Experimental Physiology

Research Paper

Neuromodulation of the luteal regression: presence of progesterone receptors in coeliac ganglion

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New Findings

- **What is the central question of this study?**
  The processes involved in luteal involution have not yet been clarified and, in general, have been studied only from a hormonal point of view. We investigated whether progesterone, from the coeliac ganglion through the superior ovarian nerve, is able to modify the luteal regression of late pregnancy in the rat.
- **What is the main finding and its importance?**
  We showed that the luteal regression might be reversed by the neural effect of progesterone and demonstrated the presence of its receptors in the coeliac ganglion. This suggests that the peripheral neural pathway, through neuron–hormone interaction, represents an additional mechanism to control luteal function in addition to the classical endocrine regulation.

The corpus luteum (CL) is a transitory endocrine gland that produces progesterone (P). At the end of its useful life, it suffers a process of functional and structural regression until its complete disappearance from the ovary. To investigate whether P is able to regulate the process of luteal regression through the peripheral neural pathway, we used the coeliac ganglion (CG)–superior ovarian nerve–ovary system from rats on day 21 of pregnancy. We stimulated the CG with P and analysed the functional regression through ovarian P release measured by radioimmunoassay, expression by RT-PCR and activity of luteal 3β- and 20α-hydroxysteroid dehydrogenase (anabolic and catabolic P enzymes, respectively). The luteal structural regression was evaluated through a study of apoptosis measured by TUNEL assay and the expression of apoptotic factors, such as Bcl-2, Bax, Fas and Fas ligand (FasL) by RT-PCR. To explore whether the effects mediated by P on the CL may be associated with P receptors, their presence in the CG was investigated by immunohistochemistry. In the group stimulated with P in the CG, the ovarian P release and the 3β-hydroxysteroid dehydrogenase activity increased, whereas the expression and activity of 20α-hydroxysteroid dehydrogenase decreased. In addition, a decrease in the number of apoptotic nuclei and a decrease of the expression of FasL were observed.

F. Ghersa and J. Burdisso contributed equally to this work.

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We demonstrated the presence of P receptors in the CG. Overall, our results suggest that the regression of the CL of late pregnancy may be reprogrammed through the peripheral neural pathway, and this effect might be mediated by P bound to its receptor in the CG.

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Introduction

The corpus luteum (CL) is a transient but dynamic steroidogenic gland that controls the output of progesterone (P). At the end of its useful life, it suffers a process of regression that starts with a decrease in P production (functional regression) and ends with its disappearance from the ovary as a consequence of the apoptosis of luteal and endothelial cells (structural regression; Stocco *et al.* 2007).

Progesterone is essential in the preparation of the uterus for the establishment and maintenance of pregnancy. Progesterone synthesis depends not only on its anabolic enzymes, but also on the level of expression of its catabolic enzyme, 20α-hydroxysteroid dehydrogenase (20α-HSD), which converts P into a progestin unable to bind progesterone receptors (PRs; Bowen-Shauver *et al.* 2003). In turn, P acts on the CL, maintaining the expression of 20α-HSD to a minimum, inhibiting the expression of interleukin-6 and protecting it from cell death by suppressing Fas expression (Sugino *et al.* 1997; Kuranaga *et al.* 2000*b*).

The ovary is innervated by the ovarian nerve plexus and the superior ovarian nerve (SON), the latter being the most relevant one because it impacts on ovarian steroidogenesis. The SON has adrenergic fibres, originating mostly in the coeliac ganglion (CG; Klein & Burden, 1988). This ganglion belongs to the sympathetic prevertebral chain and contains principal neurons, interneurons and a capillary plexus forming a microcirculation among the ganglionic structures (Eränkö, 1978; Chau *et al.* 1991; Prud'homme *et al.* 1999). We have demonstrated the ability of the CG to react to the effect of several substances, such as hormones, and to affect, through the SON, the ovarian function in the rat. Such effects may be associated with the presence of hormone receptors in the CG (Vallcaneras *et al.* 2009; Vega Orozco *et al.* 2012). It is known that PRs are widely distributed throughout the brain, but their study has been limited mainly to the hypothalamus, whereas the extrahypothalamic receptors have been neglected. In addition, some studies have demonstrated that PRs play an important neuroprotective and regenerative role in the spinal cord and the peripheral nerves (Koenig *et al.* 1995; Chan *et al.* 2000; Labombarda *et al.* 2003). According to the available literature to date, however, the presence of PRs in peripheral sympathetic ganglia has not been demonstrated.

The purpose of this study was to investigate whether P acting on the CG through the SON is able to modify the regression of the CL and to explore whether this effect is associated with the presence of the PR in CG cells. To this end, an *ex vivo* system, composed of the CG and the ovary connected by the SON, was used.

Methods

Animals

Virgin Holtzman strain female rats weighing 250 ± 50 g were used on day 21 of pregnancy. They were kept in a light- (lights on from 07.00 to 19.00 h) and temperature-controlled room (24 ± 2°C) with water and food *ad libitum*. The procedure used to induce gestation was described by Casais *et al.* (2001). Animals were handled according to the procedures approved in the UFAW *Handbook on the Care and Management of Laboratory Animals*. The experimental protocol was approved by the University of San Luis Animal Care and Use Committee (number protocol: B17/07, ordinance CD 006/02).

