Stress Promotes Development of Ovarian Cysts in Rats

The Possible Role of Sympathetic Nerve Activation

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Activation of the sympathetic innervation precedes the induction of polycystic ovaries in rats given estradiol valerate (EV). The mechanism of induction by EV may thus involve both direct and neurogenic components. We tested this hypothesis using a combined cold and restraint stress to induce an increase in sympathetic tone, including that of the ovarian sympathetic nerves. Three weeks after the start of stress we found:

1. **An increase in the content of norepinephrine (NE) in the celiac ganglion.**
2. **An increase in the release of NE from the ovary.**
3. **An unchanged NE uptake by the ovary.**
4. **An unchanged content of NE in the ovary.**

The ovarian content of neuropeptide Y (NPY) (colocalized with NE) was significantly decreased. These results suggest that NE synthesis and its secretion are increased during this period and correlate with the increase in secretion of androgens and estradiol, the development of precystic follicles, and a decrease in the ovulatory rate. After 11 wk, NE release had returned to control values, whereas the ovarian NE content had risen significantly, suggesting a maintained high rate of NE synthesis. In the ovary, NPY contents, steroid secretion, morphology, and ovulation had returned to the control state. These results suggest the participation of an extraovarian factor that might act locally to control the release of NE from the ovary, and further support the hypothesis that increased sympathetic activity plays a role in the development and maintenance of ovarian cysts.

Key Words: Stress; ovarian nerves; steroid secretion; ovarian cyst.

Introduction

The polycystic ovary syndrome (PCOS) is the most common endocrine abnormality in women during their reproductive years *(1–3)*. It is characterized by ovulatory failure, amenorrhea, increased plasma androgen concentrations, and lowered plasma capacity for binding sex hormone and, therefore, with higher concentrations of unbound estrogens *(1,4,5)*.

A major factor responsible for the acyclicity of PCO syndrome in humans is a tonic inhibition of gonadotropin secretion effected by the increased blood estrogen concentrations that result from the peripheral aromatization of androgens *(4,5)*. In fact, high doses of estradiol valerate (EV) administered to rats cause acyclicity, anovulation, and formation of ovarian cysts *(6,7)*. Thus, a primary defect underlying the causes of PCOS may be intraovarian, a possibility that has recently directed attention to catecholamines derived from the sympathetic innervation of the ovary *(8,9)*.

We have provided evidence for a direct involvement of the nervous system in the maintenance and progression of PCOS *(10)*. Actually, the ovaries of animals treated with EV exhibited an increased noradrenergic tone supported by the extrinsic innervation of the ovary. In fact, transection of the superior ovarian nerve (SON), which carries most of the sympathetic innervation to endocrine cells of the ovary *(11,12)*, leads to recovery of estrous cyclicity and ovulation *(10)*. Therefore, in addition to the direct effect of EV in the formation of cysts, the development of PCOS could have a neurogenic component involving the activation of sympathetic nerves. Clinical observations also suggest that stress may be an important factor in the development of PCOS *(9)*.

To test this hypothesis, we increased sympathetic tone using a combined cold-restraint stress procedure based on previous reports describing the effect of both procedures on

Table 1
Adrenal Gland Weight, Catecholamine Content, and Ovary Weight After Different Periods of Stress[a]

		Stress		Stress
	Control	3 wk	Control	11 wk
Rat weight (g)	252 ± 8	210 ± 7^c	310 ± 20	232 ± 13^d
Adrenal weight (mg)	15.5 ± 1.9	21.5 ± 1.3^b	16.6 ± 4.1	29.4 ± 2.2^d
Adrenal catecholamines (μg/gland)	43.5 ± 14	145.0 ± 18^c	60.9 ± 18	119.0 ± 17^c
Ovary weight (mg)	31.3 ± 6.0	31.7 ± 3.0	33.0 ± 6.3	35.4 ± 4.0

[a]Results are expressed as mean values $\pm$ SEM of 5 individual experiments in each condition. Rat weight at beginning of experiment was 204 ± 6 g (n = 10) for control group and 190 ± 15 for the stressed group. Adrenal catecholamines correspond to total catecholamines (norepinephrine and epinephrine).
[b]$p < 0.05$.
[c]$p < 0.02$.
[d]$p < 0.01$ vs control.

