Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

Brain Research

Research Report

Estrogen, but not progesterone, induces the activity of nitric oxide synthase within the medial preoptic area in female rats

Fernanda Barbosa Lima[a,c], *Fábio Honda Ota*[a], *Fernanda Jankur Cabral*[a,b], *Bruno Del Bianco Borges*[a], *Celso Rodrigues Franci*[a,*]

[a]*Universidade de São Paulo, Faculdade de Medicina de Ribeirão Preto, Ribeirão Preto, SP, Brazil*
[b]*Instituto de Ciências Biomédicas, São Paulo, SP, Brazil*
[c]*Universidade Federal de Santa Catarina, Centro de Ciências Biológicas, Florianópolis, SC, Brazil*

ARTICLE INFO

Article history:
Accepted 1 July 2014
Available online 17 July 2014

Keywords:
Estrogen
Progesterone
Tamoxifen
RU-486
Nitric oxide
Medial preoptic area

ABSTRACT

The control of gonadotropin-releasing hormone (GnRH) secretion depends on the action of ovarian steroids and several substances, including nitric oxide (NO). NO in the medial preoptic area (MPOA) stimulates the proestrus surge of luteinizing hormone (LH). We studied the effect of estrogen (Tamoxifen-TMX) and progesterone (RU-486) antagonists on mRNA and protein expression of NO synthase (NOS), the enzyme that produces NO, as well as its activity within MPOA. Female rats received s.c. injections of TMX (3 mg/animal) on first and second days of the estrous cycle (9 am), RU-486 (2 mg/animal) on first, second, (8 am and 5 pm) and third days of the estrous cycle (8 am) or oil (controls) and were killed on the third day (5 pm). Real time-PCR and western blotting were performed to study NOS mRNA and protein expressions. The NOS activity was indirectly assessed by measuring the conversion from [^{14}C]-L-arginine into [^{14}C]-L-citrulline. TMX significantly decreased neuronal NOS (nNOS) mRNA expression (90%), and the activity of NOS, but did not alter nNOS protein expression. Also, TMX significantly decreased LH, FSH, estrogen and progesterone plasma levels. RU-486 nor affected NOS mRNA and protein expressions neither the NOS activity in the MPOA, but reduced FSH levels. The nitrergic system in the MPOA can be stimulated by estrogen whereas TMX decreased NOS activity and mRNA expression. In conclusion, the involvement of the nitrergic system in the MPOA to induce the surge of LH on proestrus depends on the estrogen action to stimulate the mRNA-nNOS expression and the activity of nNOS but it does not seem to depend on progesterone action.

*Correspondence to: Departamento de Fisiologia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av. Bandeirantes, 3900, 14049-900 Ribeirão Preto, SP, Brazil. Fax: +55 16 36330017.
E-mail address: crfranci@fmrp.usp.br (C.R. Franci).

http://dx.doi.org/10.1016/j.brainres.2014.07.003
0006-8993/

1. Introduction

The control of gonadotropin-releasing hormone (GnRH) secretion depends on the interaction between ovarian steroids and several neurotransmitters, including the nitric oxide (NO) (Barnes et al., 2002; Lima et al., 2007; Moretto et al., 1993; Pinilla et al., 1999), but its relation with GnRH neurons is still unclear. GnRH neurons are dispersed from the medial septal–diagonal band complex to the rostral hypothalamus and median eminence. They are mainly found in the medial preoptic area (MPOA) and the organum vasculosum of the lamina terminalis (OVLT), and constitute a common final pathway to control the surge of the luteinizing hormone (LH) on proestrus (for review see (Jennes and Conn, 1994; Witkin, 1999)).

The bimodal effect of estrogen (E_2) on the hypothalamus controls the GnRH secretion. E_2 exerts a negative feedback effect on the hypothalamus reducing the GnRH pulse amplitude and inhibiting GnRH mRNA expression. In the end of the follicular phase, E_2 exerts a positive feedback effect stimulating synthesis and release of GnRH, which triggers the preovulatory surge of LH (for review see (Moenter and Chu, 2012; Radovick et al., 2012)). Actions of ovarian steroids may occur in either the hypothalamus or pituitary gland. In the hypothalamus E_2 can act in three ways: directly, influencing the membrane excitability of GnRH neurons; transsynaptically, regulating the activity of neurons synapsing with GnRH neurons; or indirectly, controlling glial cell-GnRH neuron interactions (Herbison and Pape, 2001). In the pituitary gland, progesterone (P4) can inhibit or stimulate the secretion of gonadotropin, depending on the estrous cycle phases. It potentiates the negative and positive feedback effects of E_2 on gonadotropin secretion (Brann et al., 1991).

Tamoxifen (TMX), an antiestrogen drug, presents selective estrogen receptor modulator (SERM) properties, with agonistic and antagonistic activity on the reproductive axis (McDonnell, 1999, 2003; McDonnell et al., 2002). It acts by inducing P receptors (PR) mRNA and protein expression, and PR-dependent GnRH self-priming in the absence of E_2 (Bellido et al., 2003; Garrido-Gracia et al., 2007), which is inhibited by administration of E_2 (Sanchez-Criado et al., 2005). The TMX-induced GnRH self-priming is exerted through its high affinity and specificity binding to intracellular ER (McDonnell et al., 2002). TMX can also display a selective antagonistic activity on GnRH self-priming of LH secretion (Sanchez-Criado et al., 2002). Mifepristone (RU-486), an antiprogestagen drug, can abolish P_4 actions on the LH secretion. It reduces the secretion of LH in response to the GnRH and this effect is enhanced in the presence of E_2 (Ortmann et al., 1989).

