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Expression of NPY-immunoreactive neurons in the hypothalamus of the cycling ewe

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Abstract: The purpose of the study was to localise neuropeptide Y (NPY) immunoreactive (ir) neurons in the hypothalamus during two phases of the oestrous cycle in the ewe. Hypothalamic tissue was collected from Polish Merino ewes (n=8) in the follicular (15th day) and preovulatory (17th day) phases of the oestrous cycle. NPY-ir neurons were detected in the hypothalamus using immunohistochemistry followed by image analysis; positive staining was expressed as the percentage of stained area and optical density. Two populations of the NPY-positive neurons were detected and evaluated in the infundibular and periventricular nuclei of the hypothalamus. The population of NPY-ir neurons located in the infundibular nucleus exhibited a prominent expression of NPY immunoreactivity in the perikarya and fibres only during the preovulatory phase. Both, percent area and the optical density of NPY immunostaining measured in this area were higher ($P<0.01$) in the preovulatory than in the follicular phase. Another population of NPY-ir neurons was localised in the periventricular nucleus and did not show any changes during the two phases of the cycle. The present study suggests that NPY-ir neurons present in the infundibular nucleus can play a role in the preovulatory GnRH discharge from the median eminence. (www.cm-uj.krakow.pl/FHC)

Key words: NPY - Neurons - Hypothalamus - Oestrous cycle - Ewe

Introduction

Neuropeptide Y (NPY), a 36-amino acid peptide is one of the major regulators of reproductive processes at the level of central nervous system in many mammalian species. In the rat, NPY is involved in the regulation of gonadotrophin releasing hormone (GnRH) secretion [7] but its specific action depends on the endocrine status. In rats, NPY stimulates GnRH release from the hypothalamus in the presence of oestrogen, but inhibits GnRH release during oestrogen deficiency [9]. In the sheep, the role of NPY in the regulation of GnRH is less clear. NPY suppressed release of LH in ovariectomized [10] and ovariectomized oestrogen-treated sheep [11]. However, in cycling ewes, the intraventricular (icv) administration of anti-NPY serum delayed the onset of the preovulatory luteinizing hormone (LH) surge, implying its stimulatory role in this process [15]. The results of our previous study confirmed that icv NPY infusion could stimulate the synthesis and storage of LH but not its release in prepubertal lambs [18] and that stimulatory action of

NPY on GnRH/LH release depended on photoperiodical ovarian activity [22].

NPY is largely distributed in the central nervous system but its role in reproductive functions is attributed to the population of neurons located in the infundibular (arcuate) nucleus (IN) [8]. NPY-containing perikarya localised in the IN innervate several sites in the diencephalon implicated in the control of the pituitary LH secretion such as preoptic area [17] and the median eminence (ME) [4]. This indicates that NPY neurons may regulate LH secretion by direct modulation of GnRH synthesis and release. It has been found that expression of prepro-NPY rises in the IN of medial basal hypothalamus (MBH) on the afternoon of proestrus in rats [16, 12]. An increase in the NPY content in ME [6] and its release [19] were observed before and during the GnRH/LH surge in rats. In addition, a stimulatory effect of NPY infusion into ME on GnRH release was observed in intact sexually active ewes, only in the follicular, but not in the luteal phase of the oestrous cycle [1].

The objective of the present study was to localise NPY-immunoreactive (ir) neurons in two subareas of the hypothalamus, the periventricular and infundibular nuclei, during follicular and preovulatory phases of the oestrous cycle in the ewe.

