Locus Coeruleus Mediates Cold Stress-Induced Polycystic Ovary in Rats

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Previous reports about the rat ovary have shown that cold stress promotes ovarian morphological alterations related to a polycystic ovary (PCO) condition through activation of the ovarian sympathetic nerves. Because the noradrenergic nucleus locus coeruleus (LC) is activated by cold stress and synaptically connected to the preganglionic cell bodies of the ovarian sympathetic pathway, this study aimed to evaluate the LC's role in cold stress-induced PCO in rats. Ovarian morphology and endocrine and sympathetic functions were evaluated after 8 wk of chronic intermittent cold stress (4 C, 3 h/d) in rats with or without LC lesion. The effect of acute and chronic cold stress upon the LC neuron activity was confirmed by Fos protein expression in tyrosine hydroxylase-immunoreactive neurons. Cold stress induced the formation of follicular cysts, type III follicles, and follicles with hyperthecosis alongside increased plasma estradiol and testosterone levels, irregular estrous cyclicity, and reduced ovulation. Considering estradiol release *in vitro*, cold stress potentiated the ovarian response to human chorionic gonadotropin. Ovarian norepinephrine (NE) was not altered after 8 wk of stress. However, LC lesion reduced NE activity in the ovary of cold-stressed rats, but not in controls, and prevented all the cold stress effects evaluated. Cold stress increased the number of Fos/tyrosine hydroxylase-immunoreactive neurons in the LC, but this effect was more pronounced for acute stress as compared with chronic stress. These results show that cold stress promotes PCO in rats, which apparently depends on ovarian NE activity that, under this condition, is regulated by the noradrenergic nucleus LC.

AUTONOMIC AND CENTRAL nervous systems play important roles in the regulation of ovarian physiology (1, 2). Viral track-tracing studies have provided morphological evidence of a direct multisynaptic neuronal pathway connecting the ovary to the brain through the autonomic nervous system and the preganglionic cell bodies of the ovarian sympathetic pathway (3, 4). A considerable amount of evidence has accumulated concerning the participation of enhanced sympathetic nerve activity in the development and maintenance of polycystic ovary (PCO) in rats (5–9). In fact, ovarian sympathetic nerve activation induced by 4 wk of cold stress seems to mediate the appearance of follicles with hyperthecosis, which precede the PCO condition (10). Cold stress also increases the expression of TRH mRNA in the magnocellular neurons of the paraventricular nucleus of the hypothalamus (PVN), correlating with biochemical indices of sympathetic activity in the ovary (11). These data raised the possibility of central nervous system involvement in cold stress-induced follicular derangement.

The noradrenergic nucleus locus coeruleus (LC), together with PVN, plays a central role in the regulation of the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system, mediating responses evoked by stressful challenges (12, 13). Accordingly, it has been demonstrated that LC neurons are activated by a variety of stressors (14–16), including cold stress (17–19), and are implicated in the neuroendocrine control of the hypothalamus-pituitary-ovary axis (20–22). Moreover, the infertility promoted by neonatal-handling stress is associated with a reduction in the number of LC neurons (23, 24), reinforcing the role of LC neurons in the regulation of reproductive function.

Because the deranged follicular development similar to PCO induced by cold stress seems to be related to the activation of the sympathetic pathway controlling ovarian function (10, 25), it is reasonable to hypothesize that the LC is implicated in the development of cold stress-induced PCO. Thus, in the present study, we evaluated 1) whether 8 wk of cold stress would induce PCO in association with correlate reproductive dysfunctions and 2) whether such reproductive alterations would be mediated by LC neurons. Ovarian morphology as well as endocrine and sympathetic functions were evaluated after 8 wk of chronic intermittent cold stress in rats with or without the LC lesion. We also evaluated the effect of acute and chronic cold stress upon the activity of LC neurons by Fos protein

Abbreviations: EV, Estradiol valerate; hCG, human chorionic gonadotropin; HPLC-ED, HPLC with electrochemical detection; LC, locus coeruleus; MHPG, 3-methoxy-4-hydroxyphenyl-glycol; NE, norepinephrine; NPY, neuropeptide Y; PCO, polycystic ovary; PCOS, PCO syndrome; PVN, paraventricular nucleus of the hypothalamus; TH, tyrosine hydroxylase.

expression in tyrosine hydroxylase-immunoreactive (TH-ir) neurons.

Materials and Methods

Animals

Adult female Wistar rats weighing 230–260 g (7–8 wk of age) from the animal house of the University of São Paulo at Ribeirão Preto were kept in a central animal care facility, housed individually in plastic cages (30 × 19 × 13 cm) under a 12-h light, 12-h dark cycle (lights on at 0600 h) and controlled temperature (24 ± 0.5 C). Air exchange rate was 10 room-volumes per hour. Food and water were provided *ad libitum*. Vaginal smears were taken daily, and only rats showing at least three consecutive 4-d regular estrous cycles were used in the experiment. For evaluation of the acute cold stress effect on LC Fos immunoreactivity, rats studied weighed 350–400 g (15–17 wk of age). All protocols used were approved by the Ethics Committee for Research Involving Animals at the Medical School of Ribeirão Preto, University of São Paulo.

Experimental design

Rats were submitted to bilateral electrolytic lesions in the LC (LC-lesion group), sham surgery (sham group), or no surgical treatment (intact group). Seven days after surgeries, rats were divided into intact (n = 6), sham (n = 5), and LC-lesion (n = 6) groups and intermittently exposed to a cold ambient temperature at 4 C for 3 h/d for 8 wk (Monday to Friday each week). Rats at room temperature were also divided into three groups: intact (n = 6), sham (n = 6), and LC-lesion (n = 5). Vaginal smears were taken daily to verify estrous cycle regularity, and food and water were provided *ad libitum*. Immediately after the end of the last stress session on the day of estrus, rats were deeply anesthetized and transcardially perfused. Before perfusion, a blood sample was collected from the left ventricle, ovaries and oviducts were removed, and ovulation was assessed. The right ovary was used for morphological analysis. The left ovary was divided in three parts; in one part, concentrations of norepinephrine (NE) and its metabolite, 3-methoxy-4-hydroxyphenyl-glycol (MHPG), were determined, and in the other two parts, estradiol release in response to human chorionic gonadotropin (hCG) stimulation was evaluated *in vitro*. The brainstem of the LC-lesion group was processed for histological analysis of the LC lesion. The effect of cold stress on the LC neuron activity was evaluated with double-label immunohistochemistry to Fos and TH in intact rats of control (n = 4) and cold-stress groups (n = 4). To assess the acute response of LC neurons to cold stress, Fos/TH-ir was also determined in rats subjected to one session of cold stress (4 C for 3 h; n = 4) or maintained at room temperature (n = 4) on the morning of estrus.

