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ORIGINAL ARTICLE

Transitory Activation of the Central and Ovarian Norepinephrine Systems During Cold Stress-Induced Polycystic Ovary in Rats

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Cold stress-induced ovarian sympathetic activation is associated with the development of ovarian cysts in rats. Although we have hypothesised that polycystic ovary (PCO) features induced by cold stress, as prevented by lesion of the noradrenergic nucleus locus coeruleus (LC), were a result of the increased activity of the ovarian norepinephrine (NE) system, this was not evident after 8 weeks of stress. In the present study, we investigated the temporal changes in LC and ovarian NE activities and steroid secretion in rats exposed to single (SS) or repeated (RS) cold stress. SS and 4 week (4W)-RS but not 8 week (8W)-RS increased c-Fos expression in the LC and ovarian NE release. Plasma oestradiol, testosterone and progesterone levels tended to increase in 4W-RS and were elevated in 8W-RS rats, which displayed PCO morphology. β-adrenergic receptor agonist increased steroid hormone release from the ovary of unstressed (US) but not from 8W-RS rats. To determine whether increased activity of noradrenergic system during the initial 4 weeks of RS would be sufficient to promote PCO, rats were exposed to 4 weeks of cold stress and kept in ambient temperature for the next 4 weeks (4W-RS/4W-US). Accordingly, PCO morphology, increased steroid secretion and decreased ovulation rate were found in 4W-RS/4W-US rats, strengthening the hypothesis that the initial increase in NE release triggers the development of PCO. The correlated activity of LC neurones and ovarian noradrenergic terminals and the induction of PCO in 4W-RS/4W-US rats provide functional evidence for a major role of NE in disrupting follicular development and causing the long-lasting endocrine abnormalities found in stress-induced PCO.

Key words: sympathetic nervous system, locus coeruleus, hyperandrogenism, polycystic ovary syndrome

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The polycystic ovary (PCO) syndrome is broadly recognised as the most common female endocrinopathy, affecting approximately 7% of women of reproductive age (1). It is a complex disorder comprising a group of signs, symptoms and biochemical features that occur in various combinations (2,3). Despite the heterogeneous phenotype, PCO syndrome has been clinically defined by the presence of enlarged polycystic ovaries in association with menstrual irregularities and hyperandrogenism (4). Several studies have highlighted that such alterations may arise primarily from abnormal ovarian steroidogenesis, as a consequence of inappropriate hypothalamic-pituitary-ovarian interaction (5,6). Although hyperinsulinaemia and hyperandrogenism may interfere in normal ovarian steroidal secretion and contribute to the severity of symptoms (7,8), the primary causes of this reproductive dysfunction remain to be determined. A disturbance in the activity of the sympathetic nervous system appears to contribute to the impaired ovarian function found in PCO syndrome (9). Increased plasma catecholamines and peripheral sympathetic nerve activity have been associated with PCO syndrome (10,11). Furthermore, the higher density of catecholaminergic nerve fibres in the ovary (12,13) and the increased levels of nerve growth factor (NGF), a hallmark of sympathetic activity, in the follicular fluid (14) of PCO patients are consistent

with the hypothesis of involvement of the sympathetic nervous system in the physiopathology of PCO syndrome.

Cold stress has been used as an experimental model to induce sympathetic activation without disturbing adrenaline or adrenocorticotrophic hormone plasma levels, in both the rat and humans (15). Thus, in this model, the interference of adrenal axis in the reproductive function is avoided. Using a chronic cold-stress model in rats, we demonstrated that cold stress caused aberrant follicular development and altered ovarian steroidogenesis similar to PCO syndrome in women (16,17). Regarding insulin resistance, we have also recently found an ovary tissue-specific insulin resistance (18), as also found in the human ovary (19). Thus, although the cold-stress procedure is not a cause of PCO syndrome, sympathetic nerve hyperactivation is associated with the syndrome, as has been demonstrated to occur in humans (11), and may contribute to the aetiology of PCO syndrome.

In rodents, stress interferes with ovarian sympathetic tonus through the activation of specific brain areas involved in the control of the autonomic nervous system (20–22). Neurones from the paraventricular nucleus of the hypothalamus and locus coeruleus (LC) project directly to the sympathetic preganglionic neurones in the intermediolateral cell column of the spinal cord (23), therefore representing feasible candidates for the modulation of ovarian sympathetic activity under stress conditions. Although we have demonstrated that the LC-norepinephrine (NE) system integrity is necessary for the development of morphological and hormonal parameters of PCO induced by 8 weeks of cold stress, ovarian NE activity was not altered and there was no evidence of functional relationship between the activities of LC neurones, ovarian NE system and hormonal secretion in these rats (16). These data, along with the results of a previous study showing that ovarian abnormalities observed after 4 weeks are associated with increased sympathetic activity (17), suggest that the LC-NE system might be transitorily activated at the beginning of long-term exposure to cold stress and that this chronic stress induced the habituation of this system.

The present study aimed to better determine the functional involvement of the LC-NE system in the development of stress-induced PCO. We investigated the temporal relationship between the activities of LC neurones and ovarian NE terminals over 8 weeks of cold stress and also whether an initial increase in the LC and ovarian NE activities would be sufficient to induce PCO. The data obtained show that cold stress induces co-activation of LC neurones and ovarian NE terminals at least during the initial 4 weeks of cold stress, which are critical and sufficient for the induction of PCO. These findings expand our knowledge of the important role played by the LC-NE system in the mechanism of stress-induced PCO in the rat.

Materials and methods

Animals

Adult female Wistar rats obtained from the animal house of the University of São Paulo at Ribeirão Preto were kept in a central animal care facility, housed under a 12 : 12 h light/dark cycle (lights on 06.00 h) at 24 ± 0.5 °C. Air exchange rate was 15-room volumes per hour. Food and water were provided *ad lib.* Oestrous cycle regularity was assessed previously and only rats showing at least three consecutive 4-day regular cycles were used in the experiments. During the experiments, vaginal smears were taken daily to monitor oestrous cycle regularity. Rats were age-paired and weighed 350–370 g (15–16 weeks of age) on the day of euthanasia. All of the protocols used were approved by the Ethic Commission for the Use of Animals at the Campus of Ribeirão Preto, University of São Paulo (protocol number 09.1.1389.53.0).