Table 2
Ovarian NE Uptake and Concentration, Ovarian NPY Concentration, and Ganglionic NE Concentration After Different Periods of Stress[a]

		Stress	
	Control	3 wk	11 wk
NE content			
(ng NE/ovary)	37 ± 8.4	38 ± 2.2	124.4 ± 20^d
(ng NE/ganglion)	92.9 ± 10.4	151 ± 20^b	191 ± 10.8^c
^{3}H-NE retention (dpm NE/mg ovary)	$33,700 \pm 2200$	$32,615 \pm 2650$	$30,040 \pm 1970$
NPY (pg/ovary)	594 ± 87	273 ± 11^b	1165 ± 204^c

[a]Norepinephrine (NE), was determined by radioenzymatic assay and NPY by radioimmunoassay. Results are expressed as mean values $\pm$ SEM of 5 individual experiments in each condition.
[b]$p < 0.05$.
[c]$p < 0.02$.
[d]$p < 0.01$ vs control.

sympathoadrenal system *(13,14)*. As an index of the activation of the ovarian sympathetic nerves, we measured the concentration and release of norepinephrine (NE) from the ovary in vitro, and examined the correlation with changes in ovarian secretion and the development of cysts.

Results

Body Weight and Adrenal Gland Catecholamines

Animals under stress grew more slowly. Despite this, the weight of the adrenal gland, a target organ during the stress response, increased steadily throughout exposure to stress. Total adrenal catecholamines (NE and epinephrine) increased threefold after 3 wk, and remained high after 11 wk of stress. Ovarian weight remained constant throughout (Table 1).

Ovarian Sympathetic Nerve Activity

The amount of NE in the ovary was unchanged after 3 wk, but had increased three times after 11 wk of stress. In the celiac ganglion, where most of the sympathetic innervation of the secretory cells of the ovary originates *(11)*, a substantial increase in the NE content had occurred by 3 wk and increased further by 11 wk of stress. The uptake of NE (measured as the amount of [^{3}H]NE retained by tissue after incubation with a known amount of NE), was unchanged during the whole 11-wk period (Table 2). NE release was calculated from the induced rates of release of recently incorporated [^{3}H]NE (Fig. 1). The release of NE had increased by 50% after 3 wk, but returned to control values by 11 wk of stress. The ovarian content of neuropeptide Y (NPY), which is cosecreted with NE in the sympathetic nerves *(15)*, decreased after 3 wk, but was increased by 11 wk of stress (Table 2).

Reproductive Function

The pattern of the estrous cycle, evaluated by daily vaginal smears, was disrupted in stressed animals (Fig. 2). Although there was no change in the percentage of time that rats stayed at the different stages of the estrous cycle, a
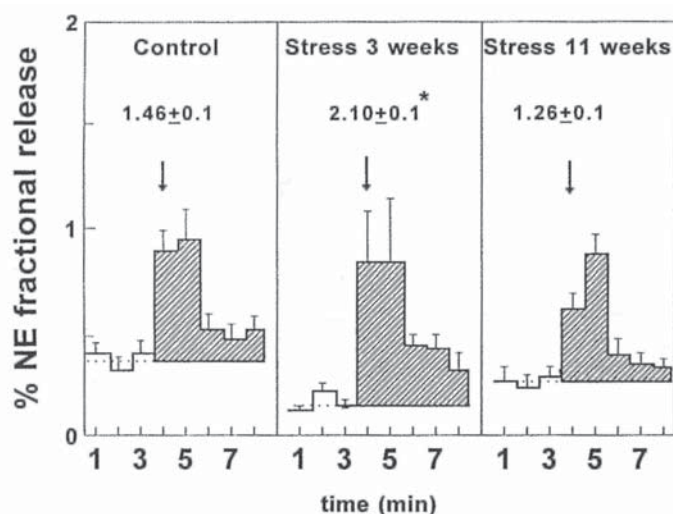
Fig. 1. Changes in the release of newly incorporated [³H]NE by transmural stimulation of the ovary in rats under 3 and 11 wk of stress. It presented the NE release when stimulating (80 V; 10 Hz; 2 ms; 1 min) pulses (arrows) were delivered. Numbers above profiles correspond to the total amount of NE released during stimulation (hatched area). Results are expressed as the percent fractional release and represent the mean ± SEM of 4 individual experiments/group. *$p < 0.05$ vs control.

