Dynamics of progesterone and estrogen receptor alpha in the ventromedial hypothalamus

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Abstract

Cyclic fluctuations of estradiol and progesterone in females influence neuronal activity in the ventrolateral division of the ventromedial hypothalamic nucleus (VMNvl), through the activation of progesterone receptors (PRs) and estrogen receptors (ERs). The expression of ER and PR in the VMNvl is influenced by their cognate ligands and is a central upstream trigger in the pathway of VMNvl-dependent modulation of endocrine responses. By studying the role played by estradiol and progesterone in PR and ERα expression in the VMNvl along the estrous cycle and how the two receptors interact in the same neuron, we aim to evaluate the synergistic action of both ovarian hormones in the regulation of VMNvl activity. In animals at all phases of the estrous cycle, the number of VMN neurons expressing PR or ERα was estimated by stereological methods, and the percentage, and rostro-caudal distribution, of neurons simultaneously expressing both receptors was determined. The highest number of PR-immunoreactive neurons was seen at proestrus, and of ERα-immunoreactive neurons was seen at proestrus and metestrus. The ERα/PR co-localization is increased at caudal levels. Approximately half the neurons expressing PR co-express ERα, a proportion that stays constant along the estrous cycle. The percentage of ERα neurons co-expressing PR changes from 60% at proestrus to 40% at metestrus. Fluctuations in circulating ovarian hormone levels promote coordinated changes in PR and ERα expression and co-localization. This may be an important mechanism in the regulation of input relayed by the VMNvl, allowing a precise modulation of endocrine responses.

Key Words

- ▶ estrous cycle
- ▶ ventromedial hypothalamic nucleus
- ▶ progesterone receptor
- ▶ estrogen receptor alpha
- ▶ female rat

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Introduction

In the female brain, cyclic fluctuations of the ovarian steroids estradiol and progesterone exert a profound influence on neuronal circuits involved in the regulation of goal-oriented behaviors fundamental to the species survival, such as food intake and sexual behavior (Pfaff *et al.* 2008, Blaustein 2009, Liu & Shi 2015). One

hypothalamic nucleus that plays a preponderant role in these behavioral answers is the ventromedial nucleus of the hypothalamus (VMN), a group of cells particularly targeted for steroid hormone action and specifically involved in the estradiol- and progesterone-dependent regulation of neuroendocrine function (Etgen *et al.* 1999,

Pfaff *et al.* 2008, Yang *et al.* 2013). The ventrolateral division of the VMN (VMNvl) expresses estrogen receptor (ER) α and β and progesterone receptors (PRs), which depend on the levels of their cognate ligands (Lauber *et al.* 1990, Shughrue *et al.* 1992, Sá *et al.* 2013, 2015). Earlier studies have demonstrated that ER α expression in the VMNvl is increased on ovariectomy and at proestrus, the stage of the estrous cycle when estradiol and progesterone levels are the highest (McGinnis *et al.* 1981, Lauber *et al.* 1990, Shughrue *et al.* 1992, Guerra-Araiza *et al.* 2003). Studies that specifically activated or inhibited ERs have shown that the activation of ER α or of ER α and ER β induces the expression of estradiol-inducible and non-inducible PRs (Moffat *et al.* 1998, Kudwa & Rissman 2003, Sá *et al.* 2013), whose activation is paramount to the complete execution of the female sexual behavior (Micevych *et al.* 2008, Mani & Oyola 2012, Snoeren *et al.* 2015). The role of progesterone in the modulation of sexual behavior is enhanced by its ability to promote post-estrous refractoriness, ending the estrous behavior and inducing a sort of reset of the neuronal circuitry involved in the regulation of female sexual behavior (Parsons *et al.* 1981, Mani & Oyola 2012).