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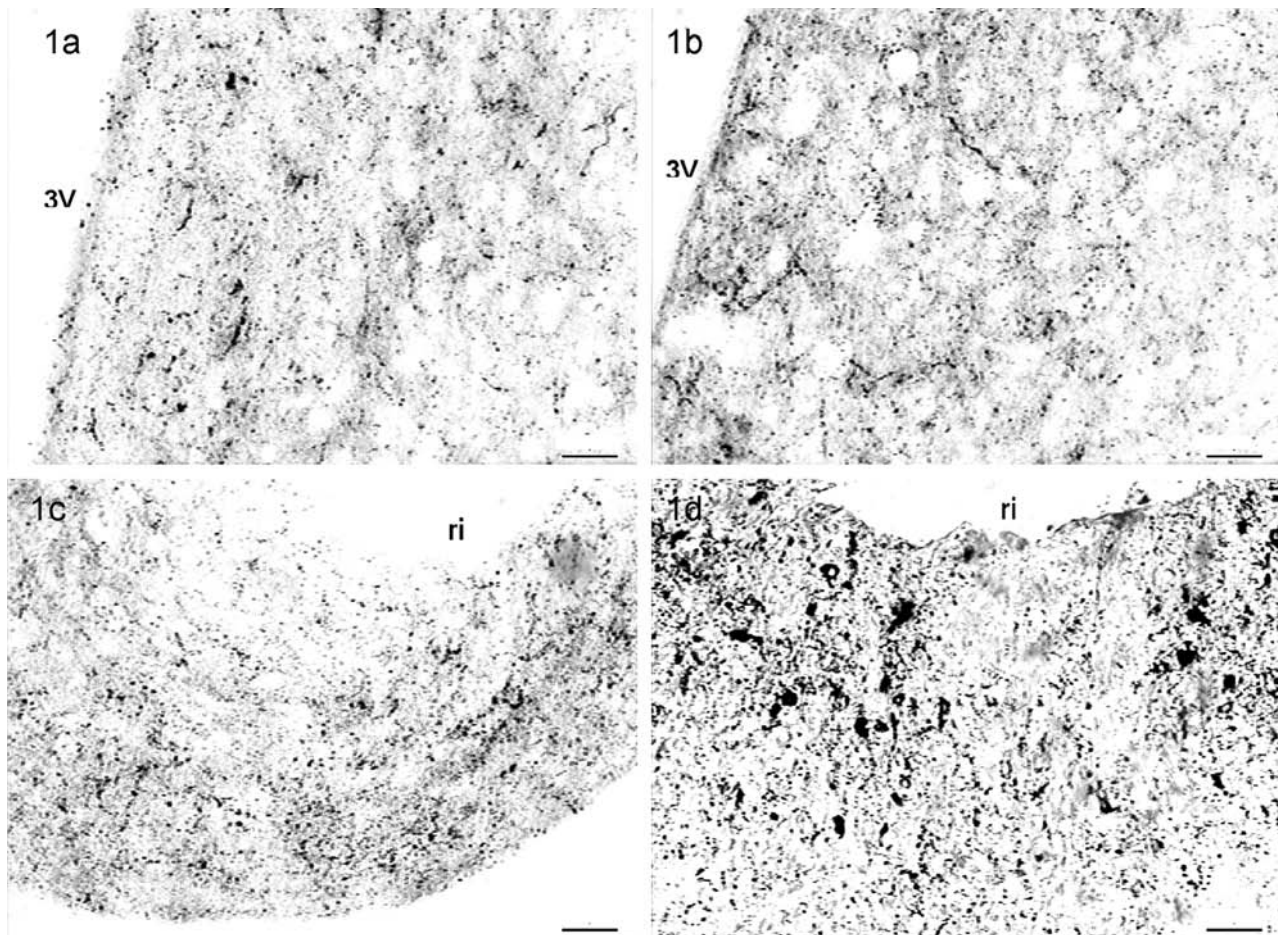


Fig. 1. Immunoreactive NPY neurons in the periventricular (**a, b**) and infundibular (**c, d**) nuclei of representative sheep from follicular (**a, c**) and preovulatory (**b, d**) phases of the oestrous cycle. Note similar density of NPY-ir neurons located in the periventricular nucleus of sheep from both phases, numerous NPY-ir perikarya and the high density of NPY neuronal elements in the sheep from preovulatory phase. ri - recessus infundibularis; 3v - third ventricle of the brain. Bars = 50 μ m.

Materials and methods

Animals. The experimental procedures were approved by the Local Ethics Committee of the Warsaw Agricultural University according to the Polish Guide of Care and Use of Animals (August 2, 1997).

Studies were performed on two-year old Polish Merino ewes ($n=8$) during the period of decreasing day length from November to December. The animals exposed to a natural day/night cycle were kept indoors in individual pens and had visual contact with their neighbours throughout the study. The animals were fed a standard diet of hay and concentrates. Food and water were available *ad libitum*. Day 0 of the oestrous cycle (standing estrus) was determined using a vasectomized ram. Oestrous behaviour (acceptance of a ram and mounting by a ram) was used as a determinant of the GnRH/LH surge [5]. During the oestrous cycle, blood samples were collected from each ewe every third day for evaluation of progesterone concentration by radioimmunoassay. The progesterone data obtained were recorded as a graph individually for each ewe. The course of each curve obtained allowed proper determination of the days of the individual oestrous cycle. Progesterone levels during the luteal phase ranged from 2 to 4 ng/ml, during early follicular phase were about 1 ng/ml and in preovulatory phase progesterone was close to 0 ng/ml. At least two oestrous cycles were completed before the experiment. Eight ewes having a regular 17-day oestrous cycles were randomly divided into two groups, one group being in the follicular phase of the cycle (15th day) and the other one in the preovulatory period (17th

day). The sheep were slaughtered by decapitation under pentobarbital anaesthesia in the local abattoir.

