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Research article

Effects of chronic tamoxifen treatment in female rat sexual behaviour

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ABSTRACT

The medial preoptic (MPN) and the ventromedial hypothalamic nuclei (VMN) modulate the estrogen receptor (ER)-dependent female sexual behavior, a response that is inhibited by tamoxifen (TAM), a modulator of the steroid receptor activation. With the objective to assess TAM action in the brain areas involved in the modulation sexual cues, an animal model on long-term TAM therapy to intact female rats, was used to mimic the 5-year prophylactic TAM therapy offered to women at higher risk of breast cancer. After three months treatment, female sexual behavior with a stud male rat was evaluated. Upon sacrifice, the brains were removed and the MPN and the ventrolateral division of the VMN were screened for the effects of TAM in the expression of ERα, ERβ and progesterone receptor. Results show that TAM inhibited the receptive component of the female sexual behavior. Even though TAM decreased estrogen and progesterone levels to values similar to the ones of estrous and diestrus rats, the biochemical data failed to demonstrate such possible causation for the behavioral response. In fact, TAM administration induced a constant low level of ovarian hormones that changed the pattern of ER and PR expression as well as receptor co-expression in the brain areas regulating the behavioral response, dissimilar to the ones seen in the cycle phases with the same low hormone levels. Nevertheless, present data suggests that by affecting ER- and/or PR-dependent mechanisms, TAM may modulate the hypothalamus, a region known to participate in several social behaviors.

1. Introduction

Breast Cancer (BC) is the most diagnosed form of cancer in women and is responsible for a high rate of morbidity and death. Changes in morbidity and mortality rates rely on various factors: treatment effectiveness, therapeutic compliance, and overall quality of life. Similarly, the determination of the histological and molecular sub-type of each BC is an important prognostic factor, which depends mostly on the expression of hormonal receptors by the cancer cells – the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor-type 2 (HER2) (Saha et al., 2019; Weigelt et al., 2010) – which makes this very heterogeneous disease dependent on hormones. In fact, about 75% of the BC types are ER-positive and this is the most common type of cancer in post-menopausal women (Saha et al., 2019; Weigelt et al., 2010).

There are two types of ERs, ERα and ERβ, which are ligand-activated nuclear factors that mediate estradiol-induced gene transcription (Björnström and Sjöberg, 2005; Hammes and Levin, 2007), and normally coexist in the same tissues. Studies in peripheral organs, such as the breast and uterus, and also in the central nervous system, has shown that both receptors interact with each other's expression and/or activation (Hsu et al., 2019; Martins et al., 2015; Sá et al., 2015; Saha et al., 2019). Because of this receptor interaction, ER-positive breast cancer patients may benefit from treatments with ERα antagonists or with ERβ agonists, depending on the stage of the BC and the expression of hormonal receptors by the cancer cells (Chang et al., 2006; Paterni et al., 2014).

For decades, Tamoxifen (TAM) – a modulator of the steroid receptor activation – has been used as an anti-estrogen in the treatment of ER-positive BC and seems to have to improve the clinical outcomes of this disease, increasing life expectancy of BC patients (Komm and Mirkin, 2014; Ribnikar et al., 2017; Yang et al., 2013). Previous studies have shown that TAM therapy stimulated the emergence of mood disorders (such as anxiety, depression and decreased libido in woman) and that it inhibits female rodent sexual behavior (Komm and Mirkin, 2014; Ribnikar et al., 2017; Yang et al., 2013). Nowadays, a 5-year prophylactic TAM therapy is offered to woman with higher risk of being diagnosed

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with BC (Davies et al., 2013; Li et al., 2016). It highlights the impact evaluation of this endocrine therapy on the neuronal systems involved in the regulation of mood and anxiety as the mechanisms underlying TAM action are currently unknown and their impact in quality of life is evident.

The medial preoptic (MPN) and the ventromedial hypothalamic nuclei (VMN) belong to the limbic system, which is responsible for the modulation of mood and anxiety. These nuclei are also known to be involved in the control of the proceptive and the receptive components of the female sexual behavior (Heijkoop et al., 2018; Madeira and Lieberman, 1995; Pfaus et al., 2015; Snoeren et al., 2015). In the female rat, the MPN, the largest cell group of the medial preoptic area, plays a main role in the regulation of proceptive as well as receptive sexual behaviors (Madeira and Lieberman, 1995; Micevych and Meisel, 2017; Morishita et al., 2021). Both the MPN and VMN have several important connections with limbic and prelimbic areas, which allow the integration of olfactory and sensitive stimulus, and, through projections with each other, regulate the behavioral response (Micevych and Meisel, 2017; Morishita et al., 2021; Pereira et al., 2021; Robarts and Baum, 2007). The female sexual behavior is dependent on the activation of ERα and PR (Heijkoop et al., 2018; Madeira and Lieberman, 1995; Pfaus et al., 2015) by estradiol and progesterone in the MPN and in the ventrolateral division of the VMN (VMNvl; Etgen and Barfield, 1986; Rubin and Barfield, 1980). Then, it will trigger the expression of neurochemical systems components that coordinate the sexual behavioral, promoting the reproductive success (Inouye and Kawamura, 1979; Liu et al., 2017; Pfaus et al., 2015).

Previous studies have shown that, opposing to rodents, sexuality in primates (including humans) is not controlled by the action of ovarian hormone levels (Baum et al., 1976, 1977), which makes the rodent models of sexual behavior not translatable to the biology of human sexuality. It is well acknowledged that psychological and social influences are paramount in human sexuality. However, the knowledge of the way a slow acting stimulus (either visceral, endocrine, or even social) promotes a neuronal output (either a thought or a behavioral action) is fundamental for the understanding of some neuronal basis of drive and motivation, which are important modulators of human social and emotional life. The present animal model of sexual behavior is of little use for the study of human sexuality, but is a good way to study several neuronal mechanisms that will act between a stimulus and a behavioral response. The main objective of this study is to evaluate the effects of long-term TAM therapy in the brain areas involved in the modulation of drive and motivation, which are neuronal programs determinant in the modulation of psychological and social cues. To achieve this goal, we have studied the effects of long-term TAM therapy in the expression of ERα and PR in the MPN and the VMNvl and associated it with behavioral outcomes.

2. Material and methods

2.1. Animals and experimental design

Young adult female Wistar rats ($N = 42$), from the animal facility of I3S (Porto, Portugal) were kept under standard laboratory conditions: l2 h light/dark cycle (lights on at 7:00 am), room temperature of 23 °C and free access to standard solid diet and water. All experiments obeyed the European Communities Council Directive 2010/63/EU, the Portuguese Act 113/13, and the ethical requirements of the review board (ORBEA) of the Faculty of Medicine, University of Porto. The clinical therapeutic settings of TAM in the prevention of recurrence of breast cancer in young fertile woman does not include the removal of the ovaries. Because in the present study we intend to mimic these settings and because ovariectomy followed by hormone supplementation is not a good comparison for females with ovaries, in the present study normally cycling young female rats (10 weeks old) were used, and allocated to one of two groups: Control and TAM treated. The rats in the TAM treated group ($N = 18$) were free fed daily, for three months, with a pellet of 150 mg hazelnut chocolate containing a solution of TAM (5 mg/kg/day) in 0.5% hydroxypropylmethylcellulose (HMC). Daily TAM dose (volume) for each animal was calculated according to each animal's weight so that the same concentration of TAM per kg was administered to the rats. The present TAM dose was proven to be the human equivalent dose known to mimic the therapeutic dose (10 mg TAM), and to present, in the rat, the equivalent serum concentration of TAM seen in humans taking the 10 mg TAM dose (Greaves et al., 1993; Jordan and Brodie, 2007; Moon et al., 1991; Resende et al., 2019). Control rats ($N = 24$) were free-fed a similar pellet of chocolate with HMC daily, for three months. The animal's health status and the estrous cyclicity were monitored daily, in order to ascertain the continuous effect of TAM and the normal physiological hormonal levels, and to evaluate any irregular cycles or some other form of cycle error. The animals body weight was determined every two days; the water and food consumption were determined weekly.

