Norepinephrine modulates the effect of neuropeptides in coeliac ganglion on ovarian hormones release: Its relationship with ovarian nitric oxide and nerve growth factor

Marisa Hilda Garraza, Myriam Forneris, Laura Virginia Gatica, Liliana Beatriz Oliveros [1]

Laboratorio de Biología de la Reproducción (LABIR), Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis. San Luis, Argentina.

Correspondence to: Dr. Liliana Beatriz Oliveros
Laboratorio de Biología de la Reproducción
Universidad Nacional de San Luis
Chacabuco 917, (5700) San Luis, Argentina.
TEL: +54 2652 424689; FAX: +54 430224/431301; E-MAIL: lolive@unsl.edu.ar

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Abstract

OBJECTIVE: Ovarian steroids are modulated by neural influences. In this work we investigate whether norepinephrine (NE) modifies the vasoactive intestinal peptide (VIP) or neuropeptide Y (NPY) actions in coeliac ganglion (CG) on the ovarian hormone release, and evaluate the participation of nitric oxide (NO), measured as nitrite, and of inducible nitric oxide synthetase (iNOS) protein, nerve growth factor (NGF) and its trkA receptor gene expression in the ovarian response.

METHODS: The study was performed in the *ex vivo* CG-superior ovarian nerve (SON)-ovary system of rats on diestrus day 2 (D2). CG and ovary were placed in separate compartments connected by the SON and incubated with Krebs-Ringer buffer. After addition of 50 ng/ml VIP, 50 ng/ml NPY, 10^{-6} M NE, or a mix of VIP+NE or NPY+NE in ganglion, samples from the ovarian compartment were taken at different times throughout 180 minutes to measure progesterone, androstenedione and nitrite levels.

RESULTS: VIP and NPY in ganglion induced an increase of progesterone release that was associated for VIP, but not NPY, with a decrease of ovarian nitrite levels, iNOS protein, and NGF/trkA receptor mRNA expression. By contrast, NE in ganglion decreased progesterone, an effect that was suppressed by addition of propranolol in ganglion, and increased nitrites/iNOS and NGF/trkA receptor expression in ovary. GABA A receptor antagonist bicuculline (20 μM) added in ovarian compartment prevented the inhibitory effect on progesterone caused by NE in CG. Androstenedione was not modified under neuropeptides or NE ganglionic stimulation.

CONCLUSIONS: Finally, results from VIP+NE or NPY+NE in ganglion showed that ovarian response on D2 induced by VIP or NPY alone is moderated by the opposite action of NE, and occurs only on progesterone, the most sensitive steroid to neural action.

ORIGINAL ARTICLE

Abbreviations :

CG	- coeliac ganglion
SON	- superior ovarian nerve
D2	- diestrus day 2
SEM	- standard error medium
NE	- norepinephrine
NPY	- neuropeptide Y
VIP	- vasoactive intestinal peptide
NO	- nitric oxide
NOS	- nitric oxide synthase
NGF	- nerve growth factor
trkA	- tyrosine kinase A receptor
RIA	- radioimmunoassay

INTRODUCTION

At present, there is strong evidence that the ovary of mammals is under the influence of direct neural factors (Burden, 1985). The superior ovarian nerve (SON) is considered as the main neural pathway related to ovarian steroidogenesis (Dissen *et al.* 2000; Forneris & Aguado 2002). Most of its fibers originate in sympathetic neuronal bodies located in the coeliac ganglion (CG) and innervate ovarian stroma cells, in particular, perifollicular theca-interstitial cells (Burden, 1985; Erickson *et al.* 1985). Furthermore, the CG constitutes a link of rapid information between the central system and the ovary (Gerendai *et al.* 1998).

The ovarian function is regulated, among others, by norepinephrine (NE) and neuropeptides such as vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), which have been found in the ovary (Papka *et al.* 1985; Ferruz *et al.* 1992) as well as in the SON and CG (Sejnowski, 1982; Dalsgaard *et al.* 1983; Morales *et al.* 1995). The presence of mRNA for VIP (types 1 and 2) and NPY (Y1 and Y2) receptors in the superior cervical ganglion and other ganglia (Tajti *et al.* 1999; Knutsson & Edvinsson 2002) has also been demonstrated.

Experiments conducted in the integrated *ex vivo* CG-SON-ovary system in rat, previously standardized in our laboratory (Sosa *et al.* 2000), have shown that the ovarian progesterone release is modified by incubation of CG with neuropeptides at diestrus (Garraza *et al.* 2004), and by occupation of ganglionic adrenergic receptors depending on the estrous cycle stage (Sosa *et al.* 2000). Noradrenaline in CG appears to increase the release of ovarian progesterone at all stages, except on diestrus day 2 (D2), when it decreases (Sosa *et al.* 2000). Also, i.c.v. injection of isoproterenol, a β-adrenergic agonist, in SON-intact rats on D2 decreases progesterone levels in ovarian vein blood (De Bortoli *et al.* 2002).

In addition to the adrenergic agents, ovarian steroidogenesis is known to be modulated by NO, the formation of which is catalyzed by different isoforms of nitric oxide synthase (NOS) present in the ovary (Tamanini *et al.* 2003). Delgado *et al.* (2004), working with CG-SON-ovary system in prepuberal rat, have associated the ovarian steroids inhibition after ganglionic cholinergic stimulus with a decrease in the release of NO and inducible NOS (iNOS) activity. Furthermore, in the culture medium of granulose/lutein cells a concentration-dependent inhibition of progesterone synthesis in the presence of the NO donors has been observed, which corresponds to an increased concentration of nitrite accumulation (Dave *et al.* 1997). In particular, iNOS expression is known to be modulated by the nerve growth factor (NGF) (Kalisch *et al.* 2003). In rat, both NGF and its high affinity tyrosine kinase A (trkA) receptor mRNA expression, as well as their respective protein products, are almost exclusively present in thecal-interstitial cells (Dissen *et al.* 1996). This neurotrophin seems to maintain a follicular phenotype and prevent the untimely differentiation of granulose cells into their luteal counterparts by production of less progesterone and more estradiol (Dissen *et al.* 2000; Romero *et al.* 2002).

