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# Role of Stress and Sympathetic Innervation in the Development of Polycystic Ovary Syndrome

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**This article presents a review of the role of the sympathetic activity in ovarian pathologies affecting reproductive function. We provide a succinct outline of the findings of our group in this area. The participation of stress as an etiological factor for ovarian pathologies throughout animal models and data in patients with polycystic ovary syndrome give strong support for participation of sympathetic nerves in the ovary function both in normal and pathological status.**

**Key Words:** Polycystic ovary; innervation; stress; neurotrophic factors.

## Ovarian Distribution of Sympathetic Nerves

Postganglionic sympathetic fibers innervating the ovary derive from neuronal cell bodies of the ovarian ganglion, which is located at the origin of the ovarian artery and from cell bodies of the celiac and renal plexuses *(1,2)*. In the rat, the ovary receives its sympathetic innervation from two sources: (a) the ovarian plexus nerve, which travels along the ovarian artery, and (b) the superior ovarian nerve, which is associated with the suspensory ligament *(3)*. In general, the superior ovarian nerve fibers innervate preponderantly the secretory components of the ovary, i.e., interstitial glands and follicles, whereas the plexus nerve fibers are mostly perivascular *(3)*.

The intraovarian distribution of sympathetic fibers is similar in all species but the density of the network varies considerably among them *(2)*. Importantly, the fibers are associated with the vasculature, travel along the interstitial tissue, and surround developing follicles, but penetrate neither the corpus luteum nor the granulosa cell layer of follicles.

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## Nature of Catecholamine Present in the Ovary

Morphological studies, in which ovarian sympathetic nerves were visualized by histofluorescence methods, suggested that the fibers were mainly noradrenergic *(4,5)*. This notion was later confirmed by selective biochemical measurement of ovarian catecholamines *(6)*, a study which also showed that epinephrine and dopamine, although detectable, constitute a minor fraction of ovarian catecholamines. However, some differences exist between species; there are high levels of dopamine found in the human ovary *(7)*. A possible physiological role of ovarian dopamine needs to be clarified because of the presence of D1-dopaminergic receptors, and the dopamine-specific vesicular transporter in the human and monkey ovaries *(8–10)*.

## Role of Ovarian Sympathetic Nerves in Ovarian Physiology

The extrinsic innervation of the gland has been shown to be involved in the regulation of ovary specific functions, such as steroidogenesis and early follicular development *(11–14)*. This regulation is exerted mainly by the norepinephrine (NE) and vasoactive intestinal peptide (VIP). These neurotransmitters may facilitate the follicular development, as seen by the inhibition of follicular growth following the ovarian denervation *(2,15,16)*.

The ovary's sympathetic innervation, however, rapidly recovered after the organ's transplantation to an ectopic site *(17)*. More recently, we found that the rat ovarian penetration of nerve fibers is accompanied by a complete biochemical biosynthetic system as ovarian norepinephrine (NE) content returns to control values 28 d after surgical denervation of the superior ovarian nerve *(18)*. The plasticity of the ovarian innervation is probably due to the trophic support exerted by abundant amounts of nerve growth factor (NGF) and its receptors present within the gland *(19,20)*. The presence of NGF—in addition to the previously demonstrated presence of an intraovarian source of neuron-like catecholaminergic cells *(21–24)*—could stimulate the formation of a network of neuronal cells whose activity could be trans-regulated in a neurotrophic-dependent mechanism and participates in the control of the neuronal-dependent

functions of the ovary. To test this possibility we recently studied the relative contribution of the intraovarian putative source of NE by measuring the changes in locally produced mRNA for tyrosine hydroxylase (TH) (marker for catecholaminergic neurons). After surgical denervation of the superior ovarian nerve to isolate the ovary from the external sympathetic control, NE concentration decreased in the ovary (8.5 ± 1.3 ng/ovary for control vs 3.1 ± 1.1 for denervated rats; $n = 11$ for control and 6 for denervated rats, mean ± SEM, $p < 0.05$), indicating the effectiveness of the surgical procedure. This condition was followed with a 13-fold increase in the expression of TH mRNA as determined by real-time PCR technique (Fig. 1A). Probably the expression of the enzyme is negatively controlled by the activity of the sympathetic nerves traveling via the superior ovarian nerve (trans-synaptic regulation), because when we activated the sympathetic nerves by the administration of estradiol valerate to produce the polycystic ovary condition (see below), the amount of mRNA for TH did not increase over control. More interesting, denervation of the superior ovarian nerve in rats treated with estradiol valerate did not increase the concentration of the TH mRNA as denervation of control rats did. It is highly probably that once the hyperactivation of sympathetic nerves is initiated, the increase in the concentration of NGF or its availability to the nerve cells (through the p75 neurotrophic receptor, p75NTR) stimulates the ovary to differentiate neurons *(18)* that can maintain their condition of activity independent of the external neural supply. As we previously found *(19)*, denervation did not change the mRNA for NGF, but it did when the procedure was performed in rats previously treated with estradiol valerate (Fig. 1B). A constant increase was found, however, in the amount of p75 NTR. This increase was manifested both by denervation and by the treatment with estradiol valerate (Fig. 1C). Because the p75 NTR is involved in the internalization of NGF into neurons, these last data could be interpreted as an increased availability of the NGF to the neuronal cells.