Experimental design

Experiment 1: Temporal evaluation of the effect of cold stress on LC neuronal activity, ovarian morphology and ovarian steroid secretion in response to β-adrenergic stimulation

Regularly cycling rats were exposed to single (SS; n = 4) or repeated (RS) cold stress for 4 (4W-RS; n = 5) or 8 (8W-RS; n = 4) weeks. Unstressed animals (US; n = 6) were maintained at room temperature. On the day of oestrus, unstressed and stressed rats, immediately after the stress session, were deeply anaesthetised and transcardially perfused. The ovaries were removed immediately before perfusion. The right ovary was processed for histological analysis in all groups. The left ovary was divided in two parts. One part was immediately incubated *in vitro* to evaluate the steroidal response to β-adrenergic stimulation in US and 8W-RS rats. In the other part, concentrations of NE and its metabolite, 3-methoxy-4-hydroxyphenylglycol (MHPG), were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ED) in US, SS and 8W-RS rats. After perfusion, the brains were removed and LC sections from US, SS, 4W-RS, 8W-RS were immunohistochemically processed for c-Fos and tyrosine hydroxylase (TH) double-labelling. The number of TH-immunoreactive (-IR) neurones expressing c-Fos was determined bilaterally in the LC.

Experiment 2: Temporal evaluation of the effect of cold stress on the activity of the ovarian NE system and the secretion of ovarian steroid hormones

On the day of oestrus, US rats (n = 7), and SS (n = 5), 4W-RS (n = 5) and 8W-RS (n = 7) rats were rapidly decapitated immediately after the stress session and trunk blood was collected and the ovaries removed. The right ovary was divided in two parts. The NGF concentration was determined in one part by enzyme-linked immunosorbent assay (ELISA) and TH expression was evaluated in the other part by western blotting. In the left ovary, NE and MHPG concentrations were determined by HPLC-ED. The plasma samples were assayed for oestradiol, progesterone and testosterone by radioimmunoassay (RIA).

Experiment 3: Comparison of two different paradigms of cold-stress exposure on the development of polycystic ovaries

Regularly cycling rats were exposed to RS for 4 weeks and then maintained at room temperature for the next 4 weeks (4W-RS/4W-US; n = 5) or exposed to RS for 8 weeks (8W-RS; n = 5). US rats were maintained at room temperature. On the day of oestrus, unstressed and stressed rats were rapidly decapitated immediately after the stress session. Trunk blood was collected, the right ovaries were removed for histological analysis and the oviducts were dissected for oocyte counting. Plasma concentrations of oestradiol, progesterone, testosterone, luteinising hormone (LH) and follicle-stimulating hormone (FSH) were determined by RIA.

Cold-stress paradigm

For RS, rats were placed individually in their cages for 4 or 8 weeks without food, water and bedding in a cold room at 4 °C from 09.00 h to 12.00 h from Monday to Friday each week. For SS, one session of the same paradigm of cold stress was performed from 09.00 h to 12.00 h on the day of oestrus. US 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 (16).

Anaesthesia

For perfusion in Experiment 1, rats were anaesthetised with ketamine (ketamine chlorhydrate, 100 mg/kg body weight, i.p.; Agner, São Paulo, Brazil) and xylazine (10 mg/kg body weight, i.p.; Coopazine; Coopers, São Paulo, Brazil).

Immunohistochemistry

Sections from the LC were immunohistochemically processed for c-Fos and TH double labelling as described previously (16, 24). Four series of 20-μm frontal sections were obtained throughout the rostrocaudal extension of the LC. Briefly, sections were incubated with anti-c-Fos rabbit antibody (Ab5; Calbiochem, Darmstadt, Germany) at a dilution of 1: 15 000 for 40 h, followed by biotinylated anti-rabbit goat immunoglobulin (Ig)G (Elite kit; Vector Laboratories, Burlingame, CA, USA) at a dilution of 1 : 600 for 2 h, and avidin-biotin complex solution at a dilution of 1 : 100 for 1 h (Elite ABC kit, Vector Laboratories). The antibody-peroxidase complex was visualised with a solution of nickel phosphate (25 mg/ml) and 3,3′-diaminobenzidine-HCl (DAB; 0.2 mg/ml; Sigma, St Louis, MO, USA). Sections were then incubated with an anti-TH mouse antibody (anti-TH2; Sigma) at a dilution of 1 : 500 000 for 40 h, biotinylated anti-mouse horse IgG (BA-2001; Vector Laboratories) at a dilution of 1 : 800 for 1 h, and ABC at a dilution of 1 : 100 for 1 h. TH immunostaining was reveled with DAB solution. As control, omission of the primary antibodies resulted in absence of Fos labelling in the LC sections incubated with normal rabbit serum (Fig. 1A). Sections were blindly analysed for experimental groups, and the number of TH-IR neurones expressing c-Fos 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 (25).

Ovarian morphology

The ovarian morphologic characterisation of the PCO was performed by the histological analysis of 8-μm serial sections stained with haematoxylin and eosin. The number of follicles with hyperthecosis, type III and cystic follicles were counted in every section through the ovary as described previously (26). Briefly, antral follicles with hyperthecosis were defined as those medium-sized antral follicles, presenting hypertrophied differentiated theca interna cells, with an increased thickness of the theca layer and a normal granulosa cell layer. Type III follicles were large, containing four or five layers of small densely-packed granulosa cells surrounding a very large antrum and thickened theca internal cell layer. Cystic follicles were defined as those follicles devoid of oocytes and displaying a large antral cavity, a thin granulosa cell layer and a thickened theca interna cell layer.

HPLC-ED

Ovarian NE and MHPG concentrations were determined by HPLC-ED as described previously (16). Briefly, ovaries were homogenised in a solution of 0.2 M perchloric acid, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 450 nM of 3,4-dihydroxybenzylamine (DHBA, internal standard) and centrifuged for 20 min at 12 000 **g**. The supernatant was acid hydrolysed by heating at 94 °C for 5 min before analysis (27). Protein content was determined using the Bradford method (28). 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. The mobile phase consisted of 100 mM sodium dehydrogen phosphate, 10 mM sodium chloride, 0.1 mM EDTA, 0.37 mM sodium 1-octanesulphonic acid and 4% methanol (pH 3.5). The flow rate was set at 0.6 ml/min and the detector potential was 0.65 V. NE and MHPG were quantified using DHBA as internal standard. All samples from a single experiment were measured in the same analysis. The intra-assay coefficient of variation was 1.2% for NE and 4.6% for MHPG. NE levels were considered to estimate neurotransmitter levels in synaptic vesicles, whereas MHPG levels reflected neurotransmitter release (27). The MHPG/NE ratio was used as a measure of the neurotransmitter turnover rate.

