Involvement of serotonin 5HT$1$ and 5HT$2$ receptors and nitric oxide synthase in the medial preoptic area on gonadotropin secretion

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Abstract

Due to the stimulatory action of serotonin (5HT) and nitric oxide (NO) on the secretion of gonadotropins and PRL, this work aimed at investigating the participation of serotoninergic receptors 5HT$1$ and 5HT$2$ of the medial preoptic area (MPOA) in the control of luteinizing hormone (LH), follicle stimulating hormone (FSH) and prolactin (PRL) secretion and the possible modulation by ovarian steroids as well as the possible participation of NO as a mediator of the stimulatory effects of serotonin in the MPOA on LH secretion. Microinjections of three different doses (0.02, 0.2, and 2 ug) of methiothepin, a serotoninergic 5HT$1$ antagonist or ketanserin, a seretoninergic 5HT$2$ antagonist, were carried out into the MPOA in ovariectomized rats treated or not with estrogen or estrogen plus progesterone. Other groups of ovariectomized rats treated with estrogen, estrogen plus progesterone or vehicle were prepared to evaluate NOS activity in the MPOA. Plasma LH, FSH, and PRL in ovariectomized rats were not altered by the microinjection of methiothepin or ketanserin in the MPOA. Methiothepin microinjection in the MPOA reduced LH but did not change plasma FSH and PRL in the animals submitted to the same steroidal treatment. NOS activity in the MPOA was significantly reduced by methiothepin or ketanserin in ovariectomized rats treated with estrogen or estrogen plus progesterone. On the other hand, ketanserin microinjection in the MPOA reduced plasma LH but did not change plasma PRL in the animals submitted to the same steroidal treatment. NOS activity in the MPOA was significantly reduced by methiothepin or ketanserin in ovariectomized rats treated with estrogen or estrogen plus progesterone. In conclusion, this work showed that in the studied conditions, serotonin in the MPOA: (1) does not work in the control of PRL secretion through 5HT$1$ and 5HT$2$ receptors; (2) integrates the control of FSH secretion by 5HT$2$ receptors, but not 5HT$1$; (3) in the presence of estrogen, stimulates LH secretion by 5HT$1$ and 5HT$2$ receptors, which can be differentially modulated by progesterone; (4) at least partly, stimulates LH secretion by nitric oxide activity.

Keywords: Serotonin; Nitric oxide; Medial preoptic area; Luteinizing hormone; Follicle stimulating hormone; Prolactin; Ketanserin; Methiotepin

1. Introduction

Gonadal steroids act directly on luteinizing hormone-releasing hormone (LH-RH) or, indirectly through excitatory or inhibitory interneurons [19]. These interneurons produce various neuromediators such as noradrenaline (NA), serotonin (5HT), y-amino butyric acid (GABA), glutamate, galanin, oxytocin, endorphin, neuropeptide Y (NPY), nitric oxide (NO), angiotensin II (AII) and the atrial natriuretic peptide (ANP). There are several studies about involvement of these neurotransmitters on regulation of LH-RH, luteinizing hormone (LH) and follicle stimulating hormone (FSH) release [2,6,9,10,12,23,27,42,44].

There is no co-localization of mRNA for 5HT$1$ or 5HT$2$ receptors in LH-RH neurons [49]. Serotonin indirectly acts on LH-RH neurons in the preoptic area through terminals that are juxtaposed to LH-RH neurons [25]. In female adult rats, 5HT has bimodal action in the release of LH-RH/LH, which is inhibitory in the medial basal hypothalamus (MBH) and stimulatory in the preoptic area, as previously suggested [26]. The action of 5HT on the LH release depends on the presence or absence of estrogen (E2) as well as on a critical period of time. The actions which alter serotoninergic neurotransmission (administration of 5HT, precursor or inhibitor of 5HT synthesis, agonist and antagonist) are effective if they occur in an appropriate period (between 10 and 12 h) on the day when the LH peak takes place. However, little is known with regard to the central site of action of 5HT, the types of participant receptors and the mediators of...
serotonergic action in LH-RH neurons [13]. The enzyme nitric oxide synthase (NOS) converts l-arginine in equimolar amounts of l-citrulline and NO. Neuronal nitric oxide synthase (n-NOS) is not co-localized in LH-RH neurons of the preoptic area [17,20] however, contacts between NOergic and LH-RH neurons were described in this area [3]. In rats, NO may stimulate the release of LH-RH by the preoptic area and medial hypothalamus [3,28,34].

