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Ovaric physiology in the first oestral cycle: Influence of noradrenergic and cholinergic neural stimuli from coeliac ganglion

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ABSTRACT

Both peripheral innervation and nitric oxide (NO) participate in ovarian steroidogenesis. The aims of the work were (1) to investigate whether ganglionic noradrenergic (NE) and cholinergic (Ach) stimulus modify the ovarian steroids and NO release and (2) to examine the effect of those stimuli on the mRNA expression of 3β-HSD and P450 aromatase in the ovary. The experiments were carried out using the *ex vivo* coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system of rats in the first oestral cycle. The system was incubated in a buffer solution for 120 min, with the ganglion and ovary located in different compartments and linked by the SON. NE and Ach were added into the ganglion compartment. Both NE and Ach predominantly induced ovarian release of androstenedione and oestradiol while inhibited progesterone release. Ovarian NO release increased after ganglionic stimulation during proestrous while its secretion decreased during the diestrous. Noteworthily, 3β-HSD and P450 aromatase expression were modulated by neural stimulation. In the follicular phase, Ach in CG increased 3β-HSD and NE increased P450 aromatase. In the luteal phase both neurotransmitters increased 3β-HSD and Ach increased P450 aromatase transcript levels. All above observations suggest that the preponderancy of an either noradrenergic or cholinergic effect would depend on the stage of the first oestral cycle in the rat. The ovarian response to noradrenergic and cholinergic stimuli on GC, via SON, is strongly linked to oestral-stage-specific ovarian structures and their secretion products.

1. Introduction

In 1973, Ramirez [1] suggested the first categorization of puberal phases in rat based on physiological parameters. This categorization was later modified by Ojeda et al. (1980) [2], with the purpose of including morphological criteria.

The juvenile period is thus considered to extend from day 22 to days 30–32, when the pulsatile release of LH begins [3], and the peripuberal period extends to the first ovulation, approximately on day 38 of age. Research carried out by Ojeda et al. (1985, 1986) [4,5] has demonstrated that this stage of ovarian development is regulated by a complex neuroendocrine mechanism. The presence of VIP and other neurotransmitters in the fetal ovarian nerves [6] stimulate the production of AMPc and induce the activity of aromatase, before the ovary is able to respond to FSH [7].

On the other hand, the sympathetic innervation of the ovary occurs before the primordial follicles start to develop [8].

These observations support the hypothesis that even though the ovary is controlled by endocrine and neural factors [9–11], the beginning of follicular growth is also influenced by neurotransmitters that reach the ovary by two routes: the ovarian plexus, which innervates the blood vessels musculature, and the superior ovarian nerve (SON), which is the most relevant for ovarian steroidogenesis [12]. The SON is mainly constituted by catecholaminergic fibers, most of which originate in the coeliac ganglion [13]. In the ovary, these fibers form a net of varicosities surrounding the theca and secondary interstitial cells, which are responsible for androgens synthesis, without entering the follicle and with little visible relation with the corpus luteum [14].

The coeliac ganglion is included in the sympathetic prevertebral chain, possesses a great variety of specific receptors and neurotransmitters, among them catecholamines [15], neuropeptides [16], nitric oxide (a gaseous neurotransmitter) [17] and

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acetylcholine, which is considered as the classic preganglionar neurotransmitter of the sympathetic ganglionic pathway [18,19].

The existence of an interaction between the parasympathetic and sympathetic nerves in prevertebral ganglia is well known, providing evidence of a vagal innervation of the ganglion cells in rat. This vagal contact may either directly modulate the postganglionic outflow or else gate some or all the potential modulatory inputs to these postganglionic neurons, thus allowing the vagal system to exert a more selective influence on sympathetic outflow. These vagal projections form varicose terminal-like structures, highly suggestive of synaptic contacts surrounding individual ganglion cells, thus demonstrating the presence of cholinergic receptors in the ganglion [19,20].

Experiments carried out in our laboratory have shown that coeliac ganglion stimulus with cholinergic and noradrenergic agents modify the ovarian steroids release through the superior ovarian nerve [10,21–23]. This may provide physiological evidence of the participation, at least in part, of the sympathetic ganglionic pathway in the response observed. Nitric oxide has been shown to participate in numerous physiological processes, which are essential for maintaining homeostasis [24,25] and is considered to be a neurotransmitter and biological mediator of the neuroendocrine axis [26,27]. Using the oestrous ganglion–superior ovarian nerve–ovary system (CG–SON–O) Delgado et al. (2004) and Casais et al. (2007) [23,28] concluded that in the prepuberal stage the neural effect on ovarian steroidogenesis was partly mediated by nitric oxide of both ovarian and ganglionic origin, based on the fact that the different isoforms of the nitric oxide synthesis enzyme have been localized in both structures. However, there is no information about the role played by the neural factors in NO release in the first oestral cycle in rat.

Therefore, the aims of this work are: (1) to investigate whether in the CG–SON–ovary system the ganglionic noradrenergic and cholinergic stimulus modifies via SON the release of steroids and nitric oxide in the ovary compartment in the absence of humoral factors and (2) to determine whether the genic expression of 3β-HSD, the synthesis enzyme of progesterone (P), and the P450 aromatase enzyme, which plays a key role in the conversion of androstenedione (A_2) to oestradiol (E_2) for the continuity of the oestral cycle, are modified at short times (120 min) by neural influence.

2. Materials and methods

2.1. Animals

Female 37 days old virgin Holtzman rats, 70 ± 10 g body weight in their first oestral cycle, were used in all the experiments. Once vaginal opening was evident, estrous cycle was monitored by vaginal smear cytology. Females in proestrous, estrous and diestrous were selected and maintained in our animal facilities under controlled conditions, with lights on from 07:00 to 19:00 h and at a temperature of 24 ± 2 °C. Animals had free access to food (rat chow Cargill SACI, Saladillo, Buenos Aires, Argentina), and tap water. Groups of six animals were used for the experimental procedure.

The experiments were performed in accordance with the UFAW Handbook on file Care and Management of Laboratory Animals, the experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number protocol: B17/04, ordinance CD 006/02).