In a previous study, it was shown that the structural and biochemical plasticity induced by estradiol in the VMNvl are differently regulated by its connections, in a way that synaptic plasticity is dependent on VMN external connections, whereas the expression of PRs was not (Sá *et al.* 2010). Calizo and coworkers (Calizo & Flanagan-Cato 2002, 2003) proposed that the VMNvl is formed by four different neuronal populations, according to its ER α expression and projection to the periaqueductal gray. However, these studies failed to perceive any correlation between neuronal phenotype and the degree of activation upon sexual sensory stimulation, suggesting that even interneurons that do not express ER α are able to regulate female sexual behavior and estrous termination. Furthermore, a previous study has shown the importance of this neuronal population in the promotion of the female sexual behavior by ablating the VMNvl neurons that express PR (Yang *et al.* 2013). Evidences reported by these previous studies suggest the existence of an intrinsic regulation of PRs by estradiol through ER α in the VMNvl, which is able to respond dynamically to different stimuli to regulate female sexual behavior. To our knowledge, only a few studies have addressed the existence of neurons co-expressing ER α and PR, showing an almost complete co-localization of PR and ER α in the ventromedial hypothalamus region (Blaustein & Turcotte 1989, Warembourg *et al.* 1989). However, those studies

were done in guinea pig, and the effects of estradiol and progesterone levels in normally cycling rats were not evaluated.

Therefore, it is hypothesized that the coordinated modulation of PR and ER α expression by cycling levels of estradiol and progesterone could change the number of neurons co-expressing both receptors exerting, in this way, a strict modulation of the pathways leading to the induction and termination of the estrous behavior. In this study, stereological methods were used to estimate the total number of VMNvl neurons that express PR and ER α along the four phases of the estrous cycle and the influence of the cycling ovarian hormones on the simultaneous expression of both receptors in the same neuron. As VMN connections are topographically organized (Sakuma & Akaishi 1987, Fahrbach *et al.* 1989, Flanagan-Cato 2000) and neurons that express PR and ER α are differently distributed along the rostro-caudal extent of the VMNvl, the aim of this study was also to determine the changes in the fraction of neurons that co-express PR and ER α along the rostro-caudal levels of the VMNvl.

Materials and methods

Animals and treatments

Female Wistar rats from the Institute for Molecular and Cell Biology (Porto, Portugal) were maintained under standard conditions with a 12-h light:12-h darkness cycle (07:00–19:00h) and ambient temperature of 23°C. Food and water were available *ad libitum*. Estrous cycles were monitored daily by vaginal smear cytology starting at the age of 8 weeks, and only animals exhibiting consecutive 4- to 5-day estrous cycles were used. At the age of 12 weeks, rats at all stages of the estrous cycle ($n=5$ per stage) were anesthetized with 2 mL/kg b.w. of a solution containing sodium pentobarbital (10 mg/mL) given intraperitoneally and killed by intracardiac perfusion of a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.6. All animal experimentation was conducted in agreement with accepted standards of animal care and in accordance with the European Communities Council Directives of 22 September 2010 (2010/63/EU) and Portuguese Act n°113/13.

Hormonal determinations

Prior to perfusion, blood samples (2000 μ L) were taken directly from the heart into the Eppendorf tubes.

After complete clot formation, each sample was centrifuged twice at 1100g for 10 min. Serum was then removed, collected in aliquots and stored undiluted at -80°C until further analysis. Estradiol and progesterone serum levels were assayed by enzyme-linked fluorescent assay using VIDAS Progesterone and Estradiol II Kits and mini VIDAS analyzer (bioMérieux S.A., Marcy L'Etoile, France). According to the manufacturer, the assay has a measurement range of 9–3000 pg/mL and 0.25–80 ng/mL for estradiol and progesterone, respectively. The analyzer was cleaned, calibrated and operated in accordance with the manufacturer's instructions. All samples were tested in singlicate.

