Effect of Exercise on Ovarian Morphology and Expression of Nerve Growth Factor and $\alpha_1$- and $\beta_2$-Adrenergic Receptors in Rats with Steroid-Induced Polycystic Ovaries

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Abstract

Oestadiol valerate (EV)-induced polycystic ovaries (PCO) in rats cause anovulation and cystic ovarian morphology. Denervation of ovarian sympathetic nerves restores ovulatory disruption. In the present study, we determined whether 5 weeks of voluntary exercise influence ovarian morphology and the expression of sympathetic markers in the EV-induced PCO rat model. The effect of exercise on (i) ovarian morphology; (ii) mRNA and protein expression of nerve growth factor (NGF); and (iii) mRNA and number of ovarian-expressing cells for the NGF receptor (p75 neurotrophin receptor) and the $\alpha_1a$-, $\alpha_1b$-, $\alpha_1d$- and $\beta_2$-adrenergic receptors (ARs) in rats with EV-induced PCO was evaluated. PCO was induced by a single i.m. injection of EV, and controls were injected with oil alone in adult cycling rats. The rats were divided into four groups: (i) control (oil); (ii) exercise group (oil + exercise); (iii) a PCO group (EV); and (iv) a PCO exercise group (EV + exercise). The exercise and PCO exercise groups ran voluntarily for 5 weeks in computer-monitored wheels placed in the cages where they were housed. The results obtained indicated that ovarian morphology was almost normalised in the PCO exercise group; NGF mRNA and protein concentrations were normalised in the PCO exercise group; high numbers of NGF receptor expressing cells in PCO ovaries were lowered by exercise; and the number of immunopositive cells of the different AR subtypes were all reduced after exercise in the PCO group, except for the $\alpha_1b$- and $\beta_2$-AR whereas the mRNA levels were unaffected, indicating transcriptional regulation. In conclusion, our data indicate a beneficial effect of regular exercise, as a modulator of ovarian sympathetic innervation, in the prevention and treatment of human PCOS.

Polycystic ovary syndrome (PCOS), the most common endocrinopathy in women of reproductive age, is a multifaceted metabolic disease associated with insulin resistance (1). The syndrome is characterised by hyperandrogenism, obesity, anovulation, hyperinsulinaemia and a higher risk of developing cardiovascular disease (1).

The abnormalities detected in PCOS have been attributed to various causes, including: (i) primary defects in the action and secretion of insulin that lead to hyperinsulinaemia and insulin resistance; (ii) a neuroendocrine defect with exaggerated luteinising hormone (LH) pulsatility; (iii) a defect of androgen synthesis that enhances ovarian androgen production, or (iv) an alteration in the metabolism of cortisol resulting in enhanced adrenal androgen production (1). Enhanced sympathetic and adrenal medullary activity are important links between defects in insulin action and the development of hypertension. Despite extensive research on 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

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suggested in several studies (2, 3). To further advance our understanding of the PCOS condition, a concerted action involving different techniques is required.

Several mechanistic and treatment studies, conducted by ourselves and others, have used an oestadiol valerate (EV)-induced rat polycystic ovary (PCO) model (4–12). PCO is induced by a single i.m. injection of EV, which leads to an anovulatory state in 13–15-week-old rats. These rats share some characteristics of human PCOS, such as the presence of cystic follicles in the ovaries and altered concentrations of LH (4). We have recently shown that rats with EV-induced PCO develop hypertension and increased sympathetic and hypothalamic-pituitary-adrenal (HPA) axis activity in line with human PCOS, but that their insulin sensitivity is unaffected and they do not develop obesity or hyperandrogenism (13). Increased sympathetic tone in the ovaries of rats with EV-induced PCO has been indicated by an increase in the enzyme activity of tyrosine hydroxylase, an increase in the concentration of norepinephrine, and a down-regulation of β2-adrenergic receptors (ARs) (5, 7, 8). The restoration of oestrous cyclicity and ovulatory capacity after transection of the sympathetic efference to the ovary (i.e. the superior ovarian nerve) suggests the involvement of the sympathetic nervous system in the development of EV-induced PCO (5). The ovarian sympathetic nerves in rats with EV-induced PCO are also associated with an increase in the production of ovarian nerve growth factor (NGF) (8), which is a target-derived neurotrophin, and its low-affinity receptor (p75 NTR) mRNA (8). Intra-ovarian blockade of NGF action restores oestrus cyclicity in EV-induced PCO in rats (8), suggesting that sympathetic input in PCO might be related to an overproduction of NGF.

In a recent study, we found that the expression of α1-AR subtypes as well as β2-AR, in the ovaries of PCO rats significantly differs from that of controls and varies at different time points after EV injection (14), which is a further indication of an imbalance in the sympathetic tone of the ovaries.