ELISA

Nerve growth factor concentration was determined by the ELISA method. Half of the right ovary was homogenised using a microultrasonic cell disrupter within 500 μl of a solution containing 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2 PO_4, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$ and 0.5 mM of $MgCl_2$ (DBPS, pH 7.35). The homogenate was centrifuged for 15 min at 13 000 **g**. Protein content was determined in the remaining pellet by the Bradford method (28). The supernatant was diluted at 1 : 100 in DBPS buffer and NGF was measured using an NGF E_{max} immunoassay system (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. Dilution curves of the ovary tissue samples were parallel to the standard curve. All samples were measured in the same analysis. The intra-assay coefficient of variation was 5.8%.

Western blotting

The expression of TH (approximately 60 kDa) in the ovary was evaluated by western blotting. Adrenals served as a positive control for TH expression. The other half of the right ovary was homogenised separately in 100 μl of freshly prepared RIPA lysis buffer (20 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride and 20 μg/ml of aprotinin). The homogenate was centrifuged for 20 min at 12 000 **g** and the supernatant retained. The protein concentration in the supernatant was estimated using the Bradford method (28). Equal amounts of protein (60 μg per lane) were denatured and size-fractionated using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Molecular weight standards (Amersham Biosciences, Little Chalfont, UK) were loaded on each gel for molecular weight assessment. Proteins were then transferred onto nitrocellulose membranes (Millipore, Bio-Rad, Hercules, CA, USA). Blots were blocked for 2 h at room temperature with 5% nonfat dry milk in PBS-T (phosphate buffer saline + 0.1% Tween-20; Sigma) before incubation with mouse anti-TH antibody (dilution 1 : 10 000, anti-TH2; Sigma) diluted in PBS-T + 1% nonfat dry milk for 24 h at 4 °C. The membrane was then washed and incubated for 1 h at room temperature with rabbit anti-mouse IgG conjugated with horseradish peroxidase (dilution 1 : 7500; Dako Cytomation, Carpinteria, CA, USA). The signals were developed by enhanced chemiluminescence using the western lightning chemiluminescence substrate (ECL; Amersham Biosciences, Piscataway, NJ, USA) and visualised by exposure to Amersham Hyperfilm ECL for 10 min. For quantification purposes, the same blot was probed with mouse anti-dynein antibody (dilution 1 : 5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a loading control. Band intensities were quantified by using IMAGEJ (NIH, Bethesda, MD, USA). TH data were normalised to dynein (approximately 72 kDa) expression in the same sample.

Ovarian response to β-adrenergic stimulation *in vitro*

The ovarian response to β-adrenergic stimulation was determined by *in vitro* release of ovarian steroids in the presence of the β-adrenergic receptor agonist isoproterenol, using a a modification of the method described previously (29). Briefly, half of the left ovaries were divided into two parts; each part was weighed and preincubated separately in Krebs-Ringer bicarbonate buffer (pH 7.4) for 30 min at 37 °C in a 95% O_2/5% CO_2 atmosphere. After the preincubation period, one part of the ovary was incubated with 2 ml of buffer alone (Vehicle group) and the other part with the same volume of buffer containing 10 μM isoproterenol (ISO group; Sigma) (29). The incubation medium was collected after 3 h of incubation, and oestradiol, testosterone and progesterone concentrations were determined. Data were normalised by tissue weight.

Radioimmunoassay

Blood samples were collected from the trunk of decapitated rats into heparinised tubes. Plasma was separated by centrifugation at 1200 **g** for 15 min at 4 °C and stored at −20 °C. Plasma and *in vitro* oestradiol, progesterone and testosterone concentrations were determined by double-antibody RIA with MAIA kits provided by Biochem Immunosystems (Bologna, Italy). The lower limits of detection for oestradiol, progesterone and testosterone were 5.0 pg/ml, 0.02 ng/ml and 5.0 pg/ml, respectively. The intra-assay coefficient of variation was 4.3% for oestradiol, 7.5% for progesterone and 4% for testosterone. Plasma LH and FSH were determined by using specific kits provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Bethesda, MD, USA). The antiserum and reference preparation were anti-rat LH-S10 and LH-RP3 for LH and anti-rat FSH-S11 and