LH-RH induces the release of both gonadotropins, however, experimental evidence has been gathered towards an alternative hypothalamic control of FSH [32,47]. There are ever, experimental evidence has been gathered towards an optic area and medial hypothalamus [3,28,34].

The medial preoptic area (MPOA) participates in the mechanisms for control of prolactin (PRL) release in female rats. The caudal transection of efferents or bilateral lesion of such area blocks the peak of PRL induced by estradiol [22].

The aim of this work was to explore the participation of serotoninergic receptors 5HT₁ and 5HT₂ in the MPOA to control of LH, FSH, and PRL secretion as well as the possible modulation by ovarian steroids and the possible participation of NO as a mediator of the stimulatory effects of serotonin on LH secretion.

2. Methods

2.1. Animals

Female Wistar (180–200 g) were housed under controlled conditions of light (07:00–19:00 h) at 22 ± 1 °C. Food and water were provided ad libitum. Two weeks after bilateral ovariectomy, a stainless steel cannula was placed into the MPOA using a stereotaxic instrument with the following coordinates: AP, 2.2 mm; L, ±0.8; V, –7.9 from ventral to the dorsum of the skull. The guiding cannula provided with a mandril to prevent obstruction was attached in place by two metal screws and dental cement. Approximately 1 week after stereotaxic surgery and 24 h before the experiment, a sylastic catheter was introduced into the external jugular vein by the previously described technique [18]. During the surgeries the animals were kept under anesthesia by thiopental (Abbott, USA; mg/100 g body weight, i.p.) or 2.5% triethanolamine (Aldrich, USA; 1 mL/100 g body weight, i.p.). After the two first surgeries, the animals received a prophylactic dose of antibiotic (Veterinary Pentabiotic, Whythe, Brazil). The animals received an injection of estradiol benzoate (Schering, Brazil; 25 μg, s.c.) for 3 days before the experiment and on the experiment day (forth day) an injection of vehicle or progesterone (Sigma, USA; 5 mg, s.c.). The control group received only vehicle (corn oil). All injections were carried out at 09:00 h.

2.2. Experimental procedure

Microinjections (1 μL) of three different doses (0.02, 0.2, and 2 μg) of methiothepin (Sigma/RBI, USA; methiothepin mesylate), a serotoninergic 5HT₁ antagonist (n = 11, 9, and 10 animals, respectively) or ketanserin (Sigma/RBI, USA), a serotoninergic 5HT₂ antagonist (n = 9, 9, and 8 animals, respectively) were carried out into the MPOA at 10:00 h in awake animals. The control animals received vehicle: isotonic saline (0.15 M NaCl) for methiothepin (n = 6 animals) or 0.1% ascorbic acid in saline for ketanserin (n = 7 animals).

Heparinized blood samples (0.6 mL) were collected from the external jugular catheter at 800 h (basal) on the experiment day and from 14:00 to 18:00 h each hour. An equal volume of isotonic saline (0.15 M NaCl) was infused through the jugular catheter after collection of each blood sample. Plasma was removed after refrigerated centrifugation and frozen for later measurement of hormones.

After decapitation at the end of the experiment, the brains were removed and fixed with formalin for histological analysis to confirm the placement of the guiding cannulae. Only animals having a cannula inserted into the MPOA were used in this work.