NO is produced by the oxidation of L-arginine to L-citruline by neuronal NO synthase (nNOS) (Southam and Garthwaite, 1993). The MPOA displays marked staining of NOS cell bodies and fibers. GnRH neurons are frequently surrounded by NOS neurons, with potential contacts between them (Bhat et al., 1996; Bredt et al., 1991). NO is involved in both surges of LH, on proestrus and that induced by ovarian steroids in ovariectomized (OVX) rats (Bonavera et al., 1996, 1993). Furthermore, NOS inhibitors and NOS antisense oligonucleotides attenuate the preovulatory surge of LH (Bonavera et al., 1994). nNOS expression and activity are regulated by E_2 in different brain areas (Gingerich and Krukoff, 2005; Grohe et al., 2004). nNOS expression is also down-regulated by E_2 in the rat anterior pituitary (Qian et al., 1999). In order to clarify the E_2 and P_4 actions on the nitrergic system in the MPOA during proestrus, we investigated whether nNOS mRNA and protein expressions or NOS activity in the MPOA would be changed by the antagonism of endogenous E_2 or P_4.

2. Results

The nNOS transcript was significantly reduced by previous treatment with TMX, but not with RU486 (Fig. 1A). The fall was near to 90% for TMX and 25% for RU-486. Western blotting analysis (Fig. 1B) did not show a significant effect of

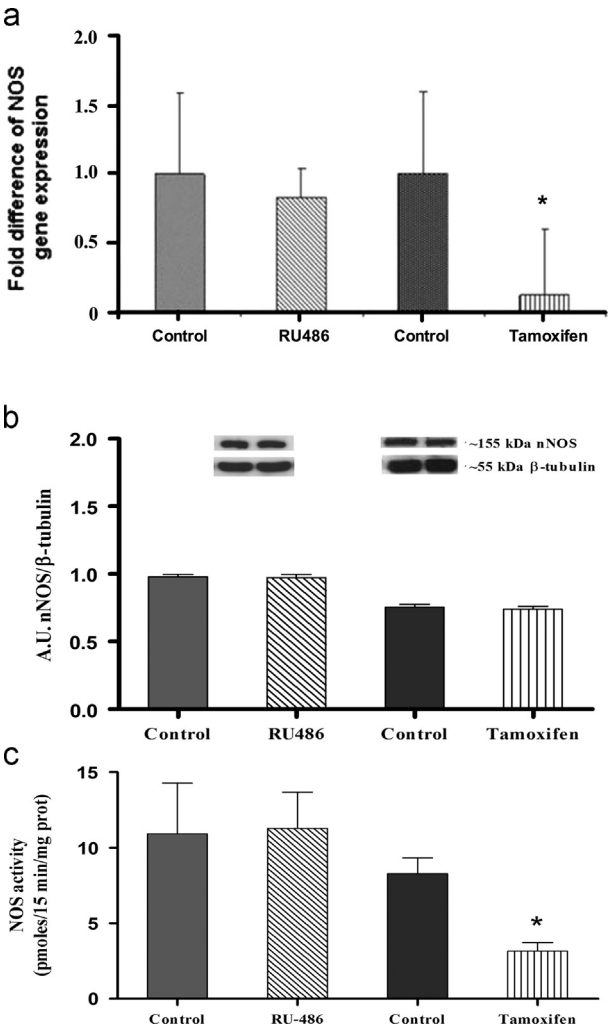

Fig. 1 – NOS mRNA levels (A), semi-quantitative analysis in the same assay for nNOS expression by Western blotting showing bands related to the molecular weight of nNOS (≈ 155 KDa) (B), NOS activity (C) in the MPOA from rats injected with corn oil (controls), Tamoxifen (3 mg/0,2 ml/animal) or RU-486 (2 mg/0.2 mL/animal). Data are presented as mean ± S.E.M. Groups size: 6–7 (A), 8–9 (B) and 9–11(C) animals. Statistical analysis: Levene test (A); Student's *t*-test (B); and One Way Anova, pos hoc Dunnett's test (C). *$p < 0.05$ versus control.