Experimental procedure

The surgical procedure to remove the coeliac ganglion–superior ovarian nerve–ovary (CG–SON–O) system and the incubation conditions were carried out according to Casais *et al.* (2001). The animals were anaesthetized with ketamine and xylazine (80 and 10 mg kg^{-1}, respectively, given I.P.). The fetuses were removed and killed in an atmosphere of carbon dioxide. The CG–SON–O system was extracted, and the mothers were killed by decapitation. The entire CG–SON–O system was removed by surgery, avoiding contact between the surgical instruments and the nerve fibres in order to prevent spontaneous nerve depolarization. The pieces of tissues removed consisted of the left ovary, the fibres that constituted the SON inserted in the suspensory ligament, and the CG accompanied by some small ganglia surrounding it (Fig. 1*A*). The whole surgical procedure was completed in 1–2 min. The CG–SON–O system was placed in a cuvette with two isolated compartments, one for the CG and the other for the ovary, both joined by the SON (Fig. 1*B*). The incubation medium

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used was Krebs–Ringer–bicarbonate buffer, pH 7.4 with the addition of glucose (0.1 mg ml^{-1}) and albumin (0.1 mg ml^{-1}) at 37°C in an atmosphere composed of 95% O_2 and 5% CO_2. The *ex vivo* system was pre-incubated for 30 min, and the end of this period was considered incubation time 0. After this time, the buffer was changed in both compartments, and ascorbic acid (0.1 mg ml^{-1}) was added to the ganglion compartment as an antioxidant agent. In the stimulated group, P at 10–5 M concentration was added to the ganglion compartment. This dose range is higher than physiological values, and it was chosen to show an effect. Control groups consisted of CG–SON–O systems that were untreated. Periodic extractions (250 μl) of the ovary incubation liquid were carried out at 30, 60, 120 and 180 min and kept at −20°C until P was assayed. The corresponding corrections were made in all cases, taking into consideration the volume extracted in each tested period. After incubation (180 min), whole ovaries were weighed, and the CLs were separated and stored at −80°C until RNA and protein extraction and analysis of enzyme activities. Corpora lutea were also fixed with 4% paraformaldehyde for further analysis by TUNEL (TdT-mediated dUTP Nick-End Labelling) assay.

To analyse the presence of PRs in CG, rats on day 21 of pregnancy were anaesthetized as described above. The CG and the nearby ganglia were fixed *in situ* with Bouin's solution for subsequent application of the immunohistochemical technique.

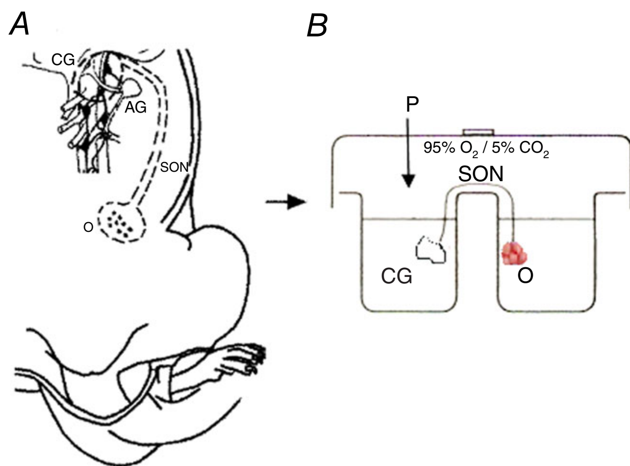

Figure 1. Schematic representation of the *ex vivo* GC–SON–O system
A, scheme of the SON pathway and the anatomical relations of the coeliac ganglion, nerve and ovary. The dashed lines indicate sites where the GC–SON–O system was dissected out. *B*, the cuvette used for the subsequent incubation of the system. Abbreviations: AG, adrenal gland; CG, coeliac ganglion; O, ovary; P, progesterone; and SON, superior ovarian nerve.

Progesterone assay

Progesterone was measured by radioimmunoassay (RIA) using antiserum produced in rabbits against P conjugated to bovine serum albumin at the 11 position, provided by Dr R. P. Deis (Laboratorio de Reproduccion y Lactancia Mendoza, Argentina). The antiserum was highly specific for P, with low cross-reactivities, <2.0% for 20α-dihydro-progesterone and deoxy-corticosterone and 1.0% for other steroids. The sensitivity was <5 ng ml^{-1} and the inter- and intra-assay coefficients of variation were <10%. Progesterone concentration was expressed as nanograms per milligram of ovary, in all the incubation times.

Enzyme activities

The activities of the enzymes 3β-hydroxysteroid dehydrogenase (3β-HSD) and 20α-HSD were measured according to Telleria & Deis (1994). The CLs from each animal were homogenized in 0.7 ml of Tris–HCl, 0.1 mM EDTA (pH 8) with a glass homogenizer. The homogenates were centrifuged at 105,000*g* for 60 min. The supernatant fluids were used for the assay of 20α-HSD activity. The precipitates were rehomogenized with 0.7 ml of 0.25 M sucrose and centrifuged at 800*g* for 5 min. The supernatants were used as the enzyme solution for the assay of 3β-HSD activity. The substrates for 3β-HSD and 20α-HSD were pregnenolone (5 μg) and 20α-hydroxypregn-4-en-3-one (12.5 μg), respectively. Both enzyme activities were assayed spectrophotometrically, dependent on the increase in NADH or NADPH in 1 min at 37°C, and the values were expressed as international units per milligram of protein per minute.

Isolation of RNA and RT-PCR analysis

Total luteal RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies, Buenos Aires, Argentina), according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed at 37°C using random primers and M-MLV Reverse Transcriptase (Promega Inc., Buenos Aires, Argentina) in a 26 μl reaction mixture. For amplification of the reverse transcription (RT) products, the reaction mixture consisted of 1× Green Go Taq reaction buffer, 0.2 mM deoxynucleoside triphosphates, 0.5 μM specific oligonucleotide primers and 1.25 U GoTaq DNA polymerase (Promega Inc., Buenos Aires, Argentina) in a final volume of 50 μl. Sequences of primers (5′–3′) were as follows:

3β-HSD: GTCTTCAGACCAGAAACCAAG and CCTTAA GGCACAAGTATGCAG;
20α-HSD: TTCGAGCAGAACTCATGGCTA and CAACC AGGTAGAATGCCATCT;

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Bax: GATTGCTGACGTGGACACGGACT and TCAGC CCATCTTCTTCCA;
Bcl-2: AGAGGGGCTACGAGTGGGAT and CTCAGTCA TCCACAGGGCGA;
Fas: GTGATGAAGGGCATGGTT and TTGACACGCAC CAGTCTT;
FasL: CCAGATCTACTGGGTAGA and ATGGTCAGCAA CGGTAAG; and
S16: CGTTCACCTTGATGAGCCCATT and TCCAAGGG TCCGCTGCAGTC.