2.2. Reagents

The following drugs: L-D-norepinephrine hydrochloride (NE), L-acetylcholine hydrochloride (Ach), dextrose, ascorbic acid, bovine serum albumin fraction V (BSA), sulfanilamide and N-1-naphthylethylenediamine were provided by the Sigma Chemical Co (St. Louis, MO, USA). 1,2,6,7-[^{3}H]-Progesterone (107.0 Ci/mmol), 1,2,6,7-[^{3}H]-androst(4-ene-3,17)dione (115.0 Ci/mmol) and 17β-2,4,6,7-[^{3}H]oestradiol (102.0 Ci/mmol) were provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

2.3. Experimental procedure

The surgical procedure used for removing the system and its characterization were performed as described previously [23]. Briefly, a piece of tissue containing the left ovary, the fibres constituting the superior ovarian nerve and the coeliac ganglion was removed. The left system was used because it has been demonstrated that this ovary is more sensitive to neural stimulus [29]. The strip of tissue was carefully dissected avoiding contact between the surgical instruments and the nerve fibres or the ganglion in order to prevent spontaneous depolarization of file nerves. The total surgical procedure was completed in 1–2 min. The coeliac ganglion–superior ovarian nerve–ovary (GC–SON–O) system was washed with the incubation medium and immediately placed in the cuvette consisting of two compartments.

Each compartment contained 1 ml of Krebs–Ringer bicarbonate buffer, pH 7.4 solution in the presence of dextrose (0.1 mg/ml) and BSA (0.1 mg/ml). The ganglion was placed in a compartment and the ovary, in the other one, both joined by the SON which had to remain humid with the work solution. The system was stabilized by a preincubation in metabolic bath at 37 °C for 15 min in an atmosphere composed by 95% O_2 and 5% CO_2. The end of the preincubation period was considered as incubation time 0. At this time, the buffer was changed in both compartments; ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent to the ganglion compartment (control group).

The experimental groups included: (a) CG–SON–O system with the addition of noradrenergic agent L-D-norepinephrine hydrochloride (NE 10^{-6} M) in the ganglion compartment, dissolved in 1 ml Krebs–Ringer plus ascorbic solution (NE group); (b) CG–SON–O system with the addition of cholinergic agent L-acetylcholine hydrochloride (Ach 10^{-6} M) in the ganglion compartment, dissolved in 1 ml Krebs–Ringer plus ascorbic solution (Ach group).

Two hundred and fifty (250) microliter samples were collected from the ovarian compartment at 15, 30, 60 and 120 min from the beginning of the incubation and were kept at −20 °C until progesterone, androstenedione and oestradiol were determined by radioimmunoassay (RIA) and nitrite a water soluble metabolite of nitric oxide was measured by Griess method. The respective corrections were made in all cases considering the volume in each period tested.

At the end of the total incubation period, the ovary was isolated and frozen at −80 °C 3β-HSD and aromatase expression were assessed.

2.4. Progesterone, androstenedione and oestradiol assay

Steroids were measured in duplicate by RIA. The antisera were kindly provided by Dr RP Deis (Laboratorio de Reproducción y Lactancia Mendoza, Argentina). Progesterone was measured in nanogram per milligram ovary per ml and assay sensitivity was <5 ng progesterone/ml. The technique sensitivity was measured in serum although in our laboratory the corresponding assays were carried out in our working solution resulting in the same sensitivity. Androstenedione and oestradiol were expressed as picograms per milligram of ovarian tissue per ml (picogram/milligram ovary per ml), all against incubation time on the left system. The assay sensitivity was <10 pg androstenedione/ml and <2.2 pg oestradiol/ml.

The inter- and intra-assay coefficients of variation in all the assays were <10.0%.

2.5. Nitrite assay

Levels of nitrites were measured spectrophotometrically [30]. Samples were immediately mixed with Griess reagent (sulfanilamide with N-1-naphthyl-ethylenediamine/HCl). After a 10-min incubation period at room temperature, they were read for absorbance of 540 nm. The assay sensitivity was <2.5 nmol/ml. The intra-assay coefficients of variation for all the assays were less than 10.0%. The results were expressed as nanomol of nitrite per milligram of ovarian tissue (nmol/mg ovary).

2.6. RNA isolation and RT-PCR real times analysis

Total RNA was isolated using TRIZOL reagent (Invitrogen Life Technologies), according to the manufacturer's instructions. Gel electrophoresis and ethidium bromide staining confirmed the integrity of the samples. Quantification of RNA was based on spectrophotometric analysis at 260 nm. Two micrograms of total RNA were reverse transcribed with 200 units of MMLV Reverse Transcriptase (Promega Inc.) using random primer hexamers in a 25-μl reaction mixture following manufacturer's instructions. Fragments coding for 3β-hydroxysteroid-dehydrogenase (3β-HSD), Aromatase and β-actin (as endogenous control) were amplified by PCR with specific primers for 3β-HSD (5′-GTCTTCAGACCAGAAACCAAG-3′ and 5′-CCTTAAGGCACAAGTATGCAG-3′), P450 aromatase (5′-TGCACAGGCTCGAGTATTTCC-3′ and 5′-ATTTCCACAATGGGGG-CTGTCC-3′), β-actin (5′-CGGAACCGCTCATTGCC-3′ and 5′-ACCC-ACACTGTGCCCATCTA-3′) in a reaction mixture consisted of 5× Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers and 1.25 IU Go Taq DNA polymerase (Promega Inc.) and RT-generated cDNA (1/5 of RT reaction) in a 50-μl final reaction volume.

2.7. Statistical analysis

Results are presented as mean ± SEM of six animals per experimental group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of variance (ANOVA I) followed by Duncan's test multiple range test was used for several comparisons. A difference was considered to be statistically significant at $p < 0.05$ [31].

3. Results

3.1. Effect of NE and Ach ganglionic stimulation on the ovarian steroids and NO release in the ex vivo coeliac ganglion–superior ovarian nerve–ovary system

3.1.1. On the first proestrus day

The addition of NE 10^{-6} M into the ganglion compartment of the CG–SON–O *ex vivo* system isolated on the first proestrous day decreased significantly the P and E_2 release at 15 min ($p < 0.001$ and $p < 0.01$, respectively) and 30 min ($p < 0.05$ and $p < 0.001$) of noradrenergic stimulation (Fig. 1a and c). On the contrary, P release was higher than in controls at 60 ($p < 0.01$) and 120 min ($p < 0.001$) while nitrites levels increased earlier and kept higher at 15, 30 ($p < 0.001$), 60 ($p < 0.01$) and 120 min ($p < 0.001$) of NE ganglionic stimulation, in comparison to controls (Fig. 1a and d). No significant effect was observed on ovarian A_2 liberation at 15 min, while A_2 release increased at 30 ($p < 0.01$), 60 ($p < 0.01$) and 120 min ($p < 0.001$) after the ganglionic noradrenergic stimulation (Fig. 1b).