Tissue preparation and immunohistochemistry

Upon killing, the brains were removed from the skulls, post-fixed for 1 h in the same fixative solution at 4°C , and then transferred to a solution of 10% sucrose in phosphate buffer at 4°C , where they were maintained overnight. Brains were transected in the coronal plane through the anterior border of the optic chiasm, rostrally, and the posterior limit of the mammillary bodies, caudally. These blocks of tissue were mounted on a Vibratome with the rostral surface up and sectioned at $40\text{ }\mu\text{m}$ throughout the rostro-caudal extent of the VMN. Each set of sections used for PR or ERa immunocytochemical detection with diaminobenzidine were sampled at regular intervals of $120\text{ }\mu\text{m}$ (1 out of 3), and each set of sections used for the fluorescent detection of PR and ERa immunoreactivity were sampled at regular intervals of $240\text{ }\mu\text{m}$ (1 out of 6).

Sections collected for the estimation of the total number of PR-immunoreactive (PR-ir) or ERa-immunoreactive (ERa-ir) neurons were processed as previously described (Sá *et al.* 2013, 2015, Leite *et al.* 2014, Martins *et al.* 2015). Briefly, sections were treated with 3% H_2O_2 , washed with phosphate buffered saline (PBS) and then blocked with 5% normal serum. The antiserum against PR (MAB462, Millipore) was used at a dilution of 1:2000, and the antiserum against ERa (MC20, Santa Cruz Biotechnology) was used at a dilution of 1:1000. Each set of sections were incubated in the respective primary antibody for 72 h, at 4°C and then in the corresponding secondary antibody, the biotinylated horse IgG anti-mouse antibody and the biotinylated goat IgG anti-rabbit antibody, respectively (Vector Laboratories, Peterborough, UK), at a 1:400 dilution for 1 h at room temperature. Afterward, sections were treated with avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector

Laboratories), at a 1:800 dilution, for 1 h at room temperature, followed by incubation, for 85 s, in 0.05% diaminobenzidine (Sigma-Aldrich) to which 0.01% H_2O_2 was added. Between each step, sections were rinsed with PBS. Stained sections were mounted on gelatin-coated slides, dehydrated and coverslipped using Histomount (National Diagnostics, Atlanta, GA, USA).

Sections collected for immunofluorescence were blocked for 1 h in a solution of 5% normal serum, and then incubated for 72 h at 4°C in a solution containing both primary antibodies at the stated dilutions. Then, sections were incubated, in the dark, in a solution containing both secondary antibodies, Alexa Fluor 568 goat anti-mouse and 488 goat anti-rabbit (A-11031 and A-11008; Life Technologies Europe BV), at a 0.5% dilution for 2 h at room temperature. Between each step, sections were rinsed with PBS. After that, sections were coverslipped with FluorSave to which 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI, D1306, Life Technologies Europe BV), was added at a 1:100 dilution.

Sets of sections of animals of all groups were processed together for either immunohistochemistry procedure to avoid variation across groups.

Antibody characterization

The ERa antibody used is a polyclonal rabbit antibody from Santa Cruz Biotechnology (MC-20, sc-542, RRID:AB_631470) prepared against a peptide mapping the amino acids 580HSLQTYYPPEAEGFPNTIS99, at the C-terminus of mouse ERa. The specificity of this antibody has been confirmed by the disappearance of signal after preabsorption with the epitope peptide and adult uterine cytosol (Omoto *et al.* 2005, Quesada *et al.* 2007, Fig. 1). In a spot blot test, the MC-20 recognized its blocking peptide on nitrocellulose paper, and after adsorption of the primary antibody to its blocking peptide, no staining was observed (Fernández-Guasti *et al.* 2000). In previous studies, we and other researchers have shown differences in distribution of the ERa using the same antibody in rat hypothalamus and preoptic area and one specific 68-kDa band in a western blot of rat hypothalamus (Kruijver *et al.* 2002, Normandin & Murphy 2008, Garcia-Falgueras *et al.* 2011, Malikov & Madeira 2012, Leite *et al.* 2014, Martins *et al.* 2015, Rebouças *et al.* 2016).

