

Adrenergic influences on coeliac ganglion affect the release of progesterone from cycling ovaries: characterisation of an *in vitro* system

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Abstract

The superior ovarian nerve (SON) arrives at the ovary through the suspensory ligament and innervates mainly the ovarian stroma. Most neurones from which the SON fibres originate are located in the complex coeliac and mesenteric ganglia. Taking into account that other ganglia have been shown to have α - and β -adrenergic receptors, and that the coeliac ganglion receives adrenergic fibres from other sympathetic paravertebral and preaortic ganglia, we utilised adrenergic agonists and antagonists specific to the ganglion, to analyse the role of the α and β receptors in ovarian physiology. To that end, it was necessary to develop and standardise an *in vitro* coeliac ganglion–SON–ovary (coeliac ganglion–SON–O) experimental system that would enable study of the release of steroids in the ovary in the absence of humoral factors. We investigated the effect of adrenergic agents on the liberation of progesterone in the different stages of the oestrous cycle. To this end we placed the coeliac ganglion and the ovary in different compartments, connected through the SON, to produce a system being studied as a whole. Combined neural and hormonal (luteinising hormone (LH)) effects were also examined.

Non-specific stimulation with KCl in the ganglion compartment evoked different responses in terms of release of progesterone, depending on the physiological conditions of the cycle; this demonstrated the sensitivity and viability of the system.

During pro-oestrus, stimulation of the ganglion compartment with adrenergic agents such as the agonist noradrenaline or the β -adrenergic antagonist propranolol, did not modify the release of progesterone. In contrast, the α -adrenergic antagonist, phentolamine, induced a strong inhibitory response. During the oestrous stage, noradrenaline was inactive, but phentolamine and propranolol exerted a strong stimulus throughout the experiment. On dioestrus

day 1 (D1), both noradrenaline and propranolol increased the release of ovarian progesterone, whereas phentolamine had the opposite effect. Finally, on dioestrus day 2 (D2), what was noteworthy was the pronounced inhibitory effect of noradrenaline, whereas phentolamine was inactive and propranolol showed its greatest stimulatory effect.

In order to compare the combined neural and endocrine effects on the ovarian release of progesterone, the experiment was carried out during stages D1 and D2, when the corpora lutea are at their peak of activity. Adrenergic agents were added to the ganglion and LH in a final concentration of 50 ng/ml was added to the ovarian compartment. Different effects were observed indicating a differential response to these agents in stimulated and basal conditions.

We conclude that the *in vitro* coeliac ganglion–SON–ovary system is a functional entity because it possesses its own autonomic tone. This is verified because different basal values of progesterone appear in the different stages of the oestrous cycle. In similar fashion, variations of progesterone induced via the neural pathway were observed under different experimental conditions. In contrast, on D2, noradrenaline added to the ganglion compartment had an inhibitory effect on the liberation of ovarian progesterone. This would indicate that, during this phase, noradrenaline may not be the neurotransmitter released in the ovarian compartment, but that other inhibitory molecules might participate in the observed effects. Finally, during D2, the neural input would condition the ovarian response to LH, facilitating the decrease in progesterone necessary to start a new cycle.

The experimental scheme is, in our opinion, a valuable tool for the study of peripheral neural participation in ovarian physiology.

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Introduction

In addition to the classic neuroendocrine system, in the past few years evidence has been found supporting a direct

neural connection between the gonads and the central nervous system (Kawakami *et al.* 1972, 1981, Chiapas *et al.* 1977, De Bortoli *et al.* 1998). Innervation of the rat ovary is through two pathways. Of these, the superior ovarian

nerve (SON) is considered to be the main neural pathway related to ovarian steroidogenesis. This view is supported by the fact that the sympathetic nerve endings are in close contact with the stromal cells, especially with the thecal and interstitial cells (Jordan 1981, Aguado *et al.* 1982, Burden *et al.* 1985, Erikson *et al.* 1985). With regard to the origin of the SON, it is known that the neuronal soma of the fibres that constitute it are located in portions T10 and T11 of the medulla (Lawrence & Burden 1980, Burden 1985). Most of the fibres that emerge from the medulla form synapses in the coeliac ganglion, thus integrating the fibres of the SON (Sarper *et al.* 1976, Lawrence & Burden 1980, Sejnowski 1982, Chávez *et al.* 1991). The remaining fibres that come from the medulla do not form synapses at the ganglion, and arrive together at the ovary (Messenger *et al.* 1994). In addition, the connection between the ovary and the coeliac plexus through the SON has been confirmed, using retrograde axoplasmic transport (Baljet & Drukker 1980, Klein & Burden 1988*a,b*).

It is known that the ganglia involved in the SON pathway are sympathetic and their stimulation releases noradrenaline and other substances such as vasoactive intestinal peptide (VIP) (Ojeda & Aguado 1985, Ojeda & Lara 1989, Gerendai *et al.* 1995). It is important to point out that β -adrenergic receptors have been found in different populations of rat ovarian cells and in membranes of whole ovaries (Jordan 1981, Aguado *et al.* 1982, Condon & Black 1976, Hsueh *et al.* 1984, Aguado & Ojeda 1986, Norjavaara *et al.* 1989). The liberation of ovarian steroids, progesterone and aromatisable androgens is regulated by the number of β -adrenergic receptors (Aguado *et al.* 1982) and by the action of peptides with inhibitory effects such as those of gonadotropin-releasing hormone (GnRH) (Hsueh & Erickson 1979, Hsueh & Jones 1981, Pieper *et al.* 1981, Sheela Rani *et al.* 1983) and GABA, the presence of which has been confirmed in ovarian tissue (Erdö *et al.* 1985), and with stimulatory effects such as that of VIP (Ahmed *et al.* 1986, Ojeda *et al.* 1989, Dissen *et al.* 1993, Kaleczyc *et al.* 1995).