The amplification of the complementary DNA was performed using a thermal cycler (My Cycler; BioRad, Buenos Aires, Argentina). For 3β-HSD, 20α-HSD, Bcl-2 and S16, the reactions were carried out at 95°C for 1 min, 59°C for 1 min and 72°C for 1 min for 35 cycles. For Bax, Fas and Fas ligand (FasL), the parameters were 95°C for 1 min, 56°C for 1 min and 72°C for 1 min for 40 cycles. All the reactions were terminated with a 5 min extension at 72°C. Reaction products were electrophoresed on 2% agarose gels, visualized with GelRed, and examined by ultraviolet transillumination. Band intensities of RT-PCR products were quantified using ImageJ (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). Relative levels of mRNA were expressed as the ratio of signal intensity for the target genes relative to that for the housekeeping gene S16.

Western blot

Protein extracts were obtained using TRIzol reagent and following the manufacturer's indications (Invitrogen Life Technologies, Buenos Aires, Argentina). Protein concentration was determined by the Lowry method. Aliquots containing 30 μg of total protein were subjected to electrophoresis in 5–10% SDS-PAGE gels and then electrotransferred to polyvinylidene difluoride (PVDF) membrane (Millipore Corporation, Bedford, MA, USA) at 100 V for 1 h in a transfer buffer (25 mM Tris, 192 mM glycine and 20% v/v methanol, pH 8.3). The membrane was immersed in 3% non-fat milk in a TTBS solution [0.2 M Tris–HCl (pH 7.6), 1.37 M NaCl and 0.5% Tween-20] for 1 h at room temperature, followed by an overnight incubation at 4°C with either goat anti-3β-HSD (SC-30820; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or rabbit anti-β-tubulin (SC-9104; Santa Cruz Biotechnology Inc.), 1:1000 dilution in a 1% solution of non-fat powdered milk in TTBS. After incubation with the primary antibody, membranes were washed in TTBS and incubated with rabbit anti-goat IgG peroxidase-linked antibody ZyMaxTM Grade (catalogue no. 81–1620; Invitrogen Laboratories), 1:5000 dilution in 1% milk for 1 h at room temperature and goat anti-rabbit IgG peroxidase-linked antibody (code 111-036-003; Jackson Immune Research Laboratories, Inc., West Grove, PA, USA), 1:5000 dilution in 3% milk for 3 h at room temperature, respectively. After washing in TTBS, blots were developed using an enhanced chemiluminescence Western blotting detection system (Thermo Scientific Super signal West Pico chemiluminescence; Pierce Biotechnology, Rockford, IL, USA) and exposed to X-ray films (Thermo Scientific CL-XPosureTM Film; Pierce Biotechnology). The mean intensity of each band was measured using the NIH ImageJ software (Image Processing and Analysis in Java from http://rsb.info.nih.gov/ij/). The 3β-HSD protein levels were normalized against β-tubulin (endogenous control).

TUNEL assay

Fragmented DNA of apoptotic cells were detected using DeadEndTM Colorimetric Apoptosis Detection System, a modified TUNEL assay (Promega, Buenos Aires, Argentina), according to the manufacturer's instructions. The detection was achieved using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine. Using this procedure, apoptotic nuclei are stained dark brown. Finally, sections were counterstained with Haematoxylin, mounted and analysed under a light microscope. The results are expressed as the mean ± SEM of the total number of apoptotic nuclei present in all fields at ×100 magnification. Three corpora lutea from different animals for each experimental group were observed.

Immunohistochemistry

The tissue sections were incubated at 60°C in a heater for 15 min, then deparaffinized with xylene and hydrated through decreasing concentrations of ethanol. After rinsing in PBS (0.01 M, pH 7.4), the sections were transferred to 0.01 M sodium citrate buffer (pH 6.0) and incubated at 97°C in a thermostatic bath for 40 min. The sections were rinsed with distilled water and PBS. Non-specific binding sites for immunoglobulins were blocked by incubation for 1 h with 5% bovine serum albumin (Sigma). Sections were incubated with rabbit polyclonal antibody raised against PRs of human origin (H-190: sc-7208; Santa Cruz Biotechnology, Inc.) diluted 1:250 and mouse monoclonal antibody raised against βIII-tubulin (Covance, Princeton, NJ, USA) diluted 1:500, overnight in a humidified chamber at 4°C. After three washes in PBS, the samples were incubated with the secondary antibodies (1:500 dilution of Alexa 488-conjugated anti-rabbit IgG [H+L] and Alexa 555-conjugated anti-mouse IgG [H+L]; Invitrogen) for 1 h at room temperature. After washing, the samples were mounted with FluorSave (Calbiochem, Buenos Aires, Argentina). Images were obtained with an Olympus FV-1000 confocal microscope. In order to confirm the

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specificity of the immunoreactive procedures, sections of mouse ovaries were used as a positive control for PR and negative control for neuronal marker βIII-tubulin. Sections of juxtaganglionic tissue were used as a negative control for PR, and sections of CG were stained according to the protocol described above, but incubation with the primary antiserum was omitted. No positive structures or cells were found in these sections.

Statistical analysis

All data are presented as means ± SEM in each group. Differences between two groups were analysed with Student's unpaired *t* test. For multiple comparisons made throughout the time of incubation, repeated-measures ANOVA followed by Tukey's test was used. For multiple comparisons not involving repeated measures, one-way ANOVA followed by Tukey's test was used. A difference was considered to be statistically significant at $P < 0.05$.

Results

Ganglionic effect of progesterone on the luteal functional regression

First, we examined whether stimulation of CG with P at 10–5 M induced changes in P ovarian release. Results showed an increase in P release in the ovarian compartment at all the studied times in relation to the control group (Fig. 2).