On the other hand, addition of Ach 10^{-6} M into the ganglion compartment of the CG–SON–O *ex vivo* system isolated on the first proestrous day, significantly decreased the ovarian progesterone release at 15, 30 and 60 min ($p < 0.001$), while increased A_2 release into the ovary compartment at 15, 30 and 120 min ($p < 0.001$) of ganglionic stimulation, in comparison to the control group (Fig. 1a and b). Ovarian liberation of E_2 and nitrites increased at 15, 30, 60 and 120 min ($p < 0.001$) of ganglionic cholinergic stimulation (Fig. 1c and d).

3.1.2. On the first oestrous day

The addition of NE 10^{-6} M into the ganglionic compartment of the *ex vivo* system isolated on the first oestrous day, significantly increased the ovarian P release at 15 ($p < 0.001$), 30 ($p < 0.01$) and 120 min ($p < 0.001$) of ganglionic stimulation, while it increased A_2 release at 15, 60, 120 ($p < 0.001$) and 30 min ($p < 0.01$) and nitrites liberation only at 15 min ($p < 0.01$), compared to the control group (Fig. 2a, b and d, respectively).

Ganglionic stimulation with Ach decreased the ovarian P liberation at 30 and 60 min of Ach addition, compared with the control group ($p < 0.001$), but it increased A_2 release at 15, 60 and 120 ($p < 0.001$) and 30 min ($p < 0.005$) and E_2 release at 15, 60 ($p < 0.001$) and 30 and 120 min ($p < 0.005$) of cholinergic stimulation (Fig. 2a–c). Ach also induced the nitrites liberation into the ovarian compartment at 15 ($p < 0.001$) and 30 min ($p < 0.01$) of cholinergic addition, compared with the control group (Fig. 2d).

3.1.3. On the first diestrous day

The addition of NE 10^{-6} M into the ganglionic compartment of the *ex vivo* system isolated on the first diestrous day, decreased the ovarian P release at 30 ($p < 0.001$) but increased it at 60 ($p < 0.01$) and 120 min ($p < 0.001$) of noradrenergic stimulation in comparison to the control group (Fig. 3a). A_2 and E_2 release into the ovarian compartment significantly increased at 15, 30, 60 and 120 min ($p < 0.001$) (Fig. 3b and c) and nitrites release decreased at 15, 60 and 120 min ($p < 0.001$) and at 60 min ($p < 0.01$) of ganglionic NE addition (Fig. 3d).

A significant decrease in the ovarian P release was observed at 30 and 120 min ($p < 0.001$) and A_2 at 15 and 120 min ($p < 0.001$), of Ach addition into the ganglion compartment. Inversely, P and A_2 release increased at 120 ($p < 0.001$) and 30 min ($p < 0.001$), respectively, of cholinergic stimulation on the first diestrous day, in relation to the control group (Fig. 3a and b). Ach also caused a significant increase in the ovarian liberation of E_2 at 15 and 30 min ($p < 0.01$), but decreased nitrites liberation into the ovary compartment at all the studied times, in relation to the control group at 15 and 120 ($p < 0.001$), 30 and 60 min ($p < 0.01$) (Fig. 3c and d).

3.2. Effect of NE and Ach 10^{-6} M ganglionic stimulation on the ovarian 3β-HSD expression

3β-HSD mRNA expression in the control group was significantly increased in the first proestrous ($p < 0.001$) (Fig. 4a).

3β-HSD mRNA expression was significantly increased in the rat ovary after ganglionic cholinergic stimulation on the first proestrous day, in comparison to the control group ($p < 0.001$, Fig. 4b). 3β-HSD transcript levels were also significantly decreased after ganglionic cholinergic stimulation on the first estrous ($p < 0.001$, Fig. 4c). 3β-HSD mRNA expression was significantly increased after noradrenergic and cholinergic stimulation on the first diestrous ($p < 0.001$) day, in relation to the control group (Fig. 4d).