The importance of the connection through the SON has been emphasised by experiments in which that neural pathway was partially or completely cut, for studies of the release of ovarian steroids in different *in vitro* systems (Aguado & Ojeda 1984*a*, Marchetti *et al.* 1987). When central adrenergic stimulation is performed on cycling rats, the effect of this stimulation on the release of progesterone surpasses the corresponding effect of luteinizing hormone (LH), which shows the greater influence of neural control on ovarian function in the adult rat (De Bortoli *et al.* 1998). Another experimental scheme utilised to evaluate neural participation in the control of ovarian function has been compensatory ovarian hypertrophy, which can be generated by unilateral ovariectomy (Burden 1978), or by chemical sympathectomy through the local action of 6-hydroxydopamine in the ovaries of rats *in situ* (Gerendai

et al. 1978). Unilateral ovariectomy produces a decrease in the steroid hormones, provoking an alteration in the negative feedback mechanism, with a consequent increase in gonadotrophins that would produce an increase in the weight of the remaining ovary (Butcher 1977). Conversely, these neural pathways must remain intact for compensatory ovarian hypertrophy to occur. As a result, it has been proposed that a neuroendocrine mechanism could be responsible for the changes observed in compensatory ovarian hypertrophy (Burden & Lawrence 1977, Gerendai *et al.* 1978). On the basis of these ideas, Chávez & Domínguez (1994) suggested that the incidence of compensatory ovarian hypertrophy attributable to neural information that reaches the ovary through the SON varies during the oestrous cycle. The mechanisms that produce these conditions are not fully understood, but it is possible that the peripheral nervous system functions as a nexus of rapid information about the endocrine status of the ovary and the central nervous system (CNS), or else that the ovarian hormones directly affect the activity of the CNS (Burden 1978).

In summary, the preceding details lead us to conclude that the sympathetic innervation of the ovary is necessary for the regulation of the function and the development of that organ, and for the rhythmic interaction of the ovary with the hypothalamo–hypophyseal axis during the oestrous cycle (Lara *et al.* 1990*a,b*). However, studies that use stimulation of the peripheral neural pathways are few (Gerendai *et al.* 1995). In addition to this, it is very difficult to determine if the effects are purely neural when *in vivo* schemes are used. These shortcomings led us to standardise a complete, integrated *in vitro* system that comprises the coeliac ganglion, the SON and the ovary, in this way avoiding the interference of humoral factors, especially the pituitary hormones. We also wanted to determine whether the occupation of the adrenergic receptors by the antagonists phentolamine and propranolol caused a response, such as the liberation of ovarian steroids. To this end, in the scheme we propose, the coeliac ganglion was stimulated with catecholamines and the release of progesterone from the ovary was studied. In addition, the existence of possible changes in the release of progesterone when LH is present in the ovary was evaluated. The experimental scheme is, in our opinion, a valuable tool for the study of peripheral neural participation in ovarian physiology.

Materials and Methods

Animals

Three-month-old female virgin rats (250–300 g), bred in our laboratory and originally of the Holtzman strain, were used in all experiments. The rats were kept under controlled conditions with lights on from 0700 to 1900 h and at a temperature of 24 ± 2 °C. Animals had free access to

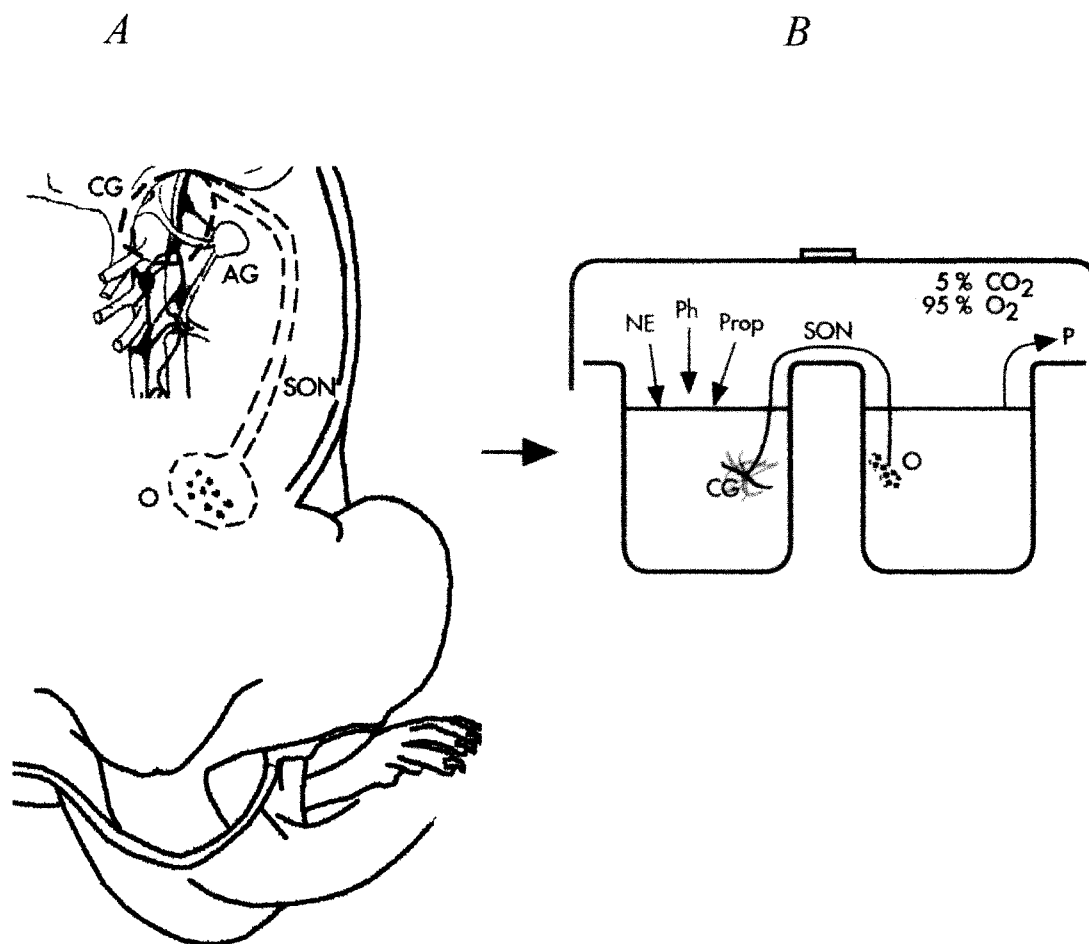


Figure 1 (A) Schematic representation of the SON pathway and the anatomical relationships of the nerve, ovary and coeliac ganglion. The dotted lines indicate sites where the coeliac ganglion-SON-ovary system was dissected out. (B) The cuvette utilised for the subsequent incubation of the system. AG, adrenal gland; CG, coeliac ganglion; NE, noradrenaline; O, ovary; P, progesterone; Ph, phentolamine; Prop, propranolol.

food (Cargill SACI, Saladillo, Provincia de Buenos Aires, Argentina), and tap water was available *ad libitum*. Vaginal smears were taken daily and only those rats showing at least two consecutive 4-day oestrous cycles were used. Groups of six to eight animals in pro-oestrus, oestrus, dioestrus day 1 (D1) and dioestrus day 2 (D2) were used for the experimental procedure.

