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Neuropeptide Y in the medial basal hypothalamus and medial preoptic area during the induction of LH surge may be controlled by locus coeruleus

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Abstract

The multiple control of gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) secretion involves locus coeruleus (LC) and neuropeptide Y (NPY). The objective of the present study was to analyze the possible contribution of the LC to the control of NPY activity in the medial basal hypothalamus (MBH) and medial preoptic area (MPOA) during the LH surge induced by estrogen (E_2) and progesterone (P_4). Ovariectomized adult Wistar rats were submitted to the hormone replacement and to the LC bilateral lesion (lesioned groups) or sham surgery (control groups). On the day of the experiment the rats were decapitated at 11:00, 13:00, 15:00 and 17:00 h for plasma and brain collection. Plasma LH was determined by radioimmunoassay. MBH and MPOA were microdissected for the measurement of NPY by enzyme immunoassay. NPY mRNA levels in MBH were assessed by the ribonuclease protection assay. The results showed that LC lesion: decreased the plasma LH; increased the content of NPY in the MBH and reduced the increase of NPY content in the MPOA during afternoon in which LH surge was induced. The increased NPY content in MBH was not associated with an increase of the respective mRNA content, suggesting the action of postranscriptional and/or postranslational mechanisms. In conclusion, the NPY activity in the MPOA on LH surge induced by estrogen and progesterone could be controlled by LC through two ways, at least: one direct way, by the release of NPY from LC neurons terminals that innervate the MPOA and they release NA and NPY; one indirect way, by the control of release but not synthesis of NPY from neurons in the MBH which innervate the MPOA.

Keywords: Locus coeruleus; Neuropeptide Y; Neuropeptide Y mRNA; Luteinizing hormone; Medial preoptic area; Medial basal hypothalamus

1. Introduction

Gonadotropin – releasing hormone (GnRH) neurons constitute the final output pathway of the neural network that regulates the gonadotropin secretion by the pituitary gland. Studies using deafferentation (Naik, 1976) and immunohistochemistry (Ibata et al., 1979; King et al., 1985) showed that GnRH is transported from other brain regions to the median eminence. Nearly 1000–1600 GnRH neuronal bodies are distributed from rostral olfactory bulb to the retrochiasmatic region of basal hypothalamus in the rat brain (Witkin et al., 1982). GnRH fibers pass through retrochiasmatic tract in way to the median eminence (Merchenthaler et al., 1984).

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There are sharp evidences to support the multiple control of GnRH/LH secretion by several neurotransmitters and neuropeptides among them catecholamines (Gallo and Drouva, 1979; Wise et al., 1981), amino acids (Brann and Mahesh, 1997; Demling et al., 1985), neuropeptide Y (NPY) (Guy et al., 1988; Kalra and Crowley, 1992), nitric oxide (Aguan et al., 1996; Ceccatelli et al., 1993; Gouveia and Franci, 2004; Rettori et al., 1993), angiotensin II (Dornelles and Franci, 1998a,b; Franci et al., 1990a; Steele et al., 1982; Steele, 1987), atrial natriuretic peptide (Franci et al., 1990b; Samson et al., 1988) and serotonin (Gouveia and Franci, 2004; Vitale and Chiocchio, 1993).

NPY, a potent mediator of GnRH secretion (McDonald, 1990), has an additive or even synergistic effect with NA on the control of LH secretion (Allen et al., 1987). It is produced in neurons located in the ARC and transported to various sites including the preoptic area, beyond to be also co-released at noradrenergic endings originating from the brain stem (Bai et al., 1985; Chronwall et al., 1985). Microinjection of NPY in the third ventricle reduced LH secretion in ovariectomized rats and stimulated LH secretion in ovariectomized rats treated with estrogen and progesterone, similar to the effect reported for noradrenaline (Kalra and Crowley, 1992; Kalra and Gallo, 1983).

The A_1, A_2, A_6 (locus coeruleus – LC) and A_7 nuclei in the brain stem control the NA content in the preoptic-hypothalamic region (Wright and Jennes, 1993). The noradrenergic endings are close to the GnRH perikarya in the MPOA as well to the GnRH terminals in the median eminence (Castañeyra-Perdomo et al., 1992). The LC promotes a more restricted innervation of the periventricular arcuate nucleus (ARC) and of the preoptic area (Everitt and Hokfelt, 1992). LC lesion did not change the basal secretion of LH but it impeded LH surge in proestrus (Anselmo-Franci et al., 1997) or estrogen–progesterone-treated ovariectomized rats (Helena et al., 2002)

The objective of the present study was to verify the role of LC on NPY content in the MPOA and MBH and on the NPY synthesis in the MBH during LH surge induced by estrogen and progesterone.