Fig. 2. Left panel: relative incidence of estrous cycle phases in control and 3- or 11-wk stressed rats. The results are expressed as a percentage (±SEM) of days in proestrus (P), estrus (E), and diestrus 1 and 2 (D) with respect to the total days examined. Right panel: disrupting effect of stress on the assumed rate of ovulation in rats. Rate of ovulation (%) represents the amount of time (expressed as percentage of cycles examined) that proestrus day was followed by estrus at examination on vaginal smear. Results are expressed as mean ± SEM of 5 individual animals in each condition. **$p < 0.02$ vs control (0 wk of stress).

significant decrease occurred in the regularity for the transition from proestrus to estrus, which most likely represents ovulation (termed as rate of ovulation in Fig. 2). Although in control rats almost 70% of proestrus days were followed by estrus (suggesting ovulation), this percentage decreased to 27% after 3 wk of stress, but recovered to near-control values after 11 wk.
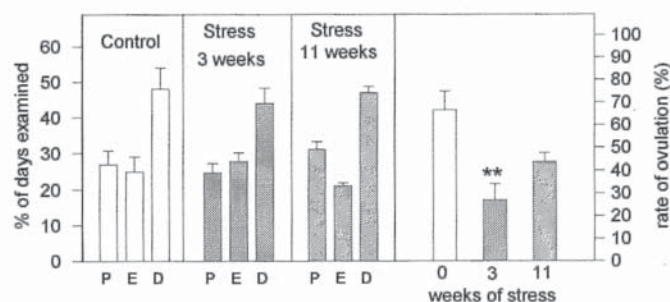
Secretion of Steroids by the Ovary

Basal progesterone secretion in vitro was unaffected by stress (Fig. 3A). The net (minus basal) release of progesterone induced by human chorionic gonadotropin (hCG) was 50 and 80% lower, respectively, after 3 and 11 wk of stress. Stimulation of secretion by isoproterenol was also less at both time periods.

In contrast, the basal secretion of androgens increased substantially after 3 wk, and it was still high after 11 wk of stress (Fig. 3B). Similarly, net release of androgens was stimulated by hCG and isoproterenol at 3 wk, but decreased to basal values by 11 wk. Net release of estradiol after hCG and isoproterenol was unchanged throughout, although basal secretion was increased after 3 wk (Fig. 3C).

Ovarian Morphology

Figure 4A shows a control ovary during diestrus; the corpus luteum and both antral and preantral follicles are clearly seen. Figure 4B shows a corresponding ovary after 3 wk of stress. There is an increase in the amount of interstitial gland, vascularization, and in the accumulation of large precystic follicles (PCF). These follicles —similar to the large type III precystic follicles described in ref. *16*—

resemble precystic follicles that display a high secretory activity and are probably responsible for the increase in ovarian secretion (well-developed theca and granulosa cell layer; *see* magnification on right of Fig. 4B). The follicles that accumulate after 3 wk of stress correspond to antral follicles 350–450 μm in diameter (stress, 8.4 ± 1.8; controls, 4.0 ± 0.3 follicles/ovary; mean ± SEM, $p < 0.05$, Fig. 5B). Figure 4C shows an ovary after 11 wk of stress. This ovary morphologically resembles that of control rats, but there is a decline in the number of total preantral and antral follicles as compared with control (stress, 111 ± 4.7; controls, 162 ± 11 preantral follicles/ovary, $p < 0.05$; and stress, 43 ± 2.5; controls, 66 ± 4.2 antral follicles/ovary, $p < 0.05$, Fig. 5A). No change in the number of preantral and antral follicles undergoing atresia was found.