LC electrolytic lesion

Under ketamine (ketamine chlorhydrate; Agner, São Paulo, Brazil; 100 mg/kg body weight, ip) and xylazine (Coopazine; Coopers, São Paulo, Brazil; 14 mg/kg body weight, ip) anesthesia, rats were positioned in a stereotaxic instrument (David Kopf, Tujunga, CA) with the incisor bar set at the zero point. The dorsal surface of the skull was exposed, and holes, 2 mm in diameter, were drilled bilaterally 1.2 mm lateral to the midline and 3.4 mm caudal to the λ-suture point. A stainless-steel monopolar electrode, 200 μm in diameter and insulated except for the tip, was angled at 15° (posteroanterior direction) and lowered to a depth of 6.8 mm below the surface of the skull. A constant anodal current of 1.0 mA was applied for 10 sec. The electrode was removed, and the skin incision was closed. Sham-operated rats were submitted to the same surgical procedure except for the depth of electrode insertion, which was 4.8 mm below the skull, and the current was not applied.

Cold stress paradigm

For chronic stress, from Monday to Friday for 8 wk, rats exposed to cold stress were placed in their cages without food, water, and bedding in a cold room at 4 C from 1000–1300 h. Control rats were moved to a location near the cold room, maintained at an ambient temperature (24 C), and returned to the animal room after 3 h (10). For acute stress, one session of the same paradigm of cold stress was performed from 1000–1300 h on the day of estrus.

Ovulation

After separation from the ovary, the oviduct was immediately squashed between two slides and the number of oocytes in each oviduct was microscopically counted in the oviductal ampullae.

Ovarian morphology

The right ovary was immersed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h, embedded in paraffin, cut in 8-μm serial sections, and stained with hematoxylin and eosin. For morphometric analysis, the number of preantral and antral follicles (healthy and atretic), antral follicles with hyperthecosis (10), type III or precyst, and cystic follicles (26) was counted in every section. The size of healthy antral follicles was also determined. Briefly, preantral follicles (mainly secondary) were defined as follicles without any antral cavity and with two or more layers of granulosa cells. Atretic follicles were defined as follicles with more than 5% of the cells having pyknotic nuclei in the largest cross-section and showing shrinkage and occasional breakdown of the germinal vesicle. Antral follicles were counted when the oocyte nucleus was visualized. Antral follicles with hyperthecosis were defined as those having hypertrophied differentiated theca interna cells, with increased thickness of the theca layer. Type III follicles were large, containing four or five layers of small densely packed granulosa cells surrounding a very large antrum and displaying an apparently normal thecal compartment. Cystic follicles were defined as those with large antral cavity, thickened theca interna cell layer, and attenuated granulosa cell layer.

HPLC with electrochemical detection (HPLC-ED)

The left ovary (one-third) was homogenized with a microultrasonic cell disrupter within 200 μl of a solution containing 0.2 M perchloric acid, 0.1 mM EDTA and 230 nM 3,4-dihydroxybenzylamine as internal standard (Aldrich, Milwaukee, WI). The homogenates were centrifuged for 20 min at 12,000 × *g*. Protein content was determined in the remaining pellet by the Bradford method (27). The supernatant was filtered through a 0.22-μm filter (Millex polyvinylidene difluoride; Millipore, Belford, MA) and injected in the HPLC-ED system by an auto injector (SIL-10Advp; Shimadzu, Kyoto, Japan). Separation was performed on a 250 × 4-mm reversed-phase C18 column (Purospher Star, 5 μm; Merck, Darmstadt, Germany) preceded by a 4 × 4-mm reversed-phase C18 column (Lichrospher, 5 μm; Merck). Ovary NE and MHPG concentrations were determined in analyses. For NE, the mobile phase consisted of 100 mM sodium dihydrogen phosphate, 10 mM sodium chloride, 0.1 mM EDTA, 1.5 mM sodium 1-octanesulfonic acid (Sigma Chemical Co., St. Louis, MO), and 15% methanol (Omnisolv; EMD Chemical Inc., Gibbstown, NJ), pH adjusted to 3.5 with phosphoric acid. The pump (LC-10Advp;, Shimadzu) flow rate was set at 0.6 ml/min and the detector potential was 0.60 V *vs. in situ* Ag/AgCl reference electrode (Decade, VT-03 electrochemical flow cell; Antec, Leiden, The Netherlands). For MHPG, supernatants were acid hydrolyzed by heating at 94 C for 5 min to deconjugate free MHPG from MHPG-sulfate before sample analysis by HPLC-ED (28). The mobile phase consisted of 100 mM sodium dihydrogen phosphate, 10 mM sodium chloride, 0.1 mM EDTA, 0.25 mM sodium 1-octanesulfonic acid, and 17.5% methanol, pH adjusted to 3.5 with phosphoric acid. The flow rate was set at 0.5 ml/min, and the detector potential was 0.65 V *vs. in situ* Ag/AgCl reference electrode. Chromatography data were plotted using Class-VP software (Shimadzu). NE and MHPG were identified based on their peak retention times. Quantification was performed by the internal standard method (3,4-dihydroxybenzylamine as internal standard) based on the area under the peak. Data were normalized by the protein content of each sample.

Ovarian response to gonadotropin stimulation in vitro

The left ovaries were divided into three parts, and two of them were evaluated for *in vitro* estradiol release. Each ovarian part was weighed and preincubated separately in Krebs-Ringer bicarbonate buffer (pH

7.4), containing 0.1 mg/ml glucose and 0.1 mg/ml albumin for 30 min at 37 C in a 95% O_2/5% CO_2 atmosphere (29). After the preincubation period, one part of the ovary was incubated with 2 ml buffer alone (vehicle group) and the other part with the same volume of buffer containing 2.5 IU hCG (Ferring Gmbh, Kiel, Germany; hCG group) (30). The incubation medium was collected after 3 h of incubation, and estradiol concentration was determined. The two parts from the same ovary were assayed simultaneously. Data were normalized by tissue weight, and the response to hCG was expressed relative to the hormonal release in the vehicle group from the same ovary.