2. Methods and materials

2.1. Animals and treatment

Adult female Wistar rats weighing 170–190 g were ovariectomized (ovx), submitted to prophylactic antibiotic therapy (Veterinary Pentabiotic, Wyeth, Brazil) and housed for three weeks under controlled temperature (20 + 2 °C) and lighting (lights on from 06.00 to 18.00 h). Food and water were provided ad libitum. The experiments were done in agreement with protocols approved by Ethics Committee of Animal Experimental of Ribeirão Preto Medical School, University of São Paulo.

A hormone replacement was begun eighteen days after ovariectomy through s.c. injection of Estradiol Benzoate (EB, 25 μg/0.1 ml in corn oil) at 9.00 h at the third, second and first days before the experiment. Progesterone (P, 2.5 mg/0.1 ml in corn oil) was injected s.c. at 9.00 h in the experiment. The surgery was carried out under sodium thiopental anesthesia (Abbott Lab, USA – 5 mg/100 g b.w., i.p.).

2.2. Locus coeruleus lesion

One day before the experiment the animals were anesthetized with tribromethanol – 2.5% (Aldrich, USA – 1 ml /100 g b.w., i.p.). In following, they were positioned in the Stereotaxic Instrument (David Kopf, USA) with the head at zero point of the incisor bar. The dorsal surface of the skull was exposed and holes (2 mm in diameter) were drilled bilaterally 1.2 mm lateral to the midline and 3.4 mm caudal to the lambda suture point. A stainless steel monopolar electrode 0.2 mm in diameter and insulated except at the tip was used. The electrode was angled 15° (postero–anterior position) and lowered to a depth of 6.8 mm below the surface of the skull. Two groups were used: control rats, which were submitted to the same surgical procedures except that the insertion of the electrode was 2 mm above that employed for lesioned animals, and that no current was applied; and LC-lesioned rats in which a constant anodal current of 2 mA was applied for 10 s. The electrode was removed, the skin incision was closed and the animals were placed in individual cages in a silent room and allowed to recover for 24 h.

2.3. Experimental procedures

Female rats were decapitated at 11:00, 13:00, 15:00 and 17:00 h on the day after LC lesion (day of the experiment). Immediately after decapitation, the brain was quickly removed and the brain stem was cut off and kept in 10% formaldehyde solution for later histological analysis. The remainder of the brain was quickly frozen on a block of dry ice and stored at −70 °C until the determination of NPY and mRNA for NPY contents. Blood samples were collected immediately after decapitation. Plasma was separated by centrifugation and stored at −20 °C for later LH determination by radioimmunoassay.

At the appropriated time, the MBH and MPOA were punched out from the frozen brains according to a previously described method (Anselmo-Franci et al., 1999; Gouveia and Franci, 2004; Palkovits, 1973).

2.4. NPY content and protein measure

The brain punches were first homogenized in 50 μl of saline with a Microultrasonic Cell Disrupter (Sonic and

Materials, USA) and 450 µl of 0.2 M hydrochloric acid was then added. The mixture was homogenized, heated at 90 °C for 10 min to inactivate endogenous proteases, and centrifuged at 4 °C. The NPY was measured in the supernatant with an Enzymatic Immunoassay Kit (Peninsula Lab, USA). The specific antibody recognizes the N-terminal portion of the NPY (36 amino acids) and it mays cross-react with proNPY (69 amino acids). NPY content was expressed as ρg/µg protein. The protein was measured in the supernatant with a Colorimetric Kit (Bio-Rad, USA).

2.5. Radioimmunoassay

Plasma LH was determined by double antibody radioimmunoassay using specific kits provided by National Hormone and Peptide Program (National Institutes of Diseases Digestive and Kidney, USA). The antiserum for LH was anti-rat LH-S10 and the reference preparation was LH-RP_3. All samples were measured in duplicate. The lower detection limit was 0.04 ng/ml and the intra-assay coefficient of variation was 3.4%.