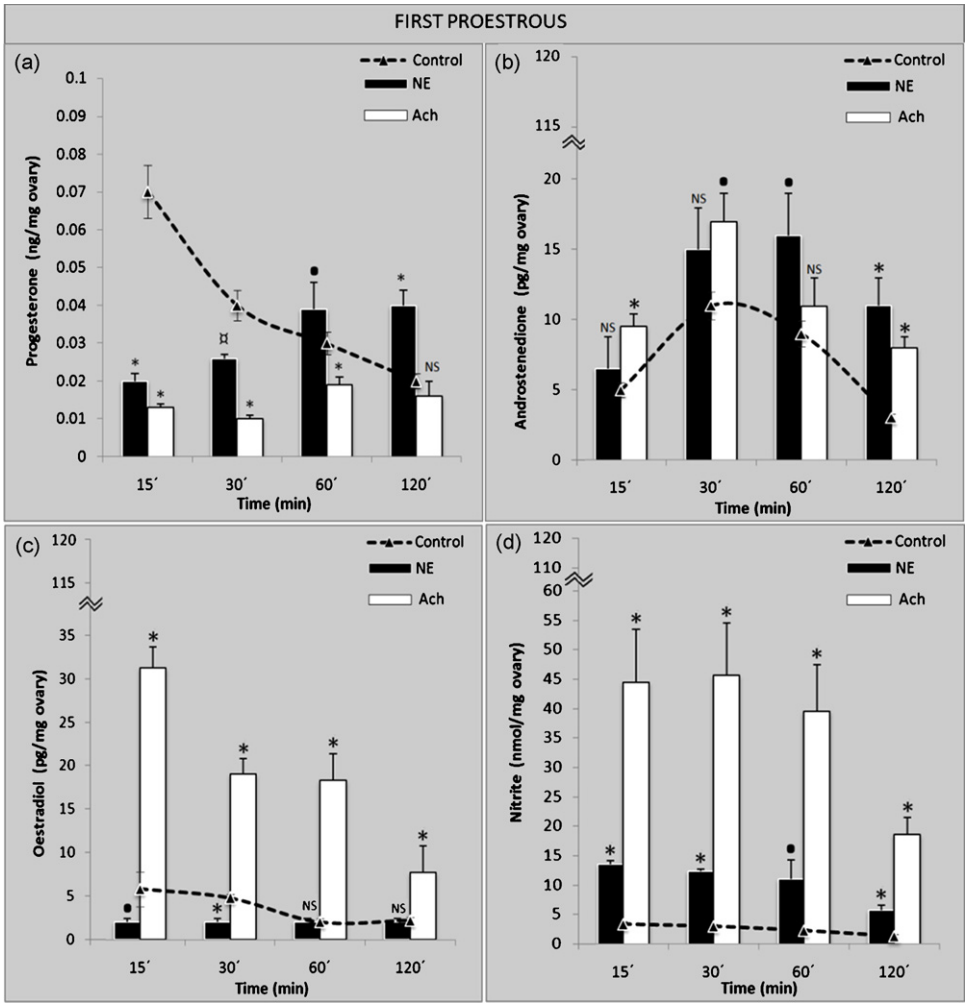

Fig. 1. Ganglionic effect of NE and Ach 10^{-6} M in the coeliac ganglion–superior ovarian nerve–ovary system on the ovarian: (a) progesterone release expressed as ng/mg ovary; (b) androstenedione release expressed as pg/mg ovary; (c) oestradiol release expressed as pg/mg ovary; (d) nitrites release expressed in nanomol/mg ovary in the first proestrous rat. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2. Values are the mean ± SEM of six animals per experimental group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of variance (ANOVA I) followed by Duncan's test multiple range test was used for several comparisons: (a) $^{*}p < 0.001$, $^{\bullet}p < 0.01$, $^{\square}p < 0.05$; (b) $^{*}p < 0.001$, $^{\bullet}p < 0.01$; (c) $^{*}p < 0.001$, $^{\bullet}p < 0.01$; (d) $^{*}p < 0.001$, $^{\bullet}p < 0.01$.

3.3. Effect of NE and Ach 10^{-6} M ganglionic stimulation on the ovarian aromatase expression

Aromatase mRNA expression in the control group was significantly increased in the first proestrous and estrous in relation to the first diestrous ($p < 0.001$) (Fig. 5a).

Aromatase mRNA expression was significantly increased in the rat ovary after ganglionic noradrenergic stimulation on the first proestrous day, in comparison to the control group ($p < 0.005$, Fig. 5b). Aromatase transcript levels were also significantly increased after ganglionic cholinergic stimulation on the first estrous ($p < 0.001$) and the first diestrous ($p < 0.001$) day, in relation to the control group (Fig. 5c and d).

4. Discussion

Ovarian sympathetic innervation is closely related to steroidogenesis, folliculogenesis and corpus luteum development and regression in various species [32,33]. This innervation not only includes the neural components that enter the ovary, as is the case of the SON and the ovarian nervous plexus, but also intermediate structures such as CG and the superior mesenteric ganglion, which are capable of receiving and integrating signals coming from the central nervous system and organizing responses that influence ovarian physiology [21,34]. Numerous studies have demonstrated that the peripheral nervous system influences the different evolution stages in rat [11,21,23,34]. However, there is little information on the action of ovarian innervation in rat during the first oestral cycle. Aguado et al. (1982) [35] demonstrated that before the first ovulation, the immature ovary of the rat already contains specific beta-adrenergic receptors located in both the granulosa cells of the growing follicles and the residual ovary and they participate in the physiological events that occur in stages close to the first ovulation. They observed that the content of beta receptors increases significantly between the anestrous and the morning of late proestrous, decreases abruptly during the pre-ovulatory peak of LH, and remains low in oestrous and highly increased in diestrous. On the other hand, Ricu et al. (2008) [36] found that although the nerves can already incorporate NE after birth, the capacity of intra-ovarian nerve terminals does not fully develop until near the time of puberty.

Considering that NE has steroidogenic activity [37,38] these results suggest that an enhanced activity of catecholaminergic ovarian nerves may contribute to the increase in ovarian responsiveness to gonadotropins that occur at puberty [39].