Although important advances have been made in the knowledge of the influence of neural factors on the ovarian esteroidogenesis, the role of neuropeptides in CG on the ovarian physiology remains obscure. The aim of this work was to investigate whether NE modifies VIP or NPY actions in CG on the ovarian progesterone and androstenedione release, and to evaluate the participation of the ON/iNOS system and NGF/trkA receptor mRNA expression in the ovarian response. For that purpose, the analysis was carried out in the *ex vivo* CG-SON-ovary system of rats on diestrus day 2 (D2) in which the ovarian corpora lutea have great importance for secretion of progesterone, and the follicles are in optimal growing conditions for secretion of androstenedione from their theca-insterstitial cells innervated by the SON.

MATERIAL AND METHODS

Animals

Virgin Holtzman strain female rats weighing 250±25 g were used in all the experiments. Animals had free access to food (Cargill, Saladillo, Buenos Aires, Argentina) and tap water. They were keep under controlled light (lights on from 0700 to 1900 h) and temperature (24 ± 2 °C). Only rats showing at least two consecutive 4 day oestrous cycles were used. After examination of vaginal smears rats in D2 were selected. Animals were handled according to the procedures approved in the UFAW Handbook on the Care and Management of Laboratory Animals, Volume 1: Terrestrial Vertebrates, 7th edition (Poole T, ed.) and experimental protocol was approved by the Committee for Animal Use of the National University of San Luis.

Experimental procedure

The surgical procedure used for removing the CG-SON-ovary system, its characterization and histological control, and the standardization of the incubation

times were performed as described previously (Sosa *et al.* 2000). The experimental scheme used allows to study the release of steroids from the ovary in absence of humoral factors. Briefly, the system containing the left ovary, the fibers constituting the SON and the CG was removed, washed with the incubation medium (Krebs-Ringer bicarbonate buffer, pH 7.4, with the addition of glucose 0.1 mg/ml and albumin 0.1 mg/ml), and placed immediately in a cuvette consisting of two compartments containing 2 ml of incubation medium each. The CG and the ovary were placed in separate compartments connected by the SON, taking care that the SON kept moisture with the incubation medium. The system was stabilized by preincubation in a metabolic bath at 37 ºC for 30 min in atmosphere of 95% O_2 and 95% 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 at 1 nM final concentration was added and periodical extractions were made from the ovarian compartment at 30, 60, 120 and 180 min for determination of the release of progesterone, androstenedione and nitrite, a water soluble metabolite of NO. The value of hormone concentrations and nitrite released under these conditions were considered to be the baseline (control groups). The specific agents tested for each experimental group were: 50 ng/ml VIP, 50 ng/ml NPY, 10^{-6} M NE and a mix of VIP+NE or NPY+NE, which were added to the ganglion compartment, respectively. Previously, the effects of different doses of VIP and NPY (20, 50 y 100 ng/ml) in coeliac ganglion on the ovarian progesterone release were determined.

In a separate experiment, the ovarian progesterone release was measured after CG incubation with 10^{-6} M propranolol as adrenergic β-antagonist, for 15 min before addition of 10^{-6} M NE, VIP+NE or NPY+NE.

The adrenergic agents were dissolved in Krebs-Ringer solution plus ascorbic acid (1 nM) to avoid its oxidization. Previous incubations of ascorbic acid with Krebs Ringer buffer had demonstrated that 1 nM of ascorbic acid did not have an effect on the basal release of ovarian hormone (Garraza *et al.* 2004). The samples of liquid from the ovarian compartment (250 µl) were collected at 30, 60, 120 and 180 min from the beginning of the incubation and kept at –20 °C until determination of hormones and nitrite concentrations. Corresponding corrections were made in all cases, taking into consideration the volume extracted in each period tested. In each group, six CG-SON-ovary systems were studied. After 180 min of incubation ovaries were kept in liquid nitrogen and maintain at –70 °C until used (1 or 2 weeks) for iNOS Western blot analysis and NGF and trkA receptor RT-PCR assays.

Steroid assay

Progesterone and androstenedione were measured in duplicate by RIA. The assay sensitivity was less than 5 ng/ml for progesterone and 0.01 ng/ml for androstenedione (Forneris & Aguado 2002). The inter- and intra-assay coefficients of variation for all the assays were less than 10.0%. The results were expressed as nanograms of progesterone and picograms of androstenedione per milligram of ovarian tissue (ng progesterone/mg tissue and pg androstenedione/mg tissue, respectively).

Nitrite assay

NO formation was measured indirectly by assaying nitrite, a stable product of NO oxidation, using the Griess reagents and absorbance was read at 540 nm (Egami & Taniguchi 1974). The intraassay coefficients of variation for the assays were less than 10.0%. The results were expressed as micromol of nitrite per milligram of ovarian tissue (µmol/mg ovary).

Western blot analysis for iNOS

Ovaries were homogenized in Tris-HCl 50 mM (pH 7.8) containing protease inhibitors. Protein was measured by the method of Lowry *et al.* (1951). 40 mg of proteins were mixed with 10 ml of sample buffer (125 mM Tris–HCl, pH 6.8, 4% SDS, 3.5 mM DTT, 0.02% bromophenol blue and 20% glycerol), boiled for 2–3 min and loaded into a 8% SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. Separated proteins were transferred to PVDF membranes (Polyscreen NEF 1000 purchased from NEN Life Science Products) using a blot transfer system (BioRad Laboratories, Hercules, CA). After being blocked with 5% BSA-TBS solution (20 mM Tris, 500 mM NaCl, pH 7.5) overnight, at 48 °C, membranes were incubated with a primary rabbit antiiNOS polyclonal antibody solution (Santa Cruz Biotechnology) (1:1000 dilution), for 1 h, at room temperature. β-Actin expression was measured as a control for protein loading using a rabbit polyclonal antibody. After washing three times with TTBS (0.1% Tween 20, 100 mM Tris–HCl, pH 7.5, 150 mM NaCl), membranes were incubated with an anti-rabbit IgG secondary antibody linked to peroxidase for 1 h at room temperature. Membranes were washed and the color was developed using a Vectastain ABCdetection system. Separated proteins were transferred to PVDF membranes (Polyscreen NEF 1000 purchased from NEN Life Science Products) using a blot transfer system (BioRad Laboratories, Hercules, CA).