The experiments were performed in accordance with The Guidelines on the Handling and Training of Laboratory Animals, The Biological Council, UFAW 1992.

Reagents

The following drugs were purchased from Sigma Chemical Co. (St Louis, MO, USA): L-D-norepinephrine hydrochloride, L-propranolol hydrochloride, phentolamine hydrochloride, dextrose, ascorbic acid, bovine serum

albumin fraction V (BSA), and LH. 1,2,6,7-³H]Progesterone was provided by New England Nuclear (Boston, MA, USA). Other reagents were of analytical grade.

Surgical procedures

A piece of tissue containing the left ovary, the fibres that constitute the SON, and the coeliac ganglion accompanied by some small ganglia that surround it, were removed (Fig. 1A); the adrenal gland and the small nerves contained in the peritoneum, oviducts, aortic tissue and the rest of the suspensory ligament were not removed. The strip of tissue was carefully dissected avoiding contact between the surgical instruments and the nerve fibres or the ganglion. This is essential in order to prevent spontaneous depolarisations of the nerves, which an inappropriate contact could provoke. The total surgical procedure was completed in

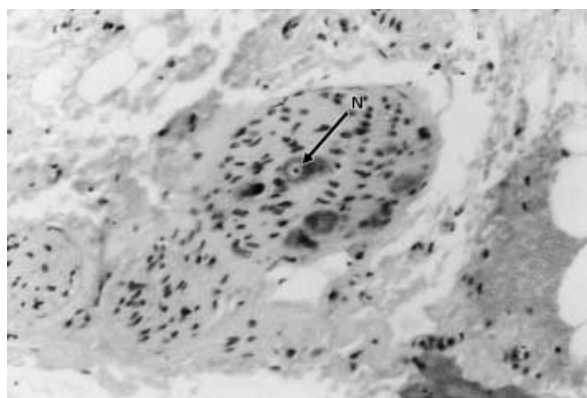


Figure 2 Survey photomicrograph of a coeliac ganglion, showing neural somas (N) and fat connective tissue. Stained with haematoxylin–eosin. Original magnification, $\times 250$.

1–2 min. We proceeded according to previous descriptions of the anatomical trajectory of this neural pathway (Lawrence & Burden 1980, Burden *et al.* 1982).

Characterisation of the system and histological control

The presence of the ganglion in the preparation used was confirmed by the application of routine histological techniques. Immediately after dissection, the material was fixed with Bouin's fluid. Serial cuts of 5 mm thickness were carried out utilising a sliding Reichert–Jung HN-40 microtome. Preparations were stained with haematoxylin–eosin and coated with sintete balsam. The micrographic image was captured using a Leitz–Dialux photomicroscope, equipped with a Leica camera. This system, using a 25' objective, was used to examine tissue sections (total magnification $\times 250$) (Fig. 2).

Standardisation of incubation times

The rats were anaesthetised with ether, described in *Surgical Procedures* and performed between 1500 and 1600 h, taking advantage of the stable concentrations of progesterone that occur at that time of day. The coeliac ganglion–SON–ovary system was removed, cleaned with incubation medium and placed immediately in a cuvette with two compartments. Each compartment contained 2 ml Krebs–Ringer bicarbonate buffer, pH 7.4, as previously described, for *in vitro* culture of whole ovaries (Selstam *et al.* 1976, Advis & Ojeda 1978, Advis *et al.* 1979, Aguado *et al.* 1982, Lara *et al.* 1990a,b, Ferruz *et al.* 1992). The ganglion was placed in one compartment and the ovary in the other, connected by the SON, which had to be kept moist with the experimental solution (Fig. 1B). The system was immediately put in a metabolic bath at 37 °C in a 95% O₂–5% CO₂ atmosphere, and the preincubation time necessary for its stabilisation was noted. A

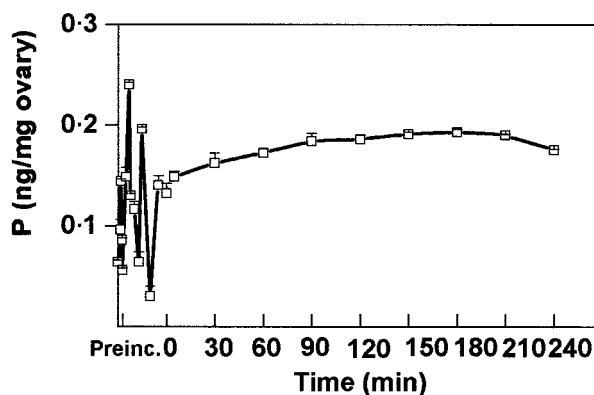


Figure 3 Incubation times. Release of progesterone (P) by the ovary in the coeliac ganglion–SON–ovary system obtained from rats in D2. The system was incubated in Krebs–Ringer plus ascorbic acid solution at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 240 min. Progesterone concentrations were measured every 1 min for the first 5 min, then every 2 mins up to and including the 60th min, and thereafter every 5 mins up to and including the 240th min. Values are the means for three experiments \pm S.E.M. from six animals per experimental group.

previously standardised volume (20 μ l) of incubation medium was extracted from the ovarian compartment for the determination of progesterone every 1 min for the first 5 min, then every 2 min up to and including the 60th min, and from then on every 5 min up to and including the 240th min. It was observed that stabilisation was achieved at 30 min (Fig. 3), which was then considered to be incubation time 0. At this time, the Krebs–Ringer solution was changed in both compartments and ascorbic acid (0.1 mg/ml in Krebs–Ringer) was added as an antioxidant agent (Koh & Hille 1997) in the ganglion compartment. The times of extraction of ovarian liquid were established at 30, 60, 120 and 180 min.