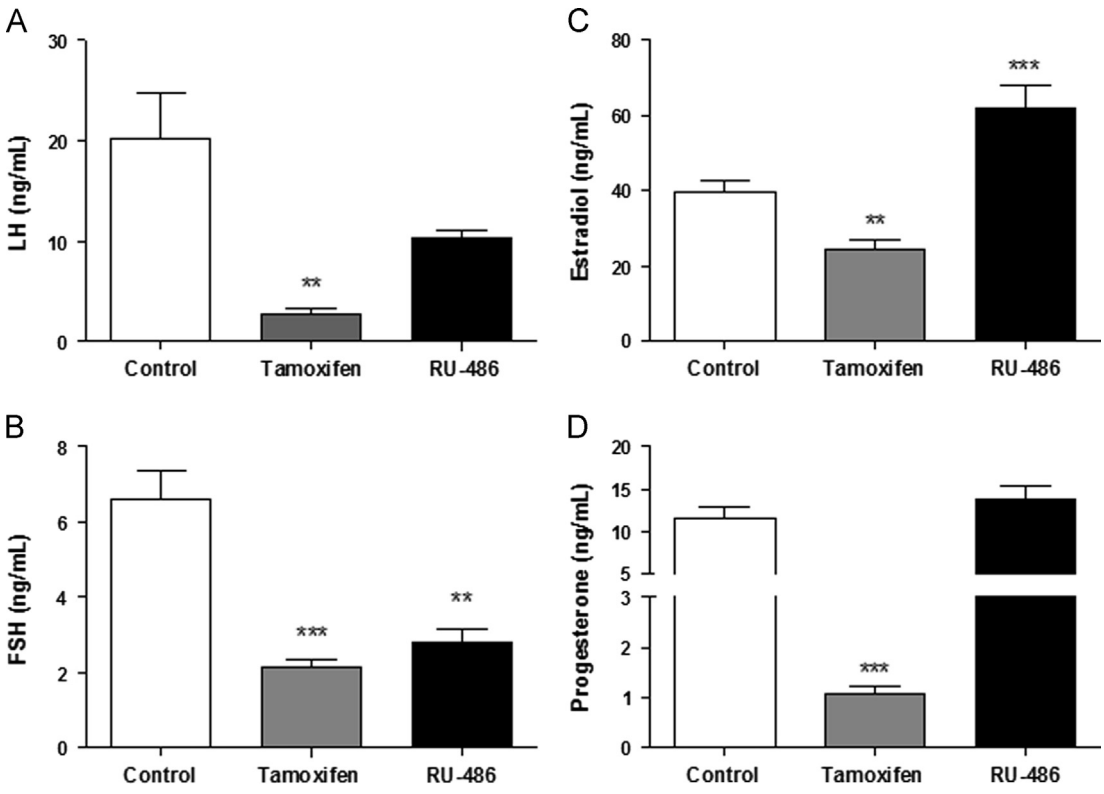

Fig. 2 – Plasma LH (A), FSH (B), E_2 (C) and P_4 (D) in rats following injections of corn oil (controls), Tamoxifen (3 mg/0.2 ml/animal) or RU-486 (2 mg/0.2 mL/animal). Data are presented as mean ± S.E.M. Groups size: 9–22 (A,B) and 5–22 (C,D) animals. $^{**}p < 0.01$, $^{***}p < 0.001$, One Way Anova, pos hoc Dunnett's test.

TMX or RU-486 treatment on the nNOS protein expression. It was detected one band of approximately 155 KDa, corresponding to the nNOS molecular weight.

TMX significantly decreased NOS activity, which was not affected by RU-486 (Fig.1C). TMX, but not RU-486, significantly decreased plasma LH (Fig. 2A), while FSH levels were decreased by both treatments (Fig. 2B). Plasma E_2 (Fig. 2C) was decreased by TMX and increased by RU-486. TMX treatment decreased plasma P_4 levels, which were not affected by RU-486 (Fig. 2D).

3. Discussion

NO is an important player in the control of GnRH neurons (Hanchate et al., 2012). Previous data have shown an increased NOS activity (Lima et al., 2007) as well as NOS mRNA expression (Lamar et al., 1999) in the MPOA in proestrus, indicating that NO stimulates the release of GnRH (Barnes et al., 2001). NO directly modulates GnRH neurons in the preoptic area, and kisspeptin–GPR54 signaling is required for E_2 to influence NOS activity in this area (Hanchate et al., 2012). Our experimental model allowed us to analyze the action of the endogenous E_2 and P_4 on the nitrergic system in the MPOA during the estrous cycle.

TMX decreased plasma concentrations of LH, FSH, E_2 and P_4. It also reduced nNOS-mRNA expression and nNOS activity in the MPOA, but it did not change the nNOS protein expression. Therefore, changes in the nNOS-mRNA did not translate to changes in the NOS protein expression. If nNOS is edited by post-transcriptional events (Huber et al., 1998), the mRNA processing would be responsible for the diversity of NOS protein pool contents to produce NOS variants. This adjustment in translational processes can maintain the nNOS protein expression within a narrow range. Parkash et al. (2010) showed that the control of NO production during the estrous cycle may not be directly linked to changes in nNOS protein synthesis, but rather would involve posttranslational modifications. Some studies have suggested that E_2 can modulate the hypothalamic nNOS gene expression (Lamar et al., 1999; Pu et al., 1998). Variations of E_2 also regulate the activation of the NOS throughout the estrous cycle (Parkash et al., 2010).

Our data suggest that the TMX-dependent reduction of NOS activity can be involved in the decrease of the secretion of gonadotropins, E_2 and P_4. NO participates in the control of the LH surge (Bonavera et al., 1996, 1993). It has been shown recently that kisspeptin neurons directly activate NOS neurons in the MPOA and this interaction modulates gonadotropins secretion (Hanchate et al., 2012). Also, it has been shown that noradrenaline and oxytocin stimulate GnRH release by activating the nNOS system. Therefore, there are some indicators of the stimulatory role of NO on the GnRH release and consequently on LH and FSH secretion. This pathway consists of oxytocin activating noradrenergic neurons in the medial basal hypothalamus (MBH), which activate NOergic neurons. The NO diffuses into GnRH terminals and induces

GnRH release (for review see (McCann et al., 1998)). Altogether, these data support our results seeing that rats treated with TMX presented a significant decrease of LH and FSH secretions. Whereas the MBH and the MPOA are under the effect of E_2, TMX may act directly on NOergic neurons or indirectly on noradrenergic and/or kisspeptinergic neurons reducing the nNOS mRNA expression/activity and consequently, GnRH and gonadotropins secretion.