Reverse transcription polymerase chain reaction for NGFand trkA receptor

Total RNA was extracted from Sp culture using TRIzol reagent (Life Technology). The RT-PCR was performed using a one-step RT-PCR method (Access RT-PCR system, Promega, Madison, USA). All components for RT and PCR were assembled in 50 µl reactions containing 5× reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3 mM $MgCl_2$, 10 mM dNTP mixture, 1 µM each of gene specific primers, 2 µg template RNA, 5 units of AMV reverse transcriptase and 5 units of Tfl DNA polimerase. The amplification of cDNA was done

under the following conditions: denaturation at 94 °C for 2 minutes followed by 35 cycles of denaturation at 94 °C for 30 seconds, annealing at 61 °C for 1 minute for both primers, and extension at 72 °C for 2 minutes. The reaction was completed with a final extension at 72 °C for 7 minutes (thermal cycler 2400, Perkin-Elmer). The following primers were used: NGF (5' TGATCGGCGTACAGGCAGAAC-3´, sense and 5'-AAGGTATGAGTCGTGGTGCAG-3´, antisense); trkA: (5´-TGCTGCTGCTGCTGATTCTAGG-3´, sense and 5´-AGGAATGAGGTAGTCGGTGGTG-3´, antisense) and GAPDH (5'-GGGCTGCCTTCTCTTGTGAC-3', sense and 5'-CGCCAGTAGACTCCACGACA-3', antisense). The predicted sizes of the PCR-amplified products were 583, 716 and 335 bases pairs (bp) for NGF, trkA and GAPDH, respectively. The PCR products were resolved on 2% agarose gel electrophoresis, visualized with etidium bromide and photographed using a Polaroid camera. The relative abundance of each band was normalized according to the housekeeping GAPDH gene.

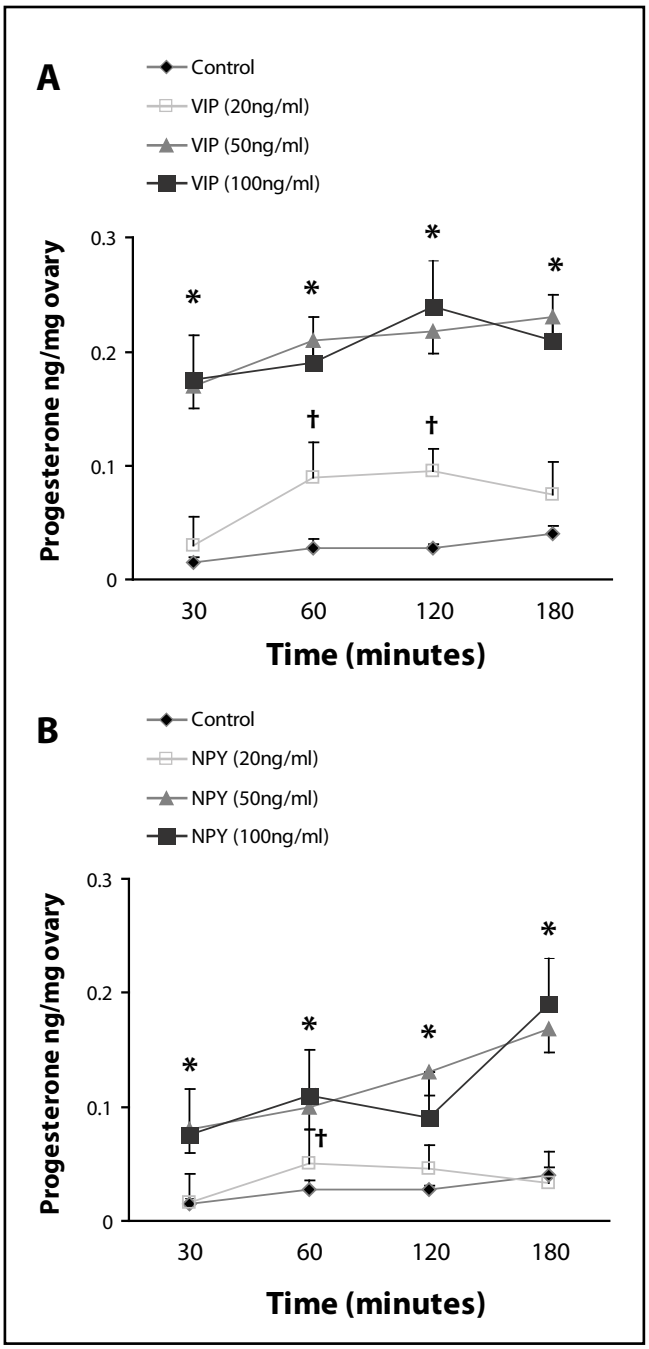

Fig. 1. Effects of different doses of VIP and NPY in coeliac ganglion on the ovarian progesterone release in the coeliac ganglion-superior ovarian nerve-ovary system removed from rats on diestrus day 2**.** The system was incubated in Krebs Ringer buffer containing 1 nM ascorbic acid at 37 ºC in atmosphere of 95% O_2-95% CO_2 for 180 min, (◆) without (control), and with (□) 20, (▲) 50 and (×) 100 ng/ml VIP or NPY added to the ganglion compartment**.** Values are the mean ± SEM of four animals per each neuropeptide concentration used. (A) and (B): † $p<0.05$ and * $p<0.001$ vs control.

Effect of a GABA A receptor antagonist in ovary

To study the possibility that gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter in mammals (Owens & Kriegstein 2001), may be involve in the ovarian progesterone response after ganglionic stimulation with NE, at the end of the preincubation period of the CG-SON-O system in metabolic bath, the buffer was changed in both compartments and 10, 20 or 50 μM final concentration of bicuculline, an antagonist GABA A receptor, was added on the ovary. Bicuculline was dissolved in Krebs Ringer solution. After 60 min of incubation (time 0), 10^{-6} M NE was added in ganglion and periodical extractions were made from the ovarian compartment at 30, 60 and 120 min for determination of progesterone and nitrites.

Data analysis

Results are expressed as mean ± SEM in each group. Significant differences among means were considered at a level of $p<0.05$ and identified by one-way ANOVA followed by Duncan's test.