Treatment of human PCOS is symptomatic, but lifestyle measures such as diet and exercise could play an important role. Recently, regular exercise was shown to significantly lower plasma homocystein in young overweight or obese women with PCOS (15). Accumulating evidence suggests that voluntary physical exercise has many positive effects on the cardiovascular system (16, 17), on the glucose transport in skeletal muscle independent of insulin, and on the insulin sensitivity of the glucose transport process (18). Furthermore, long-term voluntary exercise results in complex, adaptive changes at various levels within the HPA, as well as in the sympathetic and adrenal axis (19), and in a modulation of the immune system (20). Exercise has also been shown to affect the endogenous opioid system (21) and to act as a strong inducer of hippocampal cell proliferation in rats (22).

In the present study, we tested the hypothesis that 5 weeks of voluntary exercise inhibits the development of EV-induced PCO. This was carried out by studying the effect of voluntary exercise on: (i) ovarian morphology, mRNA expression, protein amount and the distribution of ovarian α1a-, α1b-, α1d- and β2-ARs; (i) NGF; and (iii) p75NTR in rats with steroid-induced PCO.

### Materials and methods

#### Animals

Twenty-eight virgin adult cycling Wistar-Kyoto rats (Møllegaard, Ejby, Denmark), weighing 192–210 g, were divided into four groups. They were housed one in each cage at a controlled temperature of 22 °C with a 12 : 12 h light: dark cycle for at least 1 week before and throughout the experimental periods. The rats had free access to pelleted food and tap water and were divided into four experimental groups: (i) an oil group (control, n = 7); (ii) an oil group (exercise, n = 7); (iii) a PCO group (PCO, n = 7); and (iv) a PCO exercise group (PCO exercise, n = 7). Fourteen rats (i.e. those in the PCO groups) were each given a single i.m. injection of 4 mg EV (Riedel-de-Haen, Germany) in 0.2-ml oil to induce well-defined PCO (9). Fourteen rats (i.e. those in the oil groups) received a single i.m. injection of 0.2-ml oil (arachidic oleum; Apoteket AB, Umeå, Sweden) only. On day 35 after the i.m. injection of EV, the rats were killed by decapitation. Before decapitation, the rats were anaesthetised with an i.p. injection of a mixture of Ketamin (50 mg/kg; Parke-Davis, Warner Lambert Nordic AB, Solna, Sweden) and Rompun (20 mg/kg; Bayer, Bayer AG, Leverkusen, Germany). The experiments were carried out according to the principles and procedures outlined in the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the local animal ethics committee at Göteborg University, Göteborg, Sweden.

#### Chronic voluntary exercise

A wheel (22.5 cm in diameter), to which the rat had free access, was attached to one side of each exercise group cage. Wheel revolutions were automatically registered with customised computer software. The equipment and the voluntary exercise procedure is described in detail by Shyu et al. (23). Previously, we found that 3–4 weeks of exercise is needed for the animals to reach their maximum running activity (24). Hence, to analyse the effects of voluntary exercise in rats with steroid-induced PCO compared to control animals, the rats were allowed to run for 5 weeks, starting 1 day after EV injection. The running wheels were locked 24 h before the end of the experiment to avoid any possible effects of acute bouts of exercise.

#### Tissues

The rats were decapitated on day 35 after EV injection, independent of cycle day, and the ovaries were removed and cleaned of adherent connective and fat tissues. One ovary was divided into two pieces, weighed, snap frozen in liquid nitrogen, and stored at −80 °C until extraction. The second ovary was weighed and fixed in buffered 4% formaldehyde for at least 24 h. Thereafter, the samples were dehydrated and imbedded in paraffin to await morphological analyses by one author (S.C.) and immunohistochemistry analyses by another author (L.M.).

#### Ovarian morphology

The ovaries were longitudinally and serially sectioned, with a thickness of 4 µm; every tenth section (six sections from each ovary) was mounted on the glass slide and stained with haematoxylin and eosin. The sections were analysed under a conventional birefringence microscope by one of the authors (S.C.) who was blinded to the origin of the sections. Corpora lutea and growing or atretic follicles were not quantified. To observe whether the young corpora lutea in the PCO exercise group had ovulated, all remaining ovarian tissue in this group was serially sectioned and every fifth section (4-µm thick) was taken for testing. Only corpora lutea that were completely cut from one side to the other were included.

The intention of the microscopic evaluation was to establish whether ovulation with corpora lutea formation had occurred during the 5 weeks of the experiment. According to morphometric (stereological) and statistical principles, a statistical analysis is unnecessary in this situation.

