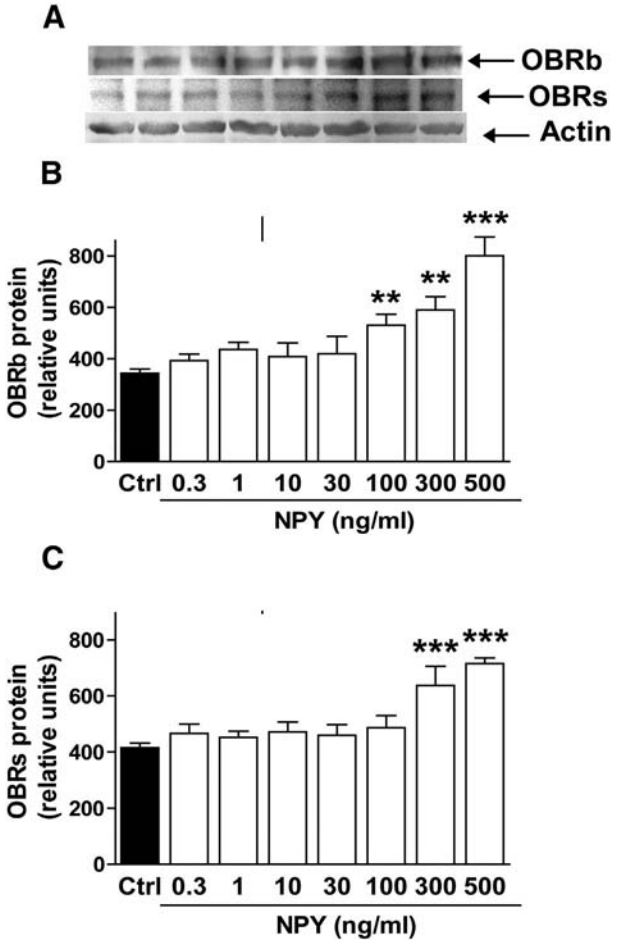

Fig. 1. Effect of neuropeptide Y (NPY) on the protein expression of leptin receptors (OBR) by cultures of hypothalamic explants. Tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 2 h in either the presence or absence (Ctrl) of different concentrations of NPY (0.3–500 ng/ml). (A) Representative bands showing the hypothalamic expression of the long (OBRb) and short (OBRs) OBR isoforms and β-actin, as protein control, by Western blot. (B–C) Quantitative analysis of immunoreactive bands for OBRb (B) and OBRs (C). Results are expressed as the mean ± SEM of six independent experiments with two replicates per experiment (n = 6). $^{**}P < 0.01$, $^{***}P < 0.001$ versus control (Ctrl) (one-way ANOVA and Dunnet's multiple comparison test).

3.3. Effect of the NPY Y1 receptor antagonist on the NPY-induced increase in OBR in hypothalamic and pituitary explants

To determine whether the Y1 receptor subtype was involved in the increased expression of OBR caused by NPY, hypothalamic and pituitary explants were incubated with NPY (100 and 300 ng/ml) or with a

Table 2
Effect of NPY on the secretion of both gonadotropin-releasing hormone (GnRH) and luteinizing hormone (LH) by cultures of hypothalamic and pituitary explants, respectively. Tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 2 or 3 h, respectively, in either the presence or absence (Ctrl) of different concentrations of NPY. Values are expressed as means ± SEM of six independent experiments with two replicates per experiments (n = 6).

NPY (ng/ml)	Ctrl	0.3	1	10	30	100	300
GnRH (pg/hypothalamus)	12 ± 1	11 ± 1	14 ± 2	15 ± 2	12 ± 1	12 ± 2	21 ± 3**
LH (ng/pituitary)	30 ± 4	29 ± 5	32 ± 5	31 ± 4	31 ± 6	56 ± 6**	39 ± 2