Thus, tonic changes (decrease or increase) in the sympathetic input to the ovary, provoked profound changes in the intraovarian neuron-to-neuron communication. All these changes agreed with the concept that the intraovarian catecholaminergic neurons present a trans-synaptic control exerted by the extrinsic sympathetic nerves of the ovary. Thus, we can suggest that one of the ways used by the ovary to maintain the sympathetic activity is a finely regulated neurotrophic-dependent control of the expression of the rate-limiting enzyme of the biosynthesis of catecholamines.

## Nerve Activation During Development of Polycystic Ovary

Polycystic ovary (PCO) syndrome is widely recognized as the most common cause of infertility in women during their reproductive years. PCO syndrome is a complex disease

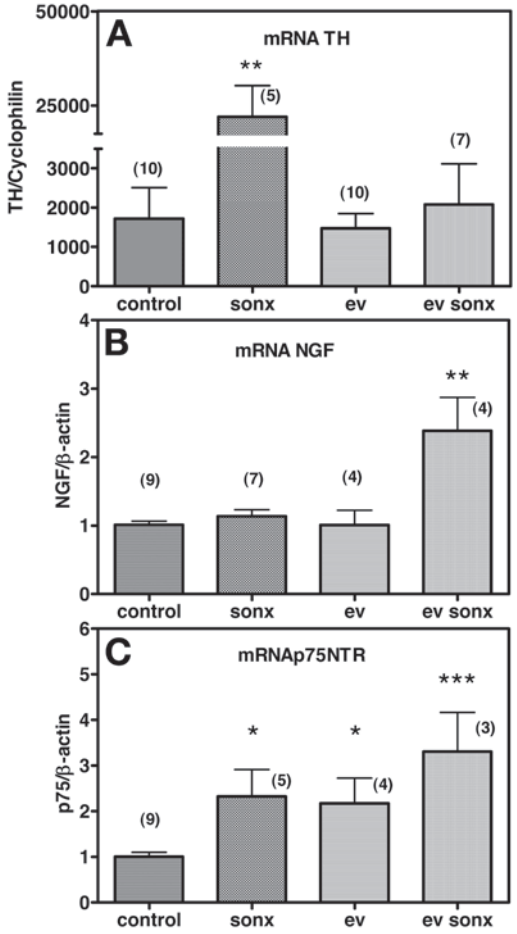


**Fig. 1.** Effects of transection of the superior ovarian nerve (SONX) on ovarian tyrosine hydroxylase (TH), nerve growth factor (NGF), and p75neurotrophic receptor (p75NTR) mRNA. (**A**) shows the changes in TH mRNA (determined by real time RT-PCR) after surgical section of the superior ovarian nerve. EV: rats treated with estradiol valerate for 60 d and EV SONX indicates rats treated with EV 60 d before and denervated during 12 d. (**B** and **C**) represent the values obtained for NGF and p75NTR mRNA in the same samples. Total RNA was isolated by the method of Chomczynski and Sacchi. PCR for TH and cyclophilin (as internal marker) was performed in a LightCycler apparatus (Roche), and products were detected with SYBR green. TH primers: forward 5' GGTCTACTGTCCGCCCGTGATT 3' and reverse 5' GAGCTTGTCCTTGGCGTCATTG 3'. Cyclophilin, primers sequences were obtained from ref. *20*. The amplification program was as follows: *Taq* Polymerase activation (95°C for 10 min), and a for TH: 45 cycles were run with 10 s denaturation at 95°C, 10 s annealing at 60°C, and 25 s extension at 72°C; b for cyclophilin: 40 cycles were run with 10 s denaturation at 95°C, 10 s annealing at 64°C, and 25 s extension at 72°C. The double-stranded PCR product was measured once every cycle immediately after the 72°C incubation (extension step) by detection of fluorescence associated with the binding of SYBR green I to the amplification product. Fluorescence curves were analyzed with the LightCycler software, version 3.5 (Roche Diagnostic). Amplification for NGF, p75NTR and β-actin was done as previously described *(30)*. Results represent the mean value ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ of the number of experiments shown in each bar.