Immunohistochemistry. Immediately after decapitation, the brain of each sheep was perfused via both carotid arteries with 1000 ml 0.1 M phosphate buffered saline (PBS) and subsequently with 2000 ml 0.1 M PBS containing 4% paraformaldehyde (w/v) and 15% saturated picric acid solution (w/v), pH 7.4. The hypothalami were dissected out 20 min after the beginning of perfusion and fixed for further 72 h by immersion in the same fixative. Hypothalamic tissues were trimmed to blocks of the same dimensions and cutting face. They were washed with 0.01 M PBS, cryoprotected in a 20% sucrose solution in 0.1 M PBS for at least two days and stored at -70°C . They material was cut in a cryostat (Jung CM 1500, Leica Instruments GmbH, Nussloch, Germany) in coronal planes at 30 μ m, throughout the anterior hypothalamic area, MBH including the ME and the pituitary stalk. Hypothalamic nuclei were identified using the atlas of sheep brain [20]. Sections were processed for immunohistochemistry (including control staining) according to the procedure described before [14] using primary antiserum against NPY (no 9528; Sigma, St Louis, USA) at 1:5000 dilution for 6 days at 4°C and secondary antibody (sheep anti-rabbit Ig[H+L] labelled with peroxidase; Institute Pasteur, France), dilution 1:40 in 0.1% normal lamb serum in PBS. The colour reaction was developed by incubating sections with 0.05% 3'3'-diaminobenzidine tetrachloride chromogen (Sigma) and 0.001% hydrogen peroxide in 0.5 M Tris buffer.

Image analysis. Nikon type 104 (Nikon Corporation, Yokohama, Japan) projection microscope was used to examine hypothalamic sections. Staining was analysed using "Lucia" version 3.51ab (Laboratory Imaging Ltd, Prague, Czech Rep.) computer-assisted image analysis system according to procedure described in [14]. Quantitative analysis was performed for each brain at both sides of the third ventricle under $\times 10$ objective. The quantification of data was based on 24 measurements in 12 sections of the periventricular nucleus and 12 sections of the IN. Each measurement was made in the subarea of interest ($0.3286 \text{ mm}^2/\text{field}$) and two parameters were analysed: area fraction (% of area that exhibited positive staining); and integral density (the sum of individual optical densities of each pixel in the area being measured, it describes the amount of substance in tissue sections, expressed in relative units). The data from each section of each hypothalamus were pooled to represent "follicular" or "preovulatory" groups. The data were analysed statistically using nonparametric Wald-Wolfowitz test using the Statistica (StatSoft, Inc, Tulsa, US) data analysis software system. The data are reported as the mean percentage \pm SEM of the total area that exhibited positive staining and mean relative units \pm SEM of integral density. All analyses were performed using Statistica™ PL computer program (StatSoft® Kraków, Poland). Significance was defined at the $P \leq 0.001$ level.

Results

Two populations of NPY-ir neurons were detected in the hypothalamus during follicular and preovulatory phases of the oestrous cycle. One population containing only NPY-ir fibres was present in the anterior hypothalamic area, mainly in the periventricular nucleus beginning from the suprachiasmatic area up to MBH (Fig. 1a-b). The second population containing NPY-ir fibers and perikarya was distributed in MBH, namely in the infundibular nucleus, periventricular subdivision of the paraventricular nucleus and in all subareas of ME including the pituitary stalk. NPY-ir perikarya were located only in the ventral and caudal parts of the infundibular nucleus close to the 3rd ventricle and in the floor of the 3rd ventricle in the area close to the mammillary recess. The distribution of NPY-ir neurons was the same in sheep from both phases of the oestrous cycle. In sheep from the preovulatory phase, dense network of NPY-ir fibres and numerous NPY-ir perikarya appeared in the infundibular nucleus (Fig. 1c-d). The number and density of NPY-ir neurones (fibres and perikarya) localised in the infundibular nucleus estimated by image analysis, demonstrated that the percentage of the area containing NPY-ir neurones and the integral density (indicator of amount of hormonal material) were greater ($P \leq 0.001$) in sheep from the preovulatory phase than in sheep from the follicular phase of the oestrous cycle (Fig. 2). The area of the positive staining and the density of NPY immunoreactivity in the periventricular nucleus were similar in follicular and preovulatory phases of the oestrous cycle (Figs. 1a-b, 2).