** $P < 0.01$ versus control (Ctrl) (one-way ANOVA and Dunnett's multiple comparison test).

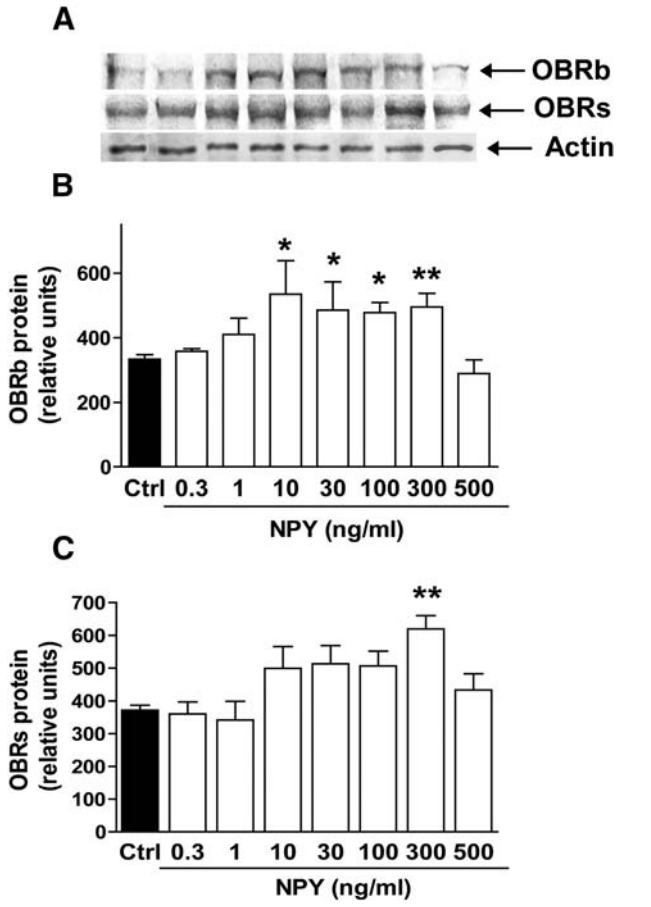

Fig. 2. Effect of neuropeptide Y (NPY) on the protein expression of leptin receptors (OBR) by cultures of anterior pituitary explants. Tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 3 h in either the presence or absence (Ctrl) of different concentrations of NPY (0.3–500 ng/ml). (A) Representative bands showing the pituitary expression of the long (OBRb) and short (OBRs) OBR isoforms and β-actin, as protein control, by Western blot. (B–C) Quantitative analysis of immunoreactive bands for OBRb (B) and OBRs (C). Results are expressed as the mean ± SEM of six independent experiments with two replicates per experiment ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control (Ctrl) (one-way ANOVA and Dunnet's multiple comparison test).

combination of NPY (100 and 300 ng/ml) and the NPY Y1 receptor antagonist (10^{-7}–10^{-6} M) in the same experimental conditions as before. We chose the NPY concentrations that were effective to increase OBR expression, especially that of OBRb, and studied the expression of both OBR mRNA and protein. In hypothalamic explants (Fig. 3), at both concentrations assayed, the NPY Y1 receptor antagonist completely blocked the increase caused by 300 ng/ml of NPY in OBRb mRNA expression (Fig. 3B) and protein content (Fig. 3E), and in OBRs protein expression (Fig. 3F). This antagonist partially inhibited the OBR transcript induced by 100 ng/ml of NPY, although this effect was not significant.

In pituitary explants, the effect of the NPY Y1 receptor antagonist was similar to that obtained in hypothalamic explants. The NPY-induced increase in both the transcript (Fig. 4, *left panel*) and protein content (Fig. 4, *right panel*) of OBR was totally reversed by 10^{-6} M of the NPY-Y1 receptor antagonist in the culture medium. However, 10^{-7} M of this antagonist was not always capable of completely blocking the increase caused by the presence of NPY. The NPY Y1 receptor antagonist alone had no effect on the expression of OBR in hypothalamic explants, but was able to increase the expression of both mRNA (95%) and protein (52%) of OBRb, and only mRNA of OBRa (47%) in pituitary explants.