RESULTS

Release of ovarian hormones: Modulation of NE on neuropeptide effects in coeliac ganglion

The addition of different doses of VIP and NPY to CG showed a maximal stimulation of ovarian progesterone release with 50 ng/ml of VIP or 50 ng/ml NPY (Figure 1A and 1B).

The ganglionic stimulation with VIP or NPY markedly increased the ovarian progesterone release at all the studied times, confirming previous reports (Garraza *et al.* 2004), while the addition of NE in ganglion produced progesterone inhibition at 60 min ($p<0.05$), 120 min ($p<0.01$) and 180 min ($p<0.001$), compared with the respective control values. To analyze whether NE modifies the neuropeptide effects on ovarian progesterone, the CG was stimulated with a mix of VIP+NE or NPY+NE. The progesterone release decreased by ~ 80% and 60% at all the studied times, as compared with

ganglionic stimulation with either VIP or NPY alone, respectively (Figure 2A and 2B).

After addition of the β-adrenergic antagonist propranolol to the CG compartment, NE, VIP+NE and NPY+NE in ganglion caused an increase of ovarian progesterone at almost all studied times, as compared with NE, VIP+NE and NPY+NE in absence of propranolol, respectively (Figure 2A and 2B).

As shown in Figure 3A and 3B, the level of androstenedione in the ovarian incubation liquid of the control group was increased at 120 min ($p<0.05$) and 180 min ($p<0.001$) in relation to the obtained value at 30 min. No differences in androstenedione response were observed at any of the studied times when VIP or NPY were added to the ganglion compartment, compared with the respective control values. Furthermore,

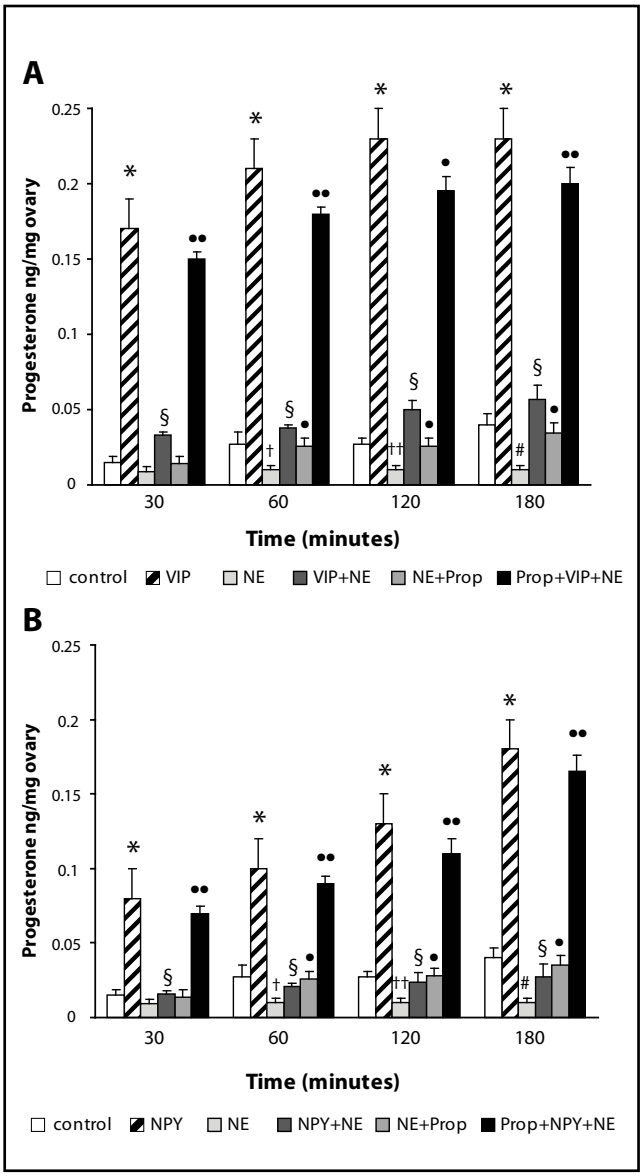

Fig. 2. Effect of norepinephrine (NE), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and antagonist β-adrenergic propranolol (Prop) in ganglion compartment on ovarian progesterone release in the coeliac ganglion-superior ovarian nerve-ovary system removed from rats on diestrus day 2. The system was incubated as indicated in Fig. 1 for 180 min without (control) and with 50 ng/ml VIP, 50 ng/ml NPY, 10^{-6} M NE, VIP+NE or NPY+NE, and with 10^{-6} M propranolol added to the ganglion compartment before NE, VIP+NE or NPY+NE (experimental groups). Values are the mean ± SEM of six animals per experimental group. (A) and (B): * $p<0.001$, $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ and $^{\#}p<0.001$ vs control; $^{\S}p<0.001$ vs VIP or NPY; $^{\bullet}p<0.05$ vs NE; $^{\bullet\bullet}p<0.001$ vs VIP+NE or NPY+NE.