Immunohistochemistry

Sections from the LC were immunohistochemically processed for Fos and TH as previously described (31). Briefly, rats under deep ketamine (ketamine chlorhydrate; Agner) and xylazine (Coopazine; Coopers) anesthesia were transcardially perfused with PBS with heparin (5 IU/ml), followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Frontal sections of 20 μm were cut in a cryostat throughout the rostrocaudal extension of the LC (32). Sections were collected in four series and stored at −20 C in culture dishes containing cryoprotectant solution (33). All sections of one series were used for double-label immunohistochemistry to Fos and TH. All steps were performed at 22 C, except for incubation with the primary antibodies, which was performed at 4 C. Sections were incubated with anti-c-Fos rabbit antibody (K-25; Santa Cruz Biotechnology, Santa Cruz, CA) at 1:2000 in PBS containing 0.3% Triton X-100 and 1% BSA (all primary and secondary antibodies were diluted in the same solution) for 40 h, biotinylated antirabbit goat IgG (Elite kit; Vector Laboratories, Burlingame, CA) at 1:600 for 2 h and avidin-biotin complex solution at 1:100 for 1 h (Elite ABC kit; Vector). The antibody-peroxidase complex was visualized with a solution of nickel chloride (25 mg/ml), 3,3′-diaminobenzidine-HCl (0.2 mg/ml; Sigma), and H_2O_2 (1 μl/ml of 30% stock solution) in 0.175 M acetate buffer (pH 7.5). Sections were incubated with an anti-TH mouse antibody (anti-TH2; Sigma) at 1:10^6 for 40 h, biotinylated antimouse horse IgG (BA-2001; Vector) at 1:800 for 1 h, and avidin-biotin complex solution at 1:100 for 1 h. TH was immunostained with a solution containing 3,3′-diaminobenzidine-HCl (0.1 mg/ml) and H_2O_2 (1 μl/ml of 30% stock solution) in 0.05 M Tris-HCl buffer (pH 7.6). Sections were blindly analyzed under a light microscope (Axioskop 2 plus; Zeiss, Hallbergmoos, Germany) with an image analysis system (Axiovision 3.1; Zeiss), and the number of Fos-immunoreactive neurons colocalized with TH was quantified bilaterally in the LC in 15 sections between −9.48 and −10.32 mm from bregma, according to the atlas of Paxinos and Watson (32).

Blood samples

One minute before the beginning of perfusion, 1-ml blood samples were collected from the left ventricle of anesthetized rats into heparinized syringes. Plasma was separated by centrifugation at 1200 × *g* for 15 min at 4 C and stored at −20 C until assayed for LH and ovarian steroids by RIA.

RIA

Plasma LH concentrations were determined by double-antibody RIA with kit provided by the National Institutes of Health, Bethesda, MD. The antiserum and the reference preparation for LH were LH-S10 and RP3. Plasma estradiol, progesterone, and testosterone concentrations were determined by double-antibody RIA with MAIA kits provided by Biochem Immunosystems (Bologna, Italy). To avoid interassay variation, all samples were assayed in the same RIA. The lower limits of detection for LH, estradiol, progesterone, and testosterone were 0.04 ng/ml, 1.0 pg/ml, 0.02 ng/ml, and 5.0 pg/ml, respectively. The intraassay coefficient of variation was 3.4% for LH, 4.3% for estradiol, 7.5% for progesterone, and 4% for testosterone.

Histological analysis of LC lesion

The brainstem of each lesioned rat was removed and immersed in 10% formaldehyde. After brain fixation, semiserial coronal 13-μm sections were cut throughout the LC, stained by Nissl technique, and analyzed by light microscopy according to the atlas of Paxinos and Watson (32). Only data from rats with the LC proper (34) totally lesioned were included in the LC-lesion group. Representative photomicrographs of an intact and lesioned LC are shown in Fig. 1, A and B, respectively.

Statistical analysis

Data are presented as mean ± SEM. Statistical differences were determined by two-way or one-way ANOVA followed by the Bonferroni *post hoc* test. $P < 0.05$ was considered statistically significant.

Results

Cold stress effect upon ovarian morphology

The morphological analyses of ovaries from control rats on estrus revealed the presence of numerous corpora lutea in different stages of development and regression, many of which clearly resulted from recent ovulation, as well as some atretic antral follicles (Fig. 2A). Eight weeks of chronic intermittent cold stress induced several ovarian morphological alterations, marked predominantly by the presence of numerous healthy antral follicles with small size, type III and cystic follicles, and follicles with enlarged theca cell layer (hyperthecosis) (Figs. 2B and 3). These alterations were, however, effectively prevented by the LC lesion (Fig. 2C). The total number of healthy or atretic preantral and antral follicles per ovary was not altered by cold stress (on average:

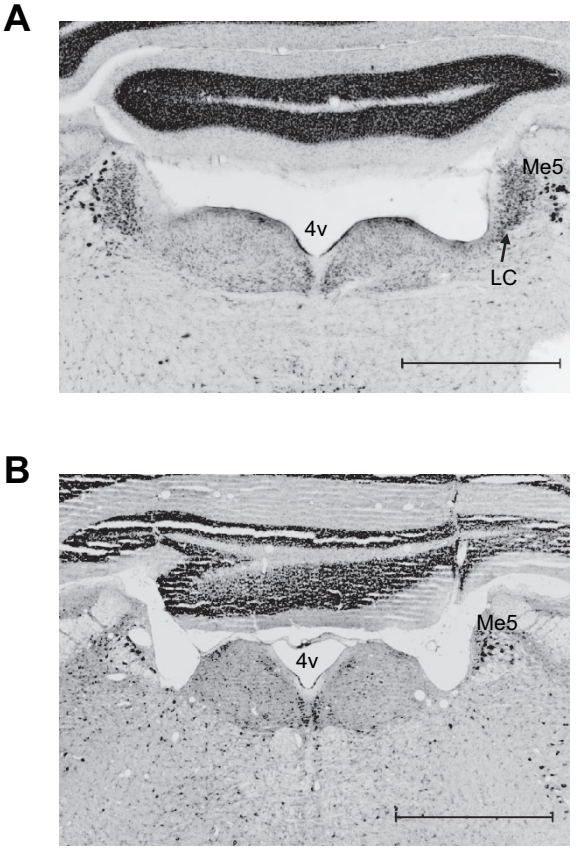

FIG. 1. Photomicrographs of Nissl-stained coronal sections of the brainstem region depicting intact (A) and lesioned (B) LC. Sections correspond to a rostrocaudal level at approximately 9.8 mm posterior to bregma (32). 4v, Fourth ventricle; Me5, mesencephalic trigeminal nucleus. *Scale bar*, 1 mm.