characterized by anovulatory failure and the presence of ovarian cyst, amenorrhea, hyperandrogenemia, and variable levels of circulating gonadotropins *(25)*. We have obtained evidence that a hyperactivation of sympathetic nerves, arriving at the ovary, participates in the development and maintenance of polycystic ovary in the rat. We found *(26)* that PCO induced by the administration of a single dose of estradiol valerate to rats results in profound changes in ovarian catecholamine homeostasis, which were initiated before the development of cyst and persisted after cysts were formed. These changes include increased ovarian NE content, enhanced NE release from ovarian nerve terminals, and downregulation of β-adrenoceptors in theca-interstitial cells, the ovarian compartment directly innervated by sympathetic nerves. The increase in NE release found in PCO rats within 30 d of estradiol administration is even more pronounced after 60 d, i.e., when ovarian cysts are fully developed. The increased activity of sympathetic nerves during development of PCO in rats is accompanied with a striking enhanced ovarian steroidal responsiveness to both β-adrenoceptor and gonadotropin stimulation, and this abnormal response can be prevented by selectively ablating the neural input to endocrine cells of the ovary *(27)*. It is noteworthy that progesterone and androgen secretory response of cystic ovaries to isoproterenol were enhanced in the face of reduced β-adrenoceptor content. A similar paradox was noted when studying the progesterone response to zinterol, a β-adrenoceptor agonist during the first proestrus at puberty *(14)* and the progesterone response to isoproterenol during adult estrous rats *(27)*. In both cases the β-adrenoceptor content was reduced, but the activation of the remaining receptors resulted in a much greater stimulation of progesterone secretion than in other phases of the cycle. Probably coupling of β-adrenoceptors to adenylyl cyclase is increased during PCO. The constitutive activation of adenylyl cyclase in McCune-Albright syndrome also produces an increased ovarian steroid output and unilateral formation of follicular cysts *(28)*. The activation of noradrenergic outflow to the ovary observed in animals with PCO suggests that an abnormally heightened sympathetic tone to the gland underlies the steroidal hyperresponsiveness of PCO. The restoration of estrous cyclicity and ovulation resulting from ablation of the superior ovarian nerve *(27)*, which carries the bulk of the sympathetic innervation to ovarian endocrine cells *(3)*, further implies a neural abnormality in the maintenance of PCO condition.

Although these data give strong support for the involvement of the nervous system in the development and maintenance of PCO, they do not provide, however, evidence for (a) whether the overdose of estradiol was the primary stimulus that initiates the syndrome and nerve activation was only a correlation between multiple changes induced by estradiol in the ovary or (b) the nerve activation is causally related to the pathology by triggering the cyst development. The activation of ovarian sympathetic nerves is driven by an increase of the intraovarian expression of the genes encoding NGF, and its low affinity receptor, p75 NTR receptor with no changes in trkA NGF receptor. Immunoblockade of NGF actions and intraovarian administration of an antisense oligodeoxynucleotide to p75NTR, partially restored estrous cyclicity and ovulatory capacity decreased during PCO induction to rats *(20)*. On the other hand, intraovarian grafting of genetically modified cells to overexpress the gene for NGF, increased the incidence of precystic follicles accompanied by a reduced number of healthy antral follicles. Therefore, an abnormally elevated production of NGF suffices to initiate many of the structural and functional alterations associated with the development of ovarian cysts *(29)*.