In a pilot experiment, we have seen that TAM administration abolished the estrous cycle and hindered estradiol and progesterone surges; however, in a previous study (Sá et al., 2018) we have seen that an acute administration of 10 mg/kg of TAM to ovariectomized rats induced neuronal atrophy and reduced synaptic connectivity, but increased the total number of VMHvl neurons expressing ERα. In addition, the administration of estradiol benzoate with TAM in that same study, prevented the morphological and physiological damage in the connectivity pattern of the VMHvl. This fact led to the suggestion that the action of TAM could be only peripheral in nature, meaning that the neuronal machinery needed for the promotion of the female sexual behavior could be intact. If that was the case, the exogenous administration of ovarian hormones would prevent the peripheral effect. In order to ascertain this assumption, for the behavioral studies, TAM-treated rats were subjected to exogenous administration of ovarian hormones. So, one set of TAM-treated rats ($N = 6$) was injected with estradiol benzoate (10 μg/rat) followed, 48 h later, by progesterone (500 μg/rat) in doses and schedule known to induce the female sexual behavior (Pfaus et al., 2015; Sá et al., 2009) and tested 4 h after the progesterone injection – TAM (EB + P) group. Because TAM can act as an estrogen agonist in some systems, another set of TAM-treated rats ($N = 6$) was tested 4 h after the administration of only progesterone in the same dose – TAM (P) group.

2.2. Behavioral testing

To access the action of TAM in female sexual receptivity, behavioral testing was done using a multi-level arena connected by a small diameter passage (approx. 5 cm) that allow only the female rats to reach the second level. During the acclimatization period, the females were placed alone in the arena and allowed to explore for 10 min. Behavioral testing was done during the first third of the dark period (between 9:00 and 11:00 pm), under a red dim light, after 3 months of TAM consumption.

The tested female was placed in the arena and allowed to acclimate for 2 min; then a stud male previously shown to always display copulation behavior (more than 10 mounts in 10 min), was added to the arena. The animals were allowed to interact for 12 min and the behavior was recorded by a video camera. The 12 min window was determined in the pilot study to be enough for a stud male to perform more than 10 mounts (a standard number of mounts analyzed in the literature) and to have a 2 min window for any event with the video camera that could reduce the 10 min behavioral analyses. At the end of the behavioral testing, the male was removed first, and the female followed. The females of the Control group were tested once, when they were at a determined phase of the estrous cycle, so that, at the end of the behavioral testing, six females in each of the four phases of the estrous cycle were tested. TAM treated rats of all three settings (only TAM, TAM with added estradiol benzoate and progesterone, TAM with added progesterone), six females per group, were also tested once.

Video analyses was done by an investigator blind to the assigned groups and the following parameters were determined: number of male mounts, lordosis number and intensity, number of exits and latency to

Table 1. Primary and secondary antibodies used.

Antibody	Antibody ID (RRID)	Host	Catalog number	Dilution
Primary antibodies				
ERα (MC-20)	AB_631470	Rabbit polyclonal	sc-542	1:1000
PR (clone 6A)	AB_2164323	Mouse monoclonal	MAB462	1:2000
PR	AB_2315192	Rabbit polyclonal	A0098	1:1500
ERB (1531)	AB_629463	Mouse monoclonal	sc-53494	1:500
Secondary antibodies				
Anti-rabbit IGG	AB_2313606	Goat	BA-1000	1:400
Goat anti-mouse Igg (H + L) Alexa Fluor 488 Conjugate	AB_138404	Goat	A-11029	1:1000
Anti-rabbit IGG (H + L) Alexa Fluor 568 Conjugate	AB_143157	Goat	A-11011	1:1000

return. The lordosis quotient (ratio of lordosis/number of mounts × 100) and the lordosis intensity (the mean of all lordosis intensities in a 4-point scale) were used as measures of receptivity. The number of mounts assessed the sexual attractiveness of the females and of exits and the latency to return measured proceptivity. Video analysis was terminated after 10 mounts or after 10 min of behavior, depending on what happened first.

2.3. Hormonal determinations

Blood samples of 2000 μl were taken directly from the heart prior to perfusion. The samples were allowed time for complete clot formation and then were centrifuged at 2000 rpm for 10 min; the supernatant serum was collected and centrifuged again in the same settings. After the second centrifugation, the supernatant serum was collected in aliquots and stored at −80 °C. Estradiol and progesterone serum levels were measured by enzyme-linked fluorescent assay using VIDAS Progesterone and Estradiol II Kits on the miniVidas analyzer (BioMerieux S.A., Marcy L'Etoile, France) in a single run. 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.

2.4. Immunohistochemical determination of ERα and PR expression

At the end of the treatment period and behavioral testing (approx. 100 days), the animals were sacrificed by perfusion with 4% PFA in phosphate buffer, under deep anesthesia with sevoflurane. The brains were removed from the skulls and post-fixed for 1 h in the same fixative solution at 4 °C and then placed in a 10% sucrose solution in phosphate buffer, at 4 °C, overnight. Dissection of the brains was performed by executing a transection in the coronal plane, at the anterior border of the optic chiasm, rostrally, and at the posterior limit of the mammillary bodies, caudally. Then, the hemispheres were separated and both blocks of tissue were mounted on a Vibratome with the caudal surface down and sectioned at 40 μm through the preoptic area and hypothalamus. The sections of both hemispheres were collected in twelve sequential sets and stored for further processing. In a previous study we have shown that estradiol does not change ERβ levels in the VMNvl; in addition, present antibodies commercially available for ERβ do not stain the nucleus. In this way, changes in ERβ expression were only determined using immunofluorescence techniques.

Two sets of sections containing the MPN and the VMN, sampled at 120 μm intervals (sampling fraction of 1:3), were processed for the immunohistochemical detection of ERα-positive or PR-positive neurons, as detailed previously (Martins et al., 2015; Sá and Fonseca, 2017; Sá et al., 2018). Then, sections were pre-treated with 3% H_2O_2 to inactivate endogenous peroxidase and blocked with 10% normal goat serum. The sections were then incubated with rabbit polyclonal ERα antibody (MC-20) or PR antibody (A0098; Table 1), for 72 h at 4 °C. After that time, the sections were incubated with biotinylated goat anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA, USA, BA-1000, Table 1), with avidin–biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories) and in diaminobenzidine (Sigma–Aldrich) to which H_2O_2 was added. The sections were mounted on gelatin-coated slides, air-dried, dehydrated in a series of ethanol solutions, cleared in xylol, and cover-slipped using Histomount (National Diagnostics, Atlanta, GA, USA).

The total number of MPN and VMNvl neurons expressing ERα-ir and PR-ir neurons was estimated using the optical fractionator method, in all sections containing the MPN and the VMNvl, comprising an average of 6 sections for MPN and 8 sections for VMNvl (Madeira et al., 1997; West et al., 1991). Using the C.A.S.T. – Grid system software (Olympus), the boundaries of the MPN or the VMNvl were determined (Figure 1) and the fields of view were sampled at regular intervals along the x and y axes. Details of the stereological analyses are provided in Table 2. Estimates were performed at a final magnification of 2000× in the computer screen using a 100× oil immersion lens with a numerical aperture of 1.40. Representative photomicrographs present in Figure 1 were taken using an 5x lens at a final magnification of 350×.