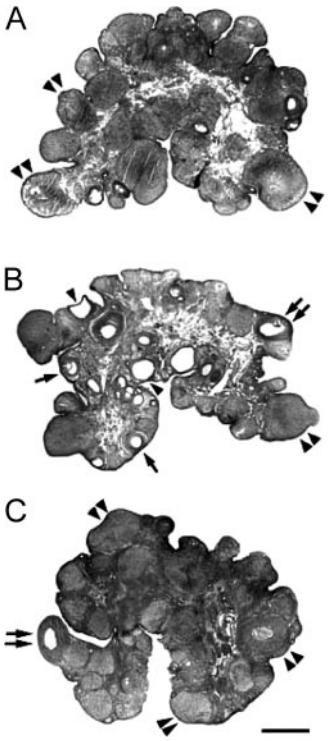

FIG. 2. Cold stress effect upon ovarian morphology. A, Ovarian morphology on estrus of rat maintained at ambient temperature (control/intact); B and C, ovarian morphology on estrus of rats exposed to 8 wk of cold stress with no surgical treatment (cold stress/intact) (B) or receiving previous LC lesion (cold stress/LC lesion) (C). *Arrow* indicates antral follicle with hyperthecosis, *double arrow* type III follicle, *arrowhead* follicular cyst, and *double arrowhead* corpus luteum. *Scale bar*, 1 mm.

healthy preantral = 27.22 ± 1.77; healthy antral = 22.73 ± 1.06; atretic preantral = 17.67 ± 1.74; atretic antral = 14.78 ± 1.63, mean of control and cold-stress rats ± SEM). However, considering healthy antral follicles categorized by size, the number of follicles ranging from 200–300 μm of diameter was significantly increased by cold stress [$F_{(1,28)} = 15.23$; $P < 0.001$], and this increase was prevented by the LC lesion [$F_{(2,28)} = 4.98$; $P < 0.05$] (Fig. 4A). The number of follicles within both 301–400 μm [$F_{(1,28)} = 40.69$; $P < 0.001$] and 401–500 μm range [$F_{(1,28)} = 5.61$; $P < 0.05$] was significantly decreased by cold stress, an effect prevented by the LC lesion [301–400 μm: $F_{(2,28)} = 5.53$; $P < 0.05$; 401–500 μm: $F_{(2,28)} = 6.03$; $P < 0.05$] (Fig. 4, B and C). Moreover, cold stress significantly augmented the number of antral follicles with hyperthecosis [$F_{(1,28)} = 70.31$; $P < 0.001$], type III follicles [$F_{(1,28)} = 60.09$; $P < 0.001$], and follicular cysts [$F_{(1,28)} = 76.52$; $P < 0.001$]. These cold stress effects on abnormal follicular development were also significantly reduced by the LC lesion [hyperthecosis: $F_{(2,28)} = 2.49$; $P < 0.05$; type III: $F_{(2,28)} = 4.92$; $P < 0.05$; cyst: $F_{(2,28)} = 3.82$; $P < 0.05$] (Fig. 4, D–F). The reduction was of approximately 40% for hyperthecosis, 46% for type III follicles, and 47% for cysts, relative to intact and sham in cold-stress group.

Cold stress effects on estrous cyclicity and ovulation rate

Estrous cycle evaluation was initiated after the reinstatement of estrous cyclicity by lesioned rats, which occurred in approximately 12 ± 2 d (mean ± SEM) after surgery, as previously reported (35). Proestrus relative incidence was significantly decreased in rats exposed to cold stress [$F_{(1,28)} = 24.89$; $P < 0.001$], mainly due to prolonged permanence on estrus-like phases. Proestrus frequency was restored to control levels in LC-lesioned rats [$F_{(2,28)} = 2.51$; $P < 0.05$] (Fig. 5A). Cold stress significantly reduced the number of oocytes in the oviductal ampullae [$F_{(1,28)} = 32.43$; $P < 0.001$], which was also restored by the LC lesion [$F_{(2,28)} = 5.34$; $P < 0.05$] (Fig. 5B).

Cold stress effect on plasma levels of ovarian steroids and gonadotropins

Plasma estradiol [$F_{(1,28)} = 33.99$; $P < 0.001$] and testosterone [$F_{(1,28)} = 22.22$; $P < 0.001$] concentrations were significantly augmented by cold stress, and these increases were blocked by the LC lesion (estradiol: $F_{(2,28)} = 7.39$; $P < 0.05$; testosterone: $F_{(2,28)} = 8.78$; $P < 0.05$] (Fig. 6, A and B). On the other hand, neither cold stress nor LC lesion altered plasma concentration of progesterone or LH (Fig. 6, C and D). Plasma FSH concentration was also determined and found to be not altered by cold stress (control: 2.43 ± 0.18 ng/ml; cold stress: 2.71 ± 0.28 ng/ml, mean ± SEM).

Cold stress effect upon ovarian noradrenergic activity

After 8 wk of cold stress, ovary NE and MHPG concentrations were not altered in intact and sham rats, whereas in LC-lesioned rats, there was a significant increase in NE levels [$F_{(2,17)} = 7.6$; $P < 0.05$] and a decrease in both MHPG levels [$F_{(2,17)} = 1.78$; $P < 0.05$] and MHPG/NE ratio [$F_{(2,17)} = 16.78$; $P < 0.05$]. In contrast, LC lesion did not alter ovary noradrenergic activity in control rats (Fig. 7).

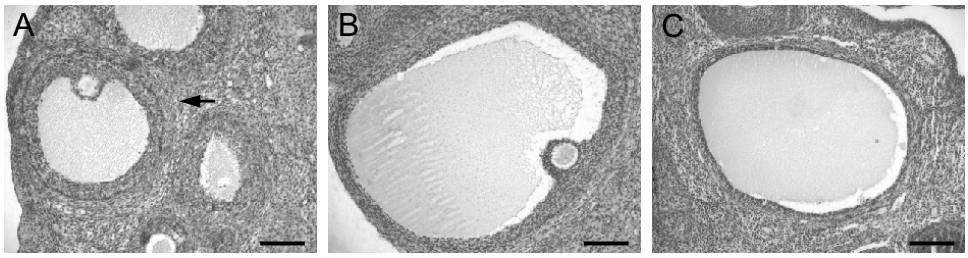

FIG. 3. Photomicrographs of antral follicle presenting a thickened theca cell layer (hyperthecosis) (A) and precystic (type III) (B) and cystic (C) follicles presented in the ovary of rats exposed to 8 wk of cold stress. *Arrow* indicates thickened theca cell layer. *Scale bar*, 100 μm.