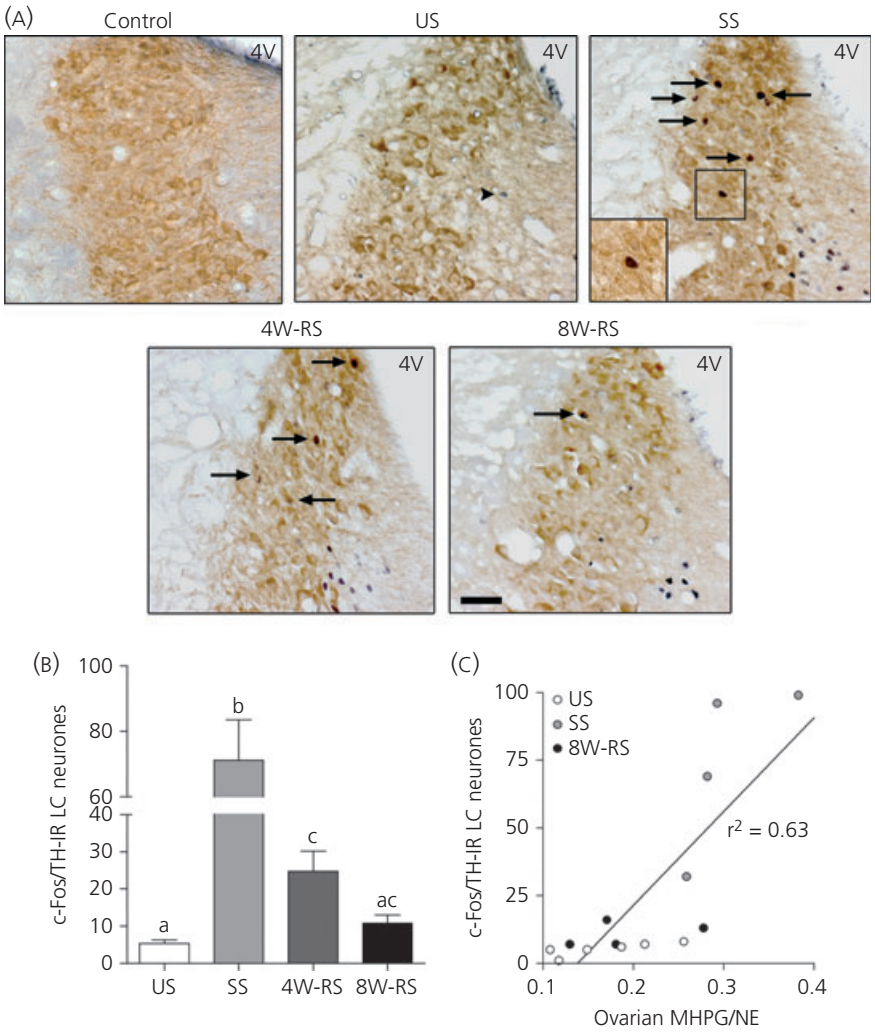

Fig. 1. Temporal effect of cold stress on locus coeruleus (LC) neuronal activity: positive correlation with the ovarian 3-methoxy-4-hydroxyphenylglycol (MHPG)/ norepinephrine (NE) ratio. Regularly cycling rats exposed to single cold stress (SS; n = 4), repeated cold stress during 4 (4W-RS; n = 5) or 8 (8W-RS; n = 4) weeks, or maintained at room temperature (US; n = 6), were perfused immediately after the end of the stress session on the day of oestrus. (A) Photomicrographs of c-Fos/tyrosine-hydroxylase (TH) double-labelled LC coronal sections from Control, US, SS, 4W-RS and 8W-RS rats. Control, negative control for c-Fos immunohistochemistry using non-immune serum instead of the anti-c-Fos antibody. Arrows indicate double-labelled neurones. Inset: double-labelled neurone shown at a higher magnification. The arrowhead indicates a single-labelled c-Fos-immunoreactive (-IR) neurone. 4V, fourth ventricle. Scale bar = 50 μm. (B) Number (mean ± SEM) of c-Fos/TH-IR neurones in the LC. Significant differences among groups are indicated by different lowercase letters ($P < 0.05$). (C) Positive correlation between the number of c-Fos/TH-IR neurones in the LC and the MHPG/NE ratio in the ovary of US (white circles), SS (grey circles) and 8W-RS (black circles) rats ($r^2 = 0.63$, $P < 0.001$).

FSH-RP2 for FSH, respectively. The lower limits of detection were 0.04 ng/ml for LH and 0.2 ng/ml for FSH. The intra-assay coefficients of variation were 3.4% for LH and 3% for FSH. To avoid interassay variation, all samples from a single experiment were assayed in the same RIA.

Statistical analysis

Data are shown as the mean ± SEM. Data were analysed by one-way ANOVA followed by the Newman-Keuls post-hoc test, except for differences in the ovarian response to isoproterenol, which were determined by two-way ANOVA followed by the Bonferroni post-hoc test. Correlation analysis between the number of c-Fos/TH-IR neurones in the LC and the MHPG/NE ratio in the ovary was performed using Pearson's correlation test. $P < 0.05$ was considered statistically significant.

Results

Temporal effect of cold stress on ovarian morphology

Morphological analyses of the ovaries showed that, as demonstrated previously (19, 20), 4W-RS as well as 8W-RS increased the number of antral follicles with hyperthecosis compared to US and SS rats ($F_{3,15} = 19.7$; $P < 0.001$). Although no change was observed in the number of type III and cystic follicles in 4W-RS (data not shown), 8W-RS significantly increased the number of type III ($F_{3,15} = 13.6$; $P < 0.001$) and cystic follicles ($F_{3,15} = 10.1$; $P < 0.001$) compared to the US, SS and 4W-RS groups.

Temporal effect of cold stress on LC neuronal activity: correlation with the ovarian MHPG/NE ratio

Figure 1A shows photomicrographs of c-Fos expression in the LC of US, SS, 4W-RS and 8W-RS rats. Although SS rats displayed a marked increase in the number of c-Fos/TH-IR neurones, a smaller increase was found in 4W-RS rats, and no significant difference was detected in 8W-RS rats, compared to US rats ($F_{3,15} = 20.1$; $P < 0.001$; Fig. 1B). In addition, a significant positive correlation was found between the number of LC c-Fos/TH-IR neurones and ovarian MHPG/NE ratio ($r^2 = 0.63$, $P < 0.001$), with those rats exhibiting the highest c-Fos expression in the LC also exhibiting the highest MHPG/NE ratio in the ovary (Fig. 1C).

Temporal effect of cold stress on the ovarian NE system

Ovarian MHPG concentration ($F_{3,20} = 14.1$; $P < 0.001$) and MHPG/NE ratio ($F_{3,20} = 24.4$; $P < 0.001$) increased in SS and 4W-RS rats but decreased in 8W-RS rats compared to the US group (Fig 2A,C). The ovarian NE concentration was not altered in SS and 4W-RS rats, although it was significantly increased in 8W-RS compared to