#### Real-time polymerase chain reaction for α1- and β2-adrenergic receptors

Total RNA from the ovary was extracted using RNAeasy Mini kits (Qiagen, Hilden, Germany). The polymerase chain reaction (PCR) analysis was performed using the ABI Prism 7700 Sequence Detection System (PE Applied
BioSystems, Stockholm, Sweden) and FAM-labelled probes specific for α1c-AR (NM 017191), α1d-AR (NM 16991), α2a-AR (NM 024483) and β2-AR (NM 024202) (PE Applied Biosystems). Designed primers and a VIC-labelled probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (NM 031144) were included in the reactions as an internal standard. cDNA Amplification conditions were: one cycle at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. The amount of mRNA of each gene was calculated using the standard curve method (following the instructions in User Bulletin no. 2; PE Applied Biosystems) and adjusted for the expression of GAPDH.

Reverse transcriptase-PCR-ELISA for NGF and p75NTR

The expression of NGF and p75NTR mRNA was evaluated using the reverse transcriptase-PCR (RT-PCR) enzyme-linked immunosorbent assay (ELISA) protocol, exactly as described by Tirasia et al. (25). Total RNA was extracted from the ovaries using the method from Chomzinsky and Sacchi (26), as modified in the TRIzol Kit (Invitrogen AB, Lidingö, Sweden). Complementary DNA was synthesised from 1 μg of total RNA using 250 ng Oligo (dT)17 primer and 200 units of M-MLV RT (Promega Italia, Milan, Italy) in 20 μl of total volume reaction. NGF, p75NTR and GAPDH genes were amplified in a single-tube PCR reaction (35 cycles: 1 min at 95 °C; 1 min at 55 °C; 2 min at 72 °C) using 5' biotinylated specific primers to generate biotinylated PCR products detectable by digoxigenin-labelled probes in an immuno-enzymatic assay. Primer/probe sequences were: NGF biotinylated forward: 5'-CAGGACTCAAAGGAGCAACG-3'; NGF reverse: 5'-GCCCTCCCTGAGCACACA-3'; NGF digoxigenin-labelled probe: 5'-TGATGTCCCGACACACCTGAAGGA-3'; p75NTR biotinylated forward: 5'-CGTGTTCCTCGGCCAGAGCA-3'; p75NTR reverse: 5'-GAGATGCTACTGCTGTTG-3'; p75NTR digoxigenin-labelled probe: 5'-ACACAGCGCAAGGAGCAATAGACAGG-3'; GAPDH biotinylated forward: 5'-CACCACACTGGAGAAGGCC-3'; GAPDH reverse: 5'-GATTGGATCCTTGGCCAGG-3'; and GAPDH digoxigenin-labelled probe: 5'-ACATCTTGTGATGTGTCATATTTCCG-3'.

The amounts of amplified products were measured at an optical density (OD) of 450/690 nm (OD 450/690) using a Microplate Reader 5000 (Dynatech Laboratories, Chantilly, VA, USA). A GAPDH level of OD 450/690 was used to normalise the relative differences in sample size, differences in the integrity of the individual RNA, and variations in RT efficiency. The exact methodological details are provided elsewhere (25).

ELISA for NGF protein quantification

The samples were sonicated in extraction buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM PMSF, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 μM aprotinin, 0.05% sodium azide, 2% bovine serum albumin and 0.5% gelatine; 1 ml/100 mg of tissue) followed by centrifugation at 10 000 g for 30 min. The supernatants were assayed. The bioactive form of NGF in the samples was determined in an immuno-enzymatic assay. Primer/probe sequences were: NGF biotinylated forward: 5'-GATGGATGCCTTGGCCAGG-3'; NGF reverse: 5'-CAGGACTCAAAGGAGCAACG-3'; GAPDH digoxigenin-labelled probe: 5'-GATGGATGCCTTGGCCAGG-3'; and GAPDH reverse: 5'-CACCACACTGGAGAAGGCC-3'. The amounts of amplified products were measured at an optical density (OD) of 450/690 nm (OD 450/690) using a Dynatech ELISA Reader 5000 (Dynatech Laboratories, Chantilly, VA, USA). A GAPDH level of OD 450/690 was used to normalise the relative differences in sample size, differences in the integrity of the individual RNA, and variations in RT efficiency. The exact methodological details are provided elsewhere (25).

Immunohistochemistry for adrenoceptors and p75NTR

Commercially available antibodies were used to detect α1a-AR [C-19]: sc-1477, Santa Cruz, CA, USA], α1c-AR [C-18]: sc-1476, Santa Cruz], α2a-AR [H-142]: sc-10721, Santa Cruz] and β2-AR [H-20]: sc-1570, Santa Cruz] by immunohistochemistry. The monoclonal antibody anti-p75NTR (clone 192) was produced and purified in our laboratory.