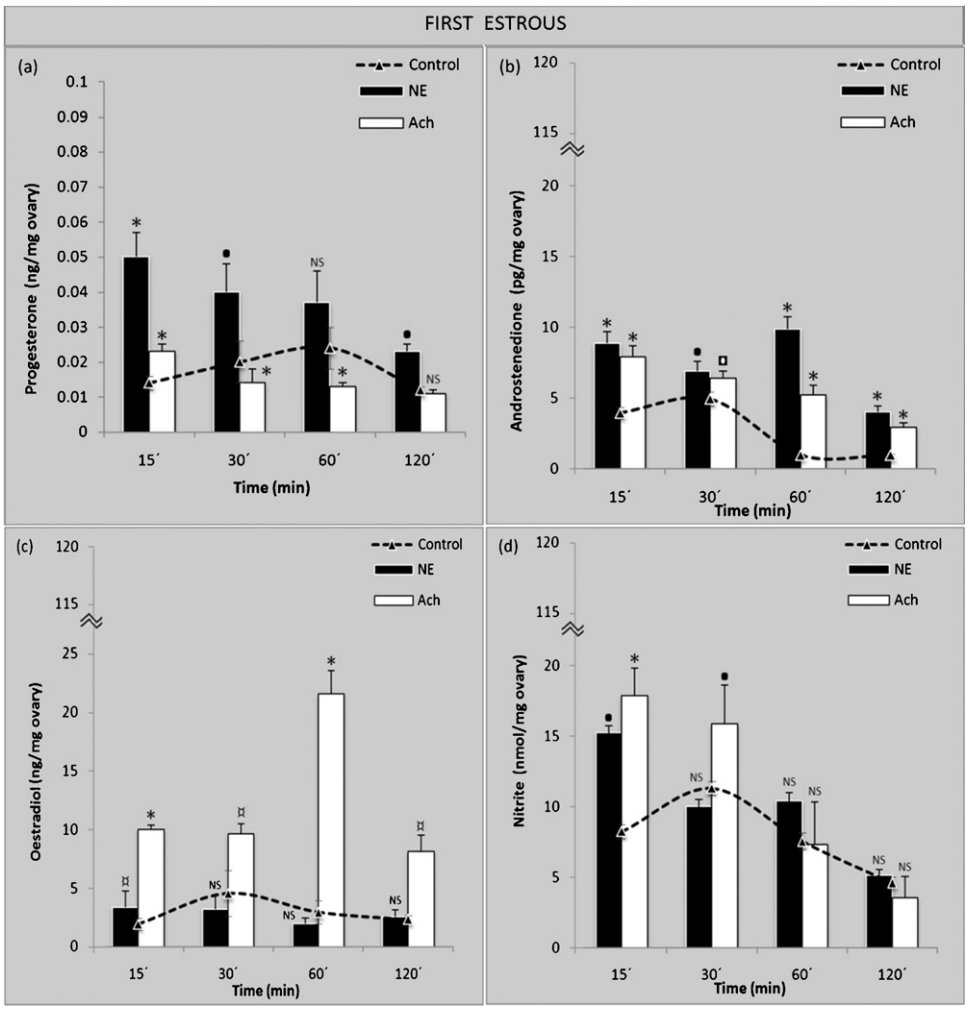

Fig. 2. Ganglionic effect of NE and Ach 10^{-6} M in the coeliac ganglion–superior ovarian nerve–ovary system on the ovarian: (a) progesterone release expressed as ng/mg ovary; (b) androstenedione release expressed as pg/mg ovary; (c) oestradiol release expressed as pg/mg ovary; (d) nitrites release expressed in nanomol/mg ovary in the first estrous rat. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2. Values are the mean ± SEM of six animals per experimental group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of variance (ANOVA I) followed by Duncan's test multiple range test was used for several comparisons: (a) $^{*}p < 0.001$, $^{\bullet}p < 0.01$; (b) $^{*}p < 0.001$, $^{\bullet}p < 0.01$, $^{\square}p < 0.005$; (c) $^{*}p < 0.001$. $^{\square}p < 0.05$; (d) $^{*}p < 0.001$, $^{\bullet}p < 0.01$.

The purpose of this work was to study the effect of catecholaminergic and cholinergic stimulus in CG and the possible participation of NO, a gaseous neurotransmitter related with steroids synthesis [40–42] on ovarian steroidogenesis during the first oestral cycle. With this aim, we investigated possible modifications in the ovarian release of P, A_2, E_2 and NO and we analyzed whether they lead to modulation of the mRNA expression of the 3β-HSD and P450 aromatase enzymes in ovary in a relatively short time (120 min). We used the *ex vivo* CG–SON–O system, previously standardized in prepuberal rats [23], which permits to emulate *in vivo* conditions, preserving the autocrine and paracrine mechanisms that occur in the ovary, without the humoral influence.

During the first proestrous, P release was inhibited by both neurotransmitters, an effect similar to that observed by Delgado et al. (2004, 2006) [23,43] in prepuberal rats using the same system. It is evident that the neurohormonal microenvironment in the ovary in the first proestrous still exhibits similarities with the prepuberal stage. The effect observed may be partly due to the fact that the ovarian noradrenergic innervation is biochemically and functionally mature only close to the onset of puberty [44].

Both neurotransmitteres stimulated ovarian release of A_2 at almost all the studied times. However, in the prepuberal stage [23,43] A_2 ovarian release was stimulated by NE but inhibited by Ach. This androgen, of major importance in rat, has a proven *per se* effect [45] and performs different actions in the ovarian physiology: as a substrate for estrogens synthesis or, through androgen receptor, as a modulator of the ovarian function, interacting with various factors to enhance granulosa cell differentiation [46–48]. However, it has also been shown that this androgen antagonizes follicular development [49–51] and causes apoptosis in granulosa cells [52]. According to Tetsuka and Hillier (1996) [53] a down-regulation of androgen receptors mRNA expression takes place in granulosa cells of pre-ovulatory follicles. Therefore, in proestrous, A_2 might act basically as a substrate for estrogens synthesis, because the presence of high levels of androgen receptors in pre-ovulatory follicles might inhibit estrogen synthesis and cause follicular atresia [51]. Our results indicate that neural stimulus via SON might favor the ovulatory process.

As regards the liberation of E_2, Ach caused a clearly stimulatory effect at all the studied times, indicating a higher sensitivity of this steroid to neural cholinergic stimulus from the coeliac ganglion to the ovary, which is the classical connection pathway between the central nervous system and the ovary.

The levels of NO increased by effect of both noradrenergic and cholinergic stimuli, which agrees with results obtained in prepuberal rats [23,43]. The increase of NO by ganglionic noradrenergic