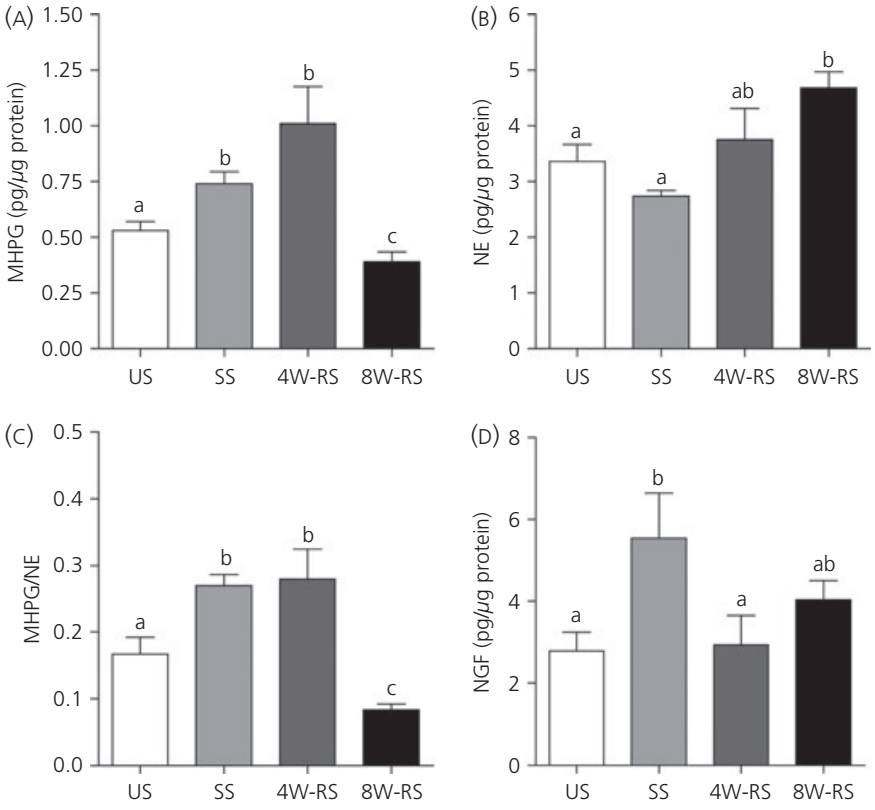

Fig. 2. Temporal effect of cold stress on ovarian norepinephrine (NE) system. Regularly cycling rats exposed to single cold stress (SS; n = 5), repeated cold stress during 4 (4W-RS; n = 5) or 8 (8W-RS; n = 7) weeks, or maintained at room temperature (US; n = 7) were decapitated immediately after the end of the stress session on the day of oestrus. Mean ± SEM (A) 3-methoxy-4-hydroxyphenylglycol (MHPG) levels, (B) NE levels, (C) MHPG/NE ratio, and (D) nerve growth factor (NGF) concentration in the ovary. Significant differences among groups are indicated by different lowercase letters ($P < 0.05$).

US rats ($F_{3,20} = 5.6$; $P < 0.01$) (Fig. 3B). Ovarian NGF levels were significantly increased in SS rats and restored to control levels in 4W-RS and 8W-RS animals ($F_{3,20} = 3.3$; $P < 0.05$; Fig. 2D), whereas TH expression was unaltered in the ovary of SS, 4W-RS or 8W-RS rats ($P = 0.79$) (data not shown).

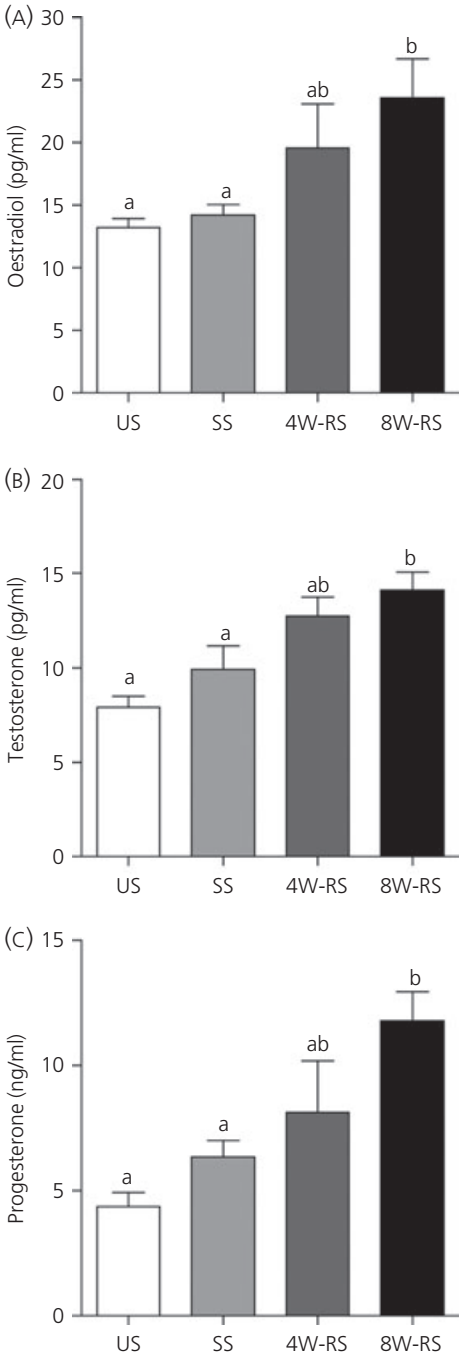

Fig. 3. Temporal effect of cold stress on plasma steroid hormone levels. Regularly cycling rats exposed to single cold stress (SS; n = 5), repeated cold stress during 4 (4W-RS; n = 5) or 8 (8W-RS; n = 7) weeks, or maintained at room temperature (US; n = 7) were decapitated immediately after the end of the stress session on the day of oestrus. Data are shown as the mean ± SEM of plasma (A) oestradiol, (B) testosterone and (C) progesterone concentrations. Significant differences among groups are indicated by different lowercase letters ($P < 0.05$).

Temporal effect of cold stress on plasma oestradiol, testosterone and progesterone concentrations

Plasma levels of ovarian steroids did not change in SS and 4W-RS rats, although there was a trend toward an increase in 4W-RS rats. Plasma oestradiol ($F_{3,20} = 4.8$; $P < 0.05$), testosterone ($F_{3,20} = 8.2$; $P < 0.001$) and progesterone ($F_{3,20} = 8.3$; $P < 0.001$) levels were significantly increased in 8W-RS compared to US rats (Fig. 3).

Effect of 8W-RS on the ovarian response to β-adrenergic stimulation *in vitro*

In vitro hormonal release from the ovaries incubated with vehicle did not differ between US and 8W-RS rats. As determined by the Bonferroni post-hoc test, incubation with isoproterenol markedly increased the release of oestradiol ($t_{15} = 3.5$; $P < 0.01$), testosterone ($t_{15} = 2.9$; $P < 0.05$) and progesterone ($t_{15} = 3.6$; $P < 0.01$) in the ovaries of US rats, whereas it had no effect in those of 8W-RS rats (Fig. 4).