4. Discussion

The reproductive function is regulated by a close relationship between the hypothalamus, the pituitary and the gonads, which form the reproductive axis. This axis is, in turn, modulated by multiple and complex metabolic and nutritional factors. Leptin and NPY are two important peptides in the regulation of the reproductive function in several species, including rats, through their action on the secretion of both hypothalamic and pituitary gonadotropins [25,37,50–52,56,57]. The leptin receptor (OBR) colocalizes with NPY gene expression in the ARC [58], which suggests that leptin and NPY interact in the regulation of gonadotropin secretion. Moreover, NPY has been shown to modulate leptin action on the hypothalamus. This is based on the fact that: i) NPY levels are increased in rats with food restriction [59]; ii) leptin administration to fasted rats causes high levels of NPY mRNA [46]; and iii) leptin administration to *ob*/*ob* mice attenuates mRNA overexpression of NPY in the ARC [44]. In previous studies, we have found that leptin is able to modulate the expression of its own receptors in the hypothalamic–pituitary (HP) axis in a differential way [27]. Therefore, and in order to investigate whether the expression of OBR in the HP axis is regulated by NPY during ovulation, rat hypothalamic and anterior pituitary explants were used because in vitro they retain the ability to secrete the corresponding hormones [27,50,52], which made it possible to study the effects of NPY directly on OBR in these tissues. Considering that NPY is able to induce both stimulatory [35,60,61] and inhibitory [62–64] effects on the HP axis depending on the physiological state, we studied the main hormones secreted by these tissues in our biological model. The secretion of GnRH and LH by the hypothalamic and pituitary explants, respectively, was increased in the presence of NPY, which confirms the positive effect of this neuropeptide on both tissues, as reported by different authors [35,57,65,66]. As proposed by other authors, this up-regulation in the secretion of gonadotropins is likely a steroid-dependent action. This is consistent with our biological model, because these tissues were obtained 4 h after hCG administration, and because it is well known that the treatment with eCG and hCG 48 h later results in an increase in the plasma concentrations of estradiol and progesterone, respectively [53,67].

Little is known about the regulatory action of NPY on the expression of OBR. Here, we aimed to study NPY effects on OBR during ovulation since both leptin and NPY seem to be involved in regulating both GnRH and gonadotropin secretions. NPY showed a stimulatory effect on the protein expression of both OBR isoforms in a tissue- and concentration-dependent manner. It has been previously reported that the expression of the long OBR isoform and NPY is negatively correlated during the estrous cycle in rats [68]. When OBRb expression is high (in estrus and di-estrus), NPY expression is low, whereas the opposite is seen in pro-estrus and met-estrus with low OBRb expression and high NPY. Other studies have reported that hypothalamic NPY gene expression in the ARC is up-regulated in obese *ob*/*ob* mice [69,70] and reduced by recombinant leptin administration [71], and that the peptide content is significantly increased [72]. In turn, the highest levels of NPY expression are detected in *db*/*db* mice, but are not influenced by leptin administration [71]. However, our studies showed no negative correlation because OBR expression was increased by different concentrations of NPY in both hypothalamic and anterior pituitary cultures after incubation for 2 or 3 h, respectively. Considering the differences between the biological models used, it is not surprising that the effect caused by a neuropeptide on the HP axis is opposite since these interactions usually depend on the species, hormonal environment, chronic or acute treatment, and use of either immature or mature animals. The actions of NPY are mediated through different receptor subtypes, being Y5 and Y1 the most important subtypes involved in NPY-induced food intake and reproduction [48,50,52]. Particularly, the activation of the NPY Y1 receptor subtype is required for the physiological amplification of the spontaneous preovulatory LH surge in rats [73], as well as for the increase in GnRH mRNA caused by NPY in the