Discussion

The present results indicate that chronic stress induces an increase in the sympathetic nerve activity of the ovary and that this increase is related to the presence of precystic follicles. Chronic cold stress has been shown to increase adrenal tyrosine hydroxylase activity, and the exposure to a heterotypic stressor (restraint) did not modify the response, but increased corticosterone plasma levels *(13)*. We also found that a combination of cold and restraint stress resulted in a clear adrenal activation, and increase in the activity of the sympathetic nerves to the ovary. The celiac ganglion is the principal neuronal relay of the sympathetic nerves that control secretion from the ovary *(12,17)*, but it is also the principal mesenteric ganglion, so the changes in ganglionic NE content indicate a general effect of stress on the sympathetic system similar to that described at the superior cer-
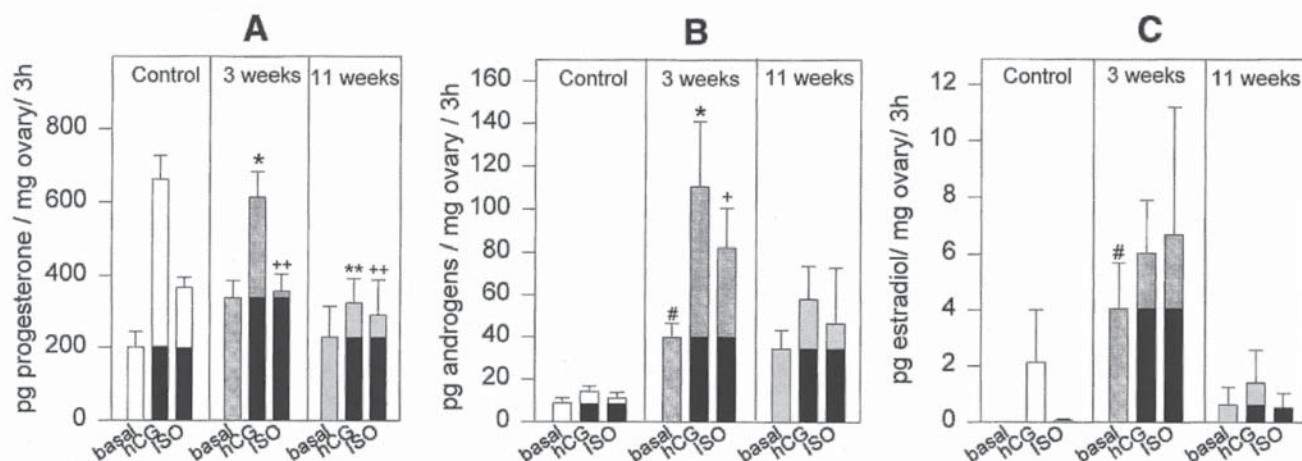
Fig. 3. Stress-dependent changes in ovarian progesterone **(A)**, androgens **(B)**, and estradiol **(C)** response to β-adrenergic and gonadotropic stimulation. The ovaries of control and after 3 or 11 wk of stress were halved and incubated for 3 h in Krebs. Ringer bicarbonate buffer alone (basal), isoproterenol (10 μM, Iso), or hCG (2.5 IU, hCG). The amount of steroids secreted into the incubation medium was measured by radioimmunoassay. Each bar represents the mean value ± SEM of 4 independent observation/group. Net release corresponds to the value of secretion in the presence of hCG or Iso minus the basal secretion (represented as a black area under each bar). *$p < 0.05$ vs net release of control + hCG. **$p < 0.01$ vs net release from control + hCG. +$p < 0.05$ vs net release of control + Iso. ++$p < 0.01$ vs net release of control + Iso #$p < 0.05$ vs basal release of control.
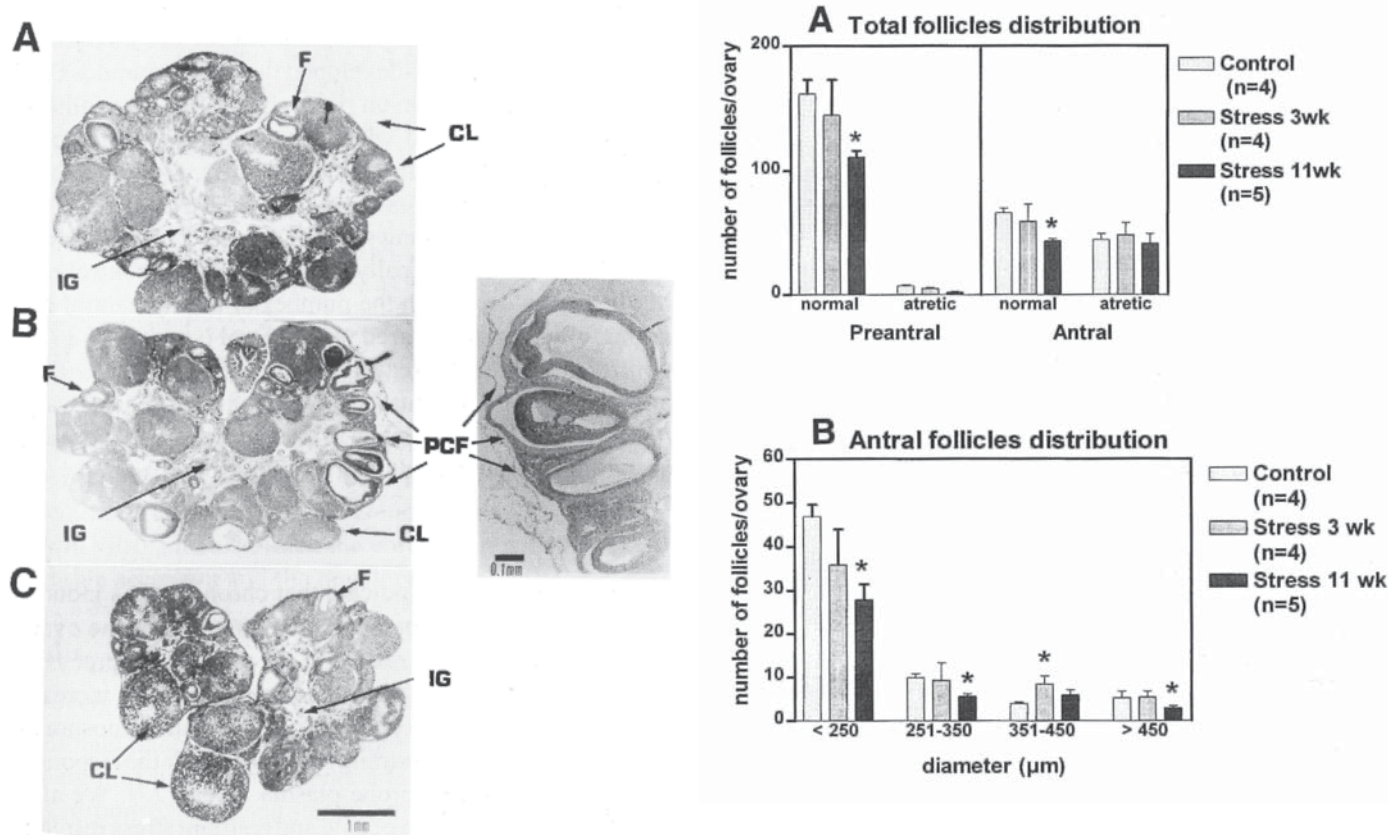
Fig. 4. *(left)* Changes in normal ovarian histology after 3 or 11 wk of stress. **(A)** Ovary from a rat in the diestrus phase of the cycle; **(B)** ovary after 3 wk of stress; **(C)** ovary after 11 wk of stress. Notice the appearance of precystic follicles (PCF) after 3 wk of stress. F, normal follicle; CL, corpus luteum; IG, interstitial gland. The section are 8-μm thick and stained with hematoxylin and eosin (magnification, ×24; insert of Fig. 4B ×100).