## Effect of Stress and β-Adrenergic System on the Formation of Ovarian Cyst

To discriminate a direct effect of estradiol as a primary etiologic factor in cyst formation from an intraovarian increase of NGF preceding the formation of cyst, we have recently studied the effect of a chronic intermittent cold stress procedure on sympathetic nerve activation and its effect on ovarian function *(30)*. Because kinetics studies using mouse, hamster, and rat have shown that the large preovulatory follicles rupturing in response to LH surge actually enter the growing pool of follicles around 20 d earlier *(31)*, we used a chronic intermittent cold stress during 3 and 4 wk (3 h/d, from Monday to Friday) to be sure that at least one cycle of follicular development was completed. This paradigm did not affect basal corticosterone plasma levels. At 3 wk of stress, we detected a decrease of NE in the ovary, but after 4 wk of stress the ovarian neurotransmitter increased over the values of control unstressed rats. Follicular development was modified during the stress procedure. A decrease in preantral healthy follicles with no atresia was found at 3 and 4 wk of stress. In parallel with the increase in NE content in the ovary at 4 wk of stress, we observed a recovery in antral follicles and the appearance of a new population of follicles presenting a hypertrophied theca cell layer characteristically seen in patients with PCO syndrome *(30)*.

Because the stress response is a multifactorial event that involves complete neuroendocrine responses, we have recently applied a method to directly stimulate β-adrenoceptors by in vivo administration of the β-adrenoceptor agonist isoproterenol *(18)*. We used the administration of isoproterenol (125 μg/kg/d) during 10 d, a procedure previously described to induce cardiac hypertrophy in the rat *(32)*, to study the changes induced by β-adrenoceptor overstimulation in ovarian follicular development. Because of the time needed to complete the follicular development *(31)*, we choose three different times to study: immediately after finishing the treatment and 20 and 30 d after the treatment. After finishing isoproterenol administration, the ovary had

already developed a small number of follicular cysts. The number of cysts was maintained 20 d after the end of the treatment, but a clear increase in the number of cysts was found 30 d after the β-adrenoceptor agonist administration. Probably the mechanism involved in this process is associated with a hyperandrogenic condition evoked by the chronic β-adrenoceptor stimulation induced by isoproterenol, because the ovary of these rats exhibited an increased capacity to secrete androgens when incubated in vitro *(18)*. It is interesting to mention that the improved secretory capacity of androgens of the ovary is only evident until the β-adrenoceptor agonist is present, because the production of androgens by the ovary returned to control levels 20 and 30 d after the treatment. Thus, a chronic β-adrenoceptor agonist–induced increase in androgen production could induce an aberrant follicular development that finally conduced the ovary to develop cysts in a process that is fully expressed after 30 d of isoproterenol administration.

### Implications of Changes in Sympathetic Nerve Activity to Polycystic Ovarian Syndrome in Humans

Polycystic ovary syndrome (PCOS) is characterized by ovulatory disorders, hyperandrogenism, and metabolic abnormalities that are consistent with the metabolic syndrome. The abnormalities detected in PCOS have been attributed to various causes: primary defects in the action and secretion of insulin that lead to hyperinsulinemia and insulin resistance, a neuroendocrine defect with exaggerated luteinizing hormone (LH) pulsatility, a defect of androgen synthesis that enhances ovarian androgen production, or an alteration in the metabolism of cortisol resulting in enhanced adrenal androgen production *(33)*. Enhanced sympathetic and adrenal medullar activities are important links between defects in insulin action and the development of hypertension. Despite extensive research seeking the pathogenesis of PCOS, there is still disagreement on the underlying mechanisms. The potential contribution of the sympathetic nervous system to the syndrome has been suggested in several studies and especially because of the role of NE to enhance androgens and progesterone secretion from the mammalian ovary including human *(7,14)*. Some believe that androgen excess early in life may provide a hormonal "insult" that results in manifestation of PCOS in adulthood *(34–36)*. For instance, polycystic ovarian morphology is highly associated with conditions in which the fetus has been exposed to high amounts of sex steroids before birth *(34)*. Studies of Apter *(35)* have also pointed to a pubertal onset of PCOS. In these studies the oligo-ovulatory adolescents, who are likely to develop hyperandrogenism, exhibit premature maturation of the GnRH–gonadotropin axis, similar to the early onset of puberty recently found to occur in the juvenile estradiol valerate–treated rats *(37)*. The relevance of this observation to pathophysiological studies is supported by our recent study in which we found that mothers with PCOS maintain their hyperandrogenic condition during pregnancy, although the hypothalamic axis has been suppressed *(38)*, thus, if this tonically increased level of androgen reach the placental tissue in which the fetus is developing, the internal milieu can "program" its reproductive axis to be disturbed at the onset of puberty and adulthood. A possibility to consider this is that increased superior ovarian nerve input may contribute toward the etiology of PCOS through a stimulatory action on androgen secretion. This would explain the effectiveness of ovarian wedge resection *(39)* or laparoscopic laser cauterization *(40)* to increase ovulatory response in women with PCOS. Both procedures are likely to disrupt superior ovarian innervation. In the same context, the transient nature of the recovery in ovulatory response in women with PCOS that follow wedge resection or laparoscopic laser cauterization may relate to the reinnervation of the ovary. In addition, the tonic increase in sympathetic activity could be directly related to the changes in the sensitivity of the ovary and other tissue to adrenergic agonist and explain the multiple expression of PCOS as a metabolic disease. Many years ago, Semenova *(41)* was the first to demonstrate that the ovary of patients with PCOS presented an increased fluorescence to catecholamines suggesting an increased activity of the sympathetic nerves. More recently, Heider et al. *(42)* showed that the ovary of PCOS women exhibited an increased number of nerve fiber immunoreactives to tyrosine hydroxylase. Moreover, sympathetic nerve activation, either by stress procedures or in vivo administration of isoproterenol, increased the number of ovarian cysts in the rat *(18)*. Our recent findings *(7)* that biopsies of human ovaries release NE in vitro and this secretion is coupled to steroid production through activation of β-adrenoceptor present in ovarian secretory cells, strongly suggest that a neural-mediated activation of ovarian steroid secretion and intraovarian neurotrophic influences on follicular development could be principal components in the development of the PCOS in humans.