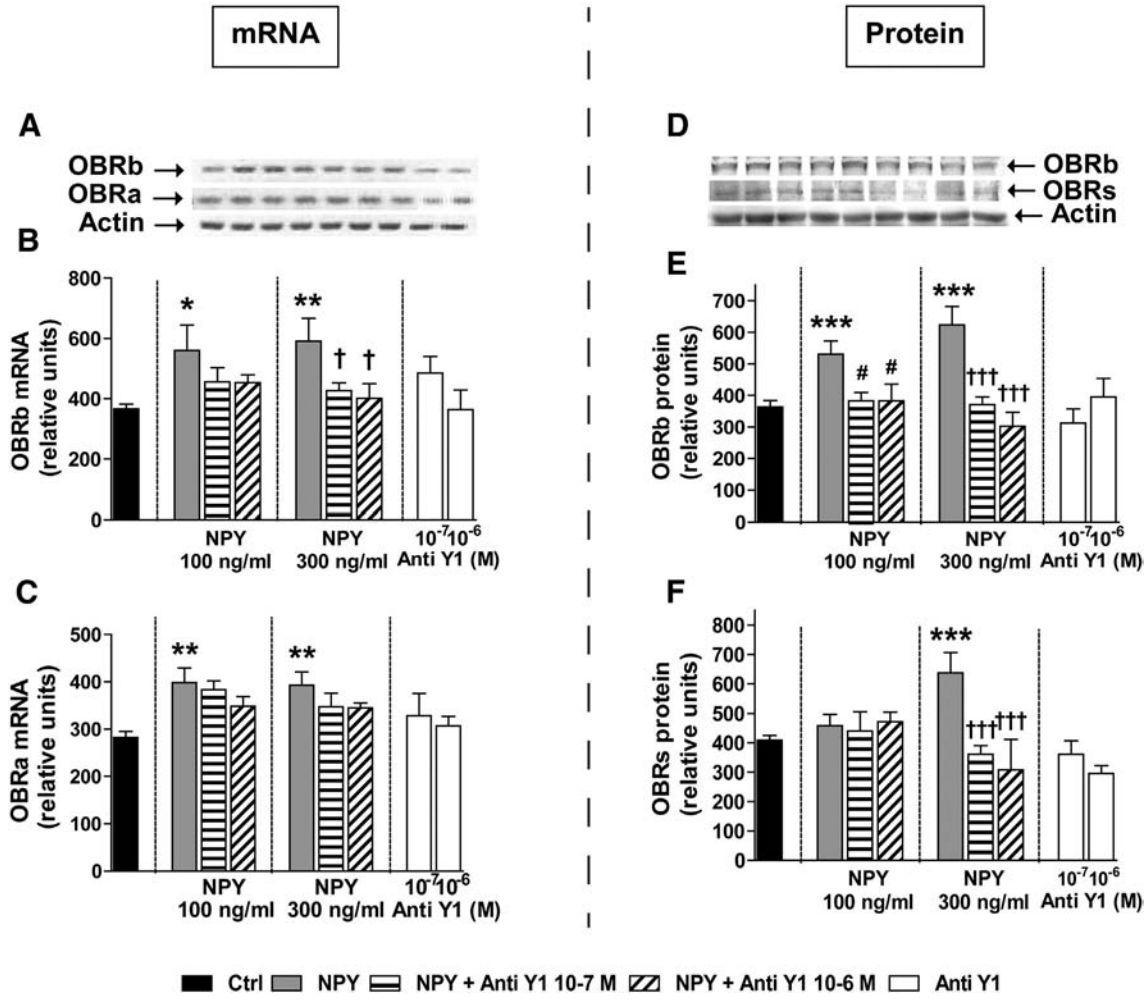

Fig. 3. Effect of BIBP3226, an NPY Y1 receptor subtype antagonist, on the NPY-induced expression of both mRNA (*left panel*) and protein (*right panel*) of leptin receptors (OBR) by cultures of hypothalamic explants. Tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 2 h in either the presence or absence of a combination of NPY (100–300 ng/ml) and BIBP3226 (10^{-7}–10^{-6} M). (A) mRNA expression of OBRb, OBRa and β-actin, as transcript control, by RT-PCR. (B and C) Quantitative analysis of OBRb (B) and OBRa (C) bands, respectively. (D) Protein expression of OBRb, OBRs, and β-actin, as protein control, by Western blot. (E and F) Quantitative analysis of immunoreactive bands for OBRb (E) and OBRs (F). Results are expressed as the mean ± SEM of six independent experiments with two replicates per experiment (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control (Ctrl); $^{\#}P < 0.05$ versus NPY 100 ng/ml; $^{\dagger}P < 0.05$ and $^{\dagger\dagger\dagger}P < 0.001$ versus NPY 300 ng/ml (two-way ANOVA and Bonferroni post-test). NPY: Neuropeptide Y; OBRa: short isoform; OBRb: long isoform; OBRs: short isoforms.