Fig. 5. *(right)* Changes in the distribution of preantral and antral follicles after 3 and 11 wk of stress. In **A** is shown the number of preantral, antral, and atretic follicles per ovary and **B** represents the morphometric analysis for antral follicles. Each bar represents the mean value ± SEM for the number of experiments shown in parentheses. *$p < 0.05$ vs control.

vical ganglia after restraint stress *(18)*. The local effect of the stress was clearly shown by the increased NE release from the ovary. We cannot discard, however, that the increase in NE release could be driven by hormones that change during stress, such as LH and prolactin. Although we have previously shown that NE release from the ovary is stimulated by luteinizing hormone (LH) *(19)*, the increase in NE release could not be attributed to LH, because in preliminary observations, we have found a decrease in LH plasma levels after 3 wk of stress. We do not have information of a local effect on NE release of prolactin. The absence of a decrease in ovarian NE content after 3 wk of stress in spite of the stimulated release could be a consequence of either an increased supply of NE from the ganglion to the ovary or an efficient reuptake of NE. Three lines of evidence support the first possibility:

1. There was a generalized increase in NE synthesis at the celiac ganglion.
2. There was no change in the amount of [^{3}H]NE incorporated by the ovary.
3. There was a decrease in the amount of NPY in the ovary at the same time (NPY has no reuptake mechanism *[20]*), and its synthesis is exclusively located in the neuronal body in the celiac ganglion).