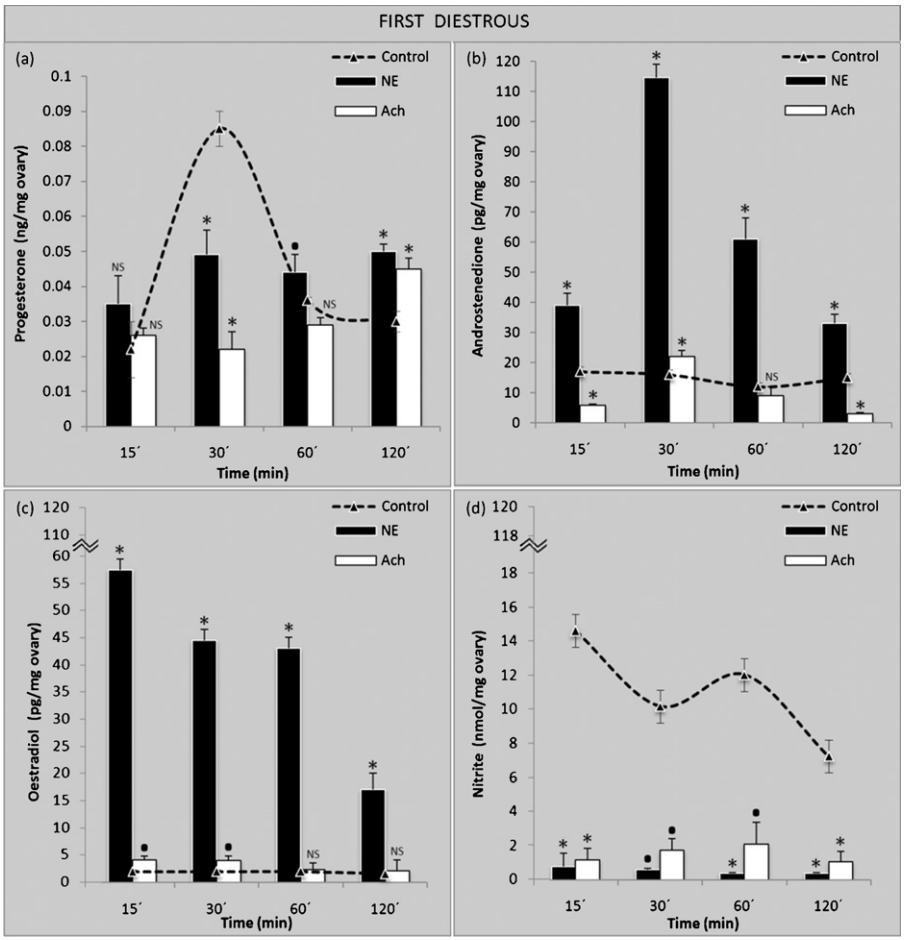

Fig. 3. Ganglionic effect of NE and Ach 10^{-6} M in the coeliac ganglion–superior ovarian nerve–ovary system on the ovarian: (a) progesterone release expressed as ng/mg ovary; (b) androstenedione release expressed as pg/mg ovary; (c) oestradiol release expressed as pg/mg ovary; (d) nitrites release expressed in nanomol/mg ovary in the first diestrous rat. The system was incubated in Krebs–Ringer solution, at 37 °C in an atmosphere of 95% O_2–5% CO_2. Values are the mean ± SEM of six animals per experimental group. Student's *t*-test was used to assay significant differences between means of two groups. Analysis of variance (ANOVA I) followed by Duncan's test multiple range test was used for several comparisons: (a) $^*p < 0.001$, $^\bullet p < 0.01$; (b) $^*p < 0.001$; (c) $^*p < 0.001$, $^\bullet p < 0.01$; (d) $^*p < 0.001$, $^\bullet p < 0.01$.

and cholinergic stimulus probably leads to an increase of blood flow thus favoring the process of ovulation and luteinization and increasing the level of E_2, which is necessary for the beginning of the oestral cycle. It must be noted that the increase of NO release by cholinergic stimulus was greater than that produced by noradrenergic stimulus at all incubation times ($p < 0.001$), which accounts for the fact that E_2 release was only increased by Ach, indicating a positive correlation between NO and E_2. The results obtained in proestrous indicate that the inhibitory effect on P release might be related with the increase of NO and agrees with the fact that P is the most sensitive steroid to neural action [35].

The oestrous stage is characterized by structural reordering and organization, two processes involved in the corpus luteum formation. In this stage it was observed that NE in ganglion provoked an increase in the release of P and A_2, while Ach increased A_2 and E_2 release and had a biphasic effect on P. These results, in which innervation is maintained and steroidogenesis is increased by neural stimulus, match reports by Kotwica and Bogachi (1999) [32], who found that ovarian denervation decreases steroidogenic activity in the corpus luteum. As regards NO, it was increased at short times by neural effect, the increase caused by Ach being more relevant. It must be noted that the greater stimulatory effect with respect to E_2 is also of cholinergic origin, indicating a positive correlation between NO and E_2. Thus, it is probable that both favor the luteinization and maturation of the newly formed corpus luteum. In agreement with our results, an increase in oestradiol release has been observed in adult rats in oestrous [29], with NO increase at short incubation times by cholinergic stimulus in the superior mesenteric ganglion, via the ovarian nervous plexus.

In the diestrous stage, the corpus luteum is functionally active and, at the same time, the new follicles destined to ovulate in the following oestral cycle are developing. This is reflected in the higher control levels of the ovarian steroid hormones P and especially A_2 as compared to oestrous. In relation to this, Weiss et al. (1982) [54] have postulated for the diestrous stage the existence of marked folliculogenesis and activity of both thecal–interstitial cells, responsible for androgens synthesis, and granulosa cells, which, together with luteal cells, are responsible for P synthesis. These authors also argue that the small P peak independent of hormonal action described by Uchida et al. (1969) [55] for that stage is probably of neural origin, being NE the main neurotransmitter involved.

In the present work, the noradrenergic ganglionic effect on P release was stimulatory at long incubation times, which does not match previous observations for the prepuberal stage [43]. The fact that the fully active corpora lutea possess beta-adrenergic receptors [35] and that catecholamines have an impact on these receptors [56] may account for our results. The most important finding is that obtained under ganglionic cholinergic effect, where the *input* produced by Ach was inhibitory at short times, in spite of the full corpora lutea functionality and of the fact that P is the main secretion product. This result matches previous results obtained for the prepuberal stage [23]. Sosa et al. (2004) [22] demonstrated