Given that ovarian release of P was increased by stimuli of P on CG, we analysed the expression (mRNA and protein) and the activity of 3β-HSD, a P anabolic enzyme, at 180 min (end of the incubation period). Progesterone from CG did not modify either mRNA or protein expression of 3β-HSD (Fig. 3*A* and *B*), but the activity of this enzyme was increased in the stimulated group with respect to the control value (Fig. 3*C*). This result suggests that stimulation by P on the CG may affect the luteal 3β-HSD activity.

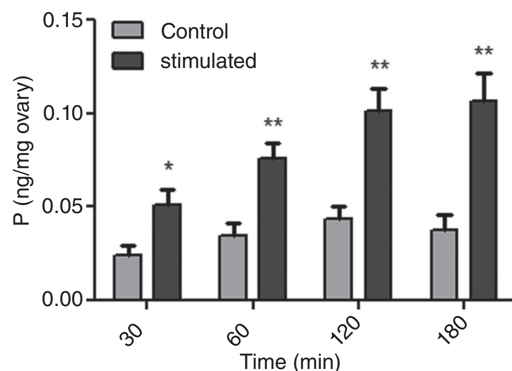

Figure 2. Ganglionic effect of progesterone (P) at 10–5 M (stimulated group) on the release of ovarian P, expressed in nanograms per milligram of ovary
The control group consisted of untreated CG–SON–O systems. Values are shown as the mean + SEM of six animals per experimental group. Repeated-measures ANOVA followed by Tukey's test was used. *$P < 0.05$; **$P < 0.01$.

Next, we studied the effect of P from the CG on the expression (mRNA) and activity of 20α-HSD, a P catabolic enzyme, at the end of the incubation period. The results showed a decrease in the enzyme expression and activity in the stimulated group compared with the control group (Fig. 4*A* and *B*). These results suggest that a P stimulus on the CG might downregulate the expression and activity of 20α-HSD in the CL on day 21 of pregnancy.

Ganglionic effect of progesterone on the luteal structural regression

After analysing the ganglionic effect of P on the factors involved in the CL functional regression, we investigated whether P, through the peripheral neural pathway, was able to modify the luteal structural regression.

Apoptotic cells were clearly identifiable after TUNEL staining of CLs obtained postincubation of CG–SON–O systems. The stimulated group showed a significant decrease in the number of TUNEL-positive cells compared with the control group (Fig. 5).

We analysed the mRNA expression of Bcl-2 and Bax by RT-PCR, and no statistically significant differences between control and stimulated groups were observed. Consequently, the ratio of Bcl-2 to Bax did not change (Fig. 6*A*, *B* and *C*).

Fas is one of the receptors that mediate apoptosis by external influence. To trigger apoptosis, Fas of luteal cells must interact with FasL. This ligand is characteristically shown on immunological cell membranes or is released as a soluble ligand by them. After stimulation with P on the CG, we analysed the expression (mRNA) of luteal Fas and FasL at the end of the incubation period. The expression of Fas remained constant (Fig. 7*A*). But interestingly, FasL expression decreased significantly in the stimulated group in relation to the control group (Fig. 7*B*).

Localization of progesterone receptors in coeliac ganglion

Finally, we investigated the presence of PRs in the CG of rats on day 21 of pregnancy by immunohistochemistry. A serum anti-βIII-tubulin was used as a neuronal specific marker, allowing observation of axons, cytoplasm and nuclei of neuronal somata (Fig. 8*A* and *B*). Figure 8*C* shows positive immunoreactivity for the PR. Figure 8*D* shows an overlay of the images in Fig. 8*B* and *C*; the PR was visible in the cytoplasm of neuronal somata (yellow arrow) and axons (pink arrow). The blue arrow shows PRs that are not colocalized with neurons; this may indicate the presence

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of glial cells that form part of the ganglionic tissue (Fig. 8*D*). The image of the CG for transmitted light microscopy (Fig. 8*E*) shows parenchyma with several neuronal somata and a thin capsule of connective tissue.

Data about the controls for immunohistochemistry are given in Fig. 9. Sections of mouse ovary were used as the negative control for βIII-tubulin immunoreactivity and positive control for PR immunoreactivity (Fig. 9*A* and *B*, respectively). Juxtaganglionic tissue immunostained with anti-PR was also used as a negative control (Fig. 9*C*). No positive structures or cells were observed in the CG sections when the anti-PR was not added

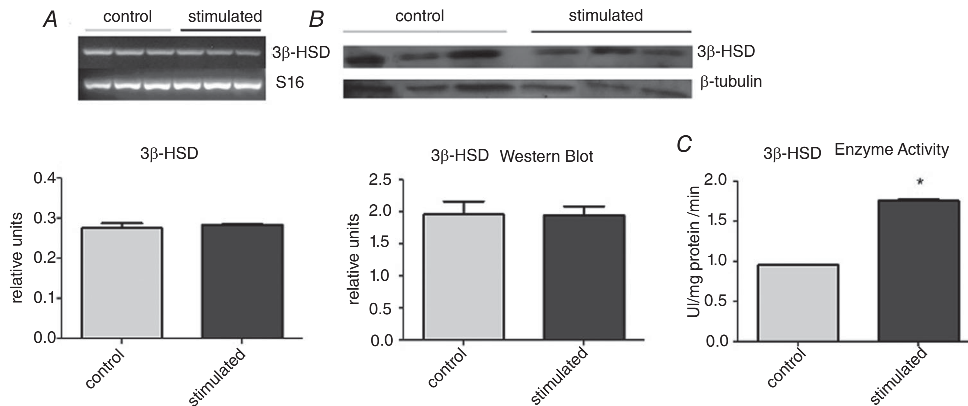

Figure 3. Ganglionic effect of P at 10–5 M (stimulated group) on the expression and enzyme activity of luteal 3β-hydroxysteroid dehydrogenase (3β-HSD)
The control group consisted of untreated CG–SON–O systems. *A*, measurement of the expression (mRNA) of 3β-HSD and S16, as housekeeping gene, by RT-PCR. The PCR products, 3β-HSD (447 bp) and S16 (100 bp), were visualized on agarose gels stained with Gel Red. *B*, measurement by Western blot of expression (protein) of 3β-HSD and β-tubulin as loading control. The gel photographs were quantified using ImageJ and expressed as relative units. *C*, measurement of enzyme activity of 3β-HSD expressed as international units per milligram of protein per minute. Results are shown as the mean + SEM of three animals per experimental group. Student's unpaired *t* test was used. $^*P < 0.05$.