Serial, 10-μm thick sections of each ovary were cut with a cryostat and processed for immunohistochemistry. Briefly, sections were incubated at 4 °C with the primary antibody diluted in phosphate-buffered saline (PBS) 0.1 M containing 0.1% Triton X-100. Sections were then incubated with biotinylated-avidin (ArPs) or antismouse (p75NTR) antibodies, diluted in PBS 0.1 M containing 0.1% Triton X-100. Diaminobenzidine was used to detect the immuno-complex. To assess staining specificity, sections were incubated with nonspecific IgG and used as controls. Immunostained sections were evaluated under the Nikon Euphle E600 microscope equipped with the Nikon DMI 1200 digital camera (Nikon, Tokyo, Japan) connected to a computer. Sections were coded, and positive cells were counted in 10 sections per experimental group. Cell count was carried out using the image processing and analysis programme Nikon-Lucia, and measurements were standardised between the experimental groups using the same calibration system and thresholding (see below). The number of immunoreactive cells (mean × SEM per section) was determined in images with a magnification of ×20 by quantifying five nonoverlapping areas of 40 000 μm² per section. Because the image analyser determines the optical density of immunoreaction using a grey scale thresholding operation, measurements were standardised between groups using the following criteria: (i) all measurements were conducted after calibration of the image analysis system with the standard calibration procedure; (ii) thresholding was carried out to the same value for each image; and (iii) the grey scale was calibrated to a range of 25–150 arbitrary units. Objects with higher or lower grey levels were not considered. A morphological programme that selects only cell bodies, but not small fragments or cells that do not have a complete soma, was also used to quantify immunopositive cells.

Statistical analysis

All statistical evaluations were performed using the software package StatView for Macintosh (Abacus Concepts Inc., Berkeley, CA, USA). Running activity and effects of EV injection and/or physical exercise on the weight of the rats were analysed using a repeated ANOVA. The effect of EV injection and/or physical exercise on the weight of the ovaries, mRNA expression, NGF ELISA and immunopositive cell counts of α1c-AR, β2-AR and p75NTR in the ovaries was evaluated using a two-way ANOVA. All differences between groups were tested using the Bonferroni-Dunn post-hoc test. P < 0.0083 was considered statistically significant. The results are reported as means ± SEM.

Results

Weights of the body and the ovaries

The animals were allowed to run voluntarily for 5 weeks. The body weights of the rats before and after the running period and the weights of the ovaries are presented in Table 1. At the end of the experiment, the body weights of the control and the exercise groups were significantly higher compared with both PCO groups. The control and the exercise group significantly

<table>
<thead>
<tr>
<th>Body and Ovary Weights.</th>
<th>Control (n = 7)</th>
<th>Exercise (n = 7)</th>
<th>PCO (n = 7)</th>
<th>PCO exercise (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before</td>
<td>201.1 ± 2.6</td>
<td>199.0 ± 1.9</td>
<td>197.0 ± 1.4</td>
<td>196.0 ± 1.1</td>
</tr>
<tr>
<td>After</td>
<td>239.0 ± 4.2</td>
<td>254.1 ± 6.3</td>
<td>196.4 ± 1.5b</td>
<td>198.1 ± 3.1b</td>
</tr>
<tr>
<td>Ovaries (mg)</td>
<td>25 ± 0.2</td>
<td>37 ± 0.2</td>
<td>13 ± 0.2</td>
<td>19 ± 0.0</td>
</tr>
</tbody>
</table>

Data are means ± SEM. Body weight: *P < 0.001 when the control and exercise groups were compared with the polycystic ovary (PCO) and PCO exercise groups. bp < 0.05 when the exercise group was compared with the control group, P < 0.001 with the PCO group and P < 0.05 with the PCO exercise group. Ovary weight: *there were a significant (P < 0.0001) effect of oestadiol valerate injection on the weight of the ovaries in the two-way ANOVA.

increased their body weight during the experimental period, whereas neither of the PCO groups did.

There were a significant (P < 0.0001) effect of EV injection on the weight of the ovaries, whereas there was no effect of exercise and no significant interaction between the two variables in the two-way ANOVA. The ovaries of both the PCO and the PCO exercise groups weighed less than the ovaries of the control and exercise groups.

**Chronic voluntary exercise**

The mean running activity during the running period is shown in Fig. 1. The running behaviour in the PCO exercise group was similar to that in the exercise group during the 5-week period with increased running for every week. However, the PCO exercise rats ran significantly less during the last 2 weeks of running (Fig. 1).