preoptic area [74]. The Y1 receptor subtype is expressed in the ARC and medial preoptic area, critical centers involved in the induction of the preovulatory LH surge [75,76]. Likewise, Y1 expression changes during the estrous cycle in rats [77] and depends on the steroid levels in the HP axis [43,73,77]. Although no complete correlation was found between mRNA and protein expression in the present study, the results obtained with the NPY Y1 receptor antagonist demonstrate that NPY is able to up-regulate OBR, at least its protein expression, through the NPY-Y1 receptor subtype.

We cannot explain the differences between the mRNA and protein expressions but many evidences indicate that the measurement of mRNA expression patterns is insufficient for the quantitative description of biological systems because protein expression is controlled by different post-transcriptional mechanisms, even in the same gene [78,79]. Although our results show that the NPY Y1 receptor is involved in the increase in NPY-induced OBR expression, its action was not effective at all concentrations. Therefore, we cannot rule out the involvement of other subtypes of the NPY receptor. Lebrethon et al. [52] have shown that leptin and NPY are involved through distinct mechanisms in the acceleration of the pulsatile GnRH secretion preceding the onset of puberty in male rats. These authors found that the GnRH interpulse interval caused by hypothalamic explants from prepubertal male rats was significantly decreased after incubation with NPY or leptin and that although the NPY Y5 receptor antagonist did not influence the effects of leptin, it totally prevented the decrease in the GnRH interpulse interval caused by other peptides such as ghrelin [52]. Although many authors have studied the interaction between leptin and NPY action, as mentioned before, here we demonstrated for the first time that NPY is able to up-regulate the leptin receptor in the HP axis, in part through the NPY Y1 receptor subtype.

The increase in OBR expression obtained in pituitary explants in the presence of the Y1 receptor antagonist is difficult to explain. This result suggests at least two possibilities: i) that BIBP3226 is acting as an agonist, as reported by other authors in different biological systems [80,81]; or ii) that this molecule may be blocking an endogenous ligand of the Y1 receptor. Further studies are required to assess these possibilities.

In conclusion, our results indicate that NPY is able to regulate the expression of both the long and short isoforms of OBR in the HP axis, at least in part, through the Y1 receptor. These results reinforce the fact that NPY and the Y1 receptor play a critical role in reproduction by modulating leptin sensitivity and thus the responsiveness to leptin, and do not discard its contribution in food intake and energy expenditure. Moreover, and considering all the data found to date, including