Experimental procedure

Rats in pro-oestrus, oestrus, D1 and D2 were used. The values for the concentrations of progesterone released in the ovary compartment under the conditions described above were taken as basal (control group). The different substances to be tested were dissolved in equal concentrations (10^{-6} M) and volumes (2 ml) of Krebs–Ringer solution plus ascorbic acid, and then added to the ganglion compartment for the measurement of progesterone release (experimental group). The adrenergic agents used in this investigation were noradrenaline as an adrenergic agonist, phentolamine as α -antagonist and propranolol as β -antagonist. KCl (56 mM) was used as a non-specific depolarising stimulus (Koh & Hille 1997). The samples of liquid from the ovarian compartment were maintained at -20 °C until progesterone concentrations were to be determined by RIA. The results were expressed as nanograms progesterone per milligram ovarian tissue (ng/mg

tissue) against time of incubation. Appropriate corrections were made in all cases, taking into consideration the volume extracted in each period tested.

Coeliac ganglion–SON–ovary system incubations with LH

In order to compare the simultaneous action of the neural effect and the endocrine effect on the release of ovarian progesterone, ovine LH was added (50 ng/ml in incubation buffer) to the ovary compartment, and incubation medium with ascorbic acid to the ganglion compartment. The values for the liberation of progesterone thus obtained were considered basal (control LH group). Groups in which the liberation of progesterone without LH was determined were considered as controls.

The experimental groups were those that had added adrenergic agents – noradrenaline, phentolamine or propranolol – in the ganglion compartment, and ovine LH in the ovary compartment.

In accordance with the established schedule, incubation liquid was collected from the ovarian compartment of the control LH groups and from that of the experimental groups, and progesterone was determined by RIA. The results were expressed as progesterone ng/mg tissue.

Progesterone assay

Progesterone was measured by RIA using antiserum raised against progesterone–11–BSA conjugate in rabbits, provided by Dr R Deis (Laboratorio de Reproducción y Lactancia, Mendoza, Argentina). The sensitivity, variability and crossreaction of this RIA have been reported previously (Telleria *et al.* 1994). The sensitivity of the assay is less than 5 ng/ml serum and the inter- and intra-assay coefficients of variation were less than 10%. This assay has been validated previously (Bussmann & Deis 1979, Donoso 1988).

Statistical analysis

All data are presented as means \pm S.E.M. Statistical evaluations of the results were done with unpaired Student's *t* test to assay significant differences between the means of the two groups. A value of $P < 0.05$ was considered statistically significant (Snedecor & Cochran 1976).

Results

Histological control

The morphological study confirmed the presence of the coeliac ganglion in the isolated *in vitro* system. The ganglion was extracted from the area proximal to the coeliac artery and from the area proximal to the renal artery. Its

elongated shape can be seen in the microphotograph (Fig. 2). It presents neural somas and is covered by a substantial amount of fatty connective tissue. This tissue probably provides additional protection against trauma, in addition to its other support functions (final magnification $\times 250$). During the standardisation of the system, we confirmed the existence of the ganglion in the preparation by means of histological studies. Afterward, we determined its spatial position in the rat for the purpose of subsequent extractions. We also analysed, immediately after incubation, preparations with vital colouration, for the purpose of proving the existence and viability of the ganglia.

Coeliac ganglion–SON–ovary system: effect of the addition of KCl to the ganglion compartment on progesterone release by the ovary in the oestrous cycle

In order to determine if stimulation of the ganglion affected the release of progesterone in the oestrous cycle, KCl 56 mM, considered to be a non-specific stimulating agent on the preganglionic nerve fibres, was added. In this case, the effect observed during pro-oestrus significantly decreased the release of progesterone at 30, 60 and 120 min (0.066 ± 0.01 compared with 0.036 ± 0.001 , $P < 0.05$; 0.072 ± 0.01 compared with 0.046 ± 0.002 , $P < 0.001$; 0.084 ± 0.01 compared with 0.062 ± 0.002 , $P < 0.05$) respectively (Fig. 4, PE). However, in oestrus it caused a stimulatory effect on the release of progesterone at all times studied ($P < 0.001$; Fig. 4, E). During D1, the release of progesterone increased significantly in comparison with the controls at all times studied ($P < 0.001$; Fig. 4, D1) The pattern of liberation of progesterone in D2 rats shows that KCl 56 mM caused a significant increase at 60 min (0.218 ± 0.01 compared with 0.14 ± 0.01 , $P < 0.001$), returning to values less than basal at 120 min and 180 min (0.132 ± 0.01 compared with 0.17 ± 0.01 , $P < 0.05$; 0.130 ± 0.008 compared with 0.206 ± 0.01 , $P < 0.001$; Fig. 4, D2).

Effect of the addition of adrenergic agents to the ganglion compartment on ovarian release of progesterone during the oestrous cycle

The presence of adrenergic agonists and antagonists in the ganglion compartment modified the release of ovarian progesterone at most times studied, depending on the stage of the oestrous cycle and the nature of the studied agent. For instance, in pro-oestrus, noradrenaline caused a significant increase in the release of ovarian progesterone only at 60 min (0.118 ± 0.01 compared with 0.072 ± 0.01 , $P < 0.001$), and when the α -adrenergic antagonist phentolamine was added to the ganglion compartment, it led to a considerable decrease (two- to threefold), in progesterone released at all times studied ($P < 0.001$). However, when the β -adrenergic antagonist propranolol was added, no significant modifications were seen

