Research Paper

Electrical and manual acupuncture stimulation affect oestrous cyclicity and neuroendocrine function in an 5α-dihydrotestosterone-induced rat polycystic ovary syndrome model

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Both low-frequency electro-acupuncture (EA) and manual acupuncture improve menstrual frequency and decrease circulating androgens in women with polycystic ovary syndrome (PCOS). We sought to determine whether low-frequency EA is more effective than manual stimulation in regulating disturbed oestrous cyclicity in rats with PCOS induced by 5α-dihydrotestosterone. To identify the central mechanisms of the effects of stimulation, we assessed hypothalamic mRNA expression of molecules that regulate reproductive and neuroendocrine function. From age 70 days, rats received 2 Hz EA or manual stimulation with the needles five times per week for 4–5 weeks; untreated rats served as control animals. Specific hypothalamic nuclei were obtained by laser microdissection, and mRNA expression was measured with TaqMan low-density arrays. Untreated rats were acyclic. During the last 2 weeks of treatment, seven of eight (88%) rats in the EA group had epithelial keratinocytes, demonstrating oestrous cycle change ($P = 0.034$ *versus* control rats). In the manual group, five of eight (62%) rats had oestrous cycle changes (n.s. *versus* control animals). The mRNA expression of the opioid receptors *Oprk1* and *Oprm1* in the hypothalamic arcuate nucleus was lower in the EA group than in untreated control rats. The mRNA expression of the steroid hormone receptors *Esr2*, *Pgr* and *Kiss1r* was lower in the manual group than in the control animals. In rats with 5α-dihydrotestosterone-induced PCOS, low-frequency EA restored disturbed oestrous cyclicity but did not differ from the manual stimulation group, although electrical stimulation lowered serum testosterone in responders, those with restored oestrus cyclicity, and differed from both control animals and the manual stimulation group. Thus, EA cannot in all aspects be considered superior to manual stimulation. The effects of low-frequency EA may be mediated by central opioid receptors, while manual stimulation may involve regulation of steroid hormone/peptide receptors.

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Chronic anovulation and hyperandrogenism are the most prominent clinical characteristics of polycystic ovary syndrome (PCOS). Polycystic ovary syndrome is also associated with obesity, hyperinsulinaemia and insulin resistance, which further aggravate the classical symptoms (Norman *et al.* 2007; Goodarzi *et al.* 2011). The aetiology of the syndrome is most likely to be multifactorial, because high concentrations of insulin and luteinizing hormone (LH) increase ovarian androgen production and contribute to impaired follicle development (Blank

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et al. 2006; Goodarzi *et al.* 2011). Women with PCOS require long-term pharmacological treatment, which is usually effective but has negative side-effects (Dronavalli & Ehrmann, 2007). Alternative therapies are often used for menstrual disorders and fertility problems, such as acupuncture with manual and/or electrical stimulation of the needles; these therapies have few negative side-effects, but their efficacy is not well established (Stankiewicz *et al.* 2007; Smith *et al.* 2010). In a randomized controlled trial, acupuncture with low-frequency electrical stimulation of the needles, so-called electro-acupuncture (EA), and physical exercise improved hyperandrogenism and menstrual frequency in women with PCOS (Jedel *et al.* 2011). Interestingly, low-frequency EA was more effective than physical exercise in relieving these symptoms. Abdominal acupuncture with manual stimulation of the needles improved endocrine and metabolic function in obese PCOS women to the same extent as treatment with metformin (Lai *et al.* 2010). In case–control studies of menstrual frequency/ovulation and endocrine measures, acupuncture with electrical (Chen & Yu, 1991; Stener-Victorin *et al.* 2000) or manual stimulation (Xiaoming *et al.* 1993) also improved these symptoms.

Little is known about the neurobiological mechanism of the effects of acupuncture on endocrine and reproductive variables in clinical trials. The effect of acupuncture with intramuscular needle insertion is mediated by activation of sensory afferents to the spinal cord and central nervous system, which could modulate the release of hormones and neuropeptides and the activity in the autonomic nervous system (Kaufman *et al.* 1983; Kagitani *et al.* 2005; McCord & Kaufman, 2010). Understanding how acupuncture affects ovulation and menstrual frequency would greatly improve the integration of acupuncture into Western medicine (Napadow *et al.* 2005). Also, the optimal acupuncture stimulation modality should be defined and used and be consistent with evidence-based medicine (White *et al.* 2008).

Manual acupuncture is performed by inserting fine needles into the skin and underlying muscle tissue and then twisting and rotating them back and forth. In EA, an electric current is passed through two or more needles attached to electrodes. Electro-acupuncture allows the frequency and intensity of stimulation to be defined objectively.

In rodents with needles placed in abdominal and hindlimb muscles, both manual acupuncture (Uchida *et al.* 2005) and low-frequency EA (2 Hz burst frequency), but not high-frequency EA (80 Hz; Stener-Victorin *et al.* 2003, 2006), modulate the ovarian blood flow response. In both cases, the response is mediated as a reflex via the ovarian sympathetic nerves and is controlled by supraspinal pathways (Stener-Victorin *et al.* 2006). The response seems to be more pronounced with low-frequency EA (Stener-Victorin *et al.* 2003, 2006). In clinical trials (Chen & Yu, 1991; Xiaoming *et al.* 1993; Stener-Victorin *et al.* 2000; Jedel *et al.* 2011), manual acupuncture and low-frequency EA have not been compared directly, to determine which regulates reproductive and endocrine functions more effectively.