2.5. Immunofluorescence detection and hormone receptor Co-localization in the MPN and VMNvl

Three sets of sections containing the MPN and the VMN, sampled at 480 μm intervals (sampling fraction of 1:6) were processed for co-localization of ERα/PR-ir, ERα/ERβ-ir and ERβ/PR-ir neurons using immunofluorescence, as detailed previously (Sá and Fonseca, 2017).

Briefly, sections were blocked using a solution of 5% normal horse serum and subsequently incubated for 72 h at 4 °C with a solution containing the three combinations of primary antibodies: ERα antibody; PR antibody and ERβ antibody (Table 1). Afterwards, the sections were incubated in the dark, for 2 h at room temperature, with a solution containing the secondary Alexa Fluor-conjugated antibodies (Table 1, Life Technologies Europe BV). Finally, the sections were cover-slipped with FluorSave to which 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, D1306, Life Technologies Europe BV), at a 1:100 dilution, was added. Sections of animals of all groups were processed together in this immunofluorescence procedure in order to avoid bias and prevent variation across groups. During the procedure, one set containing one animal of each group was damaged; in this way, only 4 animals per group were analyzed in this topic.

Immunofluorescence was detected using a Zeiss microscope (Imager.21) and the AxioVision 40 v software. For each rat, all sections containing the MPN and the VMNvl were sampled using a 40× lens (approx. 3 sections containing the MPN and 4 sections containing the VMNvl), at a final magnification of 320×. Immunolabeling images of all proteins were captured in the same optic plan of the region of interest with a mean total area of 242,240 μm^2 in the MPN and 275,440 μm^2 in the VMNvl per rat, using the three light channels, Alexa 488, Alexa 568 and DAPI. Cell counts were performed using ImageJ software after

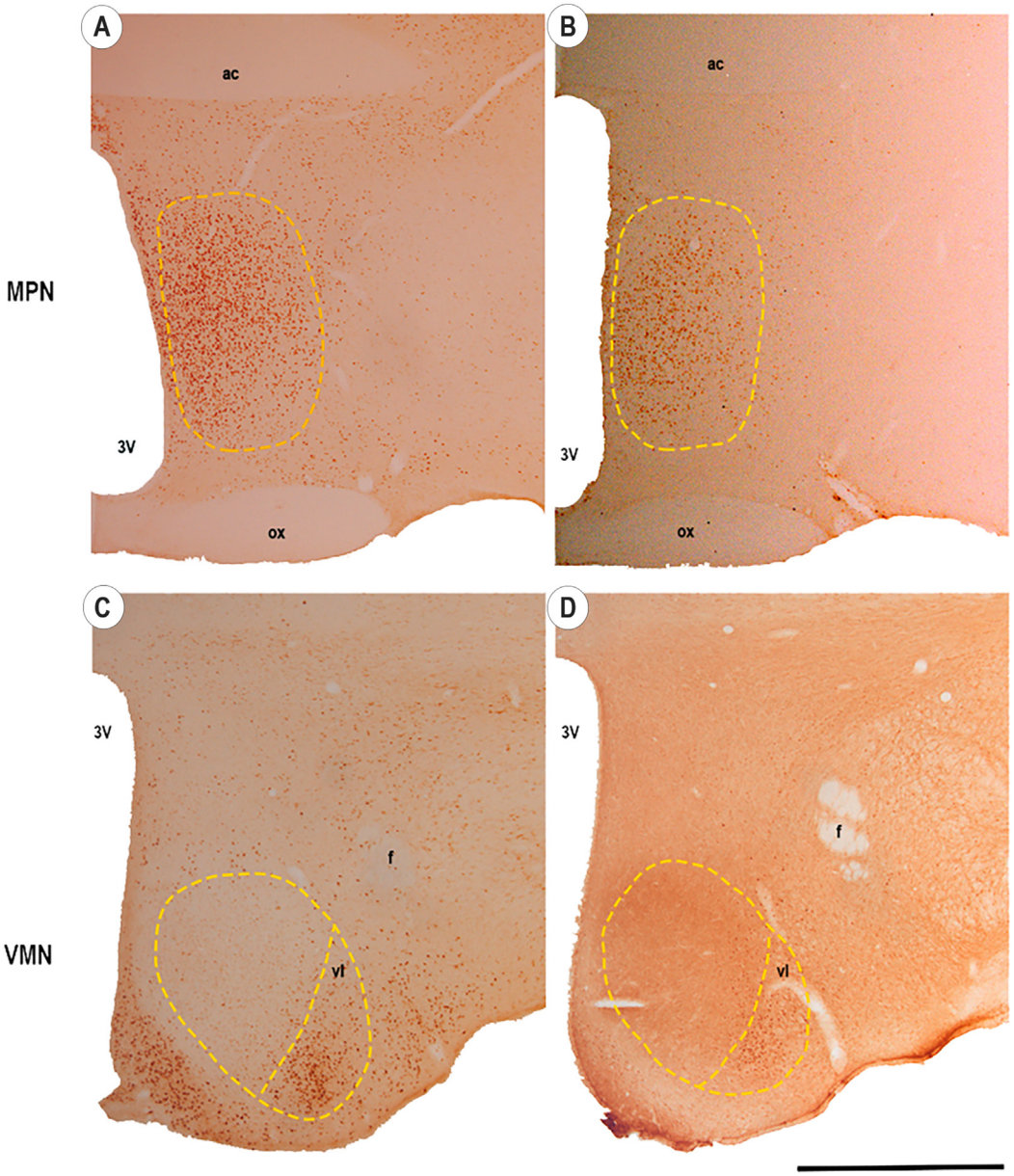

Figure 1. Representative photomicrographs of coronal sections through the MPN (bregma level – 0.70 mm; A and B) or the VMN (at bregma level – 2.40 mm; C and D) of young female rats. The sections were immunostained for ERα (A and C) or PR (B and D). Dashed lines represent the boundaries used for each nucleus quantifications. Neurons in the VMNvl were counted only in the "vl" area. The density of ERα-ir neurons is highest than the one of PR, at the presented levels. PR-ir staining is also seen the VMN neuropil. 3V, third ventricle; ac, anterior commissure; f, fornix; oc, optic chiasm; vl, ventrolateral division of VMN. Scale bar = 400 μm.

superimposition of the images of the region of interest captured with two channels (Figure 2). An average of 150 PR-ir, 200 ERα-ir and 150 ERβ-ir neurons were counted per MPN and an average of 200 PR-ir, 250 ERα-ir and 200 ERβ-ir neurons were counted per VMNvl. Quantitative data are expressed as percentage of co-localization, i.e., percentage of PR-ir, ERα-ir or ERβ-ir cells that also express the other receptor. An average of four regions of interest in the MPN and of six regions of interest in the VMN was analyzed per rat. Regions of interest in the VMN were set inside the VMNvl division and the regions of interest in MPN were distributed along the latero-medial aspect of the nucleus (Supplementary Figure S1).

2.6. Statistical analyses

Data are presented as mean ± standard deviation (SD) except for percentage of co-localization, that is presented as mean ± standard error of the mean (SEM). In all studies, the effect of treatment was assessed by one-way analysis of variance (ANOVA). When significant results were obtained from the overall ANOVA, pair-wise comparisons were consequently made using the post hoc Tukey's HSD test (GraphPad PRISM version 6.0; GraphPad Software, San Diego, CA, USA). Differences were considered significant if $p < 0.05$.

3. Results

3.1. Behavioral testing

The TAM therapy induced changes in the receptive component of female sexual behavior at different phases of the estrous cycle (lordosis quotient, $F(6, 35) = 33.84$; $p < 0.0001$; and intensity, $F(6, 35) = 13.53$; $p < 0.0001$). Female rats at proestrus presented higher lordosis quotient, when compared with female rats at any other phase of the estrous cycle, combining or not TAM with any hormonal treatment (Figure 3A). The

Table 2. Summary of the sampling schemes used for the estimation of ERα-ir and PR-ir neuron numbers in the MPN and VMHvl.