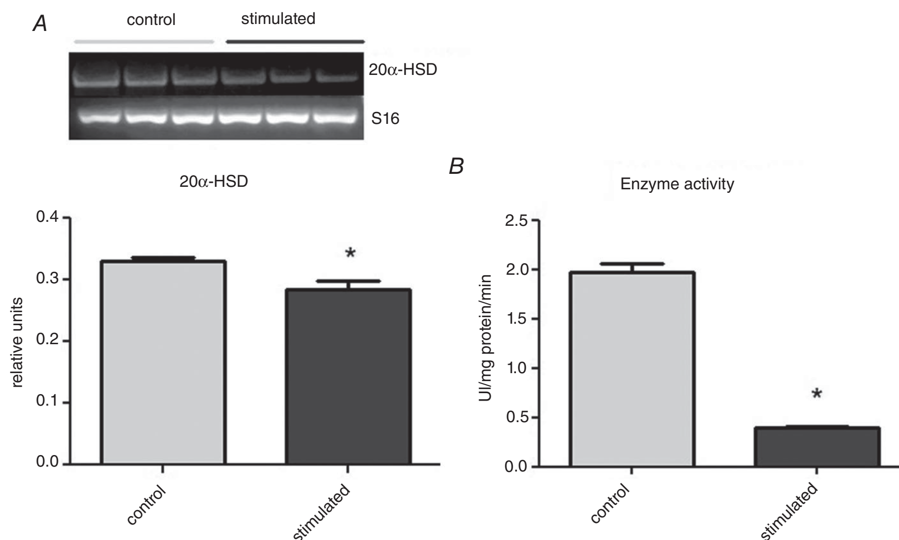

Figure 4. Ganglionic effect of P at 10–5 M (stimulated group) on the expression and enzyme activity of luteal 20α-hydroxysteroid dehydrogenase (20α-HSD)
The control group consisted of untreated CG–SON–O systems. *A*, measurement of expression (mRNA) of 20α-HSD and S16, as housekeeping gene, by RT-PCR. The PCR products, 20α-HSD (440 bp) and S16 (100 bp), were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as relative units. *B*, measurement of enzyme activity of 20α-HSD expressed as international units per milligram of protein per minute. Results are shown as the mean + SEM of three animals per experimental group. Student's unpaired *t* test was used. $^*P < 0.05$.

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(Fig. 9*E*). The results indicate that P added in the ganglionic compartment may interact with PRs present in the CG, which might mediate a response that affects the CL through the SON.

Discussion

The present study was designed to determine whether P is able to modify the regression of the CL through the peripheral neural pathway. Progesterone was added in the ganglionic compartment of the CG–SON–O system from rats on day 21 of pregnancy, when the physiological luteal regression is already established.

There are numerous studies that demonstrate the local survival effect of P on the CL. This steroid may stimulate its own secretion and protect the CL from cellular death. For instance, the intraovarian administration of an antibody against this hormone blocks the luteal production of P (Telleria & Deis, 1994). In addition, luteal cells cultured in the presence of a synthetic progestin (R5020) secrete higher amounts of P when compared with cultures of luteal cells exposed to control vehicle (Telleria *et al.* 1999). In the present study, we demonstrated that P acts in the CG and through the SON; it affects the release of P from the ovary as well as the expression/activity of its metabolic enzymes. Progesterone release increased significantly in the experimental group at the studied times when compared with the control group, and this increase was sustained throughout the incubation period. This might be due to impairment of its catabolism, an increase in its synthesis or stimulation of a releasable P pool whose existence was demonstrated by Bramley & Menzies (1994), or a combination of all these processes.

The precise expression of timing of the various enzymes/proteins required for the synthesis and

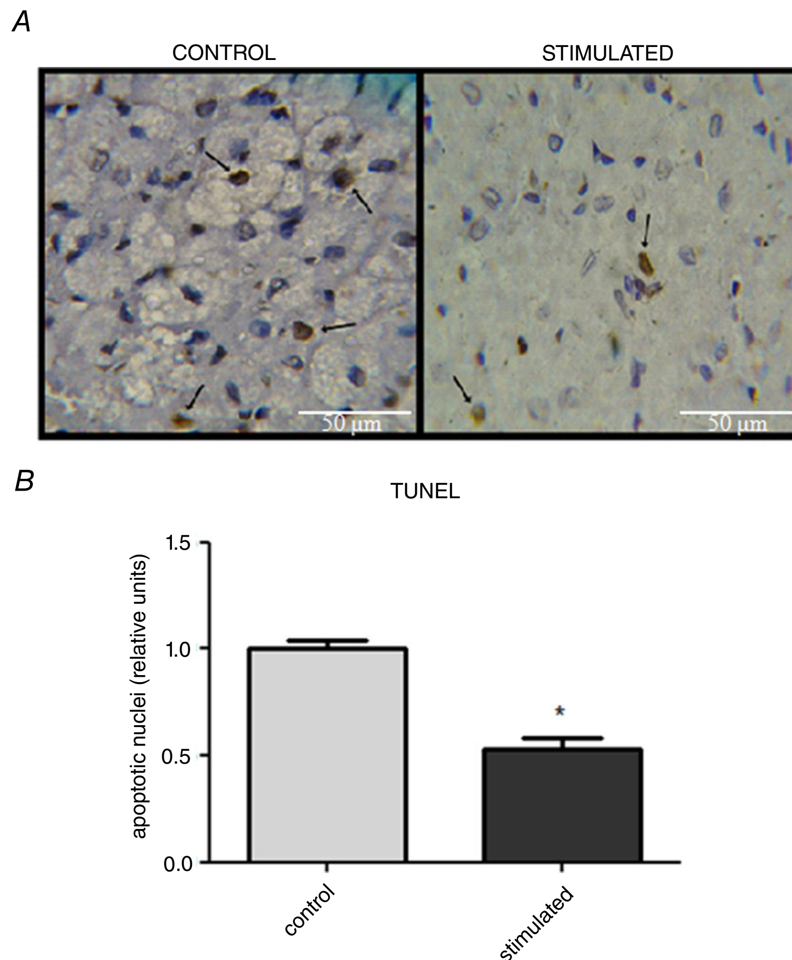

Figure 5. Ganglionic effect of P at 10–5 M (stimulated group) on the apoptosis of luteal cells
The control group consisted of untreated CG–SON–O systems. *A*, photographs of CL sections stained with TUNEL. Arrows indicate apoptotic nuclei (×100 magnification). *B*, luteal apoptosis shown as the mean + SEM of three animals per experimental group. Student's unpaired *t* test was used. $^*P < 0.05$.