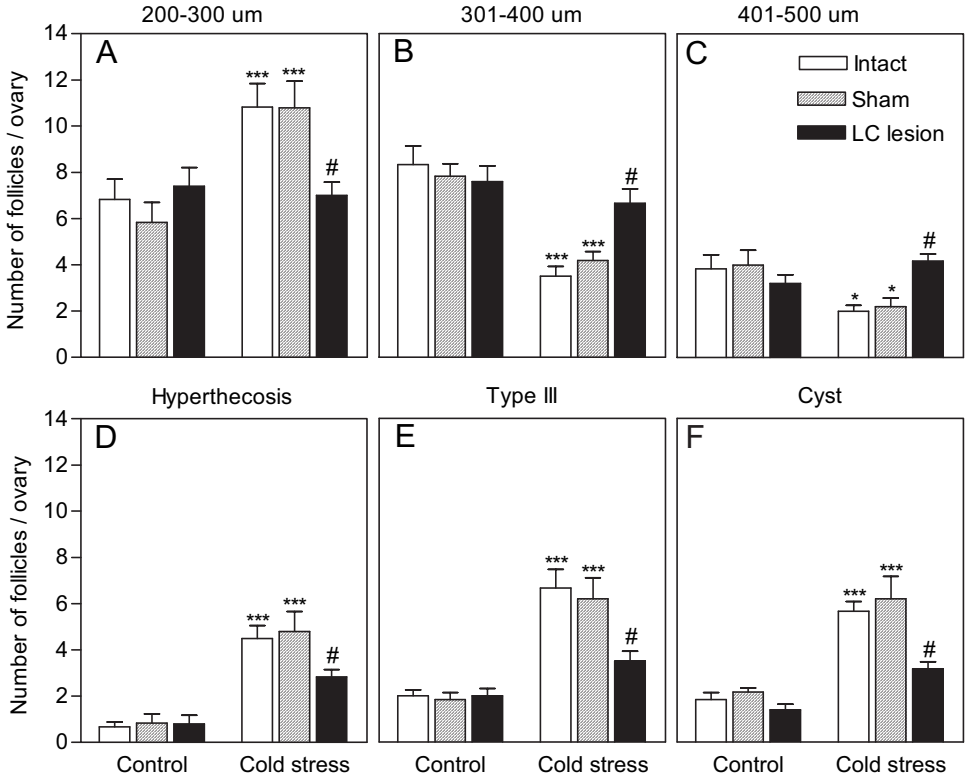

FIG. 4. LC lesion prevents cold stress-induced changes in ovarian morphology. *Upper panel* shows the number of healthy antral follicles with sizes ranging from 200–300 μm (A), 301–400 μm (B), and 401–500 μm (C). *Lower panel* shows the number of antral follicles with hyperthecosis (D), type III follicles (E), and follicular cysts (F). Adult cycling rats underwent LC lesion (n = 5–6), sham surgery (n = 5–6), or no surgical treatment (intact; n = 6) and were intermittently exposed to chronic cold stress or maintained at ambient temperature. Data correspond to the analysis in the every ovarian section and are presented as mean ± SEM. ***, $P < 0.001$; *, $P < 0.05$ *vs.* control group; #, $P < 0.05$ *vs.* intact and sham in cold-stress group.

Cold stress effect on ovarian release of estradiol in response to gonadotropin stimulation

The *in vitro* release of estradiol from ovary tissues incubated in Krebs-Ringer bicarbonate buffer alone was not different among studied groups (average value: 2.22 ± 0.15 pg/mg tissue·ml). Related to vehicle values, incubation with hCG did not alter estradiol release from control ovaries but significantly increased this hormone release from the ovaries of cold stressed rats [$F_{(1,28)} = 9.154$; $P < 0.01$]. This hCG-induced estradiol release was also prevented by LC lesion [$F_{(2,28)} = 6.21$; $P < 0.05$] (Fig. 8).

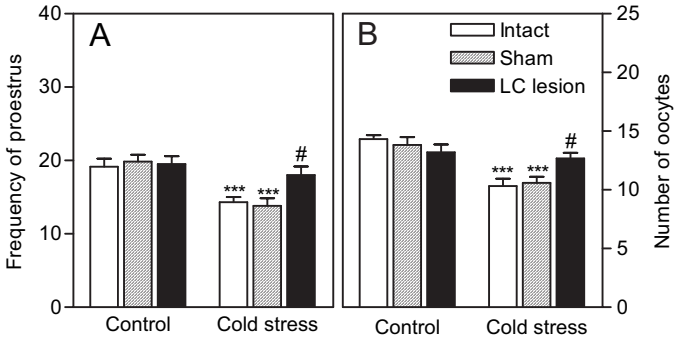

FIG. 5. LC lesion abolishes cold stress effects on estrous cyclicity and ovulation rate. A, Proestrus relative occurrence during the experimental period; B, number of oocytes found in the oviductal ampullae on the afternoon of estrus. Adult cycling rats underwent LC lesion, sham surgery, or no surgical treatment and were intermittently exposed to chronic cold stress (n = 6, 5, and 6 for intact, sham, and LC lesion, respectively) or maintained at ambient temperature (control; n = 6, 6, and 5 for intact, sham, and LC lesion, respectively). Data are presented as mean ± SEM. ***, $P < 0.001$ *vs.* control group; #, $P < 0.05$ *vs.* intact and sham in cold-stress group.

Cold stress-induced Fos immunoreactivity in the LC

Figure 9A shows photomicrographs of Fos/TH double-labeled neurons in the LC of rats subjected to acute and chronic cold stress. As determined by two-way ANOVA, the number of Fos/TH-ir neurons in the LC was significantly augmented by cold stress [$F_{(1,12)} = 12.53$; $P < 0.01$], and there was no difference between acute and chronic stress [$F_{(1,12)} = 0.79$; $P = 0.39$). However, compared with control levels, acute cold stress significantly increased Fos immunoreactivity in the LC ($t_{12} = 3.35$; $P < 0.05$), whereas cold exposure after 8 wk of chronic cold stress yielded a smaller increase in Fos immunoreactivity, being significant only for $P < 0.1$ ($t_{12} = 2.04$) (Fig. 9B).

Discussion

The present data demonstrate that 8 wk of chronic intermittent cold stress in rats are able to induce a typical PCO morphology, alongside endocrine disturbances, irregular estrous cyclicity, and reduced ovulation. Cold stress increased LC neurons activity, whereas LC lesion reduced noradrenergic activity in the ovary of cold-stressed rats and prevented all the cold stress effects evaluated. Thus, taken together, our results suggest that LC probably mediates cold stress-induced PCO in rats.