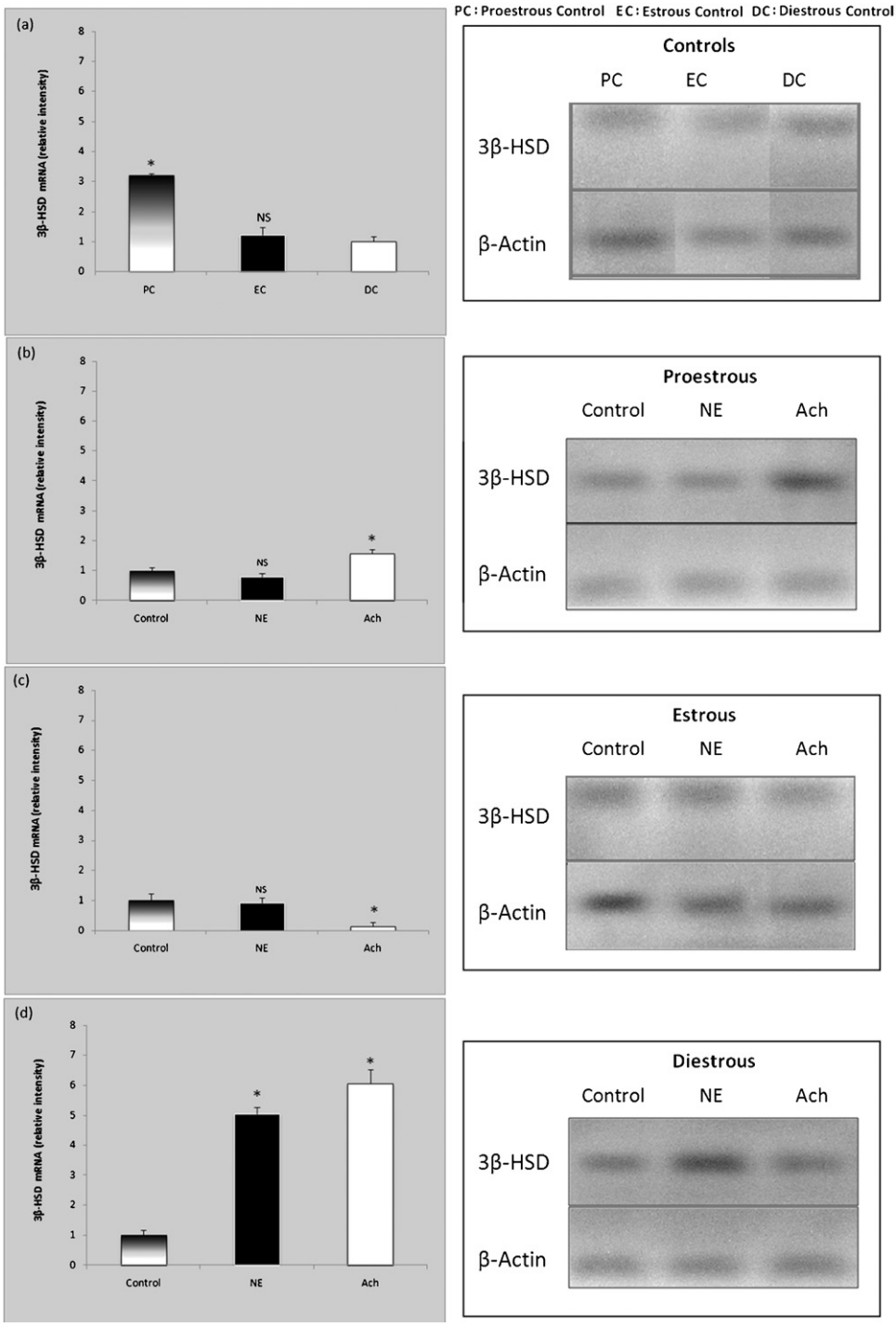

Fig. 4. (a) Expression of 3β-HSD mRNA and β-actin on the first proestrus, estrous and diestrous days, respectively, $^{*}p < 0.001$. (b) Ganglionic effect of NE and Ach 10^{-6} M in 3β-HSD mRNA and β-actin expression on the first proestrous day, respectively, $^{*}p < 0.001$. (c) Ganglionic effect of NE and Ach 10^{-6} M in 3β-HSD mRNA and β-actin expression on the first estrous day, respectively, $^{*}p < 0.001$. (d) Ganglionic effect of NE and Ach 10^{-6} M in 3β-HSD mRNA and β-actin expression on the first diestrous day, respectively, $^{*}p < 0.001$. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products. The gel photographs were quantified using Image and expressed as arbitrary units. Results are expressed as mean ± SEM ($n = 3$).

on diestrous I in adult rat that the cholinergic stimulus in ganglion caused a significant decrease of NE release in the ovarian compartment, and attributed this decrease to the inhibitory effect of Ach in ganglion. This may indicate the importance of the sympathetic neural effect in ovarian steroidogenesis. The release of A_2 and E_2 was increased predominantly by noradrenergic stimulus, in agreement with findings by other researchers for the same stage of the oestral cycle [21,57,58], who conclude that NE in ovary might play a major role in the development of the follicles of the new oestral cycle, and thus promote its continuity. It must be noted that both NE and Ach in coeliac ganglion decreased the NO release and thus favor the mature corpus luteum development. On the other hand, Snyder et al. (1996) [59] have suggested that NO inhibits the expression of P450 aromatase, while Tetsuka and Hillier (1997) [60] have proposed that A_2 promotes the activity of this enzyme only in the early stages of follicular development via receptor and together