**Ovarian morphology**

The ovaries in the control group were normal in appearance with follicles and corpora lutea in different stages of development and regression (Fig. 2A–H). The ovaries in the PCO group were smaller in size when compared with the ovaries in the control group and showed typical PCO-like changes (9) with many atretic follicles and few growing ‘healthy’ follicles in the primary, secondary, and tertiary stages (Fig. 2C–E). Only remnants of small regressed corpora lutea were observed. No young large corpora lutea were present in any ovary in the PCO group. However, the morphology of the ovaries of the PCO rats that had been allowed 5 weeks of voluntary exercise (the PCO exercise group) was almost normal, with large corpora lutea and atretic and growing primary, secondary, and tertiary follicles (Fig. 2F–H). On examination of the remaining ovarian tissue atretic and growing primary, secondary, and tertiary follicles group) was almost normal, with large corpora lutea and allowed 5 weeks of voluntary exercise (the PCO exercise group) was similar to that in the exercise group during the 5-week period with increased running for every week. However, the PCO exercise rats ran significantly less during the last 2 weeks of running (Fig. 1).

Ovarian NGF and p75NTR protein and mRNA expression

Ovarian NGF mRNA expression was significantly affected, both by EV injection (P < 0.0001) and by voluntary exercise (P < 0.0001), whereas no significant interaction between the two variables was found in the two-way ANOVA. The comparison between the study groups is shown in Fig. 3. NGF mRNA expression was significantly lower in the PCO group than in the control group. NGF mRNA expression in the PCO exercise group was significantly higher compared to the PCO group and did not differ from that of the control rats. The expression of ovarian NGF mRNA was significantly elevated in the exercise group compared to the control.

Ovarian NGF protein concentration was significantly affected, both by EV injection (P < 0.0001) and by voluntary exercise (P < 0.0032), with a significant interaction between the two variables (P < 0.0105) in the two-way ANOVA. The PCO group had significantly higher ovarian concentrations of NGF protein than the control group (Fig. 3B). The concentration of NGF protein in the PCO exercise group was significantly lower than in the PCO group and similar to that in the control group.

There were no effects of EV injection or voluntary exercise on the mRNA expression of p75NTR in the two-way ANOVA (Fig. 4A).

There was a significant effect of EV injection (P < 0.0403) and a positive interaction between EV injection and physical exercise (P < 0.0023) on immunopositive cell numbers of p75NTR in the two-way ANOVA. As shown in Fig. 4B, immunopositive cell numbers of p75NTR in the PCO group were significantly higher than in the control groups. Voluntary exercise decreased the number of expressing cells in the PCO exercise group, which was the same as in the control group.

Ovarian expression of 1α-AR

The mRNA expression of 1α-AR in the ovary was significantly affected, both by EV injection (P < 0.0055) and by voluntary exercise (P < 0.0028), whereas there was no significant interaction between the two variables in the two-way ANOVA. The mRNA expression of 1α-AR in the ovary (Fig. 5A) was significantly higher in the PCO exercise group compared to all the other groups.

The two-way ANOVA revealed a significant (P < 0.001) effect of exercise on the number of ovarian cells expressing 1α-AR. The post-hoc test indicated that the immunopositive cell count of 1α-AR (Fig. 5B) was significantly lower in the PCO exercise group compared to both the control and the PCO groups.

Ovarian expression of 1β-AR

The mRNA expression of 1β-AR in the ovary was significantly affected by EV injection (P < 0.0122), but not by voluntary exercise, and there was no interaction between the

![Fig. 1. Running distances in the exercise groups. The running distances in the two exercise groups are expressed as means ± SEMs. Running behaviour in the polycystic ovary (PCO) exercise group was similar to that in the exercise group, but the PCO rats ran less and the distances ran differed significantly during the last 2 weeks of running. *P < 0.05.](image-url)
two variables in the two-way ANOVA. No difference in the mRNA expression of $\alpha_{1b}$-AR (Fig. 6A) was found between the groups.

There was a significant ($P = 0.01$) effect of EV injection on the number of ovarian cells expressing $\alpha_{1b}$-AR, whereas no effects of exercise or interactions between the two treatments were found in the two-way ANOVA. As shown in Fig. 6B, the ovaries of PCO rats displayed significantly higher numbers of $\alpha_{1b}$-AR immunopositive cells than did the ovaries in the control groups. No difference in the number of $\alpha_{1b}$-AR stained cells was found in the PCO exercise group compared to the PCO and the control groups.

Ovarian expression of $\alpha_{1d}$-AR

The mRNA expression of $\alpha_{1d}$-AR in the ovary was significantly affected by EV-injection ($P < 0.0001$) but not by voluntary exercise, and there was no interaction between the two variables in the two-way ANOVA. As shown in Fig. 7A, the mRNA expression of $\alpha_{1d}$-AR in the ovary was significantly higher in the PCO group compared to the control and exercise groups.