It has been previously demonstrated that 4 wk of cold stress cause ovarian morphology alterations, such as the development of follicles presenting a thickened theca cell

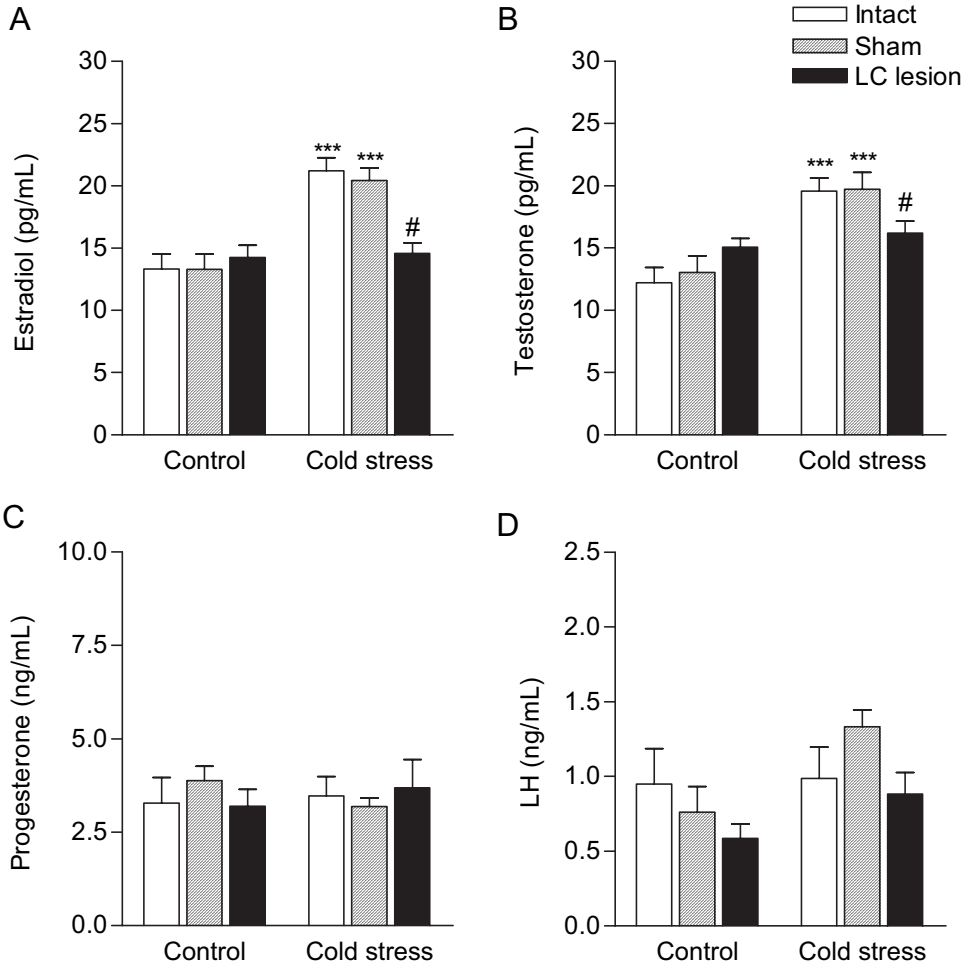

FIG. 6. Cold stress effect on plasma levels of ovarian steroids and LH. A–D, Plasma concentration of estradiol (A), testosterone (B), progesterone (C), and LH (D) on the afternoon of estrus. Adult cycling rats underwent LC lesion, sham surgery, or no surgical treatment and were intermittently exposed to chronic cold stress (cold stress) (n = 6, 5, and 6 for intact, sham, and LC lesion, respectively) or maintained at ambient temperature (control; n = 6, 6, and 5 for intact, sham, and LC lesion, respectively). Data are presented as the mean ± SEM. ***, $P < 0.001$ *vs.* control group; #, $P < 0.05$ *vs.* intact and sham in cold-stress group.

layer, as seen in human PCO (10). The primary effect of cold stress appears to be a central activation of the sympathetic nerves to the ovary (10, 11, 25). Probably, the development of precystic (type III) and cystic follicles found after 8 wk of cold stress was due to the pronounced exposure to increased ovarian sympathetic activity. Kinetic studies using mouse, hamster, and rats have shown that the large preovulatory follicles, ovulating in response to LH, actually enter into the growing pool of follicles around 20 d before (36). Therefore, 21 d are necessary for the primary changes to occur in follicular development as was demonstrated with 4 wk of cold stress (10). In support of this, the original studies of both Brawer *et al.* (26) and Barria *et al.* (30) described that the development of ovarian cysts by estradiol valerate (EV) administration requires 8 wk to be fully expressed. A similar delay in the response was found by *in vivo* administration of the β-agonist isoproterenol (6). After EV administration, rats developed precystic and cystic follicles, and the process was also causally related to increased sympathetic activity, as indicated by the augmented NE release and TH activity in the ovary (5). In these animals, surgical denervation of the superior ovarian nerve restored estrous cyclicity and ovulation (30), evidence reinforcing that increased sympathetic activity was responsible for those alterations.

The fact that ovarian NE activity was not altered after 8 wk of cold stress suggests that although cold stress modifies ovarian sympathetic activity, as was previously found after 3 and 4 wk of stress, NE response to chronic cold exposure appears to change dynamically as a function of time. In accordance with this hypothesis, Dorfman *et al.* (10) showed that 3 wk of chronic cold stress decrease ovarian NE concentration, which indicates higher NE release, whereas 4 wk augmented both NE and nerve growth factor levels, suggesting a subsequent neurotrophin-mediated increase in NE synthesis. In addition, Fiedler *et al.* (11) have reported that whereas 48 h of cold exposure reduced ovarian NE concentration, after 64 h of cold exposure, ovarian NE returned to control levels but NE concentration in the celiac ganglia was increased, suggesting a compensatory effect at the cell bodies of the sympathetic neurons innervating the ovary. This feature, therefore, seems to differ cold stress-induced from the EV-induced PCO, which, probably due to the trophic effects of estrogen, is characterized by a maintained increase in ovarian NE activity (5, 37). Thus, we suggest that once ovarian cysts develop due to the increased noradrenergic activity found at 4 wk of stress, ovarian NE is essential to maintain the follicular changes, because the LC lesion reduced NE activity in the ovary of cold-stressed rats and prevented all the morphological and hormonal alterations evaluated.