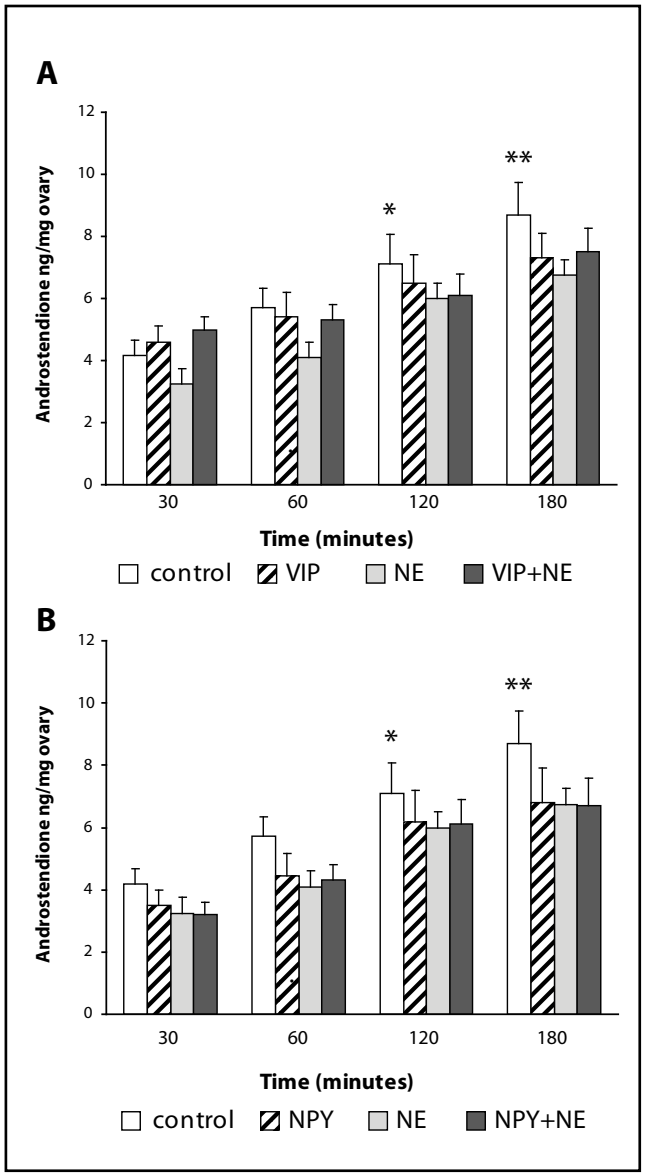

Fig. 3. Effect of norepinephrine (NE) and vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in ganglion compartment on ovarian androstenedione release in the coeliac ganglion-SON-ovary system removed from rats on diestrus day 2. The system was incubated as indicated in Fig. 1 for 180 min without (control) and with 50 ng/ml VIP, 50 ng/ml NPY, 10^{-6} M NE, VIP+NE or NPY+NE, added to the ganglion compartment (experimental groups). Values are the mean ± SEM of six animals per groups. (A) and (B) *$p<0.05$ and **$p<0.001$ vs 30 min.

the ganglionic stimulation with NE alone, VIP+NE or NPY+NE did not modify the release of androstenedione in relation to the controls.

Effect of neuropeptides and NE in ganglion on ovarian nitrite release and iNOS expression

As shown in Figure 4A, the presence of VIP in the ganglion compartment diminished the release of nitrites compared with the control group at 60, 120 and 180 min ($p<0.05$, $p<0.05$ and $p<0.001$, respectively). NE applied to CG markedly increased the release of nitrites compared with the control group ($p<0.001$), while VIP+NE reversed the decrease of nitrite induced by VIP ($p<0.001$) at all the studied times. After addition of propanolol in the CG compartment, NE and VIP+NE in ganglion caused a decrease of ovarian nitrites as compared with NE and VIP+NE in absence of propranolol, respectively.

NPY in ganglion did not modify the release of nitrites from ovary at any of the studied times in relation to controls, but ganglionic stimulation with NPY+NE induced a significant increase of nitrites ($p<0.001$), similar to that observed with NE alone. Propranolol in ganglion reversed the stimulatory effect on nitrite caused by NE and NPY+NE in the absence of propranolol, to control values (Figure 4B).

Western blot analysis showed a decreased expression of iNOS in the ovary when VIP was added in the ganglion compartment, and an increase of iNOS when the CG was stimulated with NE alone, compared with the control group. The presence of VIP+NE in ganglion restored the ovarian iNOS expression to control values. No change in the iNOS protein expression was observed when NPY alone was applied on CG, in relation to control. However, the addition of NPY+NE in the ganglion compartment caused an increase ($p<0.05$) of iNOS expression, compared with NPY alone (Figure 5).

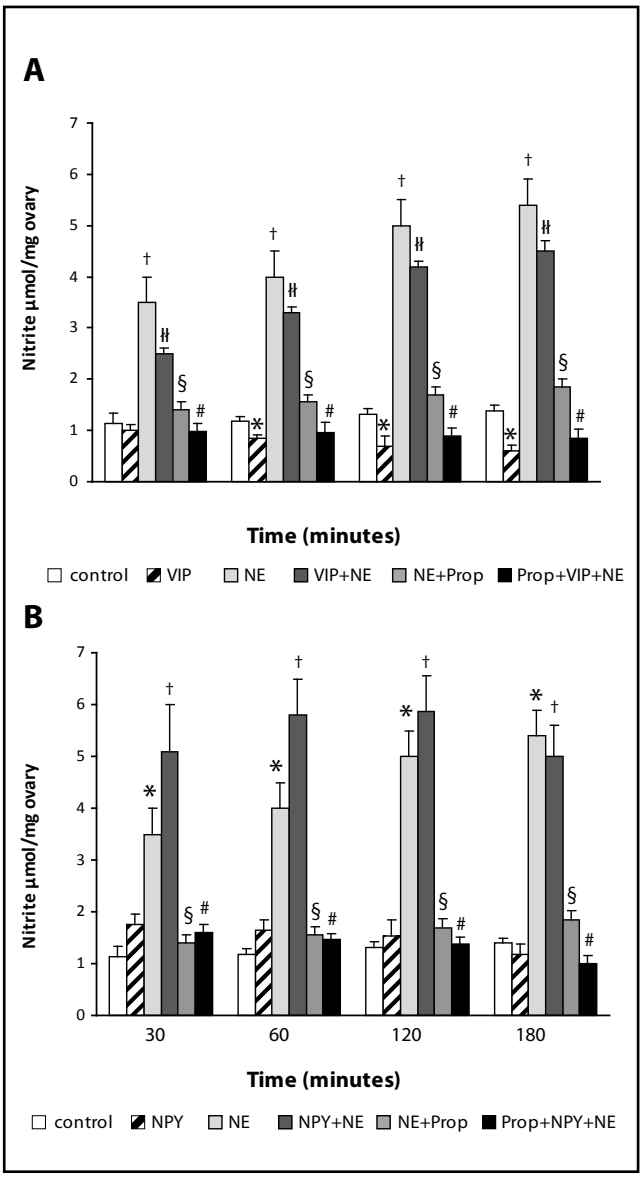

Fig. 4. Effect of norepinephrine (NE) and vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) in ganglion compartment on ovarian nitrite release in the coeliac ganglion-SON-ovary system removed from rats on diestrus day 2. The system was incubated as indicated in Fig. 1 for 180 min without (control) and with 50 ng/ml VIP, 50 ng/ml NPY, 10^{-6} M NE, VIP+NE or NPY+NE, and with 10^{-6} M propranolol added to the ganglion compartment before NE, VIP+NE or NPY+NE (experimental groups). Values are the mean ± SEM of six animals per groups. (A) * $p<0.05$, **, † $p<0.001$ vs control; †† $p<0.001$ vs VIP; § $p<0.001$ vs NE; # $p<0.001$ vs VIP+NE. (B) * $p<0.001$ vs control; † $p<0.001$ vs NPY; § $p<0.001$ vs NE; # $p<0.001$ vs NPY+NE.