The ANOVA revealed that exercise had a significant ($P < 0.0001$) effect on the $\alpha_{1d}$-AR immunopositive cell count. As shown in Fig. 7B, the number of immunopositive
The expression of ovarian NGF mRNA was significantly lower in the polycystic ovary (PCO) group than in the control group. NGF mRNA was higher in the PCO exercise group compared to the PCO group and did not differ from in the control or the exercise group. The expression of ovarian NGF mRNA was significantly higher in the exercise group compared to the control, the PCO, and the PCO exercise groups. Values are given as means ± SEMs and normalised to glyceraldehyde-3-phosphate dehydrogenase. *P < 0.05 versus the control group. #P < 0.05 versus the PCO group. (B) The ovaries of the PCO group contained significantly higher levels of ovarian NGF protein than did the control group. The concentration of NGF protein in the ovaries of the PCO exercise group was significantly lower than in the PCO group and similar to in the control group. Values are given as means ± SEMs. *P < 0.05 versus the control group. #P < 0.05 versus the PCO group.

Fig. 4. p75 neurotrophin receptor (p75NTR) mRNA expression and protein cell count in the ovaries. (A) The expression of ovarian p75NTR mRNA in the polycystic ovary (PCO) group did not differ from in the control or the exercise groups. (B) The mean immunostained cell count of p75NTR in 40 000-µm² areas of the ovary was higher in the PCO group than in the control group. The immunopositive cell count in the PCO exercise group was significantly lower than in the PCO group and did not differ from the control or the exercise group. Values are given as means ± SEMs. *P < 0.05 versus the control group. #P < 0.05 versus the PCO group.

α1d-AR cells was reduced by 5 weeks of voluntary exercise in both the exercise and the PCO exercise group compared to the control and the PCO groups.

Ovarian expression of β2-AR
The two-way ANOVA revealed that the mRNA expression of β2-AR in the ovary was not affected by EV-injection or by voluntary exercise, but there were a significant (P < 0.0116) interaction between the two variables. The comparison between specific experimental groups (post-hoc test) revealed that the mRNA expression of β2-AR in the ovary (Fig. 8A) was significantly lower in the PCO group than in the control group. The β2-AR mRNA expression in the ovary of the PCO exercise group did not differ from that in the PCO group or the control group.
The $\beta_2$-AR positive cell count in the ovary was not affected by EV injection or by voluntary exercise, but there was a significant ($P < 0.0025$) interaction between the two variables in the two-way ANOVA. The $\beta_2$-AR positive cell counts are shown in Fig. 8(B). The number of immunopositive $\beta_2$-AR cells was significantly higher in the PCO exercise group compared to the exercise group.

Ovarian distribution of $p75_{NTR}$, $\alpha_1$-AR subtypes, and $\beta_2$-AR

Ovarian $p75_{NTR}$ protein distribution was the same in all experimental groups and mainly distributed around follicles in the theca layers (Fig. 9A) with some immunoreactivity also occurring in the ovarian stroma and in the follicular and corpora lutea granulosa cells (not shown). This is in line with previous reports (6). As shown in Fig. 9(b), $\alpha_{1a}$-AR immunostaining was expressed in the theca and granulosa regions of follicles and corpora lutea. Immunostained cells were also found around blood vessels and primordial follicles (not shown). $\alpha_{1b}$-AR immunostaining was located in the granulosa cells of healthy and atretic follicles (Fig. 9c). The distribution of ovarian $\alpha_{1c}$-AR immunostaining is shown in Fig. 9(d). The $\alpha_{1d}$-AR was found expressed in the granulosa cells of healthy and atretic follicles in all of the experimental groups. $\beta_2$-AR immunoreactivity in both control and PCO ovaries was mainly found in the theca and granulosa cells of the ovarian follicles and of the corpora lutea (Fig. 9e).
Discussion

The present study addressed the question of whether physical exercise could affect morphological and biochemical manifestations in the ovary, such as mRNA expression and protein amount and distribution of the \( \alpha_1\)-AR subtypes, \( \beta_2\)-AR, NGF and p75NTR in EV-induced PCO in rats. The novel finding obtained was that ovarian morphology showed no signs of cystic formation and that ovarian mRNA expression and protein level of NGF, p75NTR, the \( \alpha_1\)-AR subtypes and \( \beta_2\)-AR were modulated by 5 weeks of voluntary exercise in rats with steroid-induced PCO.

Voluntary exercise yields many positive effects, whereas endurance training, because of an excessive physical demand, results in reproductive disturbances, impaired immunity and chronic stress-like changes in the HPA-axis, with the latter being observed in rats and mice (30, 31). Regular aerobic exercise has been associated with improved circulatory parameters in hypertensive patients (32, 33) and with a decreased general sympathetic tone (32–36). Decreased heart rate and blood pressure, as well as enhanced oxidative capacity, have been observed under resting conditions, both in exercised humans (37) and in exercised rats (17). With this in mind, we sought to investigate the effect that voluntary
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exercise exerts on the morphological and sympathetic-related features of EV-induced rat PCO.