The effect on ovarian function of exposure to 3 wk of stress was not maintained for 11 wk. Although the rates of synthesis of NE and NPY in both celiac ganglion and ovary remained high, nerve terminals were less sensitive to electrical stimulation of NE release in vitro. In addition to a generalized increase in sympathetic tone (evidenced by the increased catecholamine content in the adrenal gland, the celiac ganglion, and the sympathetic innervation of the ovary), we suggest that there is a local mechanism decreasing the availability of NE for release and acting postsynaptically (as seen from the ability of isoproterenol to induce androgen secretion). It is possible that there is an adaptive mechanism involving factors that affect sympathetic nerve release of NE by 11 wk of stress. One of this factors could be a negative regulation of NE release effected by the increased levels of NPY found at 11 wk of stress. NPY is coreleased with NE from the nerve terminals of the ovary when the firing rate of the neurons is increased *(15)*. The other factor could be corticoids secreted from the adrenal during cold and restraint stress *(13,21)* that could decrease sympathetic nerve activity *(22,23)*.

The increase in NE secretion found at 3 wk of stress occurred simultaneously with an abnormal estrous cycle, and an increase in the basal and isoproterenol-induced androgen release from the ovary. Although estradiol release from the ovary is not stimulated by adrenergic agonists *(24–26)*, the increase in basal release may indicate an increased supply of androgen precursor. This is supported by the morphological observation of precystic follicles of the type III previously described *(16)*. This fol-

licle has a well-developed oocyte, a healthy, but irregularly arranged theca cell layer, multilayered granulosa cell, and therefore, an increased capacity to secrete androgens and estradiol. Many of these characteristics have been also developed in the ovaries of the rats after 3 wk of stress. The correlation among:

1. Disruption of the ovarian function;
2. Increase in sympathetic activity;
3. Morphological changes in the ovary, such as the increased size of the interstitial gland (a target for NE secretory activity; *17*); and
4. The appearance of precystic follicles (of small diameter as the ones described in ref. *16*) supports a role for sympathetic nerves in the development of cystic follicles.

Thus, disruption of ovulation appears to be the consequence of a poorly controlled ovarian function under increased sympathetic nerve activity. It is interesting to mention that the increase in NE release by 3 wk of stress correlates with ovarian cyst formation, and the decrease in the release found at 11 wk of stress correlates with a recovery of the ovarian function to control level. Although after 11 wk of stress there was no formation of precystic follicles and the steroid secretion induced by hCG and isoproterenol from the ovary was similar to control, some changes in follicular development appeared. The decrease in the number of preantral and antral follicles without change in the number of atretic follicles could represents an increase in the rate of follicular formation without accumulation at a different stage of development (with the exception of precystic follicles that occurs after 3 wk of stress). In support of this suggestion we have previously found that immunosympathectomy by the administration of nerve growth factor antibodies to neonatal rats that completely blocks the development of sympathetic nerves leads to accumulation of small antral and preantral follicles *(35)*. Thus, overstimulation (by stress) could produce an increase in the rate of development of follicles with the exception of precystic follicles that accumulate and could originate type III follicles and/or cysts. The formation of precystic follicles at 3 wk of stress could be the result of an increased expression of an unknown ovarian growth factor induced by stress or adrenergic stimulation.

In conclusion, our results show that in addition to the well-known effect of EV administration *(7)*, exposure to stress can represent another etiological factor in the genesis of PCO, increased noradrenergic ovarian activity being a conspicuous characteristic underlying this phenomenon.

Experimental Procedures

Animals

Virgin adult cycling Sprague-Dawley rats (200–220 g), derived from a stock maintained at the University of Chile, were used. They were allowed free access to pelleted food and tap water, and were housed (2 rats/cage) in quarters

with controlled temperature (22°C) and photoperiod (lights on from 0700 to 1900 h). Only animals exhibiting regular 4-d estrous cycles were used for the experiments. Estrous cyclicity was monitored by daily vaginal smears obtained between 1000 and 1200 h. We used two experimental groups (for 3 and 11 wk of stress), of 20 rats each. In each group, half served as controls but the others were exposed to a combined cold and restraint stress procedure. Both types of stress produced increased activation of the sympathoadrenal system *(13,14,27,28)*. Rats were placed in a restriction cage (a tunnel of stainless-steel wire, 10 cm wide, 6 cm high, 30 cm long) and were kept in a cold room at 4°C for 3 h for 5 d each week (Monday to Friday). The procedure was continued for 3 or 11 wk. Previous observations in our laboratory showed that under this stress schedule, rats undergo a first phase (lasting up to the 3rd wk of stress) in which they lose their capacity to ovulate in a cyclic manner—as shown by the irregularity to reach estrus after proestrus—and a second phase (up to 11 wk) characterized by a recovery in cyclic activity. Control animals were used during the diestrus phase of the cycle. After rats were killed by decapitation, ovaries, adrenal glands, and celiac ganglia were rapidly removed. Ovaries for measurement of norepinephrine release or steroid secretion were immediately transferred to Krebs-bicarbonate buffer for preincubation. Adrenal glands and celiac ganglia were frozen at –80°C.