Effect of 4W-RS/4W-US and 8W-RS on the development of PCO

Eight weeks after the beginning of cold-stress exposure, 4W-RS/4W-US and 8W-RS rats displayed a similar increase in the number of ovarian antral follicles with hyperthecosis ($F_{2,15} = 16.24$; $P < 0.05$), type III follicles ($F_{2,15} = 4.44$; $P < 0.01$) and cysts ($F_{2,15} = 15.37$; $P < 0.05$) compared to US rats (Fig. 5). Table 1 summarises the hormone profile of US, 4W-RS/4W-US and 8W-RS rats. Plasma oestradiol levels significantly increased in 4W-RS/4W-US and 8W-RS ($F_{2,14} = 4.88$; $P < 0.05$) compared to US rats. Testosterone levels significantly increased in 8W-RS ($F_{2,11} = 7.17$; $P < 0.01$) and tended to increase in 4W-RS/4W-US ($P = 0.06$) rats. Plasma levels of progesterone were significantly increased only in 8W-RS rats ($F_{2,12} = 7.95$; $P < 0.01$). LH ($F_{2,11} = 0.12$; $P > 0.05$) and FSH ($F_{2,13} = 0.89$; $P > 0.05$) plasma levels did not change in any stressed groups. Both 4W-RS/4W-US and 8W-RS rats showed a prolonged oestrous cycle length as a result of the increased prevalence of oestrous-like phases, although they were able to display pro-oestrus followed by oestrus. A decrease in the number of oocytes ovulated was also detected in both 8W-RS (2.8 ± 1.7) and 4W-RS/4W-US (7.4 ± 2.3) rats compared to US rats (17 ± 1.2) ($F_{2,11} = 13.83$; $P < 0.01$).

Discussion

The present study demonstrates that, although single or repeated cold stress for 4 weeks increased both c-Fos immunoreactivity in the LC neurones and the activity of ovarian NE terminals, these responses were found to be absent after 8 weeks of stress, when rats display ovarian cysts, hyperandrogaenemia and a decreased ovulatory rate, which are all typical features of PCO syndrome.

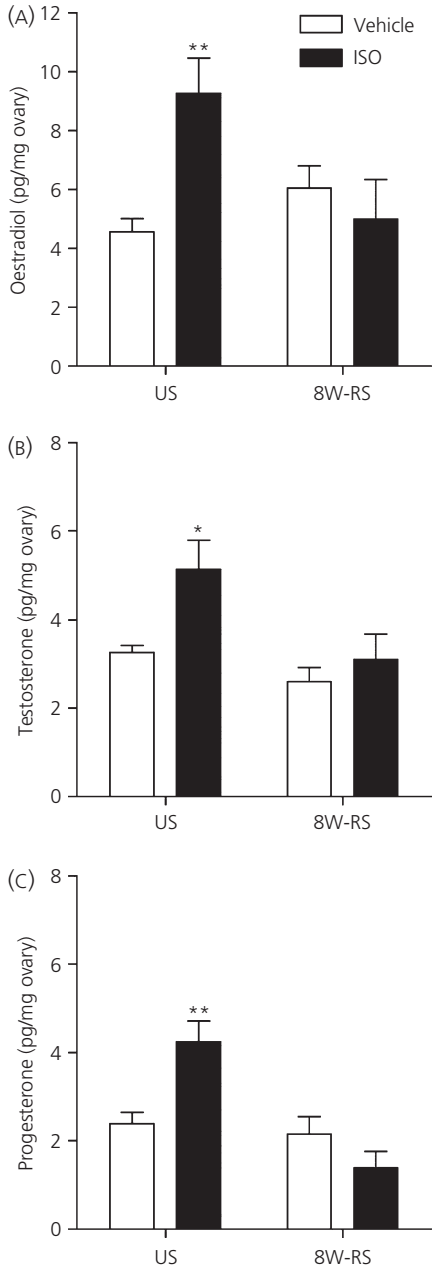

Fig. 4. Effect of 8 weeks of cold stress on ovarian response to β-adrenergic stimulation *in vitro*. Regularly cycling rats exposed to 8 weeks of repeated cold stress (8W-RS; n = 4) or maintained at room temperature (US; n = 6) were perfused immediately after the end of the stress session on the day of oestrus. Before perfusion, the left ovaries were removed, half of the left ovaries were divided into two parts, and each one was separately incubated with Krebs-Ringer bicarbonate buffer alone (Vehicle) or 10 μM isoproterenol (ISO). Data are presented as the mean ± SEM of (A) oestradiol, (B) testosterone and (C) progesterone levels in the incubation medium normalised by tissue weight. **P < 0.01 and *P < 0.05 compared to Vehicle.