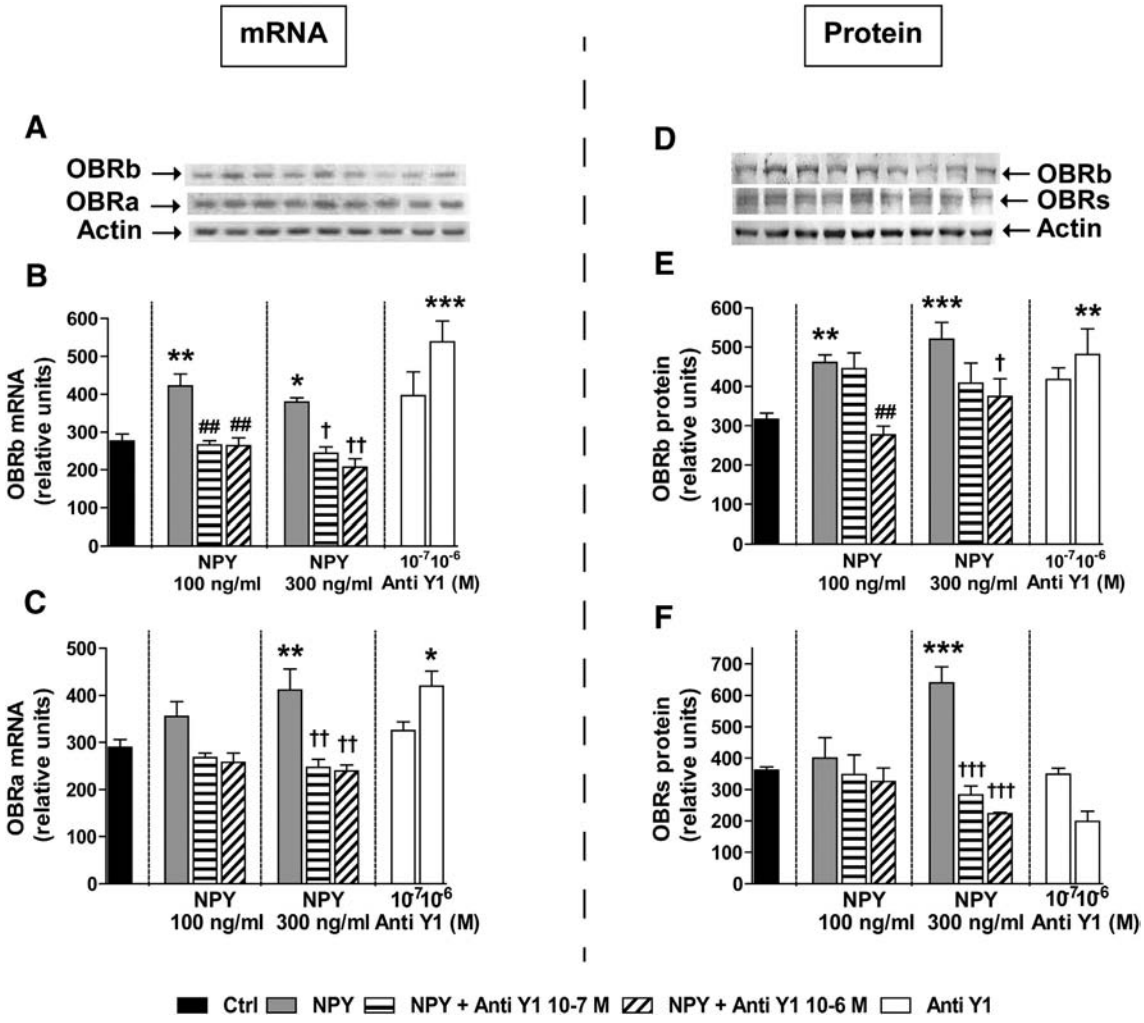

Fig. 4. Effect of BIBP3226, an NPY-Y1 receptor subtype antagonist, on the NPY-induced expression of both mRNA (*left panel*) and protein (*right panel*) of leptin receptors (OBR) by cultures of anterior pituitary explants. Tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 3 h in either the presence or absence of a combination of NPY (100–300 ng/ml) and BIBP3226 (10^{-7}–10^{-6} M). (A) mRNA expression of OBRb, OBRa and β-actin, as transcript control, by RT-PCR. (B and C) Quantitative analysis of OBRb (B) and OBRa (C) bands, respectively. (D) Protein expression of OBRb, OBRs and β-actin, as protein control, by Western blot. (E and F) Quantitative analysis of immunoreactive bands for OBRb (E) and OBRs (F). Results are expressed as the mean ± SEM of six independent experiments with two replicates per experiment (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control (Ctrl); $^{\#\#}P < 0.01$ versus NPY 100 ng/ml; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ and $^{\dagger\dagger\dagger}P < 0.001$ versus NPY 300 ng/ml (two-way ANOVA and Bonferroni post-test). NPY: Neuropeptide Y; OBRa: short isoform; OBRb: long isoform; OBRs: short isoforms.

our results, it is possible to suggest the existence of a feedback loop between leptin and NPY to regulate the reproductive function efficiently, without discarding a similar feedback mechanism to regulate the intake and energy expenditure.

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