	MPN		VMHvl	
	ERα-ir	PR-ir	ERα-ir	PR-ir
ssf	0.3	0.3	0.3	0.3
x-step (μm)	180	110	110	70
y-step (μm)	180	110	110	70
a(frame) ($μm^2$)	988	2964	1482	1482
h (μm)	8	8	8	8
$\sum Q^-$	150	160	250	280
CE (N)	0.08	0.09	0.07	0.07

ssf, section sampling fraction; x-step and y-step, predetermined distances used along the x and y axes of the section to sample, in a systematic random manner, the fields of view in which the optical dissectors were performed; h, height of the optical dissector; $\sum Q^-$, mean number of neurons counted; CE(N), mean coefficient of error of the individual estimates of total number of neurons.

addition of estradiol and progesterone to females taking TAM daily [TAM (EB + P)] allowed an increased lordosis quotient, when compared with other TAM therapies, that, however, did not reached the levels seen in Proestrus rats (Figure 3A). Both Proestrus and TAM (EB + P) groups presented higher lordosis intensity than all others (Figure 3B). There was no difference in the number of mounts (F (6, 35) = 1.11; p = 0.379; Figure 3C), the latency to return times (F (6, 35) = 1.66; p = 0.161; Figure 3F) and the percentage of exits (F (6, 35) = 0.98; p = 0.453; Figure 3E) between the females studied. ANOVA also showed no effects on the total number of exits between the groups studied (F (6, 35) = 2.23; p = 0.063; Figure 3D).

3.2. Hormonal determinations

Both estrous cycle and TAM treatment induced changes in the estradiol (F (3, 20) = 130.4; $p < 0.0001$) and progesterone (F (4, 37) = 293.7; $p < 0.0001$) serum levels. Data show that Proestrus rats present higher estradiol and progesterone levels that any other group. Metestrus rats present higher progesterone levels than the one showed by Estrous, Diestrus and TAM rats. TAM treated rats present a lower level of estrogen and progesterone levels, that are most similar with the levels presented by Estrous rats (Figure 4A, B).

3.3. Effects of TAM in the expression of ERα and PR in the MPN and VMNvl

The different phases of the estrous cycle and TAM therapy induced changes in the expression of ERα (F (4, 20) = 17.95; $p < 0.0001$) and PR (F (4, 20) = 25.50; $p < 0.0001$) neurons in the MPN and of ERα (F (4, 20) = 10.96; $p < 0.0001$) and PR (F (4, 20) = 11.38; $p < 0.0001$) neurons in the VMNvl, respectively. Current results showed that the number of MPN neurons expressing ERα is lowest at proestrus and highest upon TAM therapy (Figure 5A). Females at metestrus presented the lower number of PR-ir neurons in the MPN when compared with all other groups studied; and Proestrus rats presented more PR-ir MPN neurons than rats at estrus or treated with TAM (Figure 5B). Current results also showed that, in the VMNvl, the number of ERα-ir neurons was lower at the diestrus and estrus phases and that the number of PR-ir VMNvl neurons was higher at the proestrus phase (Figure 5C, D). In the VMNvl, TAM therapy increased the number of ERα-ir neurons to values compared with the ones seen in Proestrus rats and decreased PR-ir neuron numbers to values seen in Estrus and Diestrus rats (Figure 5C, D).

3.4. Effects of TAM in the Co-localization hormone receptors in the MPN

The different phases of the estrous cycle and TAM therapy differently affect the co-localization of ERα/PR, ERα/ERβ and ERβ/PR in the MPN.

ANOVA showed differences in the percentage of ERα-ir neurons expressing PR (F (4, 15) = 10.26; p < 0.0005) and in the percentage of PR-ir neurons expressing ERα (F (4, 15) = 7.48; p < 0.005). The percentage of ERα-ir neurons expressing PR decreased about 30% from proestrus to estrus and increased about 1.6 times from estrus do metestrus and diestrus; TAM administration increased the percentage of ERα-ir neurons expressing PR in about 1.6 times when compared with Estrus rats (Figure 6A). The percentage of PR-ir neurons also expressing ERα increased about 1.4 times from estrus to metestrus and diestrus and decreased about 30% to proestrus; TAM administration increased the percentage of PR-ir neurons also expressing ERα in about 1.4 and 1.5 times, compared with Proestrus and Estrus rats, respectively (Figure 6D).

ANOVA showed differences in the percentage of ERα-ir neurons expressing ERβ (F (4, 15) = 10.46; p < 0.0005) and in the percentage of ERβ-ir neurons expressing ERα (F (4, 15) = 3.77; p < 0.05). In the MPN the percentage of ERα-ir neurons expressing ERβ decreased about 40% from proestrus to estrus and increased about 1.7 times from estrus to metestrus; TAM administration increased the percentage of ERα-ir neurons expressing ERβ in about 1.7 times when compared with Estrus rats (Figure 6B). The percentage of ERβ-ir neurons also expressing ERα in the MPN decreased about 20% from proestrus to estrus; TAM induced no changes (Figure 6E).

ANOVA found differences in the percentage of ERβ-ir neurons expressing PR (F (4, 15) = 5.49; p < 0.05) and in the percentage of PR-ir neurons expressing ERβ (F (4, 15) = 9.57; p < 0.005). The percentage of ERβ-ir neurons expressing PR decreased about 18% from proestrus to estrus and increased about 1.2 times from estrus to metestrus (Figure 6C). The percentage of PR-ir neurons also expressing ERβ in the MPN decreased about 35% from proestrus to estrus and increased about 1.6 times from estrus to metestrus. The administration of TAM induced no changes in the percentage of ERβ-ir neurons expressing PR but increased the percentage of PR-ir neurons expressing ERβ in the MPN in about 1.5 times when compared with Estrus rats (Figure 6F).

3.5. Effects of TAM in the Co-localization hormone receptors in the VMNvl

TAM therapy differently affects the co-localization of ERα/PR, ERα/ERβ and ERβ/PR in the VMNvl at different phases of the estrous cycle, ANOVA showed differences in the percentage of ERα-ir neurons expressing PR in the VMNvl (F (4, 15) = 5.08; p < 0.05) but not in the percentage of PR-ir neurons expressing ERα (F (4, 15) = 1.20; p = 0.352; Figure 7A, D). The percentage of ERα-ir neurons expressing PR decreased about 15% in Estrus and Metestrus rats when compared with Proestrus rats; TAM administration also decreased the percentage of ERα-ir neurons expressing PR in about 15% when compared with Proestrus rats (Figure 7A).

ANOVA showed differences in the percentage of ERα-ir neurons expressing ERβ in the VMNvl (F (4, 15) = 5.12; p < 0.05) and in percentage of ERβ-ir neurons expressing ERα in (F (4, 15) = 11.68; p < 0.0005). In the VMNvl, the percentage of ERα-ir neurons expressing ERβ increased about 1.5 times from estrus to metestrus and diestrus; TAM induced no changes (Figure 7B). The administration of TAM decreased the percentage of ERβ-ir neurons also expressing ERα in the VMNvl in about 20% when compared will all phases of the estrous cycle (Figure 7E).

ANOVA found differences in the percentage of ERβ-ir neurons expressing PR (F (4, 15) = 3.47; p < 0.05) and in the percentage of PR-ir neurons expressing ERβ (F (4, 15) = 10.48; p < 0.0005). The percentage of ERβ-ir neurons expressing PR in the VMNvl decreased about 20% from proestrus to estrus (Figure 7C). The percentage of PR-ir neurons also expressing ERβ decreased about 45% from proestrus to estrus and increased about 1.8 times from estrus to metestrus; TAM administration increased the percentage of PR-ir neurons expressing ERβ in the VMNvl about 1.7 times when compared with Estrus rats (Figure 7F).