Other groups of ovariectomized rats treated with estrogen or estrogen plus progesterone were prepared as described above to evaluate NOS activity. These animals received a microinjection of methiothepin (2.0 μg, 8 and 9 animals, respectively), ketanserin (0.02 μg, 7 and 9 animals, respectively) or vehicle solutions (isotonic saline, 5 animals or ascorbic acid, 5 animals) into the MPOA at 10:00 h and were decapitated at 17:00 h. The doses of serotoninergic antagonists were chosen in conformity with the results from the first experiment. After decapitation, trunk blood, and the brain were collected to measure plasma LH and FSH activity, respectively. The brain was frozen for later microdissection of the MPOA by a previously described technique [35] using circular needle (diameter 2.0 mm) placed in the middle between anterior commissure and optic chiasm.

2.3. Radioimmunoassay

Plasma concentrations of LH, FSH, and PRL were measured by radioimmunoassay kits supplied by the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, USA) and expressed in terms of the reference preparations (RP₁ for LH and PRL; RP₂ for FSH). The intra-assay coefficients of variation were 3.4, 2.9, and 2.9% for LH, FSH, and PRL, respectively. The inter-assay coefficients of variation were 15, 10.7, and 11.5% for LH, FSH, and PRL, respectively.
2.4. Nitric oxide synthase activity into the MPOA

NOS activity in the MPOA was evaluated by the conversion of \(^{[3}\text{H}]-\text{arginine}\) to \(^{[3}\text{H}]-\text{citrulline}\) following the previously described technique [7]. A protein assay reagents kit (Bio-Rad Lab, USA) was used for protein measurement in the microdissected MPOA. NOS activity was expressed as moles of NO by 15 min for mg of protein.

2.5. Statistics

Significance of differences between plasma hormone concentrations over time was determined by analysis of variance for repeated measures and Newmann–Keuls test for multiple comparisons. NOS activity data were statistically analyzed by unpaired Student’s t-test.

3. Results

The microinjection of methiothepin or ketanserin in the MPOA of ovariectomized rats did not alter the plasma LH (Figs. 1A and 2A) or FSH (Figs. 4A and 5A) levels when compared to the basal values (08:00 h) in each group nor compared to the values in the control groups (microinjection of vehicle, saline, or ascorbic acid).

Plasma LH basal level (08:00 h) was similar in the four groups of ovariectomized rats which would receive microinjection of saline or three different doses of methiothepin in the MPOA (Fig. 1A). Basal values were significantly lower in estrogen primed ovariectomized rats (Fig. 1B) and estrogen primed ovariectomized rats, which would receive progesterone between 09:00 and 10:00 h (Fig. 1C) than basal values in control groups (Fig. 1A). The groups used for experiments with ketanserin (Fig. 2A–C) presented similar basal values that groups used for experiments with methiothepin at 08:00 h.

Estrogen primed ovariectomized rats submitted to the microinjection of saline (Fig. 1B) or ascorbic acid (Fig. 2B) in the MPOA showed an increase in plasma LH at afternoon. These responses were amplified in estrogen primed ovariectomized rats treated with progesterone (Figs. 1C and 2C).

Microinjection of methiothepin and ketanserin in the MPOA decreased the plasma LH levels in estrogen primed
ovariectomized rats treated (Figs. 1C and 2C) or not with progesterone (Figs. 1B and 2B) when compared to the basal values (08:00 h) and to the control groups (microinjection of saline and ascorbic acid, Figs. 1A and 2A, respectively).

Fig. 3 shows maximum values of plasma LH attained by ovariectomized rats treated with estrogen and estrogen plus progesterone under the effect or not of methiothepin or ketanserin into the MPOA. The three different doses of 5HT1 and 5HT2 serotoninergic antagonists reduced significantly the peak of LH in estrogen primed ovariectomized rats (Fig. 3A and C). On the other hand, in ovariectomized rats treated with estrogen plus progesterone, the lower dose of methiothepin and higher dose of ketanserin did not change the values of plasma LH.