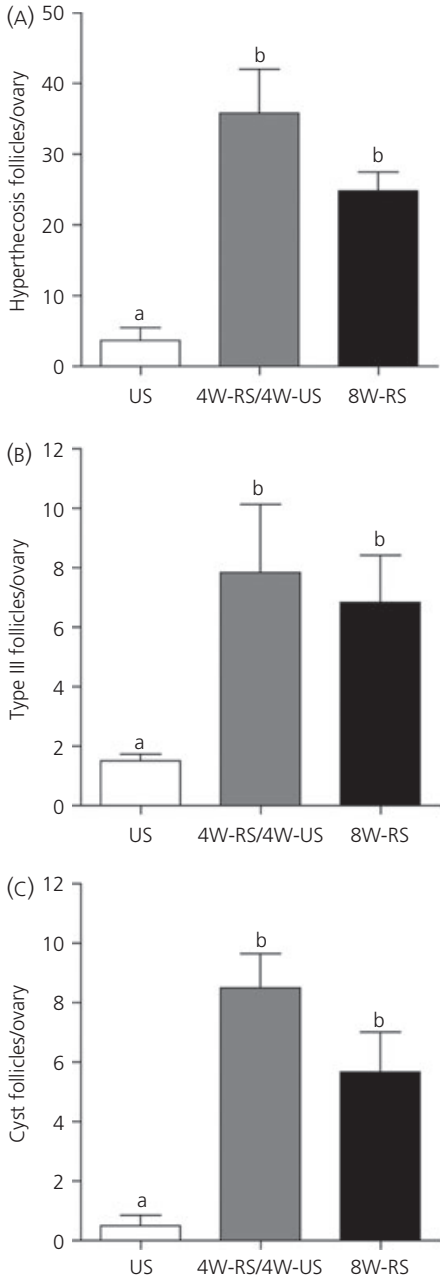

Fig. 5. Comparison of two different paradigms of cold-stress exposure on the development of polycystic ovaries. Regularly cycling rats exposed to repeated cold stress (RS) during 4 weeks and then maintained at room temperature within the next 4 weeks (4W-RS/4W-US; n = 5), exposed to RS during 8 weeks (8W-RS; n = 5) or maintained at room temperature (US; n = 5) were decapitated after the end of the experiment period on the day of oestrus. The right ovaries were processed for histological analysis of PCO morphology. The number of antral follicles with (A) hyperthecosis, (B) type III follicles and (C) cysts per ovary is shown as the mean ± SEM. Significant differences among groups are indicated by different lowercase letters (P < 0.05).

These data suggest that the initial and transitory increase in ovarian NE activity, as regulated by LC neurones, plays a crucial role in the genesis of stress-induced PCO in rats. In addition, the temporal correlation of the activities of LC neurones and ovarian NE terminals strengthen a role for central NE in the control of ovarian autonomic function under stress conditions.

Table 1. Hormone Profile of 4W-RS/4W-US Versus 8W-RS rats.

Hormones	US	4W-RS/4W-US	8W-RS
Oestradiol	12.75 ± 0.72[a]	19.58 ± 3.49[b]	17.69 ± 1.40[b]
Testosterone	11.4 ± 2.4[a]	23.38 ± 4.2[ab]	35.98 ± 7.7[b]
Progesterone	3.85 ± 0.66[a]	4.62 ± 1.30[a]	11.06 ± 2.20[b]
LH	0.24 ± 0.01[a]	0.26 ± 0.04[a]	0.25 ± 0.04[a]
FSH	10.35 ± 0.71[a]	9.74 ± 0.91[a]	8.68 ± 1.09[a]

Values are the mean ± SEM. Hormone levels are expressed in ng/ml, except for oestradiol and testosterone, which are expressed in pg/ml. Different superscript lowercase letters indicate significant differences within the rows ($P < 0.05$). LH, luteinsing hormone; FSH, follicle-stimulating hormone.

The transitory pattern of LC neurone activation under repeated cold exposure is consistent with our previous findings with respect to Fos immunoreactivity in the 8-week cold-stress paradigm (16). Because Fos expression and neuronal firing rate in the LC are considerably decreased by oestradiol (24), it is reasonable to hypothesise that the increased plasma oestradiol levels observed at 4W-RS and 8W-RS may play a role in the adaptation of the LC response to stress. This hypothesis is in agreement with the effect of oestradiol in reducing immobilisation stress-induced c-Fos expression in the LC of ovariectomised rats (30). The temporal correlation between LC and ovarian NE activities in response to single and repeated cold stress also suggests a functional interaction between the LC and ovarian NE terminals. A similar pattern of functional correlation appears to take place during the rat oestrous cycle because it has been reported, in two independent studies, that both Fos expression in LC neurones and NE release in the ovary are increased during the afternoon of pro-oestrus and unaltered during di-oestrus (24,31). The contribution of the LC to the control of autonomic nervous system appears to involve direct output to sympathetic and parasympathetic preganglionic neurones in the intermediolateral column of the spinal cord, as well as projections to other autonomic nuclei (23). Indeed, LC has been shown to be multisynaptically connected to the preganglionic cell bodies of ovarian sympathetic nerves (32,33), further supporting its involvement in the control of ovarian NE outflow, probably under both physiological and stressful conditions.

MHPG levels in dissected tissues have been shown to be a reliable marker of noradrenergic activity, reflecting the amount of NE released from synaptic terminals (27). Thus, the results obtained with respect to MHPG levels and the MHPG/NE ratio suggest an increased release of NE in the ovary at least during the initial 4 weeks of stress, and a reduced release after 8 weeks of stress. Because we found no effect of cold stress in the ovarian levels of TH, the rate-limiting enzyme in catecholamine biosynthesis, we hypothesise that the higher levels of ovarian NE found after 8 weeks of stress reflect increased levels of synaptic vesicles probably as a result of decreased release rather than increased biosynthesis. We cannot exclude the possibility, however, that an increment in NE biosynthesis may have been caused by an increase in TH activity, as elicited by enzyme phosphorylation (34). Thus, although the decrease in MHPG levels unequivocally demonstrates reduced NE release in the ovary of 8W-RS rats, the reason for the increased NE concentration needs to be clarified in further studies. Because NGF produced in the ovary is involved with the maintenance of the sympathetic nerve activity through stimulation of TH expression (35,36), we also evaluated the effect of cold stress upon ovarian NGF content and TH expression. Although the activity of NE terminals was still augmented after 4W-RS, a significant increase in ovarian NGF concentrations was only found after the SS, with recovery to basal levels within the following weeks of cold stress. Our findings of a transient effect of cold stress on ovarian NGF compared to the marked effect on NE activity during the 4W-RS support a main role for local NE release in cold stress-induced PCO. Furthermore, because we did not detect any change in ovarian expression of TH during cold stress, we assume that the higher levels of ovarian NE found after 8W-RS probably reflect levels of synaptic vesicles as a result of decreased release rather than increased biosynthesis.

Considering the main role played by β-adrenergic receptors in ovarian steroidogenesis (37,38), we also evaluated the effect of the β-adrenergic receptor agonist isoproterenol in ovarian secretion *in vitro* before and after 8 weeks of cold stress. As expected, isoproterenol increased the release of steroid hormones in the ovary of US rats, although it failed to alter the release of the same hormones in the ovary of 8W-RS rats. Because 8W-RS rats displayed high plasma levels of ovarian steroids, a down-regulation of β-adrenergic receptor expression appears to have occurred after 8 weeks of cold stress. Although we did not perform experiments investigating isoproterenol-induced steroid secretion in the ovaries of 4W-RS rats, this effect probably reflects an adaptation of the ovarian NE system to the increased release of NE during at least the initial 4 weeks of cold stress, which is sufficient for complete PCO development.