2.6. Histological analysis

The extent and location of the lesions were histologically determined in each animal. Frontal 40 µm sections were cut through the region of the LC, stained by the Nissl technique and examined microscopically using the brain atlas (Palkovits and Jacobowitz, 1974).

2.7. RNA extraction and ribonuclease protection assay

Total RNA was isolated from individual frozen MBH with Trizol® (GIBCO-BRL) according to a previously described method (Chomczynski and Sacchi, 1987). A 800 base pair fragment of preproNPY gene with T3 promoter was constructed and cloned in pBL plasmid (Stratagene, USA). Subsequently, an antisense riboprobe was transcribed with T3 RNA polymerase (Anbion, USA) by using αP^{32}–Uridine Triphosphate (Amershan-Pharmacia, USA). Ribonuclease protection assays (RPAs) were performed using the RPAII kit (Anbion, USA). Riboprobes (10^5 cpm for both preproNPY and β-actin) were hybridized with 4 µg total RNA extracted, or yeast transfer RNA at 42 °C for 18 h followed by ribonuclease A/T1 digestion at 37 °C for 30 min. Protected fragments were heat denatured and separated on 4.0% denaturing urea/polyacrylamid gels. A rat β-actin antisense probe was used as a control. Radioactive signals were obtained and analyzed with the STORM (Molecular Dinamics, USA). The densitometric analyzes were carried out using the Image Quant software (Molecular Dinamics, USA). The results are reported as the relation of the densitometric readings between the bands for NPY and for β-actin.

2.8. Statistical analysis

Data were analyzed statistically by analysis of variance followed by the Newman–Keuls test for multiple comparisons, with the level of significance set at $P < 0.05$.

3. Results

Fig. 1A presents brain sections of rats submitted to the LC lesion or sham surgery. LH surge induced by estrogen and progesterone in ovariectomized rats (Fig. 1B) was near ten (15:00 h) to thirty times (17:00 h) higher than basal values (11:00 and 13:00 h). LC lesion did not modify significantly plasma LH at 11.00 and 13.00 h but it decreased significantly the LH surge at 15:00 and 17:00 h.

The NPY content in the MBH was similar at all time points in control rats, but increased from 11:00 and 13:00 h to 17.00 h in LC-lesioned rats. The values were significantly higher in LC-lesioned rats than in control rats at 17:00 h (Fig. 2A). In the MPOA of control rats, the NPY content increased gradually from 11:00 to 17:00 h (Fig. 2B) with the values at 17:00 h being significantly higher than at 11:00, 13:00 and 15:00 h. NPY

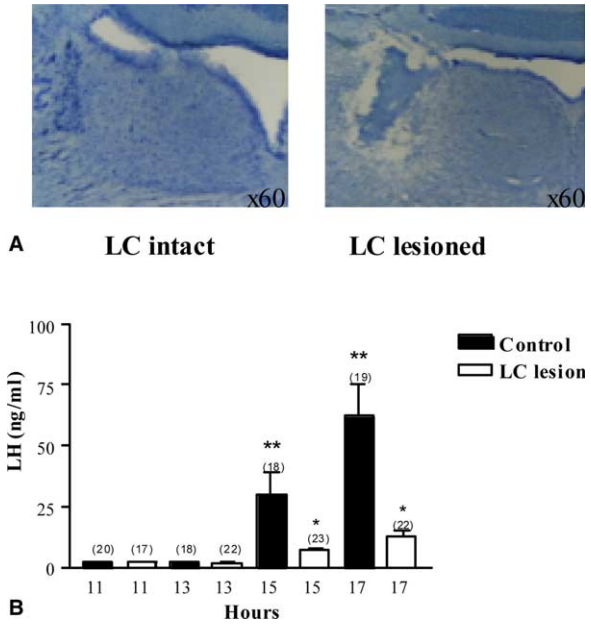

Fig. 1. (A) Photomicrograph of an intact (left) and lesioned (right) locus coeruleus, 60×. (B) Plasma LH in estradiol-and progesterone-primed ovariectomized rats. Groups of animals were decapitated at 2 h intervals from 11:00 to 17:00 h. *$P < 0.05$ versus control at the same time point. **$P < 0.001$ versus control at 11:00 and 13:00 h. The number of animals in each group is indicated in parenthesis.