Plasma FSH basal levels (08:00 h) were similar in the groups of ovariectomized rats which would be submitted to the microinjections of saline or different doses (0.02, 0.2, and 2.0 ug) of methiothepin (Fig. 4) as well as ascorbic acid or different doses (0.02, 0.2, and 2.0 ug) of ketanserin (Fig. 5). Similar basal levels were verified in estrogen primed ovariectomized rats (Figs. 4B and 5B) and estrogen primed rats which would receive progesterone between 09:00 and 10:00 h (Figs. 4C and 5C).

Plasma FSH levels were higher significantly at 17:00 and 18:00 h than 08:00 h (basal values) in estrogen primed ovariectomized rats submitted to the microinjections of saline (Fig. 4B) or acid ascorbic acid (Fig. 5B) into the MPOA. On the other hand, the increase of plasma FSH is sharper in estrogen primed ovariectomized rats treated with progesterone as showed in the Figs. 4C and 5C.

Plasma FSH was not altered by the microinjection of methiothepin into the MPOA in estrogen primed ovariectomized rats (Fig. 4C) or not with progesterone (Fig. 4B).

The effects of the microinjection of ketanserin in the MPOA on the plasma FSH are shown in Fig. 5. Plasma FSH in ovariectomized rats treated with estrogen was reduced by the three doses used (Fig. 5B). However, in ovariectomized rats treated with estrogen plus progesterone, the lowest dose (0.02 ug) caused the largest reduction (Fig. 5C).

Fig. 6 shows maximum values of plasma FSH attained by ovariectomized rats treated with estrogen and estrogen plus progesterone under the effect or not of methiothepin or ketanserin into the MPOA. Methiothepin did not change the maximum values of FSH plasma. These values were reduced by the highest dose (2.0 ug) of ketanserin in ovariectomized
rats treated with estrogen and by the low dose (0.02 ug) in
ovariectomized rats treated with estrogen and progesterone.

NOS activity in the MPOA at 17:00 h in ovariectomized
rats treated with estrogen or estrogen plus progesterone was
significantly reduced by both methiothepin and ketanserin
(Fig. 7).

The microinjection of methiothepin or ketanserin in the
MPOA in the three different doses did not alter the plasma
PRL in the various studied situations (data not shown).

4. Discussion

Plasma FSH in ovariectomized rats treated with estrogen or estrogen plus progesterone was not altered by the mi-
croinjection of methiothepin (Fig. 4), but was reduced by the
microinjection of ketanserin in the MPOA (Fig. 5). LH-RH
induces the secretion of both gonadotropins. However, there
is the evidence of an alternative hypothalamic control for
FSH [32,47], which involves a distinct releasing factor for
FSH (FSH-RH). There are few reports concerning the ac-
tion of 5HT on the release of FSH. Nevertheless, it has been
suggested that it is also bimodal, similarly to LH [22]. Our
results corroborate other data in the literature as to the exis-
tence of a duality in the neural control for LH and FSH se-
cretion. In addition, they show that 5HT2, but not 5HT1 re-
ceptors in the MPOA would participate in control of the se-
cretion peak of FSH induced by estrogen and progesterone.
Serotonin would has a stimulatory action on FSH secretion
through 5HT2 receptors in the MPOA.

Such action would be facilitated by estrogen and hin-
dered by progesterone since, in order to reduce the peak of
FSH secretion, a higher dose of ketanserin was necessary in
ovariectomized rats treated with estrogen, whereas the low-
est dose did so in ovariectomized rats treated with estrogen
plus progesterone.