Besides the development of precysts and cysts, 8 wk of

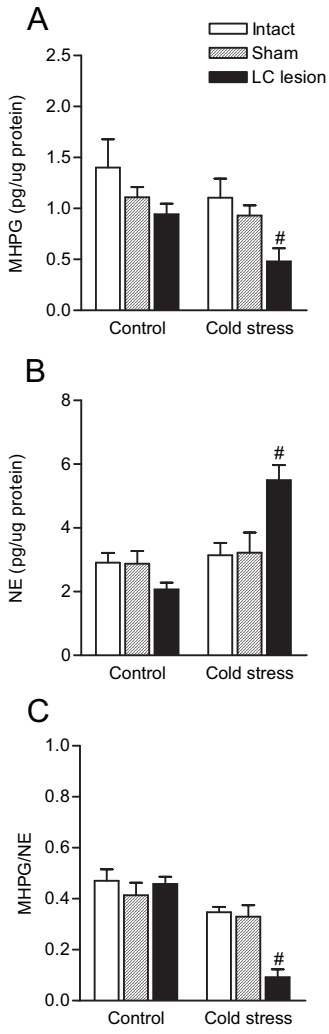

FIG. 7. LC lesion reduces ovarian noradrenergic activity in rats under chronic cold stress. MHPG (A), NE (B), and MHPG/NE ratio (C) were determined in the left ovary of adult cycling rats that underwent LC lesion (n = 3–4), sham surgery (n = 4), or no surgical treatment (n = 4) and were intermittently exposed to chronic cold stress or maintained at ambient temperature. Data are shown as the mean ± SEM. #, $P < 0.05$ *vs.* intact and sham in cold-stress group.

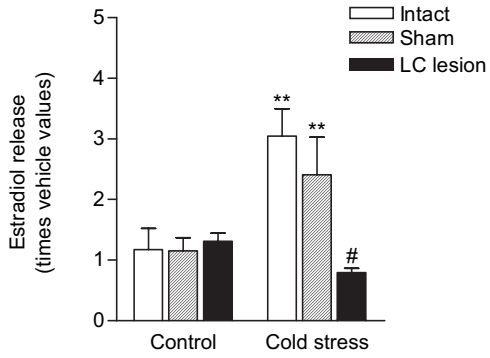

FIG. 8. Cold stress effect on ovarian release of estradiol in response to gonadotropin stimulation. Adult cycling rats underwent LC lesion, sham surgery, or no surgical treatment and were intermittently exposed to chronic cold stress (cold stress; n = 6, 5, and 6 for intact, sham, and LC lesion, respectively) or maintained at ambient temperature (control; n = 6, 6, and 5 for intact, sham, and LC lesion, respectively). Immediately after the end of the last stress session on the day of estrus, rats were deeply anesthetized and the ovaries removed. Two of three pieces of the left ovary were separately incubated for 3 h in Krebs-Ringer bicarbonate buffer alone (vehicle) or 2.5 IU hCG. Data (mean ± SEM) are expressed relative to the hormonal release in the vehicle group from the same ovary (considered to be 1). **, $P < 0.01$ *vs.* control group; #, $P < 0.05$ *vs.* intact and sham in cold-stress group.

cold stress increased the number of small healthy antral follicles (200–300 μm in diameter) and follicles with hyperthecosis, which was also found after 4 wk of stress (10). The fact that there was no change in the total number of follicles but a decrease in preovulatory follicles suggests two principal effects. One is the accumulation of small antral follicles, and the other is an increased transit from preovulatory follicles to cysts. This would decrease the rate of ovulation, as found in the present study by the decreased number of oocytes collected at the oviductal ampullae on estrus. The decrease in the number of days on proestrus with an increase in the length of estrus-like phases is consistent with the tonic increase in plasma estradiol and testosterone levels after cold stress, suggesting activation of the biosynthesis of testosterone and hence estradiol. On the other hand, the unchanged plasma levels of LH and FSH suggest a local ovarian effect of cold stress independent of gonadotropin, probably mediated by increased noradrenergic tone acting on theca cells to produce more androgenic substrates to estradiol biosynthesis (30). Both granulosa and theca cells express adrenergic receptors that seem to be implicated in abnormal steroidal response of PCO (38–40). This contribution could specifically come from the increased number of precystic and hyperthecosis follicles found in stressed rats, which present a denser granulosa and theca cell layer compared with preovulatory follicles (26). Accordingly, 2.5 IU hCG markedly increased estradiol release from the ovary of cold-stressed rats but had no effect in control ovaries, demonstrating the higher gonadotropin sensitivity in the ovary of rats exposed to cold. The possibility is open that the increased hCG-induced release of estradiol could result from a similar increase in the androgen intraovarian production.

Chronic cold exposure seems to alter the feedback regulation of the hypothalamus-pituitary-gonad axis, because the increase in plasma levels of estradiol and testosterone was not accompanied by an alteration in gonadotropin secretion. This may be explained by a reduced sensitivity of GnRH neurons to ovarian steroid inhibition, which is a frequent feature of PCO disease and seems to be related to the hyperandrogenism (41–43). In addition, although we did not find changes in plasma LH levels, we cannot exclude the possibility that changes in LH pulsatile secretion may occur after cold exposure.

In the present study, chronic exposure did not alter plasma progesterone. This is in agreement with previous studies showing that although cold stress strongly stimulates plasma NE release, it has minor effect upon the hypothalamus-pituitary-adrenal axis, resulting in slightly altered, or unaltered, secretion of ACTH, corticosterone, and progesterone (44–46). Furthermore, the unaltered release of corticosterone in cold-stressed rats is though to be essential to cyst formation. Paredes *et al.* (7) have demonstrated that chronic cold/