Effect of GABA A receptor antagonist in ovary on progesterone and nitrites release

We predicted that if progesterone inhibition induced by NE in CG is mediated by GABA A receptor, then bicuculline, a GABA A receptor antagonist, should inhibit its actions. Bicuculline in the ovarian compartment (10, 20 or 50 µM for 60 min of incubation) did not modify the basal release of progesterone compared with Krebs Ringer alone (data not shown). In comparison with control values, the decrease in the ovarian progesterone release at 30, 60 and 120 min of

Effect of neuropeptides and NE in ganglion on ovarian NGF/trkA receptor mRNA levels

To gain more insight into the regulation of ovarian steroidogenesis, we examined the gene expressions of NGF and its trkA receptor in ovary. As shown in Figure 6, the mRNA levels of both the NGF and its trkA receptor were decreased by addition of VIP and increased by addition of NE on CG, as compared with controls. The action of VIP+NE in CG caused an increase of NGF-trkA receptor gene expression in ovary, compared with that of VIP alone, restoring the control values. No differences in the mRNA levels of the NGF and its trkA receptor were observed when NPY was added to the ganglion compartment in relation to control. However, ganglionic stimulation with NPY+NE induced an increase of the NGF gene expression in relation to NPY alone.

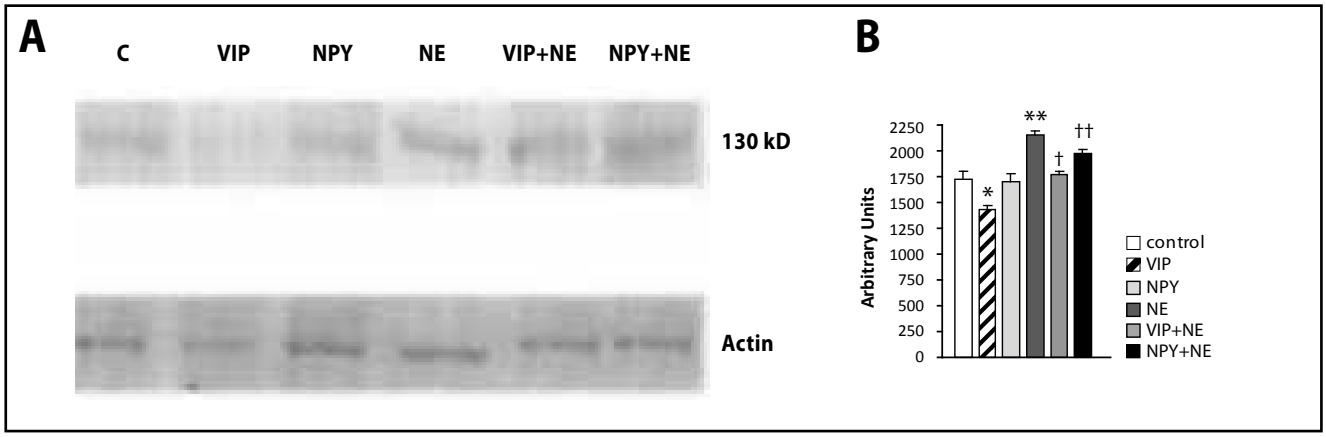

Fig. 5. Effect of vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and norepinephrine (NE) in ganglion compartment of the coeliac ganglion-SON-ovary system on the expression of ovarian inducible of nitric oxide synthase (iNOS). (**A**) Inmunoblot analyses of ovarian iNOS expression. β-actin expression was used as a control for protein loading. (**B**) Quantitative analysis of iNOS. The system was incubated as indicated in Fig. 3. The iNOS expression was determined at 180 min of incubation. Values are the mean ± SEM of four animals per group. *p<0.05 and **p<0.01vs control; †p<0.05 vs VIP; †† p<0.05 vs NPY.