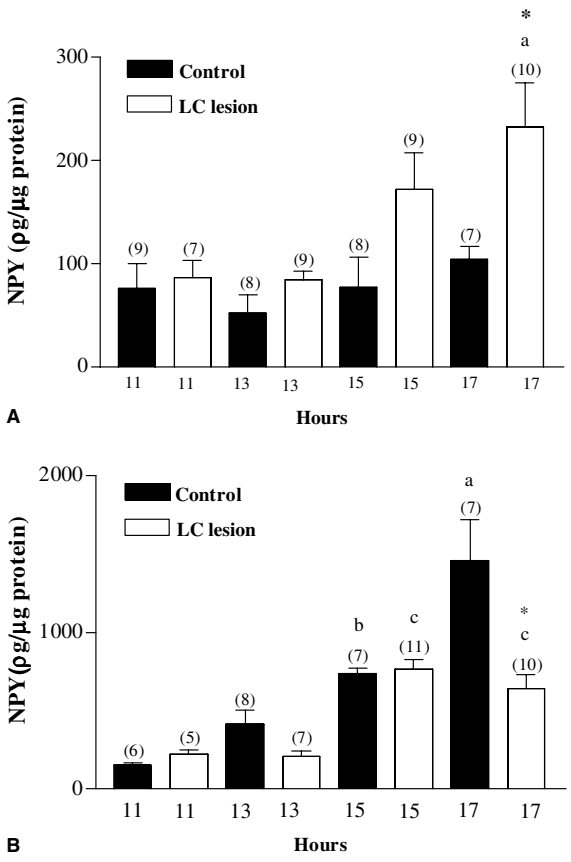

Fig. 2. Neuropeptide Y (NPY) content in the medial basal hypothalamus (A) and in the medial preoptic area (B) from ovariectomized rats treated with estrogen and progesterone, submitted to the lesion of the locus coeruleus (lesioned) or to the sham surgery (control). Significant differences within the same group (control or lesioned) at different time points are indicated by one letter for the MBH (a for $P < 0.01$ versus LC lesion at 11 and 13 h) and MPOA (a for $P < 0.001$ versus control 11, 13 and 15 h; b for $P < 0.001$ versus control at 11 and 13 h; c for $P < 0.05$ versus LC lesion at 11 and 13 h). Significant differences for comparison of different groups (control and lesioned) at the same time point $^*P < 0.001$. The number of animals is given in parenthesis.

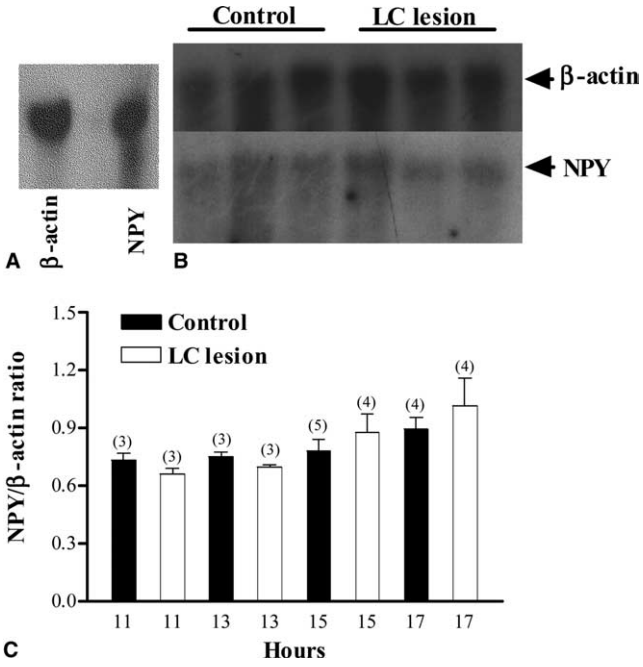

Fig. 3. (A) Illustration of β-actin and NPY probes. (B) Polyacrylamide gel developed with STORM containing specific bands of mRNA for β-actin and NPY. (C) NPY/β-actin densitometric ratio in the MBH of control and lesioned groups at different time points. There was not significant difference in NPY/β-actin ratio between groups. The number of animals in each group is given in parenthesis.

content in LC-lesioned rats (Fig. 2B) was similar at 11:00 and 13:00 h but increased at 15:00 and 17:00 h to values significantly different from those obtained at 11:00 h and 13:00 h.