Our data confirmed reports from literature (Donath and Nishino, 1998) in which TMX decreases plasma LH and FSH, blocking their preovulatory surge in proestrus. The effect of TMX on LH levels might be controversial and is dependent on the dose and time of administration. Rats treated with TMX in a late stage of the estrous cycle (proestrus) still present ovulation (Nishino et al., 2009). Also, the PR-dependent GnRH self-priming exerted by TMX only occurs in the absence of E_2. Whereas our animals were cycling, we believe that the absence of LH surge in the TMX-treated animals could be at least in part due to the absence of the GnRH self-priming. Based on our results it is difficult to clarify whether the antiovulatory effects of TMX were due to its agonistic or antagonistic activity. However, it is noteworthy that TMX also significantly decreased the P_4 release, which is essential for the preovulatory LH surge. Furthermore, in our experimental protocol, TMX was efficient at blocking the positive feedback exerted by E_2 on gonadotropins secretion.

TMX is ineffective against activating inducible macrophage NOS (iNOS) (Renodon et al., 1997). Whereas we measured total NOS activity and our procedure does not discriminate enzyme variants, the decrease of this activity could be related to the nNOS and/or endothelial NOS (eNOS). However, it is noteworthy that nNOS is the most abundant in the MPOA (Bhat et al., 1996). Sham ovariectomized rats subjected to chronic infusion of TMX for two weeks showed unaltered E_2 levels and they remained in diestrus. We treated regular cycling rats for two days. We cannot rule out other regulators modulating the NOS activity in the brain.

We observed that TMX decreased plasma E_2. This effect was previously reported (Donath and Nishino, 1998) and could be explained four different ways: first, the disruption on the GnRH-gonadotropins secretion caused by the reduced activity of nNOS would result in a decrease on E_2 and P_4 plasma levels; second, TMX would act as an ERα agonist in the pituitary gland, working in the negative feedback mechanism (Rissman et al., 1997); third, TMX would increase plasma E_2, disrupting the feedback loop between the hypothalamus and the ovaries, decreasing E_2 synthesis (Sakurai et al., 2011); fourth, TMX would act in the ovaries, blocking ERβ in the granullosa cells and decreasing E_2 production, which would also lead to the third possibility.

The reduction of plasma P_4 caused by TMX treatment corroborates previous reports (Donath and Nishino, 1998). It could be due to the inhibition of the positive feedback exerted by E_2 on the gonadotropins release.

RU-486 did not change LH or P_4 secretions. Also, it did not alter NOS mRNA/protein expression nor nNOS activity in the MPOA. However, it decreased FSH secretion and increased E_2 levels. A central action of RU-486 on the nitrergic system has been found in the paraventricular nucleus of rats (Kim et al., 2004), blocking the reduction of nNOS induced by fasting. Taken together, these data indicate that P_4 acts differently depending on the area within the brain.

RU-486 has an inhibitory effect on LH and FSH secretion (Rao and Mahesh, 1986), without abolishing ovulation (Sanchez-Criado et al. 1994). Although these authors studied cycling rats, the dose and time of administration were different from the present work. Moreover, in vitro studies demonstrated that RU-486 suppresses LH and FSH secretion at the pituitary level during proestrus, but not during metestrus (Bellido et al., 1999). Our results corroborate the inhibitory action of RU-486 on FSH but not LH secretion. This different response in the gonadotropins release has been reported and discussed in the literature (Marubayashi et al., 1999; McCann et al., 1983).

The increase of plasma E_2 induced by RU-486 also was found in humans (Weisberg et al., 2011). However, the mechanism by which these effects occur remains unknown.

RU-486 did not alter plasma P_4 as reported in literature (to review see (Levine et al., 2001)). A recent study has shown an increase of plasma P_4 during proestrus, after a single injection of RU-486 at 12:00 am (Lopez-Fontana et al., 2011). However, the dose of RU-486 was twice the amount we used in our study.

4. Conclusions

In conclusion, our results showed that TMX, but not RU-486 inhibited the nitrergic system in the MPOA. They indicate that E_2 can stimulate the nitrergic system in the MPOA, direct or indirectly, through an increase of nNOS mRNA and NOS activity to regulate the secretion of the GnRH, gonadotropins and ovarian steroids.

5. Experimental procedure

5.1. *Animals*

Thirty two female Wistar rats (180–200 g) were housed in groups of four animals in controlled environment at 22±2 °C under 12 h light–dark cycle (turn on 6 pm), and food and water provided *ad libitum*. Vaginal smears were carried out daily to determine estrous cycle phases. Only females exhibiting at least three consecutive regular cycles were used. Metestrus on a four days regular estrous cycle was arbitrarily defined as day 1 of experiment. All protocols were approved by the Ethics Committee of the Medical School of Ribeirão Preto, University of São Paulo, and they are in agreement with NIH's Guide for Care and Use of Laboratory Animal (USA).