Much evidence supports the view that android obesity and metabolic syndrome are closely related to functional alteration in the hypothalamus–pituitary–adrenal axis associated with chronic stress. Thus, to analyze the relation between chronic stress and the presence of PCOS, we have recently studied the depression and anxiety scores in patients with PCOS applying internationally accepted tests. We studied 18 patients, from the Gynecology and Endocrinology section of the University of Chile Clinical Hospital and from the Hospital San José of the Public Health System. The diagnosis of PCOS was based on clinical and biochemical features (Table 1). Twelve healthy women presenting regular menstrual cycles were studied as the control group. Goldberg's 30-item General Health Questionnaire (GHQ-30) to measure anxiety was significantly higher in patients than in controls ($10.4 \pm 5.1$ vs $4.8 \pm 5.3$, $p < 0.01$). Two PCOS patients, but none of the controls, presented depression ac-

**Table 1**
Clinical and Biochemical Parameters
in Women with PCOS and Normal Controls

| | PCOS | Controls | |
|---|---|---|---|
| Age (yr) | 25.8 ± 5 | 29.6 ± 6.5 | NS |
| BMI (kg/m$^2$) | 34.3 ± 6.2 | 26.6 ± 5.8 | 0.002 |
| Waist (cm) | 98 ± 12 | 76.4 ± 23.6 | 0.008 |
| Ferriman Score | 15.7 ± 4.9 | 8.2 ± 5.3 | 0.003 |
| SHBG (nmol/L) | 22.4 ± 12.2 | 38.9 ± 15.4 | 0.003 |
| Total testosterone (ng/mL) | 67.6 ± 26.8 | 27.4 ± 10.7 | 0.0001 |
| FAI | 8.9 ± 2.3 | 2.03 ± 0.96 | <0.0001 |

BMI: body mass index; FAI : free androgens index. Results are expressed as mean ± SEM of $n = 18$ patients with PCOS and 12 control healthy women; NS: not significant.

**Table 2**
Psychological and Hypothalamus–Pituitary–Adrenal Axis
Evaluation in Women with Polycystic Ovary Syndrome

| | PCOS | Controls | |
|---|---|---|---|
| Goldberg (GHQ-30) | 10.4 ± 5.1 | 4.8 ± 5.3 | $p < 0.05$ |
| Hamilton (HDRS) | 9.2 ± 6.2 | 5.7 ± 4.5 | NS |
| FUC (µg/24 h) | 44.2 ± 19.3 | 49.2 ± 23.3 | NS |
| FUC/g creatinine | 36.2 ± 12.6 | 43.9 ± 21.4 | NS |
| Cortisol 08:00 AM (µg/mL) | 12.4 ± 4.9 | 12.3 ± 5.8 | NS |
| Salivary cortisol 08:00 AM (µg/dL) | 0.8 ± 0.5 | 0.67 ± 0.5 | NS |
| Salivary cortisol 11:00 PM (µg/dL) | 0.12 ± 0.04 | 0.11 ± 0.02 | NS |

FUC: free urinary cortisol. Results are expressed as mean ± SEM of $n = 18$ patients with PCOS and 12 control healthy women; NS: not significant.

cording to the Hamilton Depression Rating Scale (HDRS) (Table 2). The scores for GHQ-30 were similar to the ones obtained by Goldberg in his original study in England *(43)*. The cut-off point used in our study (11/12) was chosen according to the results of the Chilean validation of the Spanish version *(44,45)*. This higher cut-off also considered the emotional expressiveness of Latino American populations *(46)* and the fact that in women higher cutting points seem to have a better discriminating capacity *(47)*.