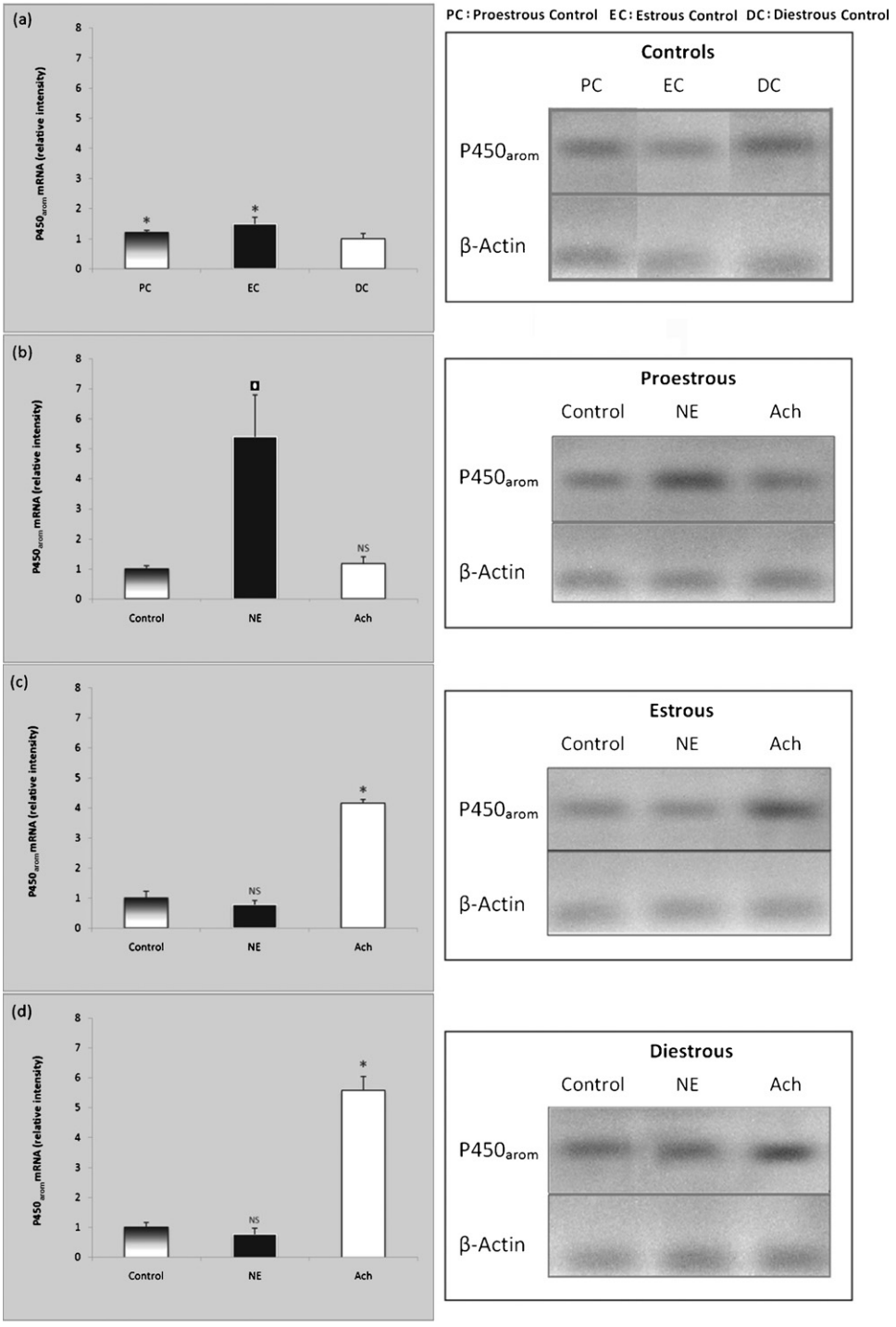

Fig. 5. (a) Expression of aromatase mRNA and β-actin on the first proestrus, estrous and diestrous days, respectively, $^{*}p < 0.001$. (b) Ganglionic effect of NE and Ach 10^{-6} M in aromatase mRNA and β-actin expression on the first proestrous day, respectively, $^{\square}p < 0.005$. (c) Ganglionic effect of NE and Ach 10^{-6} M in Aromatase mRNA and β-actin expression on the first estrous day, respectively, $^{*}p < 0.001$. (d) Ganglionic effect of NE and Ach 10^{-6} M in Aromatase mRNA and β-actin expression on the first diestrous day, respectively, $^{*}p < 0.001$. Ethidium bromide fluorescence photograph of the gel electrophoresis of the amplification products. The gel photographs were quantified using image and expressed as arbitrary units. Results are expressed as mean ± SEM ($n = 3$).

with FSH. In our case, the decrease of NO release by neural stimulus and the high basal levels of A_2 appear to favor the synthesis of E_2, as will be later discussed in relation with the increase of aromatase by cholinergic stimulus.

The expression (mRNA) of the synthesis enzyme 3β-HSD at 120 min of incubation was observed in the first three stages of the first oestral cycle under control conditions and was found to be higher in proestrous, matching the P release levels.

Even though the neural stimulation (NE and Ach in CG) predominantly decreased P release in the three stages of the first cycle, the expression of the enzyme was more sensitive to Ach. In proestrous, Ach stimulated P release; however, at 120 min, P release was not modified while NO liberation was significantly increased. The fact that P was not increased might be due to an effect of the increase of NO on the activity of 3β-HSD. During the oestrous stage, Ach inhibited the enzyme expression and caused a decrease

in P release, without a visible effect on NO liberation after 60 min. Undoubtedly, the diestrous stage is the most sensitive to the neural action as regards the expression of 3β-HSD. Both neurotransmitters stimulated 3β-HSD expression and strongly inhibited NO release throughout the studied time. This is probably why at 120 min the effect of NE and Ach on P was reversed and P increased. Our results resemble those obtained by Vega Orozco et al. (2010) [61], who worked with the GMS-PNO-ovary system during the oestral cycle in diestrous I and II. By ganglionar cholinergic stimulation, these researchers found an increase in the activity and genic expression of 3β-HSD at 120 min, in agreement with the increase of P, and with no changes in NO release.

As regards the expression (mRNA) of the P450 aromatase enzyme, it was found to be higher in proestrous and oestrous, matching results by other authors [62,63]. This also agrees with reports by Tetsuka and Hillier (1996) [53] who found an increase of the mRNA expression of P450 aromatase in the granulosa cells of bigger follicles (pre-ovulatory follicles) while in small follicles, which are present in the diestrous stage, the expression of this enzyme decreases.

In proestrous, and only by effect of NE in ganglion, the expression of this enzyme was markedly increased at 120 min. Undoubtedly, this finding indicates an important neural contribution to cycle continuity and is in agreement with Fitzpatrick and Richards (1991) [48] who observed that E_2 does not affect the expression of aromatase by itself, because there are no estrogen response elements in the enzyme gene promotor.

Ach, the conventional ganglionic neurotransmitter in this neural pathway, stimulated the expression of this enzyme in oestrous and diestrous, which matches the increased release of A_2 and E_2, highly marked in estrous and at short times in diestrous.

The major contribution of this work is that it constitutes the first demonstration, at least to our knowledge, that neural stimulus of a prevertebral ganglion (the coeliac ganglion) with both NE and Ach modifies via SON the mRNA expression of the 3β-HSD and P450 aromatase enzymes in an *ex vivo* system 120 min after stimulus. It is also noteworthy that 3β-HSD is basically more sensitive to Ach and to both neurotransmitters in the luteal phase. In turn, these neurotransmitters increase the expression of aromatase in different stages, since this enzyme exhibited sensitivity to noradrenergic stimulus in the phase of follicular development and to cholinergic stimulus in the luteal phase. The different responses are probably related with the marked difference in the neural effect on NO release between the two stages. This hypothesis will be the subject of future research.

As a whole, the results here reported demonstrate that in the first oestral cycle the response to neural stimulus strongly depends on the ovarian structures that temporarily predominate in each stage. Thus, some stages of the first oestral cycle markedly differ from the prepuberal stage, where important changes occur but only at the level of follicular maturation.

Conflict of interest statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of scientific work.

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