5.2. *Effect of TMX and RU-486 on mRNA nNOS expression and nNOS protein expression/activity in the MPOA*

Tamoxifen (3 mg/0.2 mL of corn oil/animal, s.c.; Sigma Chemical Co., St. Louis, MO) or corn oil (0.2 mL for control) was administered on the first (metestrus) and second (diestrus) days of the estrous cycle at 9 am, accordingly with previous study (Sanchez-Criado et al., 2002). RU-486 (2 mg/0.2 mL corn oil/animal s.c.; Sigma Chemical Co., St. Louis, MO) or corn oil

(0.2 mL for control) was administered on first and second days of experiment (metestrus and diestrus in control animals, respectively) at 8 am and 5 pm and in the morning of the third day (proestrus in control animals) at 8 am, accordingly with modified protocol (Tebar et al. 1994). All rats were decapitated on the third day (proestrus in controls) at 5 pm. Brains were removed and frozen at −70 °C. Trunk blood was collected for plasma LH, FSH, E_2 and P_4 measurement by radioimmunoassay. It is noteworthy that rats treated with TMX or RU-486 under the same protocol followed in the present study, present vaginal smears typically seen in estrus (Sanchez-Criado et al., 2002).

Brains were fixed in a cryostat to obtain a coronal slice of 1 mm from the MPOA region, starting at 0.26 mm posterior to bregma, according to the anatomical atlas (Swanson, 1992). MPOA was microdissected from this slice with a 2.0 mm-needle, using the previously described technique (Palkovits, 1973).

5.3. *nNOS RNA expression by real time-PCR*

mRNA expression of nNOS was analysed by quantitative real time RT-PCR. Total RNA was extracted using SV Total RNA Isolation System (Promega, USA), and treated on-column with DNase I to prevent genomic DNA contamination. About 1 μg of total RNA was reversely transcribed using an oligodT primer and Termoscript Reverse Transcriptase (Invitrogen, USA). Reverse-transcribed cDNA samples were used as templates for PCR amplification using SYBR Green Master Mix UDG-ROX® (Invitrogen, USA) and 7500 Real Time PCR System (Applied Biosystems). Primers forward (5'-AAGAGGGTCA-AGGCGACCATTC-3') and reverse (5′-CGAACACTGAGGAA-CCTCACATTGG-3') were used for amplification, designed from sequence deposited on GenBank under access number NM052799. As endogenous control were used specific primers for rat GAPDH (forward 5'-TGGAGTCTACTGGCGTCTTC-3' and reverse 5'-GCAGGATGCATTGCTGAC-3') (GenBank, number NM017008). Normalized gene expression using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) of nNOS was calculated and standardized to relative quantities of rat GAPDH using Applied biosystems 7500 System software with normalized expression calculations implemented according to the manufacturer's protocol. Negative controls were performed using all PCR components except cDNA, to evaluate the exogenous DNA contamination. After each qPCR assay we performed analysis of the transcript amplification through dissociation and amplification curves as well as the transcript amplification on agarose 1.5% gels. The efficiency for each primer set was evaluated and recorded during assay development by applied biosystems application (1 μg of cloned plasmids was diluted to 1 × -,10 × -,100 × - and 1000 × -fold; see protocol of Applied biosystems application), and Cts varied by two units. To certify the identity of the nNOS amplicon, nNOS cDNA was cloned and sequence analysis was carried out and matched the sequence of nNOS from *Rattus norvegicus* which was used for the primer design (Blastn # NM-052799).

5.4. *nNOS protein expression by western blotting*

nNOS content was studied by western blotting analysis as described previously (Laemmli, 1970). Total protein content isolated from adult cycling rats was determined by previously described method (Bradford, 1976). The same amount of total protein (30 μg) was used from each animal. Samples were separated; water and the radioactivity were measured by liquid scintillation counting. In the remaining pellets, protein content was also determined by previously described method (Bradford, 1976). Enzyme activity was reported as pmoles of [^{14}C]-L-citrulline/15 min/mg of protein using 10% SDS-poliacrylamide gel electrophoresis (SDS-PAGE). Molecular weight standards (Amersham Biosciences, UK) were loaded on gels to verify stained proteins. Proteins were electroblotted onto nitrocellulose membranes (Millipore, BioRad, Hercules, USA). Monoclonal antibodies for nNOS (sc-5302, Santa Cruz Biothecnology. Inc., USA-1:10,000) and for β-tubulin (T4026, Sigma-Aldrich-1:5000) were used to detect nNOS proteins and for constitutive control, respectively. HRP-conjugated anti-mouse antibody (1:5000; Dako Cytomation, USA) was used for both nNOS and β-tubulin primary antibodies. Blots were exposed to the film (Kodak, USA) and nNOS protein expression was determined by multiplying the optical density for each band by its areas (Image J analyses program-NIH, USA). Loading consistency was also confirmed by Comassie Blue staining.