One of the novel findings in the present study is that voluntary exercise prevented the development of an ovarian PCO-like morphology in rats with steroid-induced PCO. To determine the functional capacity of the ovaries in the PCO exercise group, we made a partial serial sectioning of all remaining tissue from all ovaries. This examination showed that all ovaries, except one, contained no complete corpora lutea, and there was no 'trapped' oocyte in the tissue. Hence, the corpora lutea were formed after ovulation. However, it cannot be excluded that the oocytes were released into the stroma (interstitial ovulation). In a subsequent study, this will be explored by checking whether oocytes can be found in the tubes/ampoules. It is known that sympathetic hyperactivity, such as that achieved in rat stress models, can modify follicular development and morphology (38, 39). It is also known that reducing ovarian sympathetic input by cutting the supraoptic nucleus reverses PCO changes in ovarian morphology and the features of the ovaries begin to revert to normal (5). It is reasonable to hypothesise that the features of normal ovarian morphology in the PCO exercise group could be due to the exercise-mediated sympathetic down-regulation. The physiological basis for the effects of physical exercise on the modulation of sympathetic output are most likely similar to those of electro-acupuncture (EA) because both are believed to act at the spinal segmental and central level via activation of muscle sensory Aδ and probably C fibres (40). In previous studies, we evaluated the effect of low-frequency EA on morphology and biochemical features of the ovary in PCO rats (9–11). Nevertheless, repeated low-frequency EA, although effective in preventing EV-induced biochemical abnormalities such as high concentrations of NGF, corticotrophin-releasing factor and endothelin-1, did not induce any morphological changes in PCO ovaries. One explanation for the efficacy of physical exercise and the lack of an effect after EA treatment might be that prolonged and sustained stimulation of muscle afferents, such as those achieved with regular physical exercise, are necessary to elicit an observable effect on ovarian morphology. The sympathetic nerves are involved in the control of ovarian secretory activity (5, 41) and play an important role in the regulation of ovarian function (39, 41, 42). In the last 30–40 years, evidence that supports the hypothesis that the sympathetic nervous system is important for ovarian function in women with PCOS has accumulated (3, 43–45). In addition, the effectiveness of ovarian wedge resection (46) or laparoscopic laser cauterisation (47) in inducing ovulation and increasing ovulatory responses in women with PCOS raises the possibility that an increase in sympathetic input to the ovary may play a role in the development of PCOS. Changes in sympathetic-related parameters in rats with EV-induced PCO have been demonstrated by elevated tyrosine hydroxylase and norepinephrine concentrations and by down-regulation of the β2-AR (5, 7, 8). The restoration of oestrous cyclicity and ovulatory capacity after transection of the superior ovarian nerve further confirms the involvement of sympathetic nerves in the development of PCO (5). Overall, this evidence indicates that physical exercise might be a therapeutical intervention for overcoming ovulatory dysregulation in women with PCOS, and for possibly preventing the occurrence of PCOS itself (48).

In the present study, body weight increased in the control and exercise groups during the course of the experiment. After the running period, both the control and exercise groups had significantly higher body weights than either PCO group. The PCO and the PCO exercise group gained no weight during the experiment. Although we did not measure food intake, we can hypothesise that rats in the exercise group, who achieved normal weight gain, balanced their augmented metabolic requirements with appropriate food intake behaviour, whereas the PCO rats did not. The reason for lack of weight gain in the PCO groups is most probably related to EV-induced weight loss via the central nervous system. Confirming previous reports, we found that EV injection increases NGF protein levels in the ovary (8). In light of the well-described effects of NGF on sympathetic innervation and nerve recruitment (49), this finding indicates increased sympathetic innervation in the ovaries following induction of PCO. We also found that voluntary exercise maintains NGF protein levels at around control levels in PCO rats. Previous studies have demonstrated that exercise has a neuroprotective role; it raises NGF concentrations in the brain (50, 51) and increases plasma levels of NGF (52). To the best of our knowledge, this is the first time that the effect of physical exercise on NGF expression in peripheral organs has been demonstrated. It could be hypothesised that the down-regulation of sympathetic innervation to the ovaries elicited by physical exercise results in lower ovarian NGF synthesis. A modulation of NGF synthesis by challenging β2-AR with selective agonists has been demonstrated both in vitro (53) and in vivo (54). These studies, together with the present study, demonstrate that a sympathetic-dependent regulation of NGF production in the target organ is a pathophysiological mechanism, possibly active in the steroid-induced rat PCO model.