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metabolism of P constitutes an important variable in the regulation of the CL function. Thus, the mRNA and protein expression of 3β-HSD did not seem to be affected by P added to the CG, but its enzymatic activity was increased. In the case of 20α-HSD, the mRNA expression and enzymatic activity were significantly diminished in the experimental group compared with the control group. These results are in agreement with the fact that in rodents, P is capable of inhibiting mRNA expression of 20α-HSD enzyme in CL incubated *in vitro* and in a luteal

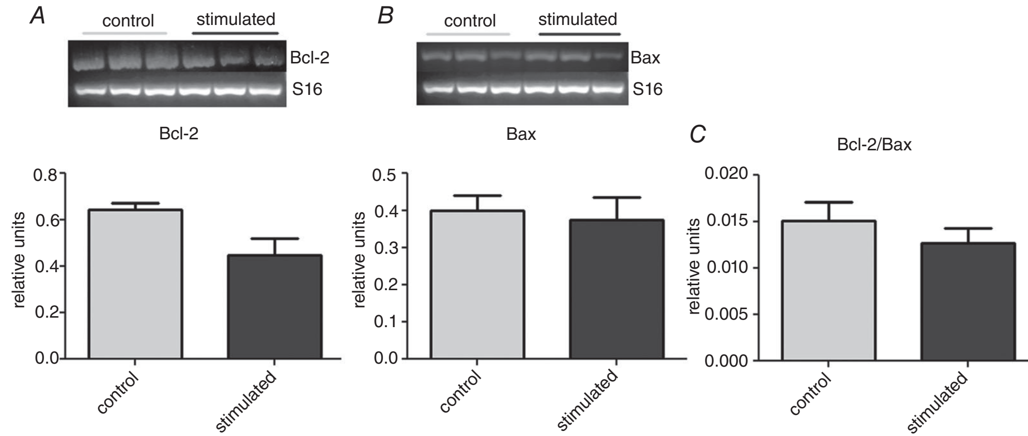

Figure 6. Ganglionic effect of P at 10–5 M (stimulated group) on the expression of intrinsic apoptosis factors Bcl-2 and Bax
The control group consisted of untreated CG–SON–O systems. *A*, analysis of Bcl-2 relative to S16 by RT-PCR. *B*, analysis of Bax relative to S16 by RT-PCR. *C*, ratio of Bcl-2 to Bax. The PCR products, Bcl-2 (454 bp), Bax (473 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are shown as the mean + SEM of three animals per experimental group. Student's unpaired *t* test was used.

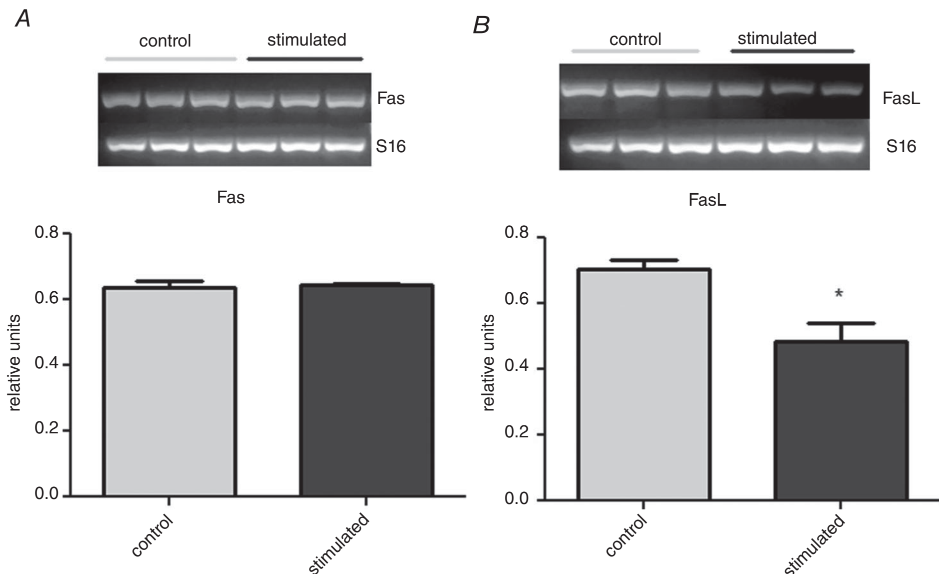

Figure 7. Ganglionic effect of P at 10–5 M (stimulated group) on Fas and FasL expression
The control group consisted of untreated CG–SON–O systems. *A*, analysis of Fas relative to S16 by RT-PCR. *B*, analysis of FasL relative to S16 by RT-PCR. The PCR products, Fas (190 bp), FasL (178 bp) and S16 (100 bp) as endogenous control, were visualized on agarose gels stained with Gel Red. The gel photographs were quantified using ImageJ and expressed as arbitrary units. Results are shown as the mean + SEM of three animals per experimental group. Student's unpaired *t* test was used. $^{*}P < 0.05$.

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cell line (Sugino *et al.* 1997). In addition, the ovarian administration of the hormone delays the decline in the activity of 3β-HSD during the functional regression of the CL, as well as the stimulation of the expression of 20α-HSD triggered by luteinizing hormone or prostaglandin $F_{2\alpha}$ (Stocco & Deis, 1998; Telleria *et al.* 1999).