Discussion

The present results demonstrated two populations of NPY neurons in the hypothalamus of the sheep. Similar

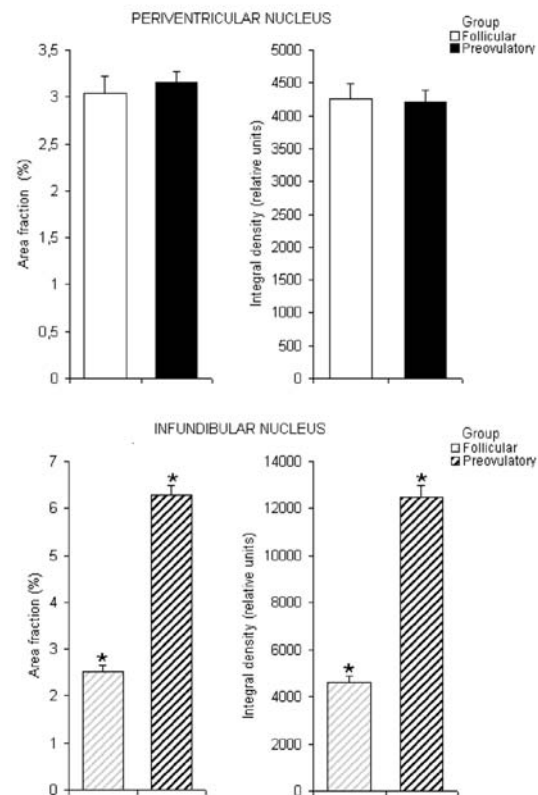


Fig. 2. Percentage of area exhibiting positive staining (area fraction %) and integral density (relative units) for NPY immunoreactivity in the periventricular and infundibular nuclei of sheep from follicular and preovulatory phases of the oestrous cycle. Mean \pm S.E.M. * $P \leq 0.001$.

distribution of NPY-ir perikarya and fibres in the hypothalamus was described by Chaillou *et al.* [3]. The NPY-ir perikarya were found after colchicine treatment which prevented the axonal transport of the secretory products and thus enhanced the amount of secretory product in the cell soma [3]. In addition, secretory activity of NPY-ir perikarya was enhanced by starvation [3] or long-term undernutrition [14] in sheep. In the present work, NPY-containing neurons in IN of the ewe exhibited the enhanced NPY expression during the preovulatory phase of the oestrous cycle. This suggests that the increase in the number of NPY-ir neurons and visualisation of perikarya may result from their augmented secretory activity during the preovulatory phase of the cycle, and thus from their participation in the preovulatory GnRH/LH surge. It has been suggested that the presence of NPY-ir terminals in ME can be attributed mainly to reproductive functions [2]. In the sheep, the common localization of GnRH and NPY containing neurons especially in IN and ME was shown [2, 14]. This neuroanatomical colocalization implicates some physiological interactions which are associated with hormone release. Recently, Advis *et al.* [1] showed, using the push-pull technique, that NPY release from ME was

positively correlated with GnRH and LH release in the follicular but not in the luteal phase of the cycle. Moreover, it was demonstrated that NPY did not modulate the frequency or amplitude of LH pulses at the level of GnRH cell bodies, so their action on the pulsatile LH secretion could be attributed to the level of GnRH release rather than its synthesis [2].

Expression of NPY-ir neurons in IN during the preovulatory phase of the cycle could be due to the increased synthesis of the peptide. However, the activity of mRNA for NPY during the oestrous cycle is not known in sheep. In the rat, prepro-NPY mRNA levels increased only on the afternoon of proestrus [12, 16] and on the day of oestrus [12] but were stable during the diestrus. The correlation between variations in the pre-pro-NPY mRNA and those of GnRH mRNA throughout the oestrous cycle in this species supported the hypothesis that NPY might be involved in the regulation of GnRH secretion.

In the present study, we have demonstrated that the population of NPY-ir neurons in the periventricular nucleus does not change during the two phases of the oestrous cycle. This area of the hypothalamus was established as the centre of somatostatin synthesis in many species including sheep [reviewed by 13]. This suggests that NPY terminals located in this area can be involved in some other regulations associated with the somatotrophic axis. Such implications were shown in our previous study in which nutritional manipulations increased activity of this population of NPY-positive neurons and a decrease in hypothalamic somatostatin output was observed [14, 21].

In summary, the neurosecretory activity of the hypothalamic NPY-ir neurons located in the infundibular but not in the periventricular nuclei was enhanced in the preovulatory phase of the oestrous cycle in the ewe. This suggests the participation of this population of hypothalamic neurons in the preovulatory events at the level of the GnRH release.

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