5.5. *NOS activity*

Nitric oxide and L-citrulline are produced in equimolar amounts. NOS enzyme activity was quantified by measuring the conversion from [^{14}C]-L-arginine into [^{14}C]-L-citrulline (Bredt and Snyder, 1989). Microdissected MPOA was homogenized (Micro Ultrasonic Cell Disrupter Kontes) in HEPES buffer (20 mM HEPES, 50 mM L-valine, 0.45 mM $CaCl_2$, 100 mM dithiothreitol) containing approximately 1 μCi/ml [^{14}C]-L-arginine and 1mM NADPH. After 15 min of incubation, samples were centrifuged for 10 min at 10,000*g*. They were then applied in duplicates to 1 mL DOWEX AG500-X8 column (Na^+ form). The elution of the [^{14}C]-L-citrulline was made in 2.5 mL of distilled water.

5.6. *Radioimmunoassay*

Plasma LH and FSH concentrations were determined by double antibody radioimmunoassay (RIA) using specific standards and antibodies from the National Program of Pituitary Hormones (Harbor, USA). All samples were measured in the same assay. The nonspecific antibody, anti-rabbit gamma-globulin, was produced in goat by our laboratory. Lowest detectable doses were 0.05 ng/ml for the LH-RP_3 standard and 0.2 ng/ml for the FSH-RP_2 standard. Intra-assay coefficients of variations were 4% for LH and 3% for FSH. Plasma E_2 and P_4 were determined by double antibody RIA using commercial kits (Biochem Immunosystems, Serotec, Italy). Lower limits of detection for E_2 and P_4 were 7.5 pg/ml and 4.1 ng/ml, respectively. Intra-assay coefficients of variation were 2.5% for E_2 and 3.5% for P_4.

5.7. *Statistical analysis*

Statistical analysis of RT-PCR data was performed using Statistical Package for the Social Science (SPSS, Inc., Chicago,

IL, USA-version 6.0). Levene's test was used for testing variance homogeneity. When samples were not homogeneous, it was applied logarithmical transformation to become homogenous samples. All results are showed as mean+S.E.M. Results from western blot were analysed by Student's *t*-test. For other results, statistical differences were determined by One Way Anova, followed by a pos hoc Dunnett's Multiple comparison test. Differences were considered significant for $p<0.05$.

Acknowledgments

The authors are grateful to FAPESP (Grant nos. 09/54887-0 and 04/09638-9), and CNPq (Grant nos. 473234/2007-6; 303195/2007-0) from Brazil for financial support. We wish to thank Sonia Aparecida Zanon for technical assistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.brainres.2014.07.003.

REFERENCES

Barnes, M.J., Lapanowski, K., Rafols, J.A., Lawson, D.M., Dunbar, J.C., 2001. GnRH and gonadotropin release is decreased in chronic nitric oxide deficiency. Exp. Biol. Med. (Maywood) 226, 701–706.

Barnes, M.J., Lapanowski, K., Rafols, J.A., Lawson, D.M., Dunbar, J.C., 2002. Chronic nitric oxide deficiency is associated with altered leutinizing hormone and follicle-stimulating hormone release in ovariectomized rats. Exp. Biol. Med. (Maywood) 227, 817–822.

Bellido, C., Gonzalez, D., Aguilar, R., Sanchez-Criado, J.E., 1999. Antiprogestins RU486 and ZK299 suppress basal and LHRH-stimulated FSH and LH secretion at pituitary level in the rat in an oestrous cycle stage-dependent manner. J. Endocrinol. 163, 79–85.

Bellido, C., Martín de las Mulas, J., Tena-Sempere, M., Aguilar, R., Alonso, R., Sánchez-Criado, J.E., 2003. Tamoxifen induces gonadotropin-releasing hormone self-priming through an estrogen-dependent progesterone receptor expression in the gonadotrope of the rat. Neuroendocrinology 77, 425–435.

Bhat, G., Mahesh, V.B., Aguan, K., Brann, D.W., 1996. Evidence that brain nitric oxide synthase is the major nitric oxide synthase isoform in the hypothalamus of the adult female rat and that nitric oxide potently regulates hypothalamic cGMP levels. Neuroendocrinology 64, 93–102.

Bonavera, J.J., Kalra, P.S., Kalra, S.P., 1996. L-Arginine nitric oxide amplifies the magnitude and duration of the luteinizing hormone surge induced by estrogen: Involvement of neuropeptide Y. Endocrinology 137, 1956–1962.

Bonavera, J.J., Sahu, A., Kalra, P.S., Kalra, S.P., 1993. Evidence that nitric oxide may mediate the ovarian steroid-induced luteinizing hormone surge: involvement of excitatory amino acids. Endocrinology 133, 2481–2487.

Bonavera, J.J., Sahu, A., Kalra, P.S., Kalra, S.P., 1994. Evidence in support of nitric oxide (NO) involvement in the cyclic release of prolactin and LH surges. Brain Res. 660, 175–179.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Brann, D.W., Putnam, C.D., Mahesh, V.B., 1991. Validation of the mechanisms proposed for the stimulatory and inhibitory effects of progesterone on gonadotropin secretion in the estrogen-primed rat: a possible role for adrenal steroids. Steroids 56, 103–111.

Bredt, D.S., Glatt, C.E., Hwang, P.M., Fotuhi, M., Dawson, T.M., Snyder, S.H., 1991. Nitric oxide synthase protein and mRNA are discretely localized in neuronal populations of the mammalian CNS together with NADPH diaphorase. Neuron 7, 615–624.