The antibody used for PR labeling, the mouse monoclonal antiserum a.a. 922–933, clone 6A, prepared against a peptide mapping the amino acids 922AGMVKPLLFHKK933 (MAB462, RRID:AB_2164323,

Figure 1

Representative photomicrographs of adjacent coronal sections through the medial preoptic nucleus (A, B) and the VMN (C, D) showing the peptide-inhibition experiment of the ER α antibody MC-20 with the corresponding blocking peptide (sc-542 P). A normal IHC procedure using the MC-20 antibody at 1/1000 showing immunostained neurons in the medial preoptic nucleus (A) and in the VMN and arcuate nucleus (C). No immunostaining signal is seen when the antibody is incubated with the peptide 10 $\times$ concentrated (B, D). Scale bar = 150 μ m. 3v, third ventricle; ARC, arcuate nucleus; MPN, medial preoptic nucleus; ox, optic chiasm; VMN, hypothalamic ventromedial nucleus.

Chemicon, Millipore Iberica), recognizes both the A and B isoforms of the human progestin receptor. The specificity of this antibody has been demonstrated by preadsorption with the immunizing peptide (Tetel *et al.* 2007). This antibody recognizes PRA and PRB bands on western blots of estrogen-treated hypothalamic cell lines and rat uteri (Fitzpatrick *et al.* 1999) of T47D-Y breast cancer cells and human uterine myosarcoma cell line SKUT-1B (Samalecos & Gellersen 2008, Lee *et al.* 2010). In our laboratory, the distribution of PR-positive neurons in the VMN (Sá *et al.* 2010, 2013, 2014) matched previous observations made by other researchers using the same antibody (Blaustein & Gréco 2002, Tetel *et al.* 2007).

Estimation of the total number of PR- and ER α -immunoreactive neurons

The optical fractionator method (West *et al.* 1991, Madeira *et al.* 1997) was applied to estimate the total number of PR- or ER α -ir neurons in the VMNvl. Sections were analyzed using a modified Olympus BH-2 microscope interfaced with a color video camera and equipped with a Heidenhain ND 281 microcator (Traunreut, Germany), a computerized stage, and an object rotator (Olympus). A computer fitted with a frame grabber (Screen Machine II, FAST Multimedia, Germany) was connected to the monitor. By using the C.A.S.T. – Grid system software (Olympus), the fields of view of both receptors were systematically sampled at regular intervals of 100 μ m along the x and y axes. The counting frame area of both dissectors was 1486 μ m² at the tissue level with a fixed depth of 10 μ m. The estimations were performed with

a 100 $\times$ oil immersion lens with a numerical aperture of 1.40, which produced a final magnification of 2000 $\times$ in the computer screen. Cell profiles were considered immunopositive for PR or ER α whenever a dark brown reaction product was present within the cell nucleus of either immunolabeling (Fig. 2). On an average, 200 PR- and 200 ER α -ir cells were counted per VMNvl; the mean coefficient of error (Gundersen *et al.* 1999) of the estimates was 0.07 for PR and 0.06 for ER α . All counts were made by an observer who was blinded to group assignment.

Fluorescence quantification

Fluorescence image acquisition was made using a Leica DC 300F color video camera (Leica Microsystems Imaging Solutions) connected to a Leica DMR light microscope (Leica Microsystems Imaging Wetzlan GmbH), using a 63 $\times$ /1.32–0.6 objective lens (HCX PL APO). The double-labeled neurons were detected by observing the cells through an N2.1 filter (excitation wavelength 515–560 nm) for PR immunoreactivity detection and a L5 filter (excitation wavelength 480/40 nm) for ER α -immunoreactivity detection (Fig. 3). DAPI was detected using an A filter (excitation wave length 340–380 nm). For each rat, three sections, level match, containing the VMNvl were sampled, at the rostro-caudal levels approximately Bregma –2.3; –2.8; –3.3 (Paxinos & Watson 1998). Images of either immunolabeling were captured in a optic plan in one region of interest with an area of 57,766.42 μ m² in each of the three VMNvl sections (with a total area of 173,299.26 μ m² per rat), by switching from one filter cube to the other, using the