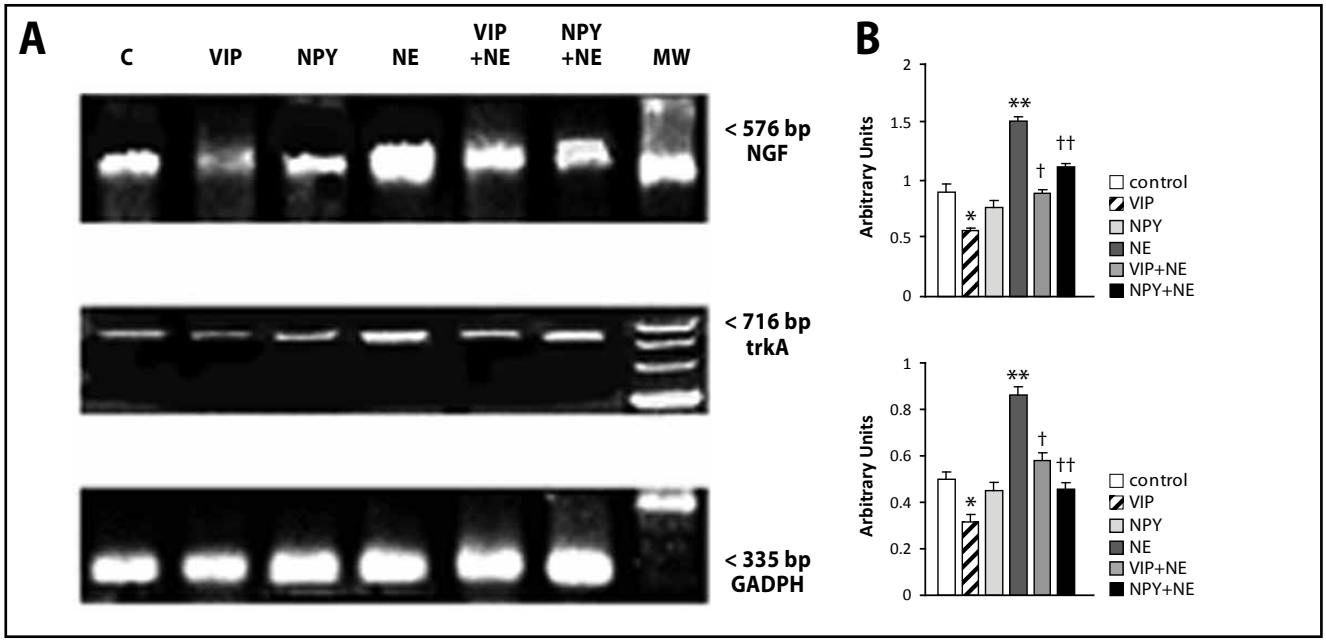

Fig. 6. Effect of vasoactive intestinal peptide (VIP), neuropeptide Y (NPY) and norepinephrine (NE) in ganglion compartment of the coeliac ganglion-SON-ovary system on the expression of ovarian nerve growth factor (NGF) and trkA receptor genes. (**A**) Representative RT-PCR analysis. (**B**) Quantification of the intensity of the fragment bands in relation to the intensity of the internal control bands (GADPH). The system was incubated as indicated in Fig. 3. Expression of genes were determined at 180 min of incubation. Values are the mean ± SEM of four animals per group. NGF: *p<0.01 and **p<0.001 vs control; †p<0.05 vs VIP; ††p<0.05 vs NPY. trkA receptor: *p<0.05 and **p<0.01 vs control; †p<0.01 vs VIP.

incubation induced by NE in CG was not observed when the ovary was previously incubated with 20 and 50 μM bicuculline (Figure 7). In addition, the release of ovarian nitrites caused by NE in ganglion at 60 and 120 min was reduced by previous incubation of the ovary with bicuculline (20 μM) (2.6 ± 0.2 and 2.4 ± 0.3 vs 4.2 ± 0.3 and 3.8 ± 0.4 μmol nitrite/mg ovary, p<0,01, respectively). All these results suggest a participation of GABA neurotransmitter in the ovarian response when CG is under NE stimulation.

DISCUSSION

Although existing evidence suggests a role for neuropeptides and NE in the control of ovarian steroidogenesis, it is unclear to what extent they are involved in the maintenance of the ovarian cyclic steroid production through their effects on the sympathetic ganglions. In this study, we examined whether ovarian progesterone and androstenedione release induced by neuropeptides in CG is modified by ganglionic NE in rat on D2.

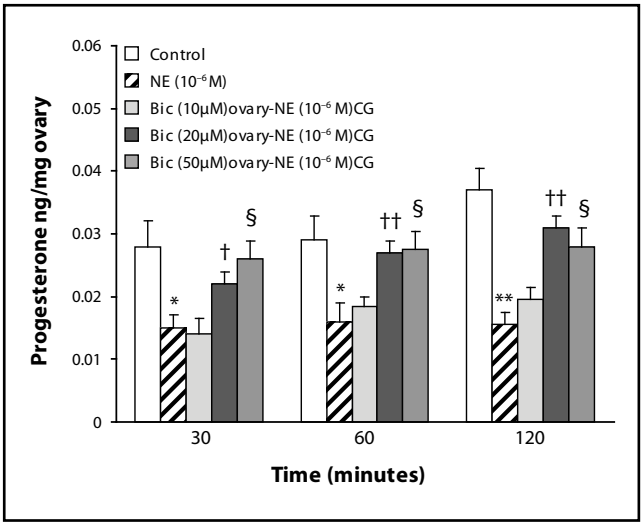

Fig. 7. Effect of GABA A receptor antagonist bicuculline in the ovary of coeliac ganglion-superior ovarian nerve-ovary system on progesterone release. 10^{-6} M NE was added on ganglion after 60 min of incubation (time 0) of the system in metabolic bath without and with 10, 20 or 50 μM final concentration of bicuculline (Bic) added to the ovarian compartment. Values are the mean ± SEM of four animals per group. *p<0.01 and ** p<0.001 vs control; †p<0.05 vs NE; ††, § p<0.01 vs NE.

It is known that at diestrus the corpora lutea are the functionally active structures, with progesterone being the main secretion product. In our experimental model, ganglionic stimulation with NE alone or plus either VIP or NPY induced a decrease of progesterone release from the ovary in contrast to the excitatory effect on this hormone by the neural input produced by either VIP or NPY alone in ganglion.

We have previously shown that VIP or NPY added to the ovary cause an increase of progesterone release (Garraza *et al.* 2004). Also, catecholamines have been shown to exert a stimulating effect on the release of progesterone when they impact on the beta adrenergic receptors in ovary (Jordan 1981). In turn, previous results obtained in our laboratory have shown that, in the CG-SON-ovary system in control conditions, NE is released in the ovarian compartment at values significantly lower on D2 as compared to D1 (Sosa *et al.* 2004). Therefore, it would appear that VIP, NPY or NE are not responsible for the clear inhibitory effect on ovarian progesterone caused by NE in ganglion. It is likely that such effect may be due to the action of some other factor, such as GABA, a neurotransmitter with inhibiting characteristics that reaches the ovary through the SON (Gladkevicha *et al.* 2006). In fact, an inhibitory effect of GABA on progesterone production from cultured luteal cells has been shown to be mediated by GABA (A) receptor (Zhang *et al.* 2000). In the CG-SON-O system under study the GABA A antagonist receptor bicuculline, reversed the inhibitory effect on progesterone induced in the ovary by NE in CG, indicating that GABA may be a factor that modulates the ovarian progesterone response. It is probable that, *in vivo*, NE action in CG contributes to facilitate the decrease in progesterone concentration that is necessary to start a new cycle.