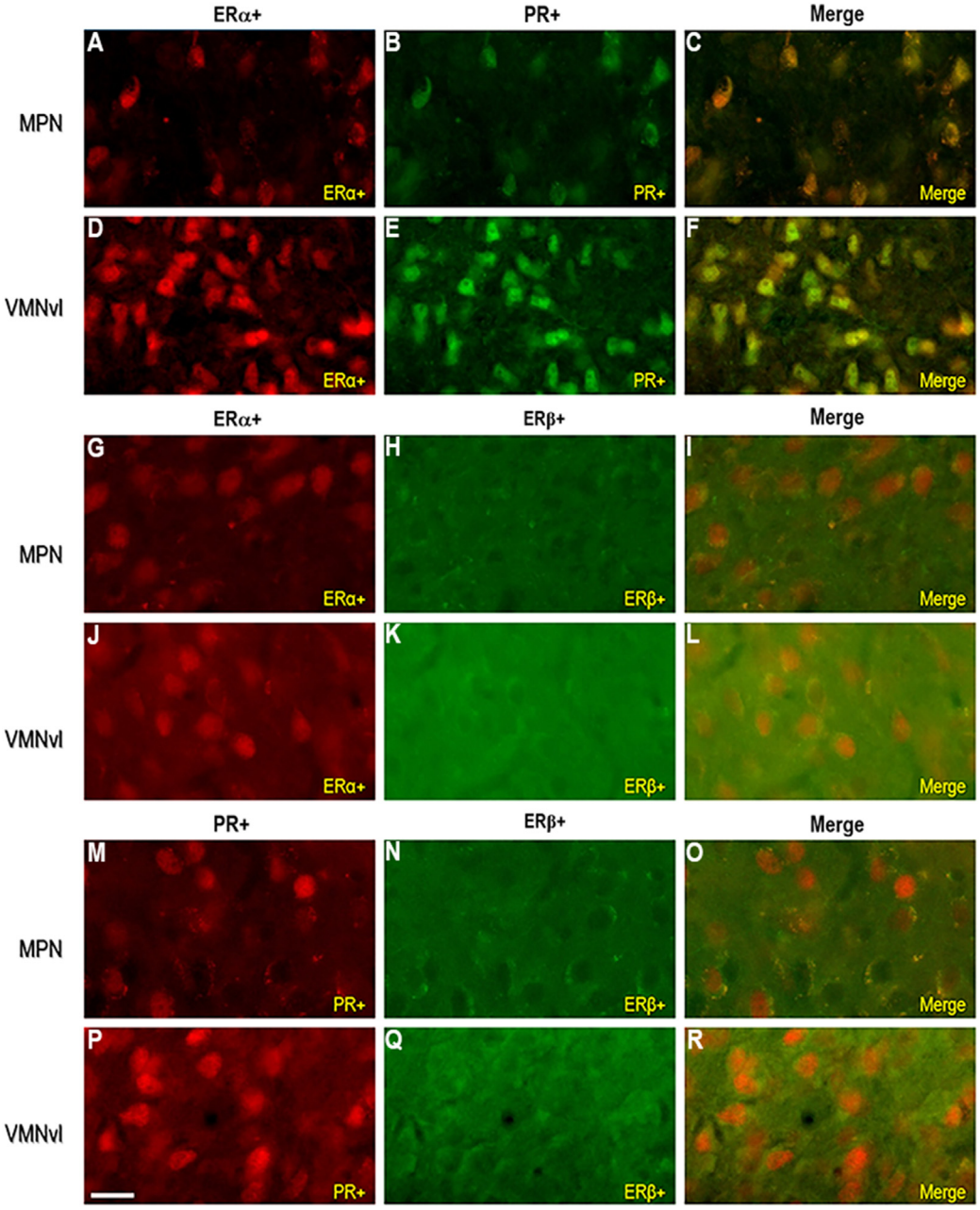

Figure 2. Fluorescence photomicrographs illustrating the co-localization of immunoreactive staining of ERα (red, stained with Alexa Fluor 568; A, D, G, J), ERβ (green, stained with Alexa Fluor 488; H, K, N, Q) and PR (green when co-localized with ERα; B and E; and red when co-localized with ERβ; M and P) in the MPN and VMNvl. Merged photomicrographs show in yellow the co-localization of both receptors in each pairing. The micrographs were adjusted only for brightness and contrast with Adobe Photoshop CS2 to optimize the quality of the images. Scale bar = 50 μm.

4. Discussion

The existence of a direct correlation between high ovarian hormone levels, the activation/inactivation of the MPN and VMNvl, and the promotion of female rat sexual behaviour is consensual (Etgen and Barfield, 1986; Heijkoop et al., 2018; Pfaus et al., 2015; Rubin and Barfield, 1980). Present-day data suggests that TAM disrupts females' estrous cyclicity maintaining estradiol levels very low and unable to stimulate the expression of PR in the MPN and VMNvl – both critical neuronal areas where estradiol and progesterone promote proceptive behavior and lordosis reflex, respectively.

One would think that TAM affects sexual receptivity by hampering the surge of serum levels of ovarian hormones that, by failing to activate ovarian hormone receptors, hindered the behavioral response. A previous study done in our lab (Sá et al., 2018) showed that TAM in fact increases the number of neurons expressing ERα in the VMNvl and does not counteract estradiol dependent increase in neuronal connectivity. The fact that the administration of estradiol benzoate after TAM, in that previous study (Sá et al., 2018), has increased the expression of ERα in 30% of new VMNvl neurons could suggest that TAM-dependent ERα manipulation could be a mechanism to trigger sexual behavior on rats under taking TAM plus estradiol. Data showing that exogenous administration of estradiol followed by progesterone in doses known to promote the female sexual response in ovariectomized rats (Heijkoop et al., 2018; Pfaus et al., 2015; Sá et al., 2009), induced the behavioral response, although still presenting lordosis intensity lower than the ones showed by Proestrus rats. This data suggests that TAM only partially impairs the neuronal circuitry that promotes the receptive behavior. Data from the observations made on behavior proceptive component (which show that TAM treated rats displayed behavioral responses similar to the ones seen in Metestrus rats) suggests that the effects of TAM in the MPN are less dependent on the external administration of ovarian hormones, suggesting an impaired central mechanism.