The effects of chronic stress on the hypothalamus–pituitary–adrenal axis have previously been described as lost of circadian secretion of cortisol with higher night cortisol levels compared with low morning cortisol levels and increased post prandial peak *(48,49)*. We did not find differences in salivary and plasma levels of cortisol at 08:00 AM and, in salivary cortisol at 11:00 PM compared with controls, but, analyzing the 24 h free urinary cortisol (FUC) corrected by creatinine, as a measure of daily cortisol secretion, the proportion of patients with lower values was significantly

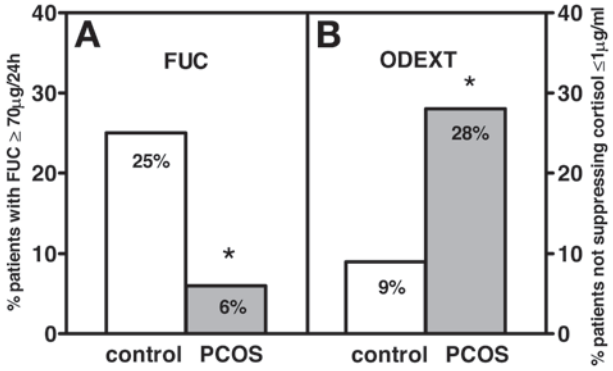


**Fig. 2.** Increased levels of stress in patients with PCOS. (**A**) shows the percentages of patients with values of free urinary cortisol (FUC) lower than controls. (**B**) demonstrates the percentages of patients unable to suppress cortisol at levels below 1 µg/mL as compared to controls after a test of an overnight 1 mg dose of Dexametasone (ODEXT). Graphic correspond to the results found after the $\chi^2$ test used to analyze differences in the different groups of patients followed by a Fisher test to a number of 18 patients with PCOS and 12 controls. *$p < 0.05$.

higher than in controls (25% vs 6%) (Fig. 2A). We also found that, according to the actual discriminating cut point between Cushing syndrome and normal subjects, all patients and controls, suppressed below 1.8 µg/mL the morning plasma cortisol levels after an overnight 1 mg dexamethasone test. Nevertheless, we found a significant increase in the population of PCOS patients showing incapacity to suppress plasma levels of cortisol below 1 µg/mL as control patients did (Fig. 2B).

The higher level of stress not associated with depression in patients with PCOS, in addition to the lack of capacity to suppress the effect of dexamethasone and the increase of PCOS patients that secrete less urinary cortisol, strongly suggest that patients with PCOS presented a higher level of stress that could be causally related to an increased sympathetic tone.

## Concluding Remarks

The variety of evidence presented in this article coming from biochemical, physiological, and functional studies in animal models and in humans, strongly suggests that chronic increase in ovarian sympathetic nerve activity is related to changes in follicular development, producing non-cyclic anovulatory ovary that develops cysts. The process seems to be reversible if the sympathetic activity is attenuated. These data could offer new alternatives to treat the PCO syndrome by using methods to attenuate the sympathetic activity or the expression of neurotrophic factors as has been published by Stener-Victorin et al. *(50)* with acupuncture methods to treat women with PCOS. Their studies were based on the acupuncture-dependent decrease of the high levels of NGF that accompanies follicular cyst development in the rat treated with estradiol valerate *(51)*. On the other hand, using β-adrenoreceptor antagonists for patients

resistant to the standard procedure of clomiphene citrate treatment could provide a inexpensive means to reinstate ovulatory cycles in PCO syndrome–affected women. The confirmation in our population of the positive correlation between anxiety and the incidence in PCOS, reinforces the concept originally described by Gerendai et al. *(52)*, that the ovary presents direct neural communication with the hypothalamus. Chronic activation of this pathway could affect ovary function, warning of the importance to control stress in these patients to decrease the impact in the maintenance of PCOS and its well-recognized metabolic complications associated with the disease.

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