Figure 2

Representative photomicrographs of adjacent coronal sections through the rostro-caudal extent of the VMN, arranged from rostral to caudal levels, showing the VMNvl (arrow) immunostained for PR or ERa (Bregma level approximately -2.30 , -2.80 and -3.30 mm). Scale bar = $150\mu\text{m}$. Inset: higher magnification of the area delineated by a box in the upper image, showing PR-ir or ERa-ir neurons with a dark brown immunostained nucleus and a relatively unstained cytoplasm. Scale bar = $5\mu\text{m}$. 3v, third ventricle; ARC, arcuate nucleus; f, fornix; VMN, hypothalamic ventromedial nucleus.

same image settings for each filter cube. Cell counts were performed using ImageJ software after superimposition of the two images of the same region of interest. An average of 135 PR-ir and 135 ER-ir neurons were counted per VMNvl. Quantitative data were expressed as percentage of co-localization, i.e., percentage of PR- or ERa-ir cells that also express the other receptor. As diaminobenzidine immunohistochemistry revealed different rostro-caudal distribution of PR- and ERa-ir neurons, the percentage of PR/ERa and ERa/PR co-localization at rostral (Bregma

level approximately -2.16 to -2.28), medial (Bregma level approximately -2.64 to -2.76) and caudal levels (Bregma level approximately -3.12 to -3.24) (Paxinos & Watson 1998), was also calculated. An average of six regions of interest was analyzed per rat.

Statistical analyses

The influence of ovarian hormone changes along the estrous cycle on uterine weight, hormonal levels and

Figure 3

Fluorescence microscopy images of VMNvl neurons stained with Alexa Fluor 568 (red, PR-ir), with Alexa Fluor 488 (green, ERa-ir) or with both (yellow, co-localization). The upper set shows ERa-ir neurons that do not express PR (asterisk) and the lower set shows PR-ir neurons that do not express ERa (cross). Scale bar = $10\mu\text{m}$.

Table 1 Uterine weights and hormonal determinations.

	Diestrus	Proestrus	Estrus	Metestrus
Uterine weight (g)	0.47 (0.03)	0.84 (0.06)*	0.47 (0.02)	0.44 (0.04)
Estradiol levels (pmol/L)	79.83 (10.03)**	270.72 (9.67)	37.86 (2.24)** ϕ	49.70 (7.36)**
Progesterone levels (nmol/L)	30.07 (1.38)** $\#$	126.53 (4.61)	24.11 (1.91)** $\#$	93.45 (8.94)*

Values represent means (s.e.m.).

* $P < 0.001$ compared with all other groups; ** $P < 0.001$, * $P < 0.01$ compared with proestrus; # $P < 0.001$ compared with metestrus; $\phi P < 0.01$ compared with diestrus.

receptor expression in the VMNvl was assessed by one-way analysis of variance (ANOVA). The influence of the estrous cycle in the rostro-caudal distribution of the receptor co-localization was assessed by two-way ANOVA. Pairwise comparisons were made using the post hoc Tukey's HSD test (GraphPad Prism, version 6.0; GraphPad Software). Differences were considered significant if $P < 0.05$.

Results

Uterine weight and hormonal determinations

As expected, a significant overall difference was observed on uterine weights ($F(3,16)=20.97$, $P < 0.0005$) as well as in estradiol ($F(3,16)=187.3$, $P < 0.0005$) and progesterone ($F(3,16)=92.91$, $P < 0.0005$) levels. Uteri of rats were 2-times heavier at proestrus than at any other stage of the cycle. There were no differences in the uterine weight of rats at diestrus, estrus or metestrus (Table 1). Estradiol levels were 3-, 7- and 5-times higher at proestrus when compared with diestrus, estrus and metestrus phases, respectively, and 2-times higher at diestrus than at estrus phase (Table 1). Progesterone levels were 4-, 5- and 1.4-times higher at proestrus than at diestrus, estrus and metestrus phases, respectively, and 3- and 4-times higher

at metestrus when compared with diestrus and estrus phases, respectively (Table 1).