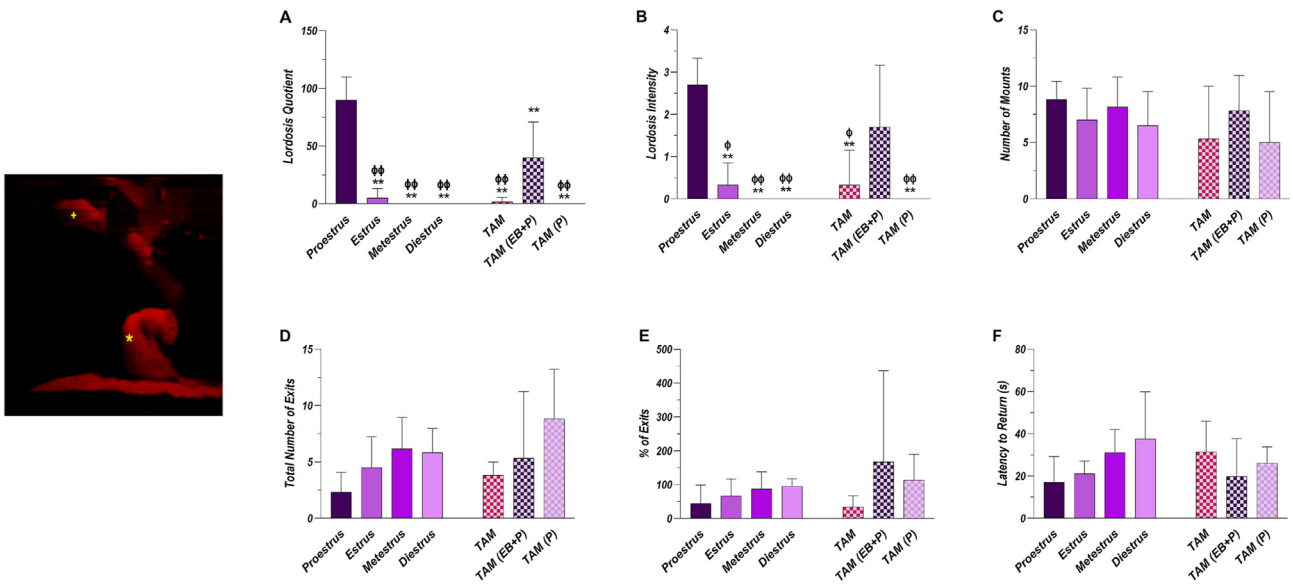

Figure 3. Graphical representation of the measurements of behavioral tests applied to females in all phases of the estrous cycle or supplemented with TAM. Since TAM inhibited estradiol and progesterone surges, TAM-treated rats were supplemented with exogenous estradiol benzoate (EB; 10 μg/rat) and progesterone (P; 500 μg/rat) 48 h later - TAM (EB + P) or with progesterone alone - TAM (P). A. Lordosis quotient, B. Lordosis intensity, C. Number of mounts, D. Total number of exits, E. Percentage of exits, F. Latency to return. Bars represent mean ± SD ($N = 6$/group). Tukey HSD test: $^{**}p < 0.0005$; $^{*}p < 0.005$, compared with Proestrus; $^{\phi\phi}p < 0.005$; $^{\phi}p < 0.05$, compared with TAM (EB + P).

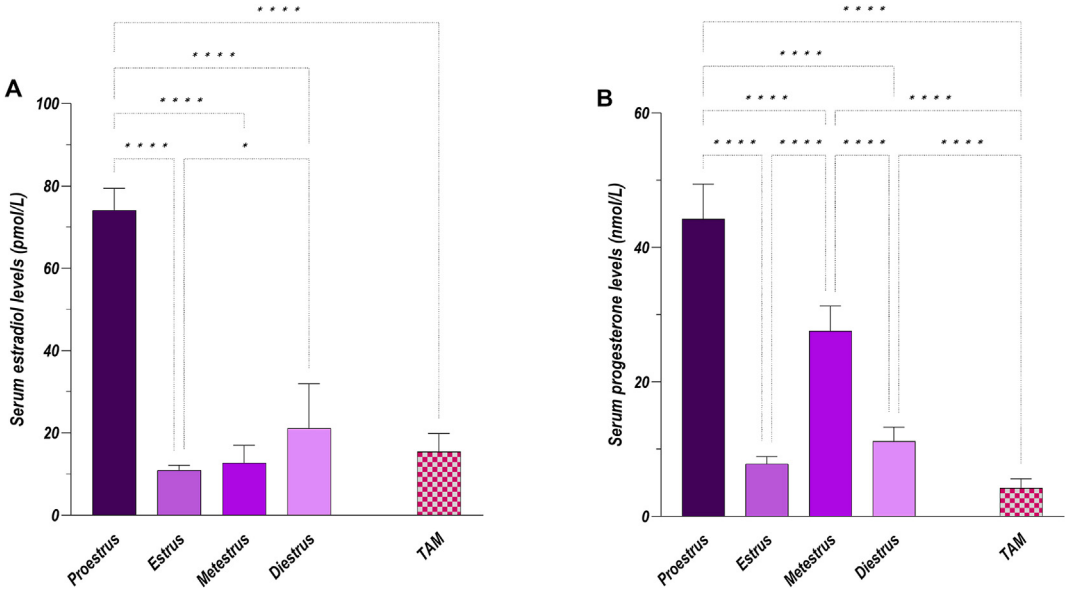

Figure 4. Graphical representation of hormonal determinations of A. serum estradiol levels and B. progesterone levels. Circles represent the four phases of the estrous cycle ($N = 6$/group) and the square represents TAM-treated rats ($N = 18$). TAM inhibited hormonal fluctuation; data were aggregated in the same group. Symbols represent mean ± SEM. Tukey HSD test: $^{\blacklozenge\blacklozenge}p < 0.0005$, compared with all other groups; $^{\S\S}p < 0.005$, compared with Diestrus rats.

As it was previously suggested, the successive administration of estradiol, followed by progesterone, is able to reduce the estrus termination induced by vagino-cervical stimulation and increases the contact-return latency (Georgescu et al., 2009). In this study, the same could be induced by TAM, which would prevent this hormonal action, and, in this case, the mounts would induce the anticipation of the estrous end. The fact that the average number of mounts did not change between groups suggests that, in average, female attractiveness was not compromised by the phase of the estrous cycle nor by TAM administration. Data concerning the latency to return refers to all moments of exits and time to re-enter the arena: either after contact with the male or in moments where the female was just running away from the male. Taken together, current results agree with previous ones by suggesting that, since the amount of vagino-cervical stimulation is related with contact-return latency (Georgescu et al., 2009), there is no difference between the latency to return from the animals studied.

The behavioral response follows the expression of ERα and PR in the VMNvl and MPN, areas known to be involved in the regulation of female sexual behavior (Pfaus et al., 2015; Sá and Fonseca, 2017; Sá et al., 2009, 2018). Previous studies have shown the induction of the ERα-dependent expression of PRs. In the VMNvl this promotes a facilitative action, while in the MPN promotes an inhibitory action in triggering the behavioral