Death of the luteal cells by apoptosis is the main cause of the CL structural regression (Stocco *et al.* 2007). The histological analysis of luteal tissue sections revealed that there was a significantly smaller number of apoptotic nuclei in the experimental group when P was added in the CG compartment when compared with the control group. There are numerous factors involved in the apoptosis of the CL of pregnancy, of which the Bcl-2–Bax and Fas–FasL systems are the most relevant ones (Sugino & Okuda, 2007). According to the present results, P acting on the CG did not modify Bcl-2 or Bax expression in the ovary. Bcl-2 activity is determined by its interaction with Bax, an antiapoptotic protein of the Bcl-2 family. Previous studies have demonstrated that the ratio of Bcl-2 to Bax determines cell death by apoptosis, considering that elevated Bcl-2 is a cell survival factor and elevated Bax is a cell death factor (Oltvai *et al.* 1993; Williams & Smith, 1993). No change occurred in the expression of both factors in the experimental and control groups; therefore, no modification was observed in the ratio of Bcl-2 to Bax during the studied time (180 min).

Previous studies reveal the participation of the Fas–FasL system in CL regression (Kuranaga *et al.* 2000*a* and 2000*b*). Fas is a cell surface molecule that mediates apoptosis induced by FasL or Fas antibody (Nagata & Golstein, 1995). Roughton *et al.* (1999), using RT-PCR, found that both Fas and FasL mRNA are expressed throughout pregnancy and pospartum in rats. The study demonstrated that FasL mRNA is significantly upregulated from day 16 to 22 of pregnancy, coinciding with the physiological decrease of P production at the end of gestation (Rothchild, 1981). Our results demonstrated a downfall in FasL mRNA expression in the experimental group. This expression was determined at 180 min of incubation, and the results coincided with elevated ovarian P release, suggesting P to be the final intracrine executer.

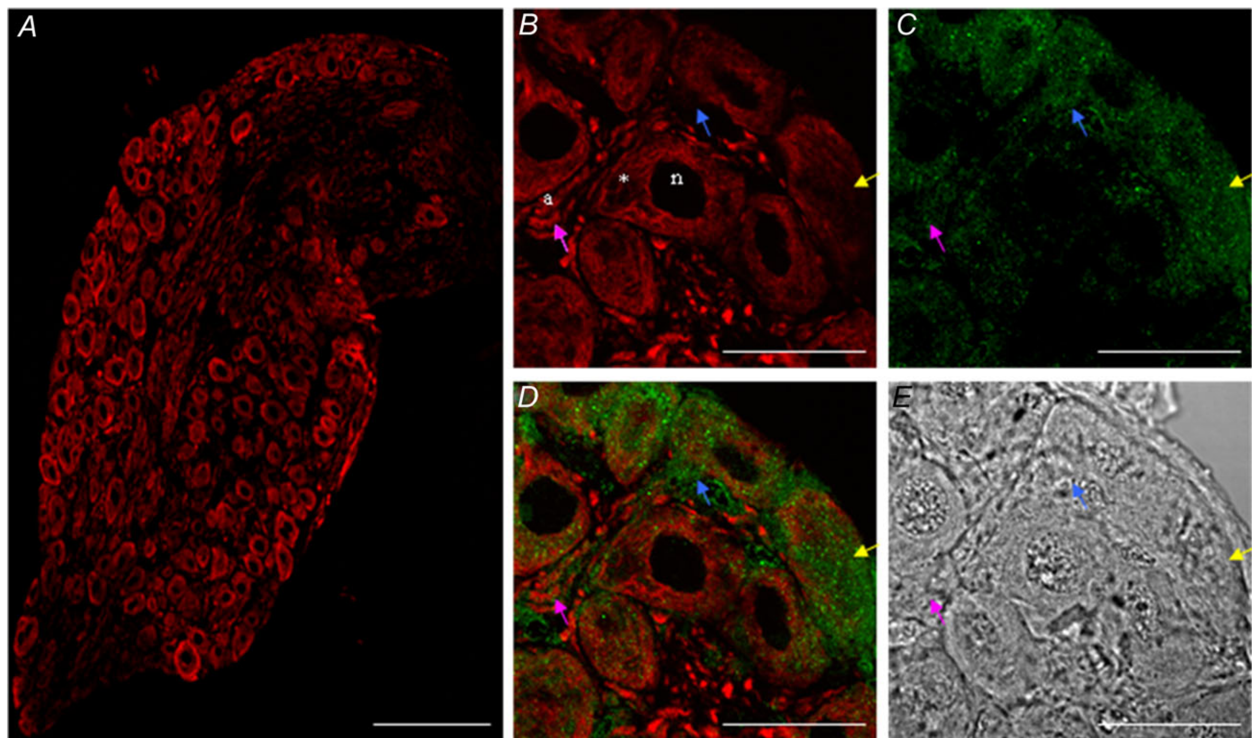

Figure 8. Localization of progesterone receptor (PR) in CG of rats on day 21 of pregnancy by immunohistochemistry
A, photograph of the CG immunolabelled with serum anti-βIII-tubulin (neuronal specific marker). *B*, region of CG showing axons (a), cytoplasm (*) and nucleus (n) of neuronal soma. *C*, CG immunostained with anti-PR (H-190: sc-7208). *D*, overlay of images *B* and *C*. Note that localization of PRs was observed in the cytoplasm of neuronal somata (yellow arrow) and axons (pink arrow). The blue arrow shows PRs that were not colocalized with neurons. *E*, image of the CG for transmitted light microscopy. Scale bars represent 100 μm (*A*) and 25 μm (*B*–*E*). Images were obtained with an Olympus FV-1000 confocal microscope.

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In this manner, P might mediate a protective effect through the CG over the CL regression. In support of this hypothesis, Kuranaga *et al.* (2000*b*), working on ovarian cycling in rats, demonstrated that P suppressed luteal cell apoptosis induced by prolactin because Fas expression was reduced in the CL, indicating that P might change the sensitivity of the CL to prolactin.