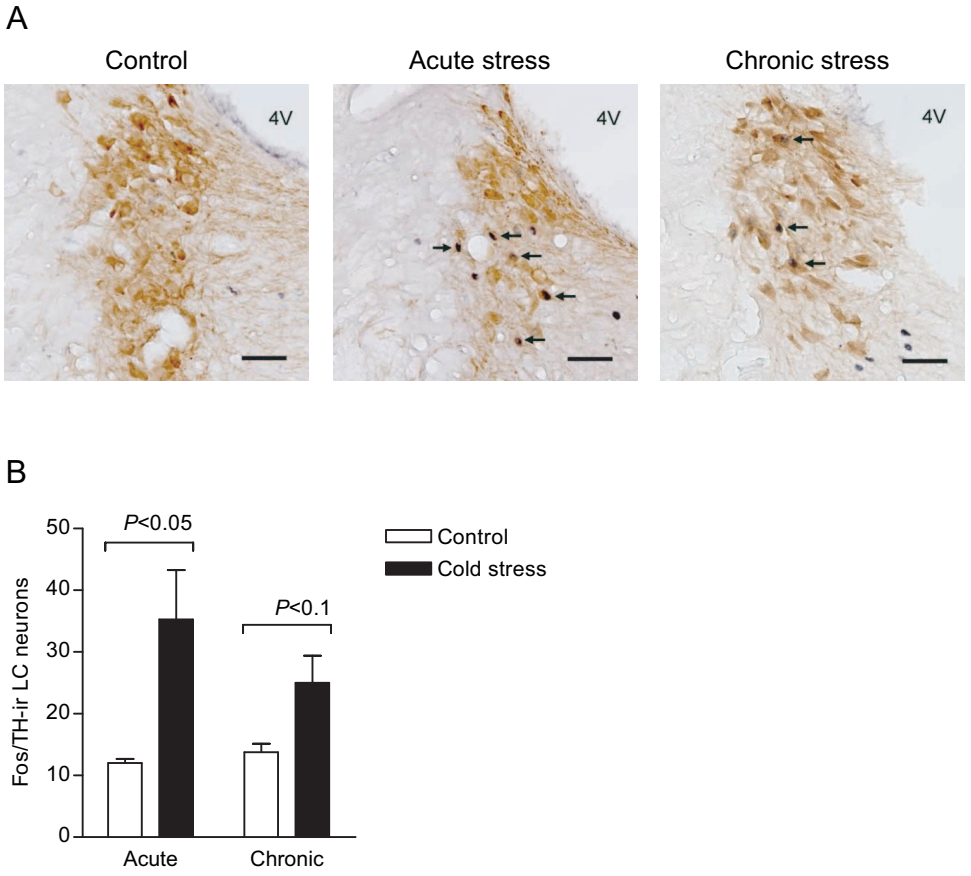

FIG. 9. Cold stress-induced Fos immunoreactivity in the LC. A, Photomicrographs of Fos/TH double-labeled LC coronal sections of control, acutely cold-stressed, and chronically cold-stressed rats. *Arrows* indicate double-labeled neurons (TH staining is cytoplasmic, and Fos is nuclear). B, Number of Fos/TH-ir neurons in the LC of rats exposed to acute (control, n = 4; stress, n = 4) or chronic (control, n = 4; stress, n = 4) cold stress. Data are presented as mean ± SEM. 4V, Fourth ventricle. *Scale bar*, 50 μm.

restrain stress does not promote PCO, which is probably due to the increased secretion of corticosterone in this model of stress.

The fact that LC lesion restored all of the changes found in follicular dynamic and the progression of cystic follicles induced by stress suggests at least two possibilities. One is that the lesion could alter gonadotropin secretion, and the other possibility is that it could affect the neural communication from brain to periphery. We did not find changes in plasma LH and FSH levels among all experimental groups studied, but noradrenergic neurons of the LC were profoundly affected by cold stress.

The acute and chronic activation of noradrenergic neurons demonstrated by Fos immunoreactivity and the reversion of all physiological and endocrine reproductive parameters in stressed rats bearing the LC lesion confirmed to us that noradrenergic neurons of the LC are a central component of the stress-induced alteration in ovarian morphology.

However, ovarian NE activity was not found to be altered after 8 wk of cold exposure. Two mechanisms could be operating in this response. One is an intraovarian mechanism to maintain a constant local neurotransmission, which could be mediated by a negative effect of neuropeptide Y (NPY) on NE release and concentration as has been recently found to occur in the mesenteric artery after chronic cold stress (47). Although we did not measure the amount of ovarian NPY in the present experiment, this mechanism could be operative in the ovary because NE regulation by NPY has been previously demonstrated in the ovary of both stressed and unstressed rats (7, 48). The second possibility is a long-term adaptation of stress circuitry at the LC level. In this support, cold stress-induced Fos expression in LC neurons was less pronounced after 8 wk of chronic cold stress compared with a single acute session, indicating an attenuation of the LC responsiveness in long-term exposure to cold stress. In addition, TH mRNA expression was reported to decrease in the LC after 21 d of chronic cold stress, which was correlated with an increase in the α_{2A} adrenergic receptor subtype mRNA (49). The α_{2A} receptor is known to regulate the activity of LC neurons, reducing NE release (50). In accordance with our data, adaptation of both Fos and TH mRNA expression in the LC has been reported after repeated sessions of foot-shock or immobilization stress (51–53).

These findings are, therefore, consistent with the hypothesis of a LC-mediated adaptation of ovarian NE activity after 8 wk of cold stress. The fact that NE turnover, as determined by MHPG/NE ratio, was decreased in stressed rats bearing a LC lesion supports this last suggestion. Thus, although ovarian NE activity returned to control level after 8 wk of stress, it seems likely that it was a definitive component in the changes in follicular dynamics induced by stress. The fact that the LC lesion did not alter NE activity in the ovary of control rats suggests that LC may regulate ovarian sympathetic activity only under specific conditions, such as after cold stress exposure. This finding is corroborated by previous studies from our laboratory showing that LC does not participate in steady-state homeostatic regulation but is ap-

parently important in situations of physiological challenge (54–56).

That cold stress-induced ovarian sympathetic activation is modulated by the central nervous system, with an important role for the PVN, has been suggested by previous studies (11, 25). Indeed, other brain areas such as raphe obscurus, raphe pallidus, and parapyramidal region have also been shown to be involved with sympathetic spinal nuclei activity during cold exposure (57–59). Several brainstem catecholaminergic neurons are known to innervate the sympathetic preganglionic neurons and thereby are potential modulators of the sympathetic nervous system response to stress (60, 61). Although some studies suggest that alterations in catecholamine metabolism and psychological stress are prevalent in women with PCO syndrome (PCOS) (62–64), the involvement of stress in the etiology of PCOS has been poorly understood. The present study is the first to report that chronic stress promotes PCO in rats, which seems to be initiated by an increased central noradrenergic tonus dictated by an augmentation in the LC activity.

Although our data strongly suggest that an increase in sympathetic nerve activity is a definite component in the changes in ovarian follicular development induced by stress, we cannot discard the effect of cold stress on thyroid function through increased TRH release. Because hyperthyroid function mimics PCO in women and is an exclusion criterion preventing the diagnosis of PCOS, it is important to dissect the effects derived from sympathetic innervation from those of hyperthyroidism. We did not measure plasma thyroid hormones, but the fact that stressed and/or LC-lesioned rats did not evidence alterations in body weight supports a preferential effect of stress on ovarian sympathetic nerve activity rather than on thyroid function in the paradigm of cold exposure used in the present study. Moreover, LC neurotoxic lesion has been shown to have no effect on either basal or stimulated TSH secretion (65), and we have found that LC electrolytic lesion does not alter TSH secretion in response to ether stress (unpublished data). Therefore, it is unlikely that PCO prevention by the LC lesion was due to reduction in thyroid function.

These data open a new field to investigate the etiology of the PCOS and suggest that attenuation of the stress effects, such as decrease in the central noradrenergic tonus, could help in diminishing the symptoms of PCOS and increasing fertility in women presenting this syndrome.

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