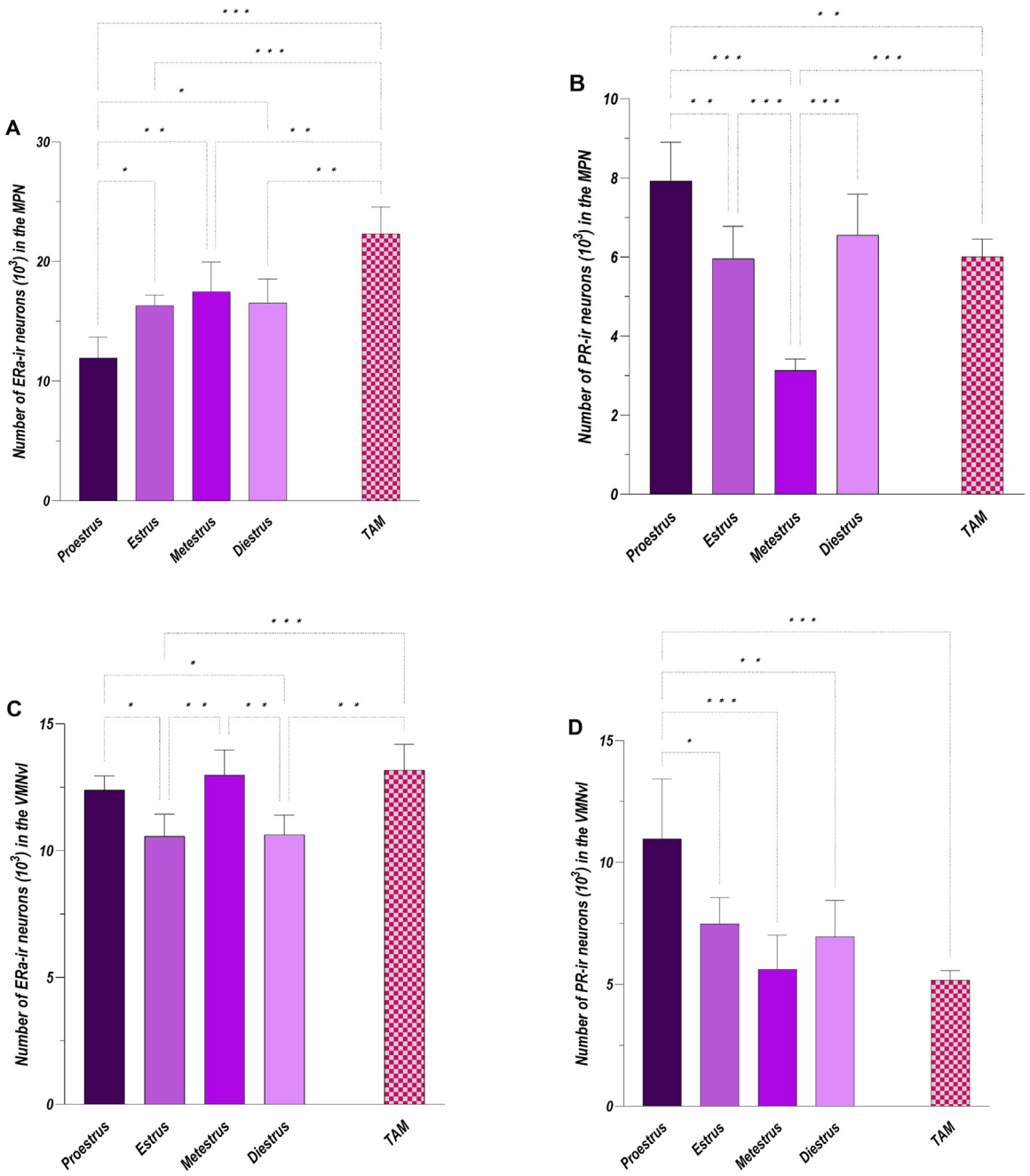

Figure 5. Graphical representation of the total number of ERα-ir and PR-ir in the MPN (A, B) and in the VMNvl (C, D) of rats at the four phases of the estrous cycle or treated with TAM. Columns represent means ± SD ($N = 5$/group). Tukey HSD test: ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$, according to the comparisons made by the dashed lines.

response (Heijkoop et al., 2018; Madeira and Lieberman, 1995; Pfaus et al., 2015). Data from previous studies shows that in the VMNvl, the activation of ERα increases the expression of PRs of female rats (Ogawa et al., 1998; Sá et al., 2013, 2018), whereas in the MPN, estradiol decreases the number of neurons expressing ERα and that progesterone administration stimulates its renewal (Martins et al., 2015; Pfaus et al., 2015). The analyses of behavioral data could suggest that TAM is not able to promote the sexual behavior because it reduces ovarian hormone levels. In fact, this seem to not be case; if the effects of TAM were only attributed to reduced hormone levels, then, the receptor expression induced by TAM would be similar to the one induced by Estrus rats; which was not the case. In this way, the differences seen in the behavioral response and in the expression of ERα between TAM and Estrus rats point to other causes than only hormone reduction. This fact suggests that the changes seen in the expression of both ERα and PRs in the VMNvl and MPN may be a possible mechanism of TAM action in the inhibition of the sexual response. In fact, TAM increases the number of ERα both in VMNvl and in MPN, when compared with estrus (despite the low levels of estradiol in both circumstances) but fails to promote the expected ERα-dependent increase in PR expression. Thus, it suggests that the abundance of ERα expression is not complemented with receptor activation and the concomitant transcription activation. This contra-intuitive increase in ERα protein is in line with the previous suggestion that TAM has a mechanism of action that not only blocks the ERα-dependent response, but it also inhibits the destruction of these receptors (Martinkovich et al., 2014; Sá et al., 2018). In this way, TAM increases the amount of ERα expression even though the concentration of estradiol is diminished.

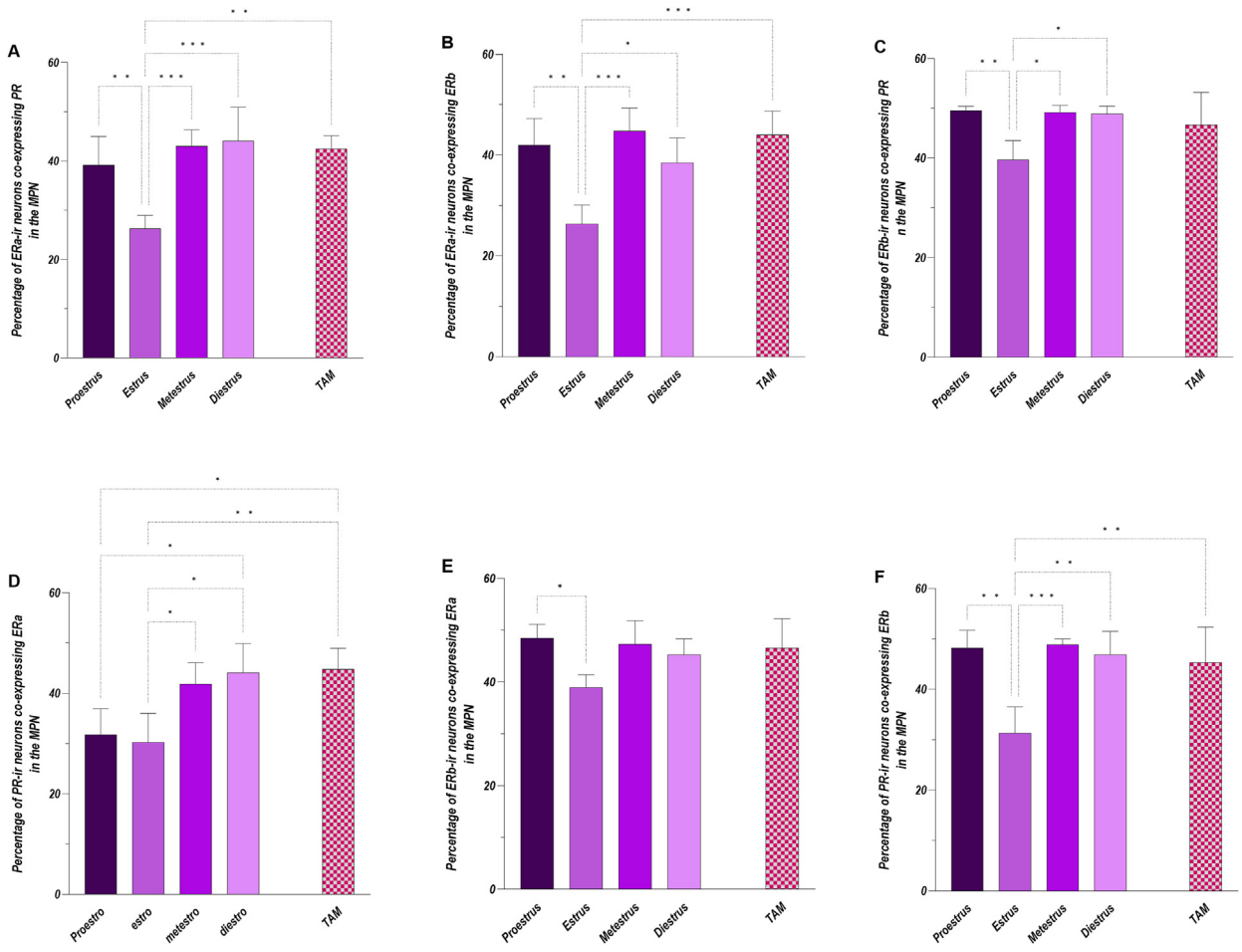

Figure 6. Graphical representation of the percentage of MPN neurons co-expressing ERα/PR (A); ERα/ERβ (B); ERβ/PR (C); PR/ERα (D); ERβ/ERα (E); PR/ERβ (F) of rats at the four phases of the estrous cycle or treated with TAM. Columns represent means ± SEM (n = 4/group). Tukey HSD test: ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$, according to the comparisons made by the dashed lines.