It should be noted that the neurotransmitters released from the SON nervous terminals, such as nitric oxide or noradrenaline (Vallcaneras *et al.* 2009, 2011), or ovarian P itself may also be the final element responsible for the observed effects.

The presence of PRs in CG neurons is demonstrated for the first time in this study. Progesterone receptors are a class of proteins belonging to a large superfamily of nuclear receptors that act as powerful transcription factors. In presence of the hormone, these receptors translocate to the cell nucleus, regulating gene expression. The immunohistochemical analysis showed positive immunoreactivity for the PR in the cytoplasm of neuronal somata and axons of the CG. We have previously demonstrated the presence of androgen receptors in the CG, and their distribution was also cytoplasmic (Vallcaneras *et al.* 2009). There is enough supporting evidence that PRs are also located in extranuclear sites. A study by immunoelectron microscopy in neurons outside the hypothalamus revealed abundant PR labelling within axons, dendrites and at the level of synapses (Waters *et al.* 2008). Blaustein *et al.* (1992), using conventional immunohistochemistry, have reported P and estrogen receptors in axon terminals and dendrites. In neuronal compartments distant from the nucleus, PRs might influence neurotransmission, possibly by interacting with membrane proteins, rather than acting as transcription factors (Schumacher *et al.* 2014). It is interesting to note that a subcellular distribution in axons and dendrites has also been reported for the androgen receptor in neurons outside the hypothalamus (DonCarlos *et al.* 2003, 2006). However, how these receptors located in axons, dendrites or at the level of synapses may influence the activity and excitability of neurons remains to be explored.

We propose that the peripheral neural pathway, through neuron–hormone interaction, represents a mechanism to control ovarian function in addition to the classical endocrine regulation. Progesterone may access the ganglionic neurons, bind to the specific receptors and induce release of neurotransmitters via the SON, possibly modulating ovarian function.

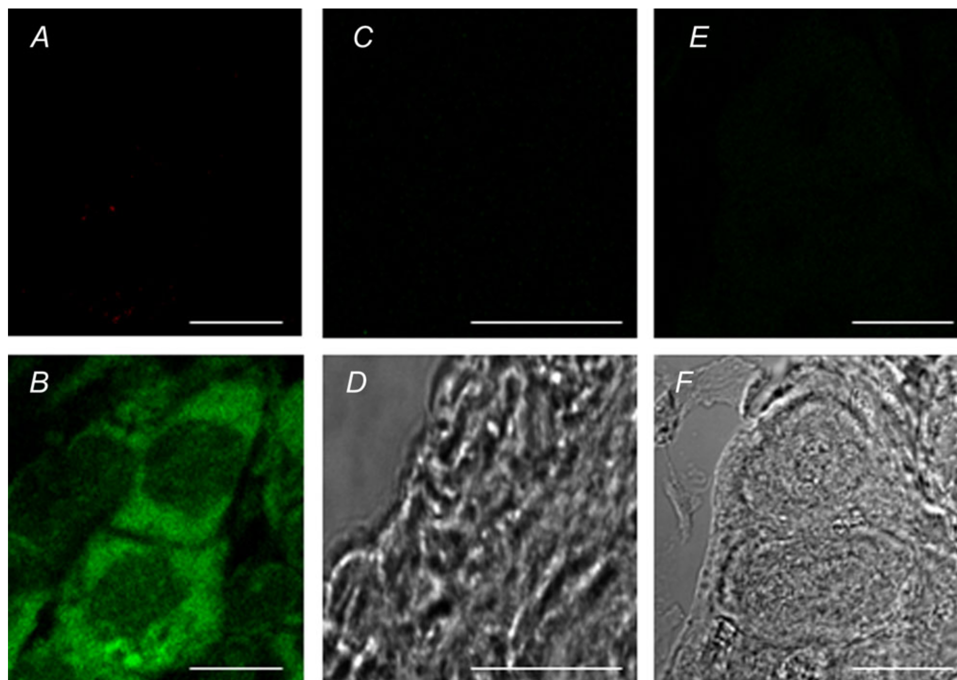

Figure 9. Controls for immunohistochemistry
Images showing ovary of a mouse as a negative control for staining with anti-βIII-tubulin (*A*) and as a positive control for staining with anti-PR (*B*). Images of juxtaganglionic tissue immunostained with anti-PR (*C*) and by transmitted light microscopy (*D*). Immunohistochemistry of the CG without incubation with anti-PR (*E*) and its corresponding image by transmitted light microscopy (*F*). Note that no positive structures or cells were found in *A*, *C* and *E*. Scale bars represent 5 μm (*A* and *B*) and 15 μm (*C*–*F*). Images were obtained with an Olympus FV-1000 confocal microscope.

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In relation to the origin of the P that may act on the CG, it is feasible that P arrives through the microcirculation that exists between the different ganglionic structures (Chau & Lu, 1995) or that synthesis of P occurs *in situ*. Supporting the latter hypothesis, a study using *in situ* hybridization and RT-PCR reported an increase in the expression of 3β-HSD mRNA in the dorsal root ganglion after the injury in the peripheral nerve (Hashimoto *et al.* 2003).

Previous studies and our present results indicate that the CL lifespan is programmed, but can be reprogrammed with the appropriate stimuli, such as P, through the peripheral nervous system. Understanding the involvement of the peripheral nervous system on the regulation of CL regression might help in devising approaches to prevent miscarriages, which are very frequent in the first trimester of pregnancy in humans. The knowledge of which stimuli cause or prevent CL regression might be essential to find new therapeutic targets to prevent or provoke luteal insufficiency.

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Additional information

Competing interests

None declared.

Author contributions

M.C. and C.M.T. were responsible for the conception and design of the study. F.G., J.B., S.S.V. and M.C. collected, analysed and interpreted the data. F.F. was responsible for the immunohistochemistry. M.V. was responsible for the Western blot. S.S.V., S.M.D. and M.C. drafted and/or critically reviewed the manuscript. All authors approved the final version of the manuscript, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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