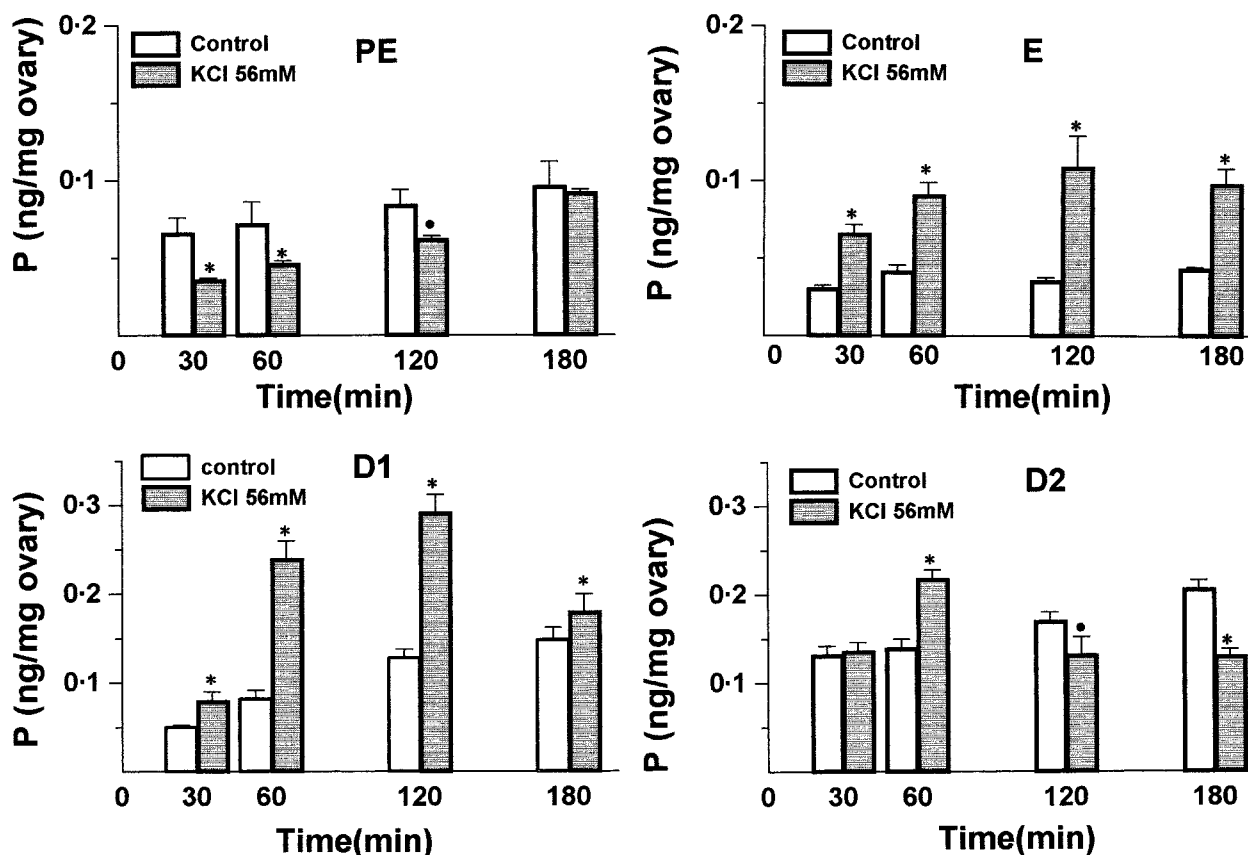


Figure 4 Effect of KCl (56 mM) in ganglion on release of progesterone by the ovary in the coeliac ganglion–SON–ovary system, obtained from rats in pro-oestrus (PE), oestrus (E), D1 and D2. The systems were incubated in the ganglion compartment in presence or absence of KCl in Krebs–Ringer plus ascorbic acid solution at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 180 min. Values are the means ± s.e.m. from six to eight animals per experimental group. * $P < 0.001$ and • $P < 0.05$ compared with respective control groups (Student's *t* test).

compared with the controls, for all the times studied (Fig. 5, PE). During oestrus, noradrenaline induced a significant increase in the release of progesterone compared with the controls only at 30 min (0.095 ± 0.001 compared with 0.031 ± 0.002 , $P < 0.001$), whereas phentolamine and propranolol induced a very significant increase in comparison with the controls at all times studied ($P < 0.001$; Fig. 5, E). When animals in D1 were examined, noradrenaline caused a significant increase (from two- to threefold) in the release of ovarian progesterone at all times studied ($P < 0.001$), phentolamine decreased the release of progesterone with respect to controls ($P < 0.001$), and propranolol induced a significant increase at all times analysed ($P < 0.05$; Fig. 5, D1). During D2, an inhibitory response was observed with noradrenaline at all times ($P < 0.001$) and with phentolamine there were no significant changes; with propranolol there was a very significant increase in the release of progesterone from the ovary at all times studied ($P < 0.001$; Fig. 5, D2).

Effect of LH during D1 and D2 in the ovary, with and without adrenergic agonist and antagonist stimulation in the ganglion

As the presence of LH is essential to the processes of ovulation and the luteinisation, its effect was evaluated during two stages in which corpora lutea were present and it was determined to have the main responsibility for the release of progesterone (Hsueh *et al.* 1984).

Without the presence of the stimulus in the ganglion, when LH was added to the ovary compartment a noticeable increase in the release of progesterone was observed, more prominent during D2 than D1 ($P < 0.001$) compared with the respective control groups (Fig. 6, D1 and D2). The presence of noradrenaline in the ganglion compartment caused a considerable increase (two- to threefold) in the release of progesterone in D1 compared with the control LH ($P < 0.001$). In contrast, phentolamine showed an inhibitory effect only at 30 and 60 min (0.124 ± 0.004 compared with 0.081 ± 0.001 , and 0.152 ± 0.008