In our experiments, the plasma PRL was not altered by the
microinjection of methiothepin or ketanserin in the MPOA
in ovariectomized rats treated with estrogen or estrogen
plus progesterone. Serotoninergic receptors participate in the
stimulatory control of PRL secretion, but the mechanisms in-
volved and the action sites are not completely clarified [27].
Our results indicate that serotonin action in the control of PRL secretion must occur in other cerebral structures than the MPOA or it may act through other receptors than 5HT₁ and 5HT₂. On the other hand, the participation of the MPOA in such control involves other different neuromediators. Plasma LH (Figs. 1A and 2A) and FSH (Figs. 4A and 5A) in ovariectomized rats was not altered by the microinjection of methiothepin or ketanserin in the MPOA. Therefore, the control of LH and FSH secretion in the condition of deficiency of ovarian steroids would not involve the action of 5HT in 5HT₁ and 5HT₂ receptors in the MPOA. We cannot rule out a possible action of 5HT through other types of receptors or an action of 5HT on pulsatile release of gonadotropin. The experimental protocol of this work did not allow evaluate these questions.

In female adult rats, 5HT has bimodal action in the secretion of LH-RH/LH, which is inhibitory in the MBH and stimulatory in the preoptic area [25,26,46]. However, E₂ is the determinant factor in the type of action performed by 5HT in LH secretion. Secretion is inhibited when plasma E₂ is low, but it is stimulated when plasma E₂ is high [27]. Therefore, in the condition of low estrogen concentration or in ovariectomized rats, 5HT would have inhibitory action on LH secretion and MBH would be the site or one of the sites for such action. However, it would have no action in the MPOA at least on 5HT₁ or 5HT₂ receptors, whose inactivation did not alter the plasma LH.

On the other hand, the treatment with ovarian steroids would make the MPOA a site for the stimulatory action of 5HT for LH secretion and the inactivation of 5HT₁ or 5HT₂ receptors would cause the reduction of LH secretion induced by ovarian steroids, as shown by our results.

The following questions then arises: what factors would make MPOA a site for the stimulatory action of 5HT on LH secretion and how would they be modulated by gonadal steroids? Among such factors, there may be: density of receptors; 5HT metabolism and the activity of 5HT neurons in the brain stem.

According to some authors, E₂ can reverse the action of 5HT in reproduction, changing it from inhibitory to stimulatory by changing the density of 5HT receptors [4]. It was shown that E₂ has a diphasic effect on the density of 5HT receptors in the preoptic area of ovariectomized rats, that is, a decrease after 24 h of treatment and an increase after 48–72 h [5]. It was also shown that the ovulatory peak of LH induced by E₂ as well as ovulation depends on 5HT receptors [45]. Ketanserin blocks the peak of LH [11] and of LH-RH [13] on proestrus and in ovariectomized rats treated
with estrogen. The use of $^3$H-ketanserin showed that there is an increase in the density of 5HT$_2$ receptors in the pre-optic area of ovariectomized rats treated with estrogen and progesterone [16].

Another alternative action of E$_2$ would be to alter the metabolism of 5HT in the MPOA or to modulate the amount of 5HT produced by the serotoninergic neurons of the brain stem. However, it was shown that the turnover of 5HT is not altered in the preoptic area [8,21] during the peak of LH induced by E$_2$. On the other hand, the increase in the synthesis of 5HT in the dorsal raphe nucleus (DRN) induces the peak of LH in ovariectomized rats treated with estrogen or estrogen plus progesterone [39] while the destruction of the 5HT terminals in the preoptic area—stria terminal region by neurotoxin 5,7-DHT in ovariectomized rats treated with estrogen or estrogen plus progesterone [21] or a lesion in the DRN on the proestrus morning blocks LH secretion [31]. Little is still known with regard to the direct or indirect action of E$_2$ and P$_4$ in raphe nuclei [33].