Bredt, D.S., Snyder, S.H., 1989. Nitric oxide mediates glutamate-linked enhancement of cGMP levels in the cerebellum. Proc. Natl. Acad. Sci. USA 86, 9030–9033.

Donath, J., Nishino, Y., 1998. Effects of partial versus pure antiestrogens on ovulation and the pituitary-ovarian axis in the rat. J. Steroid Biochem. Mol. Biol. 66, 247–254.

Garrido-Gracia, J.C., Gordon, A., Bellido, C., Aguilar, R., Barranco, I., Millan, Y., de Las Mulas, J.M., Sanchez-Criado, J.E., 2007. The integrated action of oestrogen receptor isoforms and sites with progesterone receptor in the gonadotrope modulates LH secretion: evidence from tamoxifen-treated ovariectomized rats. J. Endocrinol. 193, 107–119.

Gingerich, S., Krukoff, T.L., 2005. Estrogen modulates endothelial and neuronal nitric oxide synthase expression via an estrogen receptor beta-dependent mechanism in hypothalamic slice cultures. Endocrinology 146, 2933–2941.

Grohe, C., Kann, S., Fink, L., Djoufack, P.C., Paehr, M., Van, E.M., Vetter, H., Meyer, R., Fink, K.B., 2004. 17 Beta-estradiol regulates nNOS and eNOS activity in the hippocampus. Neuroreport 15, 89–93.

Hanchate, N.K., Parkash, J., Bellefontaine, N., Mazur, D., Colledge, W.H., d'Anglemont, T., Prevot, V., 2012. Kisspeptin-GPR54 signaling in mouse NO-synthesizing neurons participates in the hypothalamic control of ovulation. J. Neurosci. 32, 932–945.

Herbison, A.E., Pape, J.R., 2001. New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Front. Neuroendocrinol. 22, 292–308.

Huber, A., Saur, D., Kurjak, M., Schusdziarra, V., Allescher, H.D., 1998. Characterization and splice variants of neuronal nitric oxide synthase in rat small intestine. Am. J. Physiol. 275, G1146–G1156.

Jennes, L., Conn, P.M., 1994. Gonadotropin-releasing hormone and its receptors in rat brain. Front. Neuroendocrinol. 15, 51–77.

Kim, Y.M., Lee, J.Y., Choi, S.H., Kim, D.G., Jahng, J.W., 2004. RU486 blocks fasting-induced decrease of neuronal nitric oxide synthase in the rat paraventricular nucleus. Brain Res. 1018, 221–226.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Lamar, C.A., Bhat, G.K., Mahesh, V.B., Brann, D.W., 1999. Evidence that neuronal nitric oxide synthase but not heme oxygenase increases in the hypothalamus on proestrus afternoon. Neuroendocrinology 70, 360–367.

Levine, J.E., Chappell, P.E., Schneider, J.S., Sleiter, N.C., Szabo, M., 2001. Progesterone receptors as neuroendocrine integrators. Front. Neuroendocrinol. 22, 69–106.

Lima, F.B., Szawka, R.E., Anselmo-Franci, J., Franci, C.R., 2007. Pargyline effect on luteinizing hormone secretion throughout the rat estrous cycle: correlation with serotonin, catecholamines and nitric oxide in the medial preoptic area. Brain Res. 1142, 37–45.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2 ($^{-\Delta\Delta Ct}$). Methods 25, 402–408.