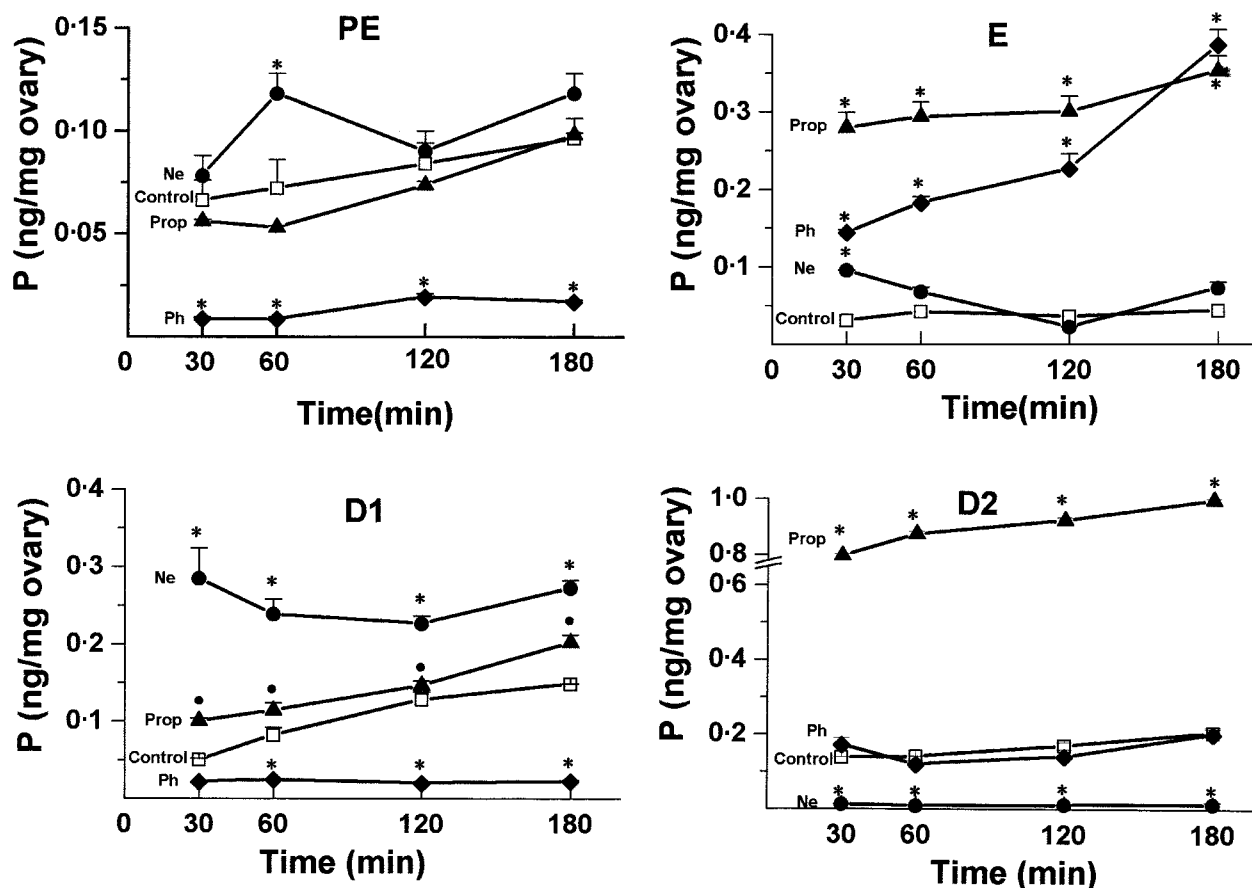


Figure 5 Effect of agonist and antagonist adrenergic agent in ganglion on ovarian release of progesterone (P) in the coeliac ganglion–SON–ovary system obtained from rats in pro-oestrus (PE), oestrus (E), D1 and D2. The systems were incubated in the presence or absence of agonist and antagonist adrenergic agents at 10^{-6} M in Krebs–Ringer ascorbic acid solution at 37 °C in an atmosphere of 95% O_2 –5% CO_2 for 180 min. Values are the means \pm S.E.M. from six to eight animals per experimental group. Ne, noradrenaline; Prop, propranolol; Ph, phentolamine. * $P < 0.001$ and $\bullet P < 0.05$ compared with the respective control groups (Student's *t* test).

compared with 0.104 ± 0.008 , $P < 0.05$) compared with its control LH, whereas propranolol caused a significant decrease in the release of progesterone at all times studied ($P < 0.001$; Fig. 7, D1).

During D2, the presence of noradrenaline was associated with a diminution of the release of progesterone at 60 and 120 min (0.400 ± 0.04 compared with 0.280 ± 0.02 , $P < 0.001$; 0.420 ± 0.02 compared with 0.260 ± 0.02 , $P < 0.01$) compared with its control LH. The addition of phentolamine and propranolol decreased the release of progesterone even more (two- to threefold) at all times compared with its control-LH ($P < 0.001$; Fig. 7, D2).

Discussion

Several authors have provided evidence of a functional neural connection among different hypothalamic centres and the ovary, which contributes to its endocrine regu-

lation (Kawakami *et al.* 1972, 1979, 1981, Weiss *et al.* 1982, Seto *et al.* 1988, Saito *et al.* 1990, Gerendai *et al.* 1995). However, at present there is not much information on the peripheral neural control during the different ovarian physiological states of the oestrous cycle, and consequently little is known about such control during two very important stages, D1 and D2, when the corpora lutea have great importance for the secretion of progesterone (Weiss *et al.* 1982). *In vitro* experiments involving incubating the ovary in the presence of adrenergic agonists show that, in the rat, the β agonist induces the release of progesterone during the first oestrous cycle, including D2 after puberty (Aguado *et al.* 1982, Aguado & Ojeda 1984a,b). In addition, it has been observed that both electrical stimulation and the dissection of the SON cause modification of both follicular development and ovarian steroidogenesis (Aguado & Ojeda 1984a,b, Chávez *et al.* 1991, Chavez & Domínguez 1994). This led us to study the physiological role of the entire peripheral nervous pathway (from the

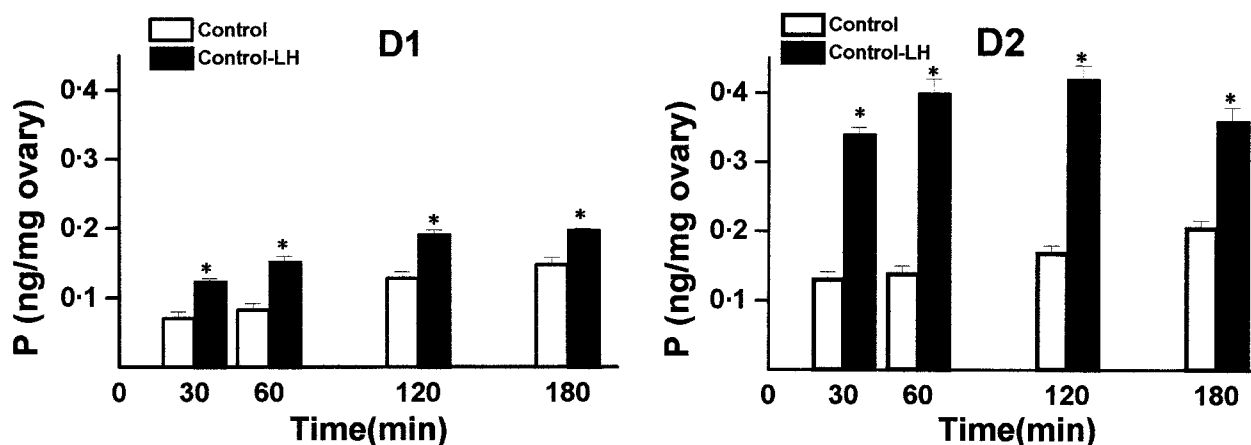


Figure 6 Progesterone (P) release by the ovary in a coeliac ganglion–SON–ovary system obtained from rats in D1 and D2 with or without LH (50 ng/ml) in the ovarian compartment. The systems were incubated in the ovarian compartment in the presence or absence of LH in Krebs–Ringer at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 180 min. Values are the means ± S.E.M. from six to eight animals per experimental group. *P < 0.001 compared with the respective control groups. (Student's *t* test).

coeliac plexus through the SON to the ovary), not only under the control of the corpora lutea, but also under regulation by steroid secretion during all phases of the oestrous cycle.