Fig. 3A shows a representative gel with the β-actin and NPY probes in the MBH and Fig. 3B shows a polyacrylamide gel developed with the STORM containing specific bands of mRNA for β-actin and NPY in the MBH. Fig. 3C shows the levels of mRNA for preproNPY expressed in the MBH by the rate of the densitometric readings for NPY and β-actin. It can be seen that there was no difference in the levels of mRNA for preproNPY between 11:00 and 17:00 h before or during the induced LH surge in estrogen- and progesterone-primed ovariectomized rats. Also, LC lesion did not cause changes in mRNA for preproNPY at the same time points studied.

4. Discussion

In the present study, the high levels of LH obtained at 15:00 and 17:00 h in estrogen and progesterone-treated ovariectomized rats were significantly decreased by LC previous lesion (Fig. 1) as previously described (Anselmo-Franci et al., 1997). The catecholaminergic neurons from A_1, A_2, A_6 (LC) and A_7 nuclei in the brain stem are responsible for NA release in the hypothalamus and MPOA (Honma and Wuttke, 1980; Palkovits and Jacobowitz, 1974) and their terminals are quite close to the GnRH perikarya in the MPOA and to the GnRH terminals in the median eminence (Castañeyra-Perdomo et al., 1992). Studies to identify sites of origin of noradrenergic fibers that project to areas containing GnRH perikarya showed retrograde label in catecholaminergic neurons of A_1, A_2 and A_6 nuclei (Wright and Jennes, 1993).

Previous data from our laboratory have shown that FOS expression is increased in the MPOA and LC during proestrus afternoon (Martins-Aférri et al., 2003). Besides, the electrolytic lesion of the LC impedes the proestrus LH surge (Franci and Antunes-Rodrigues, 1985), blocks the pulsatile LH release in ovariectomized rats (Anselmo-Franci et al., 1999), increases GnRH content in the MPOA and median eminence from rats in proestrus and ovariectomized

rats treated with estrogen and progesterone (Helena et al., 2002), and it causes a fall in NA content into the MBH and MPOA on the morning of estrus (Anselmo-Franci et al., 1997).

Other researchers showed earlier FOS expression in the GnRH neurons during proestrus from 1600 to 2200 h but not during diestrus, estrus or proestrus morning. Nearly 50% of the GnRH neurons were FOS positive in the preoptic-anterior hypothalamic region (Lee et al., 1990). GnRH mRNA levels increase by 28% in FOS positive GnRH neurons when compared with FOS negative GnRH neurons. This suggests that GnRH neurons are transcriptionally more actives during proestrus LH surge (Wang et al., 1995). In spite of, the noradrenergic system acts in the neuronal chain leading to the LH surge and it is not responsible for the FOS transcriptional changes in GnRH neurons that accompany the natural LH surge (Le et al., 1997).

The NPY content in the MPOA was increased near tenfold between 11:00 and 17:00 h in estrogen and progesterone-treated ovariectomized rats (Fig. 2B) but it did not change in the MBH (Fig. 2A). NPY is a potent modulator of GnRH secretion and its content in the hypothalamus and preoptic area originates from arcuate nucleus (ARC) in the MBH and brainstem neurons. Neurons from brainstem can co-release NA and NPY while NPY neurons with cell bodies localized in the ARC do not co-release NA (Bai et al., 1985; Chronwall et al., 1985). There is morphological evidence for interaction between ARC–NPY neurons and GnRH neurons (Li et al., 1999). ARC neonatal lesion significantly reduced the NPY fibers in the preoptic area as well as their contacts with GnRH neurons. Moreover, NPY pathways ascending from noradrenergic/adrenergic cells of the brain stem were identified as a second important source for NPY fibers regulating GnRH neurons (Turi et al., 2003).

NPYergic cells in the ARC accumulate estrogen, indicating that the NPY secretion induced by gonadal steroids in females depends on the action, at least in part of ARC cells (Kalra and Crowley, 1992). The NPY content fluctuations may reflect alterations of NPY synaptic release within hypothalamic circuits controlling GnRH release (Crowley et al., 1985; Sahu et al., 1989) or of the NPY neurosecretion to the hypophysial portal vessels (Bauer-Dantoin et al., 1992). NPY produced in neurons located in the ARC is transported to several sites including the preoptic area (Chronwall et al., 1985) and this is the probable site of action of NPY to control the GnRH secretion (Norgreen and Lehman, 1989).