Co-localization results agree with previous ones (Sá and Fonseca, 2017). From diestrus to proestrus and estrus, the estradiol fluctuations increase or decrease the expression of both receptors in the same neurons. On the contrary, at metestrus the sole increase in progesterone, promotes the expression of ERα on neurons not expressing PR. This disruption of co-localization may be due to an increase in ERα expression, induced by progesterone activation, and a reduction of PR expression, because ERα is not activated by the lack of estradiol. Interestingly, the same results are seen in TAM-treated rats. Previous studies have suggested that TAM increases the expression of ERβ upon concomitant increase in ERβ mRNA but the increase in ERα expression is not preceded by increased ERα mRNA (Hall and McDonnell, 1999; Patisaul et al., 2003; Sá et al., 2018). Present results suggest that in the MPN, TAM induces no differences in the concomitant expression of both ERs, compared with Proestrus rats, showing that almost half of ERα-ir neurons express ERβ and vice-versa. On the contrary, in the VMNvl, TAM administration decreases the number of ERβ-ir neurons that express ERα but not the other way around. The observed reduction in the co-expression of both receptors, seems to corroborate previous studies (Hall and McDonnell, 1999; Kojetin et al., 2008; Martinkovich et al., 2014; Sá et al., 2018) and further suggest that TAM sequestering of nonfunctional ERα is done in a different neuronal population. By increasing the expression of ERβ, that does not promote the behavioral response (Mazzucco et al., 2008; Pfaus et al., 2015; Sá et al., 2013), and inactivating the ERα that could, TAM not only does not promote the lordosis response, but also counteracts the ability of the exogenous administration of ovarian hormones to do so. This proposal if further substantiated by the data showing that TAM induces no change in the percentage of neurons ERβ-ir expressing PR and vice versa in both areas studied, indicating that ERβ-ir neurons only present the constitutive expression PR.

The current study did not evaluate TAM occupation of ERs nor its interaction as an estradiol antagonist. On the contrary, the results point to a differential mechanism of action of TAM that prevents ligand induced ERα degradation, known to be a requirement for ERα transcription activity (Alarid et al., 1999; Martinkovich et al., 2014; Sá et al., 2016; Wijayaratne and McDonnell, 2001). In this way, present data supports the notion that the behavioral response is not dependent on receptor expression but more in receptor activity. Altogether, our data suggests that TAM presents a complex mechanism of action. It is not fully comprehended nor reversed by the exogenous administration of estradiol and progesterone, since the sexual response is only partially increased with that administration.

It was demonstrated that women taking TAM as an adjuvant therapy for breast cancer treatment report more mood disorders, such as anxiety and depression, known to depend on ovarian hormone modulation of the limbic system, containing the hypothalamus and preoptic areas. By using a rodent model of long-term TAM therapy, we did not intend to unravel the neuronal mechanisms underlying the reported TAM-dependent loss of libido. In fact, the main goal of this study is to understand some of the neuronal mechanisms that are involved in the ability of an endocrine disruptor (that is also a common therapeutic agent) to prevent or inhibit the neuronal mechanisms underlying behavioral responses. This knowledge will contribute to the discussion about alternatives that could help

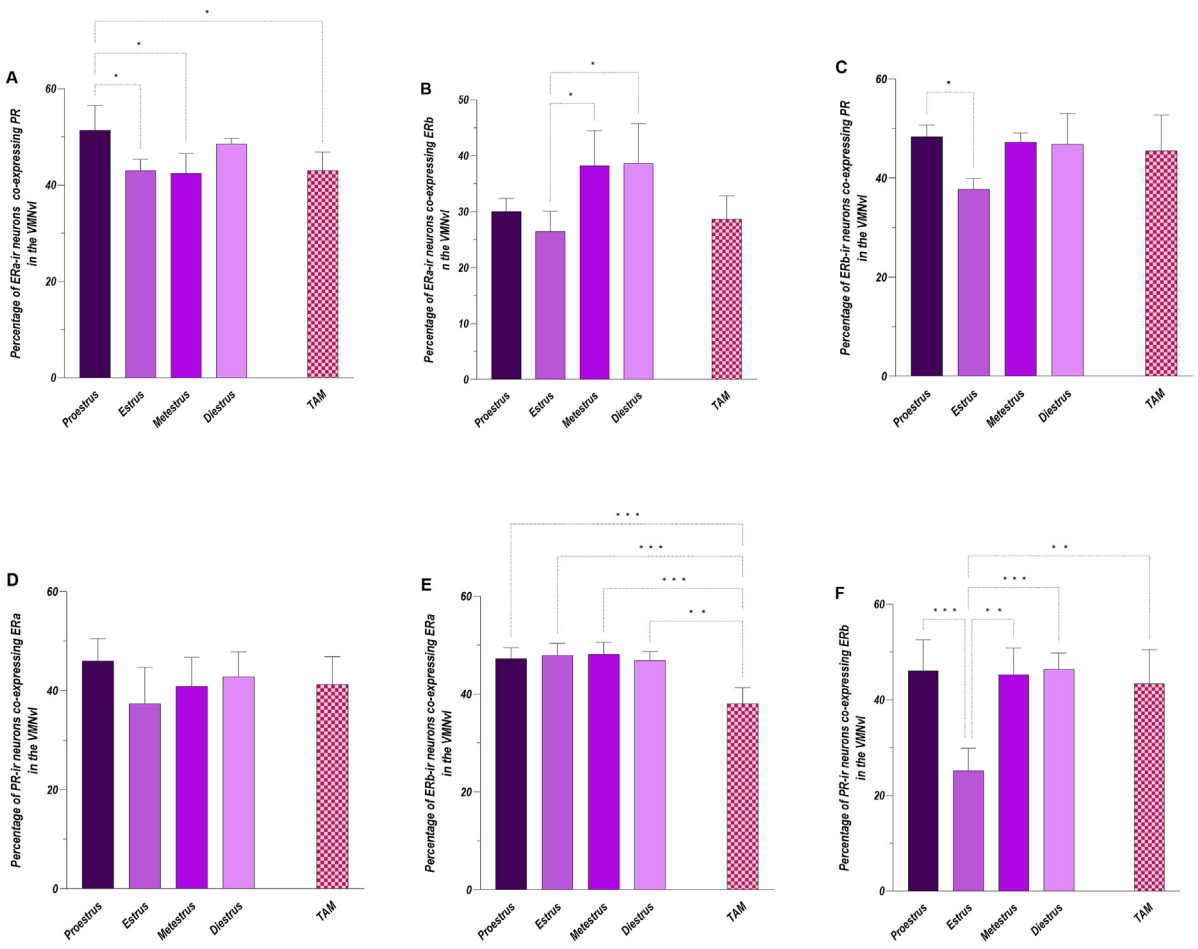

Figure 7. Graphical representation of the percentage of VMNvl neurons co-expressing ERα/PR (A); ERα/ERβ (B); ERβ/PR (C); PR/ERα (D); ERβ/ERα (E); PR/ERβ (F) of rats at the four phases of the estrous cycle or treated with TAM. Columns represent means ± SEM (n = 4/group). Tukey HSD test: ***$p < 0.0005$; **$p < 0.005$; *$p < 0.05$, according to the comparisons made by the dashed lines.

to mitigate some of these effects. Nevertheless, further studies are required to assess the mechanism of action of TAM and to fully realize it.

Declarations

Author contribution statement

Cláudia A. Pinto: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Bruno M. Fonseca: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Susana I. Sá: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

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