Release of NE

The experimental procedure was as previously described *(14,15,19)*. Ovaries, removed through an abdominal midline incision, were cut in half. Two halves, one from each ovary, were stored together at –80°C for NE determination. The other two halves were preincubated for 20 min in Krebs-bicarbonate buffer, pH 7.4, and incubated for 30 min at 37°C with 2 µCi of [^{3}H]NE (SA 40.1 Ci/mmol, New England Nuclear, Boston, MA). After washing (to remove any radioactivity not incorporated), the two halves were transferred to a thermoregulated superfusion chamber and perifused at a flow rate of 2.5 mL/min. One-minute fractions were collected; after 3 min, the ovaries were subjected to a train of monophasic electrical pulses (80 V, 10 Hz, 2 ms, 1 min). After the stimulation protocol was finished, four 1-min sample were collected. For details, *see* ref. *(8)*. At the end of the experiment, the halves were homogenized in 0.4 M HClO$_4$, the homogenate was centrifuged (15,000g, 10 min), and [^{3}H]NE remaining in the tissue was determined by scintillation counting. Overflow of radioactivity was calculated as a percentage of the fractional release *(15,19)*.

Steroid Response to β-Adrenergic and/or Gonadotropin Stimulation

Ovaries from control and stressed rats were halved. Halves were incubated in vitro in 2 mL Krebs-Ringer

bicarbonate buffer, pH 7.4, for 3 h at 37°C *(24,25,29)* with D,L-isoproterenol-HCl (10^{-5} M; Sigma Chemical Co., St. Louis, MO), hCG (2.5 IU; Sigma), or without drug (basal). The experimental design was such that three ovarian halves were used simultaneously. Progesterone, androgen, and estradiol released into the incubation medium were measured by radioimmunoassay *(24,29)*. The values for testosterone were reported as androgen, because the antiserum used crossreacts with 5-dihydrotestosterone *(30)*.

Determination of NE, Protein, and Total Adrenal Catecholamines

The remaining ovarian halves from the NE release experiments and celiac ganglia were homogenized in 0.2 M HClO$_4$. The suspensions were centrifuged (15,000g, 10 min) and catecholamines present in the supernatant were determined by a specific radioenzymatic method *(31)* as previously described *(15,19)*. Pellets were dissolved in 1 M NaOH, and protein content was determined *(32)* with BSA as standard. Owing to the high amount of catecholamines present in the adrenal gland, the supernatants homogenized in the same way as ovaries were used for colorimetric determination of total catecholamines *(33)*. This method measures NE and epinephrine as a whole by the formation of noradrenochrome and adrenochrome when the sample is oxidized with iodine at pH higher than 6.0.

NPY Determination

Ovaries were homogenized in 0.1 M acetic acid, boiled at 100°C for 10 min and centrifuged (15,000g, 10 min). NPY was measured in the supernatants by radioimmunoassay *(34)*.

Histology

Ovaries were cleaned of adherent fat, fixed in Zamboni's fixative, embedded in paraffin, sectioned at 8 µm, and stained with hematoxylin-eosin. All ovarian structures were analyzed every fifth section as previously described *(35)*. Antral follicles were classified by size; cystic follicles were defined as the population of follicles devoid of oocyte with a well-developed theca cell layer and a very thin granulosa cell compartment (mostly monolayer). Precystic follicles, similar to the type III follicles described in ref. *16*, were defined as big follicles with healthy oocytes, a well-developed theca cell layer, and thick granulosa cell layer. Atretic follicles were classified by the appearance of more than 5% pyknotic cells in the largest cross-section, shrinkage of oocyte, and sometimes with breakdown of germinal vesicle. For the morphometric analysis, we considered the maximal diameter of follicles in which the oocyte was present.

Statistics

Differences between control and experimental groups were analyzed with Student's *t*-test. Data were normalized by arc-sine transformation before statistical evaluation when percentages were compared. Comparisons between

several groups were made by one-way analysis of variance, followed by the Student-Newman-Keuls multiple-comparison test for unequal replications *(36)*.

Acknowledgments

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