Effect of the estrous cycle on the total number of PR- and ER α -immunoreactive neurons

ANOVA revealed significant variations induced by the stage of the estrous cycle in the total number of PR- ($F(3,16)=16.19$, $P < 0.0005$) and ER α -ir neurons ($F(3,16)=10.91$, $P < 0.0005$) in the VMNvl. The total number of PR-ir neurons increased nearly 70% from diestrus to proestrus and decreased nearly 30% from proestrus to estrus phase (Fig. 4A). The total number of ER α -ir neurons increased nearly 30% from diestrus to proestrus phase and nearly 20% from estrus to metestrus phase. ER α -ir neuron numbers decreased nearly 15% from proestrus to estrus phase and nearly 25% from metestrus to diestrus phase (Fig. 4B).

Percentage of PR and ER α co-localization in VMNvl neurons

The phase of the estrous cycle significantly influenced the percentage of ER α -ir VMNvl neurons that co-express PR ($F(3,16)=17.03$, $P < 0.005$) but not the percentage of

Figure 4

Graphical representation of the total number of PR-ir (A) and ER α -ir (B) neurons in the VMNvl of rats at four phases of the estrous cycle (diestrus, proestrus, estrus and metestrus). Columns represent means $\pm$ s.d. Tukey HSD test: ** $P < 0.001$, * $P < 0.01$, compared with proestrus groups; ** $P < 0.001$, * $P < 0.05$, compared with proestrus and estrus groups.

Figure 5

Graphical representation of the percentage of PR-ir neurons that co-express ER α (A) and of ER α -ir neurons that co-express PR (B) in the VMNvl of rats at the four phases of the estrous cycle (diestrus, proestrus, estrus and metestrus). Columns represent means $\pm$ s.e.m. Tukey HSD test: ** $P < 0.01$, * $P < 0.05$, compared with proestrus groups.

Figure 6

Graphical representation of the rostro-caudal distribution of the percentage of PR-ir neurons that co-express ERa (A) and of ERa-ir neurons that co-express PR (B) in the VMNvl of rats at four phases of the estrous cycle (diestrus, proestrus, estrus and metestrus). Columns represent means. Tukey HSD test: *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, compared with proestrus groups in the same VMNvl level; + $P < 0.05$, compared with VMNvl medial level at the same phase of the estrous cycle, # $P < 0.001$, compared with VMNvl caudal level at the same phase of the estrous cycle.

PR-ir VMNvl neurons that co-express ERa ($F(3,16)=2.71$, $P=0.080$; Fig. 5A). At estrus and metestrus phases, the percentage of ERa-ir neurons that express PR was 30 and 35% lower, respectively, than at proestrus phase (Fig. 5B).

Percentage of PR and ERa co-localization along VMNvl rostro-caudal levels

As revealed by the two-way ANOVA, the percentage of PR-ir neurons expressing ERa (Fig. 6A) was influenced by the estrous cycle ($F(3,48)=2.94$, $P < 0.05$) and by the rostro-caudal levels of the VMNvl ($F(2,48)=3.79$, $P < 0.05$). No significant interaction between the estrous cycle and the rostro-caudal level was detected ($F(6,48)=1.52$, $P=0.19$). In proestrus rats, the percentage of PR-ir neurons expressing ERa was 1.6 times higher at mid-levels than at rostral or caudal levels of the VMNvl. There were no changes in the percentage of PR-ir neurons expressing ERa along the rostro-caudal levels in diestrus, estrus and metestrus rats.

The two-way ANOVA revealed a significant effect of estrous cycle ($F(3,48)=16.79$, $P < 0.0005$) and the rostro-caudal levels of the VMNvl ($F(2,48)=28.11$, $P < 0.0005$) on the percentage of ERa-ir neurons expressing PR (Fig. 6B). A significant interaction between the estrous cycle and the rostro-caudal levels was detected ($F(6,48)=2.98$, $P < 0.05$). At caudal levels, the percentage of ERa-ir neurons expressing PR was higher at proestrus than at any other phase of the estrous cycle. Specifically, it was approximately 1.4, 1.7 and 2 times higher when compared with estrus, diestrus and metestrus rats, respectively. At proestrus, the percentage of ERa-ir neurons expressing PR was 1.5 and 2 times higher in caudal levels than in the mid and the rostral levels, respectively.