For this purpose we sought an experimental design that could simulate *in vitro* the conditions that exist *in vivo*, preserving in particular the paracrine and autocrine mechanisms of regulation that take place in the ovary, and without humoral influence. For this, an integrated coeliac ganglion–SON–ovary system was designed and standardised, in which adrenergic stimulation took place in the ganglion, and neither ovarian structures nor innervation were affected. The presence of the ganglion was confirmed by histological studies moments before its surgical

removal from the animal, and after the incubations. It did not present significant modifications with regard to *in vivo* conditions.

We assume that we have a system that is a functional entity, with its own autonomic tone, the latter point having been verified because the liberation of basal progesterone increases or decreases during the different stages of the oestrous cycle. We wanted to see if the occupation of the adrenergic receptors by the antagonists phentolamine and propranolol caused a response such as liberation of ovarian steroids. If this were not so, liberation of only basal progesterone would be stimulated in the presence of noradrenaline in the ganglion; however, the basal value was independent of the addition of the neuro-

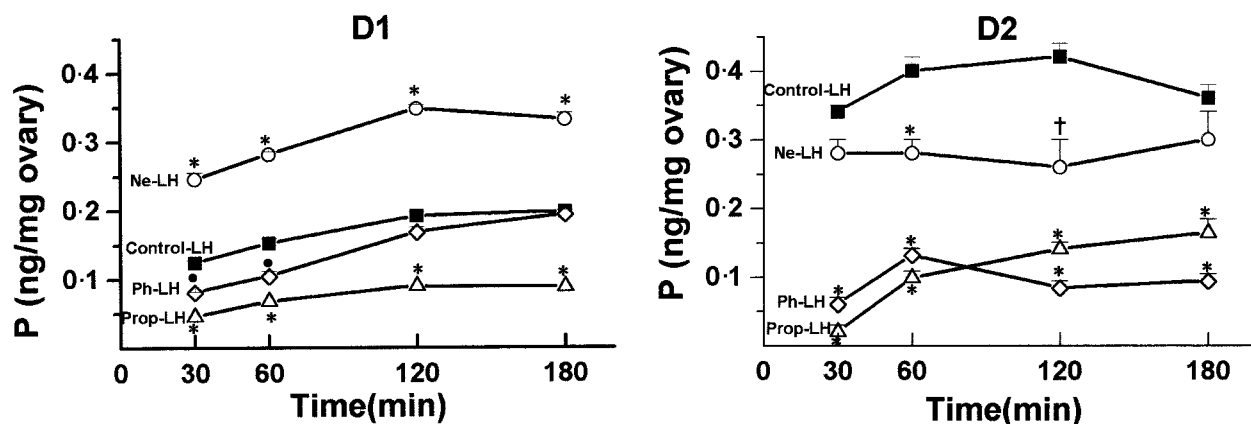


Figure 7 Effect of agonist and antagonist adrenergic agents in ganglion on release of progesterone (P) by the ovary in a coeliac ganglion–SON–ovary system obtained from rats in D1 and D2 with LH (50 ng/ml) in the ovarian compartment. The systems were incubated in the presence or absence of agonist and antagonist adrenergic agents at 10⁻⁶ M in Krebs–Ringer plus ascorbic acid solution at 37 °C in an atmosphere of 95% O₂–5% CO₂ for 180 min. Values are the means ± S.E.M. from six to eight animals per experimental group. Ne, noradrenaline; Ph, phentolamine; Prop, propranolol. *P < 0.001, †P < 0.01, ●P < 0.05 compared with the respective control groups (Student's *t* test).

transmitter to the ganglion compartment. The effect is verified, also with the addition of LH in the ovary, in this system as well as in the CNS.

In order to verify if the coeliac ganglion–SON–ovary system responds to non-specific stimulation, a moderate concentration (56 mM) of KCl was added to the ganglion compartment (Koh & Hille 1997). As expected, different responses were obtained according to the physiological conditions of each stage.

After establishing the spatial location of the system by means of histological studies, we demonstrated, by means of non-specific stimulations, that the ganglia have their own tone, and that the system was viable. Furthermore, knowing also that, in the superior cervical ganglion as in other sympathetic ganglionic systems, *in vitro* studies have detected α - and β -adrenergic receptors (Shivachar & Eikenburg 1999), we wanted to observe, in our system, whether the occupation of adrenergic receptors by agonists (noradrenaline) and antagonists (phentolamine and propranolol) produced the response of release of ovarian steroids; if so, this would confirm the presence of such receptors (Eccles & Libet 1961, Christ & Nishis 1971, Frankö 1978).

When the stimulation was performed with adrenergic agents, the addition of noradrenaline 10^{-6} M to the ganglion compartment during pro-oestrus did not cause significant changes in the release of progesterone from the ovary, except after 60 min of incubation. At this stage of the cycle, the preovulatory peak of LH takes place and, possibly, in the face of this stimulus, the action of noradrenaline is not as noticeable as it is during pregnancy (unpublished data). In oestrus, a similar response was observed, perhaps because during this stage, after ovulation, the corpus luteum had only just been organised and was therefore unresponsive. The stimulation during D1 showed a remarkable increase in the release of ovarian progesterone at all times studied. This is to say that during this stage the corpora lutea are in full activity, which would be the reason why the adrenergic effect on the secretion of progesterone is greater. In addition, in *in vivo* studies, a peak in the release of progesterone has been observed during D1 that is probably neural in origin (Kawakami *et al.* 1972, Weiss *et al.* 1982, Gerendai *et al.* 1995). During D2, a very noticeable inversion occurs that is concordant with results that were obtained by CNS stimulation (Kawakami *et al.* 1981, De Bortoli *et al.* 1998). It cannot be ignored that during pro-oestrus, oestrus and especially during D1, noradrenaline is being released from the nerve endings, as the response is the same as that obtained when the ovary is stimulated directly with noradrenaline in *in vitro* systems (Aguado & Ojeda 1984a). In contrast, during D2, noradrenaline would not be liberated, as the effect is inhibitory and, when the ovary is stimulated with noradrenaline, the release of progesterone generally increases (Aguado & Ojeda 1984a). With respect to phentolamine, during pro-oestrus and D1 the release

of progesterone is inhibited, whereas in oestrus it is stimulated. During D2, phentolamine does not modify basal concentrations of progesterone.