Moreover, although a primary function of the NGF/NGF receptor system is the regulation of sympathetic metabolism and activity, they also directly affect the function of the ovarian secretory compartment and the process of follicle maturation and ovulation (55, 56). Thus, at least some of the abnormalities in steroid production observed in EV-induced PCO (5), and possibly in human PCOS, could be elicited by...
the sympathetic-driven ovarian up-regulation of NGF synthesis and biological action on ovarian cells. Further studies are necessary to clarify this point.

Our data demonstrated that the increased synthesis of ovarian NGF in the PCO group is associated with a decreased expression of NGF mRNA. The cause of this discrepancy was not investigated in the present study. However, enhanced translational activity might be responsible for this EV-induced effect. Physical exercise most probably normalises this outcome because an increase of NGF mRNA in parallel with a decrease of NGF protein was found in PCO exercise ovaries.

The augmentation of ovarian p75NTR content is thought to be one of the causes of NGF-mediated sympathetic recruitment in the PCO rat (8, 14). In the present study, we confirmed this hypothesis and demonstrated that physical exercise reduces the overexpression of p75NTR in PCO rats. Because p75NTR synthesis is controlled by its ligand NGF (57), it is possible that an NGF-mediated mechanism is active in the modulation of p75NTR by exercise. Overall, our findings indicate that physical exercise can modulate interactions between the sympathetic system and the ovary, regulating both the availability of the target-derived trophic support and, via modulation of p75NTR, the responsiveness of the sympathetic neurones to the neurotrophin.

An original finding emerging from the recent work of our group is that α1-ARs are expressed in normal ovaries and undergo significant changes after EV treatment and PCO induction in rats (14). However, the functional significance of the different α1-ARs in the ovaries of PCO rats has not been clearly identified. These receptors have been described as being mainly involved in the regulation of vascular tone and in the response of the cardiovascular system to pharmacological challenges (58). In ovarian physiology, the function of these ARs has traditionally been characterised as being primarily related to the regulation of ovarian blood flow. Recently, α1-AR-agonist stimulation was shown to modulate the release of progesterone in cultured granulosa cells by potentiating of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide (59). In the present study, the α1-ARs in the ovary were found to be distributed not only around blood vessels, but also in the secretory compartments, suggesting that α1-AR subtypes could have more specific functions in the physiology of the ovary than previously assumed.

Furthermore, we demonstrated that the expression and synthesis of the α1-AR subtypes are modulated by physical exercise in the PCO rat. Our results show that the mRNA expression of α1a- and α1d-AR mRNA is higher in exercised EV-treated rats than in EV-treated rats that have had no exercise whereas the mRNA expression of α1d-AR is lower in EV-treated rats and does not differ from controls. The protein levels of all α1-AR subtypes was lower in exercised PCO rats than in PCO rats that had not exercised and the levels were normal (α1b-AR) or lower (α1a- and α1d-AR) compared to control rats. Thus, different regulatory mechanisms, acting at both the transcriptional and the translational level, could be active in the PCO- and exercise-mediated modulation of the α1-AR subtypes. Nevertheless, our data demonstrate that physical exercise is effective and reverses all PCO-induced changes in ovarian α1-AR synthesis to normal levels.

Moreover, the EV-induced reduction in ovarian β2-AR content was counteracted by physical exercise. The expression and activity of β2-AR in the PCO rat ovary have been reported to be associated with increased ovarian sympathetic input (5). The main role of β2-AR in ovarian physiology is to regulate steroidogenesis (5, 60). Our data on β2-AR ovarian distribution are in accordance with these findings and indicate an alteration in ovarian sympathetic input, and possibly also a higher content of ovarian norepinephrine in the regulation of β2-AR. It is possible that this mechanism is also involved in the regulation of the α1-AR subtypes.

Different models of PCO are inevitable, and the most suitable model for a particular study depends on the goals of the study. We have demonstrated that rats with EV-induced PCO develop increased sympathetic activity and have found evidence of increased HPA axis activity (13). Furthermore, we have also demonstrated that the insulin sensitivity of rats with EV-induced PCO is not lower than that of control rats, and PCO rats do not develop obesity or hyperandrogenism, which might be later signs of sympathetic hyperactivity (13). These findings have implications for future research with respect to the effect of different interventions.

In conclusion, in the present study, we demonstrate that EV-induced PCO in rats is associated with an increased synthesis of ovarian NGF and p75NTR, which may in turn be linked with increased ovarian sympathetic innervation, increased nerve recruitment, or both. We also demonstrate that voluntary physical exercise almost normalises ovarian morphological and biochemical features in rats with steroid-induced PCO. Our data suggest that regular exercise, which tends to modulate sympathetic outflow, could prove to be effective in the treatment of anovulation and possibly in the prevention of human PCOS.

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