Our results show that LC lesion caused a 40% lower increase of NPY in the MPOA at 17:00 h in comparison with the control (LC sham surgery). On the other hand, this lesion increased the NPY content into the MBH near 2.5-fold between 11:00 and 17:00 h (Fig. 3B). The lower NPY content in the MPOA may be due to the decreasing of noradrenergic/NPY neurons input from the locus coeruleus since it was lesioned and/or of NPY neurons input from ARC in the MBH. In this latter case, the NPY releasing from MBH to MPOA could be decreased because it could depend on signaling from LC input. Thus, the higher storage of NPY in the MBH from lesioned animals could be due the fall of NPY releasing to MPOA.

It has been showed that synthesis and release of NPY are high before proestrus surge (Herbison, 1998; Kalra and Crowley, 1992; McDonald, 1990; Sahu and Kalra, 1998). In situ hybridization studies showed a significant increase of NPY mRNA content in the MBH at 16, 18, 20 and 22:00 h of proestrus, therefore during and after the preovulatory peak of LH (Bauer-Dantoin et al., 1992). Other researchers using ribonuclease protection assay did get an increase of NPY mRNA content in the MBH before (12 and 14:00 h) and during (16 and 18:00 h) the LH surge induced by estrogen and progesterone in ovariectomized rats (Sahu et al., 1994). The different results may be due to the differences of animal conditions and/or of methodologies. The former researchers used rats in proestrus and in situ hybridization (Bauer-Dantoin et al., 1992) while the latter researchers used ovariectomized rats treated with steroids and ribonuclease protection assay (Sahu et al., 1994). We used also ovariectomized rats treated with steroids and ribonuclease protection assay but did not get to verify changes of NPY mRNA in the MBH. The divergence in the results may be due to the differences in the experimental protocol such as the time for use of animals after ovariectomy and the time and doses of hormone replacement. Furthermore, our values of NPY mRNA are relative to the basal value (11:00) from brain removed 2 h after progesterone injection in estrogen primed ovariectomized rats while other researchers (Sahu et al., 1994) presented NPY mRNA values relative to the basal value (10:00) from brain removed of estrogen primed ovariectomized rats before progesterone treatment.

The increase of NPY content in the MPOA (about threefold) was not accompanied by an increase of mRNA levels for preproNPY in the MBH (Fig. 3), suggesting the predominance of posttranscriptional control mechanisms over a real transcriptional activity. The present results suggest that mRNA levels were constant during this period, indicating mRNA stability, as previously reported (Lerchen et al., 1995; Minth-Worby, 1994). Posttranslational control mechanisms may also be involved since the increase or decrease in NPY content with no change in mRNA levels has already been described (Broberger et al., 1997; Karvonen et al., 1998), suggesting modulation of the protein precursors of NPY. The involvement of mechanisms of mRNA stabilization for the maintenance of prepro-mRNA levels has been reported (Day and Tuite, 1998; Good, 2000; Ross, 1996). The evidence

obtained thus far has not permitted the identification of a region involved in the recognition of the estrogen-receptor complex at the NPY gene promoter site, supporting the hypothesis of the absence of a direct effect of estrogen on the transcription of this gene. The present results show that during the induced LH surge the levels of the respective mRNA were maintained constant.

In conclusion, the NPY activity in the MPOA on LH surge induced by estrogen and progesterone could be controlled by LC through two ways, at least: one direct way, by the release of NPY from LC neurons terminals that innervate the MPOA and they release NA and NPY; one indirect way, by the control of release but not synthesis of NPY from ARC neurons in the MBH which innervate the MPOA. If taken together, the evidences indicate that: NPY gene expression seems to be controlled at least in part by estrogen (Sahu et al., 1989); NPY neurons in the MBH but not in the brainstem express estrogen-receptor-alpha (Simonian and Herbison, 1997); NPY activity in the MPOA could be controlled by LC, direct or indirectly (the results of the present work); we may build a hypothesis that to occur GnRH/LH surge, the estrogen could stimulate the NPY synthesis in ARC neurons of the MBH and so this synthesized NPY would be discharged to the MPOA by action of LC noradrenergic neurons that innervate the MBH neurons.

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