Discussion

For the first time, data in this study show that ERa/PR co-localization changes along the rostro-caudal levels of

the VMNvl of rats in all stages of the estrous cycle in a way that co-expression is increased in neurons at all levels of the VMNvl. In fact, co-localization increases in neurons in the rostral levels, where the expression of the receptors is smaller, from estrus to proestrus and in the caudal levels, where it is higher, from metestrus to proestrus. Results have also shown that only half the neurons expressing estradiol-dependent and non-dependent PR express ERa that does not change along VMNvl rostro-caudal levels. Contrariwise, the majority of neurons expressing ERa at proestrus also express PR, and this increase is most evident at caudal levels.

Results on uterine weights and hormone levels in the present study are in agreement with previous ones (Butcher *et al.* 1974, Smith *et al.* 1975, Leite *et al.* 2014, Martins *et al.* 2015) by showing an increased uterine weight and hormone levels at proestrus with subsequent decline along the phases of the estrous cycle. As the animals were allocated to each estrous cycle phase according to the results of the vaginal cytology done at the day of killing, present hormonal determinations confirm the use of vaginal smear cytology for the recognition of the different phases of the estrous cycle in the Wistar rat.

The estimated total number of VMNvl neurons expressing PR is increased at proestrus, which is in agreement with previous studies on the estrous cycle (McGuinnins *et al.* 1981, Guerra-Araiza *et al.* 2003, Blaustein 2009) and also with previous studies that used ovariectomized (OVX) rats injected with estradiol (Etgen *et al.* 1999, Blaustein 2009, Sá *et al.* 2010, 2013). In a recent study that used genetic strategies to visualize PR-ir neurons in mice, Yang and coworkers (Yang *et al.* 2013) reported a higher percentage of PR/ERa co-localization (approximately 90%) than the present results. The gold standard for co-localization studies is confocal microscopy, because it ensures sensitive detection of labeled cells and accurate focusing on the same confocal plane. In this study an epifluorescent microscope has been used, which may have been a limitation as it

could introduce some underestimation in the overlap of ERα and PR signaling. However, the discrepancies between the present and previous studies (Blaustein & Turcotte 1989, Yang *et al.* 2013) could also be accounted for by other methodological differences, such as the hormonal milieu of the females studied and the rostro-caudal level of the sections screened, because present data suggest co-localization changes along the estrous cycle and along the rostro-caudal levels of the VMNvl. The fact that the number of VMNvl neurons expressing PR increases on natural or exogenous estradiol surges suggests that progesterone has no effect in the amount of neurons expressing PR. However, when the present data estimated at proestrus were compared with previous results obtained from OVX rats injected with estradiol benzoate using the same method (Sá *et al.* 2010, 2013), 3500 less PR-ir neurons were estimated. This difference could be accounted for the different circulating estradiol levels at proestrus versus OVX rats injected with estradiol benzoate as the dose-dependent effect of estradiol on PR expression in the VMN is well known (Clark *et al.* 1982, Etgen *et al.* 1999, Blaustein 2009). Nevertheless, at metestrus, where a second, smaller, surge of progesterone occurs, there is a higher decrease in the number of PR-ir neurons when compared with proestrus. Taken together, these observations provide additional support to the notion that progesterone–PR binding induces the receptor degradation and downregulation (Funabashi *et al.* 2000, Qiu & Lange 2003, González-Flores *et al.* 2004), which could account for the less exuberant increase in the number of PR-ir VMNvl neurons at proestrus. Present results shown at estrus and metestrus phases support the concept that the downregulation of PRs is an important mechanism in the pathway of estrous termination (Parsons *et al.* 1981, González-Flores *et al.* 2004).