Initially, due to the insufficiency of estrogen receptor (later called estrogen receptor alpha) in 5HT neurons of the DRN, a possible indirect action by such steroid mediated through the locus coeruleus (LC) was suggested [39]. The peak of LH secretion depends on the integrity of both nuclei, LC [2,14], and DRN [31]. Recent work studied the effect of the desipramine, a NA re-uptake inhibitor, on the rate of serotonin synthesis in the rat brain. It showed that serotonin synthesis could increase in the terminals and it could decrease in the soma of these neurons as consequence of a direct increase of NA [40]. Therefore, the LC, a noradrenergic neurons group, could be modulating the serotoninergic neurons to control of LH secretion. Another form of indirect action would be in neurons with receptors for estrogen (alpha type) and progesterone, which are neighboring of 5HT neurons in the DRN [1]. Recently, direct estrogen action on 5HT neurons of the DRN was suggested due to the identification of beta estrogen receptors in such neurons that project to the MPOA [30].

In ovariectomized rats treated with estrogen, the three doses of methiothepin or ketanserin used reduced the peak of LH secretion (Fig. 3). However, in ovariectomized rats treated with estrogen plus progesterone, the reduction of the peak of LH secretion was more intense with the highest dose of methiothepin and with the lowest dose of ketanserin. The serotoninergic system is very sensitive to progesterone. During the estrous cycle E$_2$ and P$_4$ act synergistically by increasing the fluctuation in the activity of 5HT in the hypothalamus and integrating them with the neural signal for the peak of LH secretion [46,48]. The amplifying of the LH peak by progesterone in ovariectomized rats treated with estrogen is eliminated by 5HT antagonists and restored by 5HT agonists [15]. Our data are in accordance with those concerning the interaction of progesterone and serotonin for LH secretion and, furthermore, they indicate a possible differentiation of the activity of 5HT$_1$ and 5HT$_2$ receptors in the MPOA mediated by progesterone. The action of proges-
Serotonin may take place by altering the affinity of the neuro-
mediator by different receptors since other researchers [49]
showed that it does not alter the increase in the density of
receptors induced by estrogen.

In this work, we also observed that the decrease in the
plasma LH after the microinjection of methiothepin or ke-
tanserin in the MPOA is followed by the reduction of ac-
tivity in the NOS of the MPOA. Correlation does not mean
causality. However, it was shown that the release of NO in
the MPOA presents circadian rhythm with longer release in
the afternoon than in the morning. Ovarian steroids not only
increase and anticipate the time of occurrence of the peak of
NO production in the MPOA, but also synchronize it with
the activation of neural systems that depend on estrogen for
the activation of the LH-RH/LH peak [41]. Therefore, the
stimulatory action of 5HT on 5HT 1 and 5HT 2 receptors, in
their turn, stimulate LH-RH neurons in the MPOA indicate that 5HT can act directly or indirectly on
NO neurons, which, in their turn, stimulate LH-RH neurons
in the MPOA for LH secretion may be at least partly medi-
ated by NO. The increase in plasma E2 and P4 during the
estrous cycle would inhibit the inhibitory components of
LH-RH neurons (such as opioid neurons) as well as acti-
activate the excitatory neurons that use glutamate, NO and neu-
ropetide Y as mediators [6]. Our results associated with the
non-localization of 5HT1 and 5HT2 receptors [49] or
coco-localization of NOS [3,17,20] in LH-RH neurons in the
MPOA indicate that 5HT can act directly or indirectly on
NO neurons, which, in their turn, stimulate LH-RH neurons
as previously proposed [6,43].

In summary, this work showed that in the studied condi-
tions, serotonin in the MPOA: (1) does not work in the con-
tral of PRL secretion through 5HT2 and 5HT3 receptors; (2)
integrates the control of FSH secretion through 5HT2 recep-
tors, but not 5HT1; (3) in the presence of estrogen, stimulates
LH secretion through 5HT1 and 5HT2 receptors, which can be
differentially modulated by progesterone; (4) at least partly,
stimulates LH secretion by nitric oxide activity.

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