Lopez-Fontana, C.M., Maselli, M.E., de Di Nasso, F.E., Telleria, C.M., Caron, R.W, 2011. Regulation of prolactin secretion during the estrus in rats: possible role of glucocorticoids. Reproduction 142, 477–485.
Marubayashi, U., Yu, W.H., McCann, S.M., 1999. Median eminence lesions reveal separate hypothalamic control of pulsatile follicle-stimulating hormone and luteinizing hormone release. Proc. Soc. Exp. Biol. Med. 220, 139–146.
McCann, S.M., Mizunuma, H., Samson, W.K., Lumpkin, M.D., 1983. Differential hypothalamic control of FSH secretion: a review. Psychoneuroendocrinology 8, 299–308.
McCann, S.M., Kimura, M., Walczewska, A., Karanth, S., Rettori, V., Yu, W.H., 1998. Hypothalamic control of gonadotropin secretion by LHRH, FSHRF, NO, citokines and leptin. Domest. Anim. Endocrinol. 15 (5), 333–344.
McDonnell, D.P., 1999. The molecular pharmacology of SERMs. Trends Endocrinol. Metab. 10, 301–311.
McDonnell, D.P., 2003. Mining the complexities of the estrogen signaling pathways for novel therapeutics. Endocrinology 144, 4237–4240.
McDonnell, D.P., Connor, C.E., Wijayaratne, A., Chang, C.Y., Norris, J.D., 2002. Definition of the molecular and cellular mechanisms underlying the tissue-selective agonist/antagonist activities of selective estrogen receptor modulators. Recent Prog. Horm. Res. 57, 295–316.
Moenter, S.M., Chu, Z., 2012. Rapid nongenomic effects of oestradiol on gonadotrophin-releasing hormone neurones. J. Neuroendocrinol. 24, 117–121.
Moretto, M., Lopez, F.J., Negro-Vilar, A., 1993. Nitric oxide regulates luteinizing hormone-releasing hormone secretion. Endocrinology 133, 2399–2402.
Nishino, T., Yamanouchi, H., Ishibashi, K., Hirtreiter, C., Nishino, Y., 2009. Antiovulatory effect of a single injection of pure antiestrogen ZK 191703 at early stage of rat estrus cycle. J. Steroid Biochem. Mol. Biol. 114, 152–160.
Ortmann, O., Emons, G., Knuppen, R., Catt, K.J., 1989. Inhibitory effects of the antiprogestin, RU 486, on progesterone actions and luteinizing hormone secretion in pituitary gonadotrophs. J. Steroid Biochem. 32, 291–297.
Palkovits, M., 1973. Isolated removal of hypothalamic or other brain nuclei of the rat. Brain Res. 59, 449–450.
Parkash, J., d'Anglemont de Tassigny, X., Bellefontaine, N., Campagne, C., Mazure, D., Buee-Scherrer, V., Prevot, V., 2010. Phosphorylation of N-methyl-D-aspartic acid receptor-associated neuronal nitric oxide synthase depends on estrogens and modulates hypothalamic nitric oxide production during the ovarian cycle. Endocrinology 151, 2723–2735.
Pinilla, L., Tena-Sempere, M., Gonzalez, D., Aguilar, E., 1999. The role of nitric oxide in the control of basal and LHRH-stimulated LH secretion. J. Endocrinol. Investig. 22, 340–348.
Pu, S., Kalra, P.S., Kalra, S.P., 1998. Ovarian steroid-independent diurnal rhythm in cyclic GMP/nitric oxide efflux in the medial preoptic area: possible role in preovulatory and ovarian steroid-induced LH surge. J. Neuroendocrinol. 10, 617–625.
Qian, X., Jin, L., Lloyd, R.V, 1999. Estrogen downregulates neuronal nitric oxide synthase in rat anterior pituitary cells and GH3 tumors. Endocrine 11, 123–130.
Radovick, S., Levine, J.E., Wolfe, A., 2012. Estrogenic regulation of the GnRH neuron. Front. Endocrinol. (Lausanne) 3, 52.
Rao, I.M., Mahesh, V.B., 1986. Role of progesterone in the modulation of the preovulatory surge of gonadotropins and ovulation in the pregnant mare's serum gonadotropin-primed immature rat and the adult rat. Biol. Reprod. 35, 1154–1161.
Renodon, A., Boucher, J.L., Sari, M.A., Delaforge, M., Ouazzani, J., Mansuy, D., 1997. Strong inhibition of neuronal nitric oxide synthase by the calmodulin antagonist and anti-estrogen drug tamoxifen. Biochem. Pharmacol. 54, 1109–1114.
Rissman, E.F., Wersinger, S.R., Taylor, J.A., Lubahn, D.B., 1997. Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects. Horm. Behav. 31, 232–243.
Sakurai, K., Matsuo, S., Enomoto, K., Amano, S., Shiono, M., 2011. Menstruation recovery after chemotherapy and luteinizing hormone-releasing hormone agonist plus tamoxifen therapy for premenopausal patients with breast cancer. Surg. Today 41, 48–53.
Sanchez-Criado, J.E., Bellido, C., Aguilar, R., Garrido-Gracia, J.C., 2005. A paradoxical inhibitory effect of oestradiol-17beta on GnRH self-priming in pituitaries from tamoxifen-treated rats. J. Endocrinol. 186, 43–49.
Sanchez-Criado, J.E., Guelmes, P., Bellido, C., Gonzalez, M., Hernandez, G., Aguilar, R., Garrido-Gracia, J.C., Bello, A.R., Alonso, R., 2002. Tamoxifen but not other selective estrogen receptor modulators antagonizes estrogen actions on luteinizing hormone secretion while inducing gonadotropin-releasing hormone self-priming in the rat. Neuroendocrinology 76, 203–213.
Sanchez-Criado, J.E., Hernandez, G., Bellido, C., Gonzalez, D., Tebar, M., Diaz-Cruz, M.A., Alonso, R., 1994. Periovulatory LHRH, LH and FSH secretion in cyclic rats treated with RU486: effects of exogenous LHRH and LHRH antagonist on LH and FSH secretion at early oestrus. J. Endocrinol. 141, 7–14.
Southam, E., Garthwaite, J., 1993. The nitric oxide-cyclic GMP signaling pathway in rat brain 32, 1267–1277Neuropharmacology 32, 1267–1277.
Swanson, L.W., 1992. In: Brain Maps: Structure of the Rat Brain. Elsevier, Amsterdam.
Tebar, M., Bellido, C., Aguilar, R., Sanchez-Criado, J.E., 1994. Inappropriate ovarian feedback in basal gonadotropin secretion in 4-day cyclic rat treated with mifepristone: role of endogenous estradiol. J. Endocrinol. Investig. 17, 425–430.
Weisberg, E., Croxatto, H.B., Findlay, J.K., Burger, H.G., Fraser, I.S., 2011. A randomized study of the effect of mifepristone alone or in conjunction with ethinyl estradiol on ovarian function in women using the etonogestrel-releasing subdermal implant, Implanon(R). Contraception 84, 600–608.
Witkin, J.W., 1999. Synchronized neuronal networks: the GnRH system. Microsc. Res. Technol. 44, 11–18.