It is known that α and β adrenergic receptors have been detected in the superior cervical ganglion and other ganglia (Shivachar & Eikenburg 1999). In particular, in the CG-SON-ovary system of D2, the α-adrenergic receptor antagonist phentolamine does not modify the basal concentrations of ovarian progesterone, while the occupation of β-adrenergic receptors by propranolol increases it (Sosa *et al.* 2000). Our results indicate that after incubation of the CG with propranolol the ganglionic NE stimulation did not inhibit the progesterone release, maintaining the control values. This fact confirms that ganglionic β-adrenergic receptors are involved in the ovarian progesterone response on D2. Vásquez & Lewis (2003) have shown that the β2-adrenergic receptor agonist isoproterenol abolishes the signaling by G(s) protein-coupled receptors for VIP in neurons of rat superior cervical ganglion. Thus, it is possible that in the CG-NOS-system the NE acting on β-adrenergic receptor of CG prevents VIP receptors from transducing their biological signals, inhibiting the ovarian progesterone release. Although it has been shown in mouse bone marrow cells that NPY blocks the increase elicited by the β-adrenergic receptor agonist isoprenaline in the production of cAMP (Amano *et al.* 2007), it is not yet know whether there is an interaction between NPY and adrenergic receptors in CG.

In recent years NO has been recognized as a paracrine molecule that plays a physiological role in the ovarian function (Tamanini *et al.* 2003; Delgado *et al.* 2004). In the CG-NOS-ovary system, the induced release of progesterone by VIP in ganglion was associated to a decrease in the levels of nitrite, a soluble metabolite of NO, and iNOS expression, in the ovary. In contrast, the decrease of progesterone caused by ganglionic stimulation with NE, alone or plus VIP, occurred simultaneously with an increase of nitrites and iNOS expression in the ovary. An inverse relationship between NO and progesterone production has been reported, although in cell culture systems, by other researchers who found that NO inhibits ovarian release of progesterone (Dave *et al.* 1997; Estevez *et al.* 2002). The involved mechanisms are probably related to NO inhibition of the activity of progesterone synthesis-limiting enzyme, cytochrome P450 side chain cleavage (Olson *et al.* 1996). All these results suggest that ganglionic actions of VIP and NE modulate the NO/NOS ovarian system on D2 rat through the SON. In addition, it is known that NO modulates the uptake and/or release of neurotransmitters through a variety of cellular mechanisms. In particular, NO activates the expression of GABA A receptor by S-nitrosylation/oxidation of thiol groups either directly on the GABA A receptor subunits or on a regulatory protein tightly associated with the GABA A receptor in rat brain and frog pituitary melanotrophs (Kim & Oh 2002; Castel & Vaudry

2001). On the other hand, the depolarization following the GABA A receptor activation, through activation of the transcription factor CREB, can induce nNOS expression in rat cortex (Mantelas *et al.* 2003). Although we do not know from our results whether a functional modulation occurs between GABA A receptor activity and NO synthesis in the ovary, this cannot be discarded since the level of nitrites was observed to decrease after GABA A receptor was blocked with bicuculline in ovary.

It is now clear that NGF is not only important for the differentiation and survival of neuronal cells, but is also required as an intragonadal molecule for promoting steroidogenesis (Dissen *et al.* 2000; Romero *et al.* 2002). Furthermore, it has been reported that NGF increases the expression of NOS isoenzymes and NO production expression in PC12 cells (Kalisch *et al.* 2003). Considering this evidence, the fact that the expressions of both NGF and its high affinity trkA receptor mRNA as well as their respective protein products are almost exclusively present in thecal-interstitial cells (Dissen *et al.* 1996) that are preferentially innervated by SON fibers, and on the basis of our results in the CG-NOS-ovary system under study, we investigated the participation of NGF and its trkA receptor in the ovarian response. An inverse relationship between intraovarian mRNA levels of the NGF and its trkA receptor and progesterone release was observed when CG was stimulated with VIP or NE. Possibly, the decrease of mRNA levels of NGF/trkA receptor might be associated to the inhibition found in the ovarian NO/iNOS system induced by VIP in CG, as the increase of NGF/trkA receptor to the stimulation of NO/iNOS system caused by NE in ganglion. On the other hand, it can be assumed that the decrease of NGF gene expression is related to that of the trkA receptor, since NGF itself participates in the regulation of its receptor expression in ovary (Dissen *et al.* 2000). In addition, the ability of NGF to modify human granulosa cell steroidogenic function requires the activation of trkA (Salas *et al.* 2006). The opposite effect induced by NE in ganglion on the ovarian mRNA NGF/trkA levels could explain the restoration of their gene expressions to control value when ganglion was stimulated with VIP+NE. Also, the inhibitory NE action on the ovarian NGF expression was observed with NPY+NE in ganglion. However, from our results it can be suggested that the increase of ovarian progesterone induced through the SON by the ganglion stimulation with NPY alone is associated to some other mechanism distinct from the ovarian NO/iNOS system and NGF/trkA receptor gene expression. At the moment, there is no information about a functional modulation of GABA on NGF in ovary, however, it has been reported that the depolarization following GABA A receptor activation induces brain-derived neurotrophic factor in rat brain (Mantelas *et al.* 2003).

Androstenedione plays an important role in the ovarian physiology since it is involved in the synthesis of oestradiol and in the maintenance of the corpora lutea by its transformation into intraluteal oestradiol (Gibori 1993). Since ovarian androstenedione response was not modified under ganglionic stimulation with either neuropeptides or NE, it can be suggested that their steroidogenic effects on D2**,** in our experimental model, only occurs on progesterone, the most sensitive steroid to the neural action (Aguado *et al.* 1982). Other authors have reported that NO inhibits (Dunnam *et al.* 1999) and NGF stimulates (Dissen *et al.* 2000) the ovarian release of androstenedione, although in different experimental schemes.

This study permits us to conclude that the CG modifies its activity when it is stimulated with VIP and NE and that these influences reach the ovary through the SON and modify the release of progesterone at D2, associated to changes in NO/iNOS system and NGF/trkA gene expression. Also, it constitutes the first evidence of a participation of GABA neurotransmitter in the ovarian response when CG is under NE stimulation.

From a physiological point of view, the obtained results contribute to confirm that the CG-SON-ovary system could provide a good resemblance of *in vivo* conditions to evaluate the neuropeptide actions on ovarian function. More detailed knowledge will be needed to clarify the mechanisms by which neuropeptides in CG modulate the ovarian steroidogenesis. This might help to further understand certain ovarian dysfunctions that are associated to neural and hormonal causes.

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