In general, it is assumed that estradiol downregulates ERα (Laubert *et al.* 1990, Etgen *et al.* 1999, Blaustein 2009). Nonetheless, studies have reported some discrepancies by showing no effect (Chakraborty *et al.* 2003) or an increase in ERα upon estradiol administration to OVX rats in the VMN (Devidze *et al.* 2005, Malikov & Madeira 2012). Present observations at proestrus show an increase in the total number of ERα-ir neurons, which may be due to the increased levels of estradiol and not progesterone. In fact, it was previously shown that progesterone downregulates ER binding in the preoptic–hypothalamic area and VMN (Blaustein & Brown 1984, Brown & MacLusky 1994), and previous studies in the medial preoptic nucleus and bed nucleus of the stria terminalis (Leite *et al.* 2014, Martins *et al.* 2015) further corroborate

this finding. Nevertheless, it was also shown that in VMNvl neurons, progesterone promotes ERα expression (Malikov & Madeira 2012), which is further supported by present data showing an increase in the total number of ERα-ir neurons at metestrus, when a second surge of progesterone occurs. However, at this phase of the cycle, PR-ir neurons are reduced to 60%, which averts a direct action of progesterone and further corroborates the notion that progesterone is able to activate other pathways, namely membrane mechanisms and electrophysiological responses in some neurotransmitter systems (Petitti & Etgen 1992, Etgen *et al.* 1999, Mani & Oyola 2012), increasing ERα expression.

It was reported that approximately all PR-ir neurons in the VMN region co-express ERα (Blaustein & Turcotte 1989, Warembourg *et al.* 1989). On the contrary, present results show that only nearly 50% of PR-ir VMNvl neurons also express ERα and that 40–60% of ERα-ir VMNvl neurons co-express PR. Furthermore, no differences were shown along the four stages of the cycle in the percentage of PR-ir neurons co-expressing ERα, suggesting that only half of the neurons expressing estradiol-induced PRs co-express ERα, reinforcing the notion that estradiol-induced PR expression is activated both directly and transsynaptically (Blaustein 2009, Sá *et al.* 2013). The small co-expression of both receptors further support the concept that the VMNvl neurons that do not express ERα but are involved in the promotion of the female sexual behavior express PR and are directly involved in the regulation of estrous initiation and termination.

Data in this study show that the percentage of ERα-expressing neurons that also express PR increases at proestrus, suggesting that the majority of neurons expressing ERα *de novo* are also PR-ir. Proestrus is the stage of the estrous cycle when the VMN is activated and prepared to relay all sorts of sensorial information into activated motor responses (Etgen *et al.* 1999, Guerra-Araiza *et al.* 2003, Pfaff *et al.* 2008). These results suggest that the high ERα/PR co-localization may be a downstream effect of neuronal activation that triggers molecular mechanisms necessary for the promotion of the behavioral response. In addition, at metestrus, there is the combination of a high number of ERα-ir neurons with the lowest percentage of ERα/PR co-localization due to the lowest number of PR-ir neurons, suggesting that at this stage of the estrous cycle, the low combination of PR and ERα expression averts sexual responsiveness triggering estrous termination.

Previous studies have shown that VMNvl connections are topographically organized in a way that inputs reach

the caudal VMNvl to be integrated and relayed to the rostral VMNvl, where outputs are sent to the midbrain (Sakuma & Akaishi 1987, Fahrbach *et al.* 1989, Flanagan-Cato 2000). The present observations show a higher increase in the percentage of ERα/PR co-localization at VMNvl caudal levels at proestrus, suggesting that combined expression of both receptors warrants the responsiveness of these VMNvl neurons allowing them to modulate the extrinsic input into relayed outputs fundamental to the behavioral response.

With this study, we have shown that successive changes in circulating estradiol and progesterone levels along the estrous cycle induce coordinated changes in the expression of PR and ERα and in the percentage of their co-localization. This variant co-localization of PR and ERα could be an important mechanism in the regulation of received information by the VMNvl that is relayed to the midbrain. In this way, the female sexual behavior may be methodically modulated by ovarian hormones in VMNvl neurons through intrinsic and extrinsic mechanisms, triggering the induction and estrous termination.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S I S designed the research. S I S and B M F performed the techniques. All authors were involved in drafting and all approved the manuscript.

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