The addition of propranolol to the ganglion compartment during oestrus, D1 and D2 stimulates the liberation of progesterone, achieving its maximum effect in D2, having been inactive in pro-oestrus.

With respect to similar changes in progesterone output caused by both phentolamine and propranolol, we have not been able, using our experimental scheme, to confirm that the observed effect is, or is not, specific. It can only be noted that this effect is observed in oestrus, when phentolamine and propranolol produce significant changes – in this case stimulatory – in the liberation of progesterone, although of different magnitude. This could suggest the presence of α - and β -adrenergic receptors in the ganglionic neurones, and possibly different response of these receptors, depending on the antagonists used and the ganglionic neurones in which the receptors are located. Alternatively, in oestrus the corpus luteum is being organised, in contrast with di-oestrous, when different responses are observed in the presence of antagonists in the ganglion. We are, however, able to affirm that the presence of these agents in the coeliac ganglion modifies the release of progesterone according to the stage of the oestrous cycle.

In order to compare the combined neural and endocrine effects on the ovarian release of progesterone, the experiment was carried out during stages D1 and D2, when the corpora lutea are at their peak of activity. When LH was added to the ovarian compartment, concentrations of progesterone increased significantly with respect to their basal values, the greatest values being obtained in D2 (Richardson 1986). In contrast, when LH was added to the ovarian compartment and the adrenergic agonist and antagonists were added to the ganglion compartment, the most noteworthy aspect of the experiment was that, during D1, the effect of noradrenaline increased the release of progesterone even more, surpassing the increase obtained in the presence of LH alone in the ovary. With regard to phentolamine and propranolol, the effect of noradrenaline was reversed, the β antagonist having a more significant effect.

During D2, noradrenaline, phentolamine and propranolol produced a marked inhibition of the effect of LH. These results prove the functionality of the system, as the hormonal action produced by the gonadotrophin is modified by the neural action, facilitating the decrease in progesterone concentration that is necessary to start a new cycle.

The fact that, at all stages, noradrenaline almost always increased the release of progesterone, only diminishing in D2, may be interpreted as an indication that ganglionic stimulation causes the discharge of a substance that stimulates the release of progesterone in the ovary during

pro-oestrus, oestrus and D1; the possibility that the substance is noradrenaline cannot be disregarded (Dissen *et al.* 1993, Aguado & Ojeda 1984*a,b*). Meanwhile during D2, the observed inhibitory effect is not consistent with the effect of the adrenergic agonist on the ovary. Whenever adrenergic receptors are stimulated in ovarian incubations, or in culture of granulosa cells, liberation of progesterone increases, and never diminishes (Hernandez *et al.* 1988). In view of the preceding observations, we cannot interpret in the same way the decrease in the values of ovarian progesterone with respect to its controls on D2 when noradrenaline is added to the ganglionic compartment. Neither can we explain the decrease in progesterone when it is phentolamine that is added to the ganglionic compartment in pro-oestrus and on D1. These results lead us to believe that it could be another neurotransmitter that is finally released in the ovary. We could also postulate that other synaptic circuits are established in the ganglion – that is, it is not only noradrenaline that is released, but also other neurotransmitters, such as neuropeptide Y or VIP, which could themselves regulate the release of noradrenaline (Ojeda *et al.* 1989, Ferruz *et al.* 1992). This is a possibility that remains to be investigated.

Of the known peptidergic neurotransmitters located in the ganglion that are transported to the ovary through the SON, none has an inhibitory effect on the release of progesterone. In general, their effect is stimulatory, as in the case of VIP (Ahmed *et al.* 1986, Dissen *et al.* 1993, Kaleczyc *et al.* 1995). On the basis of what has been stated, we can only speculate about the possible release of other substances with inhibitory effect, in the ovary during D2, in response to stimulation of the ganglion. One such substance, GnRH, in addition to its classical releasing action at the pituitary level, acts on multiple extrapituitary sites to regulate various reproductive functions. This peptide has shown inhibitory effects on the release of progesterone in luteal cells, in both *in vitro* and *in vivo* conditions (Clayton *et al.* 1979, Jones & Hsueh 1980, 1981, 1982*a,b*, Massicote *et al.* 1981). In the rat ovary, specific high-affinity GnRH receptors in granulosa and theca cells and a GnRH-like substance have been identified, although the presence of an ovarian GnRH-like substance still remains difficult to confirm (Birnbaumer *et al.* 1985, Séguin *et al.* 1982). In addition to their action at the gonadal level, GnRH or GnRH-like peptides may have important roles as neurotransmitters in the CNS (Hsueh *et al.* 1983). Thus GnRH and GnRH-like peptides may have important paracrine and neurotransmitter roles in the regulation of various reproductive functions in extrapituitary sites (Berhman *et al.* 1980, Smith-White & Ojeda 1983). These substances have been found in sympathetic ganglia, for which reason we suppose that, from here, they could reach the ovary via the SON, or that their presence in the ganglia could modulate the release of noradrenaline in the ovary (Jan *et al.* 1979,

1980, Jan & Jan 1982, Sejnowski 1982, Reprintseva *et al.* 1986).

Other factors that might be exerting an influence during D2 could include GABA (Erdö *et al.* 1985, Castelli *et al.* 1999), as there is some evidence to indicate that GABA can increase ovarian blood flow, and increase the secretion of oestradiol while decreasing the secretion of progesterone (Erdö *et al.* 1985). The epidermal growth factors have an inhibitory action on ovarian and testicular steroidogenesis (Hsueh *et al.* 1981); however, the physiological significance of these peptides is not yet known.

With this experimental design we cannot know, for the time being, which is the neurotransmitter that is finally released in the ovary. What we are sure of is that the coeliac ganglion modifies its activity when is stimulated by adrenergic agonists or antagonists, and that these influences arrive at the ovary through the SON and modify the release of progesterone. In conclusion, by utilising an experimental design that allows us to make observations independent of the humoral factors that affect the ovary, we have been able to confirm that the coeliac ganglion has a direct neural effect on activation of the corpus luteum, and thus on the release of progesterone. The experimental scheme is, in our opinion, a valuable tool for the study of the participation of peripheral neural influences in ovarian physiology.

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