Accordingly, down-regulation of β-adrenergic receptors has been described after a long period of sympathetic activation in oestradiol valerate-induced PCO in rats (39). Because NE acts through β-adrenergic receptors to induce secretion of ovarian steroids in both rodents and humans (40,41), and also to amplify gonadotrophin-stimulated androgen biosynthesis in theca-interstitial cells (42), it is likely that the higher NE release in the ovary during the initials weeks of cold stress elicits cyst development through stimulation of irregular steroid hormone secretion. Indeed, isoproterenol treatment has been shown to enhance androgen secretion and elicit cyst development in the rat ovary (43).

A higher number of type III follicles and follicles with hyperthecosis has been associated with hyperandrogenism (44), which is probably the main cause of the rise in plasma levels of steroid hormones found after 8 weeks of cold stress. Granulosa and theca cells from the follicles of anovulatory women with PCO syndrome also release higher amounts of steroids *in vitro* than those from ovulatory women (45–48). Although plasma oestradiol, testosterone and progesterone levels tended to increase in 4W-RS rats, they were only significantly elevated in 8W-RS rats, which is consistent with a role for the ovarian abnormal follicular structures as the main source of steroid hormone secretion in cold-stressed rats.

Considering that NE has been associated with acquisition of FSH receptor by the newly-formed follicles (49) and that increased folli-

cle recruitment is another distinction of PCO syndrome (50,51), we suggest that the higher NE release in the ovary during the initial weeks of cold stress enhances follicle recruitment and thus increases the probability of going on to develop cysts in the follicular cohort. Accordingly, our previous report of a two-fold increase in the number of small antral follicles and a higher sensitivity to human chorionic gonadotrophin stimulation in the ovary of 8W-RS rats (16) supports a role for NE in the stress-induced alteration of ovarian responsiveness to gonadotrophin.

Because there was an adaptation of the noradrenergic system after continuous stress exposure, we hypothesised that the initial 4 weeks would be sufficient to trigger the development of PCO. As we observed previously (17), 4 weeks of repeated cold exposure were sufficient for the development of antral follicles with a hypertrophied theca cell layer but not for cysts or type III follicles. Thus, the presence of these anomalous follicles 8 weeks after the initiation of stress suggests that a longer time window, independent of NE, is required for the full development of PCO syndrome features induced by the initial 4 weeks of cold stress. Indeed, 4W-RS/4W-US rats displayed an ovarian morphology identical to that of 8W-RS rats. In the oestradiol valerate-induced PCO model, an 8-week delay is also needed for the appearance of ovarian cysts (26). In accordance with our results, early increases in ovarian sympathetic activity are strongly associated with deranged follicle development and cyst formation in oestradiol valerate-induced PCO (43,52). The re-establishment of oestrous cyclicity and ovulatory capacity after transection of the ovarian sympathetic nerve reinforce a role for NE in oestradiol valerate-induced PCO (29).

In addition to morphological alterations, a second important criterion for PCO syndrome diagnosis, hyperandrogenaemia (4), was also observed in the 4W-RS/4W-US rats. This inherited trait of PCO syndrome is currently viewed as a heterogeneous disorder in which androgens may be originated in the ovary or adrenals. Thus, plasma levels of testosterone have been used as an endocrine marker of this condition in women (53, 54). Hyperandrogenaemia in 4W-RS/4W-US rats and 8W-RS rats was characterised by increases in the concentration of testosterone plasma. These data are in agreement with our previous report of the increased secretion of oestradiol and testosterone after long-term cold exposure (16); however, differences in progesterone plasma levels were only detected in the present study. This discrepancy may be explained by the use of anaesthetised rats, which may interfere with progesterone secretion (55), compared to the use of unanaesthetised rats for hormonal and neurochemical evaluation in the present study. The increased plasma progesterone concentration, however, differs from the endocrine findings observed in human PCO syndrome. This is probably because the morphology of rat PCO includes a distinct class of type III follicles that are not present in the woman PCO syndrome. This comprises a large follicle with a dense granulosa cell layer, which is not apoptotic and is a precursor of cystic follicles (26). When the ovaries of stressed rats are incubated *in vitro*, they show a higher release of progesterone, oestradiol and testosterone (29). Thus, we hypothesise that type III follicles may represent the origin of the increased plasma levels of progesterone observed in the cold-stress model of PCO.

Elevated LH or LH/FSH ratios have been described as a common endocrine feature in the classic (chronic anovulation and hyperandrogenism) PCO syndrome phenotype; nevertheless, these are not elevated in the ovulatory PCO syndrome (hyperandrogenism and polycystic ovaries but ovulatory cycles) phenotype (56) and, according to the Rotterdam guidelines, this endocrine feature is not an essential criterion for the diagnosis of PCO syndrome (4). Although stressed rats showed a decreased ovulation rate, they were able to exhibit ovulatory cycles and normal plasma baseline LH and FSH concentrations. We have also previously demonstrated that cold-stress signalling initially activates the paraventricular nucleus, and then projects through sympathetic nerves originating centrally to the sympathetic peripheral celiac ganglia, arriving in the ovary through the superior ovarian nerve (20–22). Thus, this descending pathway does not appear to involve the endocrine response involving GnRH secretion from the reproductive hypothalamus. This explains why there is ovarian activation as a result of cold stress without direct alteration of gonadotrophin. Thus, because this paradigm of stress elicited morphological ovarian features typical of PCO, hyperandrogaenemia and a reduced ovulation rate, it fulfills the criteria for a diagnosis of PCO syndrome. We therefore suggest that the initial increase in ovarian NE release is one determinant for the development of PCO in the rat.

Taken together, our present data demonstrate a functional and temporal interaction between the activities of LC neurones, ovarian NE terminals and steroid hormone secretion during the development of cold stress-induced PCO in the rat. The increased ovarian NE activity during at least the initial 4 weeks of cold stress appears to elicit deranged follicular development leading to the long-lasting endocrine abnormalities related to PCO. In the present study, we provide further evidence supporting the hypothesis that ovarian NE outflow under control of the LC plays a role in the genesis of stress-induced PCO, and we propose a modified paradigm that may facilitate future studies of PCO in animal models. These findings improve our knowledge of the complex interrelationship between stress, disturbed follicular development and steroid secretion in the pathophysiology of PCO syndrome.

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