In rat models of PCOS, low-frequency EA modulates the hypothalamic β-endorphin system (Stener-Victorin & Lindholm, 2004); it also restores oestrous cyclicity by modulating the hypothalamic–pituitary–ovarian axis (Feng *et al.* 2009; Mannerås *et al.* 2009) and by reducing high-level expression of hypothalamic gonadotrophin-releasing hormone (GnRH) and the androgen receptor (Feng *et al.* 2009), as well as other endocrine measures (Zhao *et al.* 2004, 2005). Moreover, oestrous cycle changes are more prominent when low-frequency EA is given 5 days per week instead of 3 days per week.

In the present study, we sought to determine whether low-frequency electrical stimulation is more effective than manual stimulation in regulating oestrous cyclicity in rats with 5α-dihydrotestosterone (DHT)-induced PCOS treated 5 days per week for 4–5 weeks. To elucidate the central mechanisms of the effects of EA *versus* manual acupuncture, mRNA expression of key molecules that regulate reproductive and neuroendocrine function was measured in arcuate (Arc), medial preoptic area (MPOA) and anteroventral periventricular (Avpv) nuclei in the hypothalamus. Nuclei were obtained by laser microdissection and pressure catapulting (LMPC), and mRNA expression was measured with custom TaqMan low-density arrays designed to assess 24 selected genes.

Methods

Animals

Three lactating Wistar dams, each with 10 14-day-old female pups, were purchased from Charles River (Sulzfeld, Germany). At 21 days of age, pups were separated from their lactating dams and housed five per cage in controlled conditions (21–22°C, 55–65% humidity, 12 h light–12 h dark cycle). All rats had free access to commercial chow (Harlan Teklad Global Diet, 16% protein rodent diet; 2016, Harlan Winkelmann, Harlan, Germany) and tap water. The study was approved by the Animal Ethics Committee of the University of Gothenburg (approval ID, 23-2008) and conducted in accordance with the Guide to the Care and Use of Experimental Animals (www.sjv.se).

Study procedure

At 21 days of age, female pups were randomly distributed from each lactating dam to the three experimental groups (PCOS, $n = 8$; PCOS EA, $n = 8$; and PCOS manual, $n = 9$). Under light general anaesthesia with isoflurane (2% in a 1:1 mixture of oxygen and air; Isoba Vet; Schering-Plough, Stockholm, Sweden), a 90 day continuous-release

pellet (Innovative Research of America, Sarasota, FL, USA) containing 7.5 mg of DHT (daily dose, 83 μg) was subcutaneously implanted in the neck. This dose of DHT results in PCOS characteristics, including reproductive and metabolic disturbances at adult age (Manneråas *et al.* 2007). Microchips (AVID, Norco, CA, USA) were inserted along with the pellets for numbering and identification. Body weight was monitored weekly. Treatment with low-frequency EA or manual acupuncture started at 70 days of age, 7 weeks after the start of DHT exposure. The study was concluded after 11–12 weeks of DHT exposure, including 4–5 weeks of treatment. If rats started to show signs of regular cyclicity during the treatment period, they were euthanized in the oestrus phase.

Treatment

Rats were handled and treated daily from Monday to Friday for 4–5 weeks (20–25 treatments in total). The duration of each treatment was 15 min during week 1, 20 min during weeks 2 and 3, and 25 min during weeks 4 and 5. Before handling or needle insertion, rats were lightly anaesthetized as described above for 2–3 min. During treatment, rats were suspended in a fabric harness above the desk and remained conscious throughout the episode. To control for environmental factors, untreated rats were handled in the same way as rats in two treatment groups, but without needle insertion or electrical or manual stimulation of the needles.

Acupuncture needles (HEGU Svenska, Landsbro, Sweden) were inserted bilaterally in the rectus abdominis and triceps surae muscles at points in somatic segments corresponding to the innervation of the ovaries (i.e. from spinal levels T10 to L2 and at the sacral level); EA stimulation at these points improves oestrous cyclicity in our rat PCOS model (Feng *et al.* 2009; Manneråas *et al.* 2009). The needles were inserted 0.5–0.8 cm. In the EA group, needles were attached to an electric stimulator (CEFAR ACU II; Cefar-Compex Scandinavia, Malmö, Sweden) and stimulated at 2 Hz in 0.1 s, 80 Hz burst pulses (Stener-Victorin *et al.* 2003; Manneråas *et al.* 2008, 2009; Feng *et al.* 2009; Johansson *et al.* 2010). The stimulation amplitude (intensity) was adjusted to produce tolerable and visible local muscle contractions (0.8–1.4 mA). Rats generally tolerated higher amplitudes towards the end of each treatment owing to receptor adaptation. In the manual acupuncture group, the needles were rotated back and forth (five rotations) at the beginning, middle and end of each treatment.

Vaginal smears

Cyclicity was analysed from daily vaginal smears obtained during the final 2 weeks of the experiment. The stage of cyclicity was determined by microscopic analysis of the predominant cell type (Marcondes *et al.* 2002). Rats who responded to treatment by estrus cycle change were finalized during estrus cycle.

Biochemical analyses

Serum concentrations of 17β-oestradiol, progesterone and testosterone were determined with enzyme-linked immunoassay kits (1244-056, A066-101 and A050-201; PerkinElmer Life and Analytical Sciences, Wallac Oy, Turku, Finland) as recommended by the manufacturer. The sensitivity of the 17β-oestradiol was 0.05 nmol l^{-1}, and the intra- and interassay coefficients of variation were 3.8–10 and 3.6–9.7%, respectively; the sensitivity for progesterone was 0.08 nmol l^{-1}, and the intra- and interassay coefficients of variation were 3.3–7.3 and 2.7–10.1%, respectively; the sensitivity for testosterone was 0.3 nmol/l and the intra- and interassay coefficients of variation were between 5.6–6.0% and 5.6–14.2%, respectively. Measurement of LH concentration in serum was performed by a radioimmunoassay according to the manufacturer's protocol (RK-552; IZOTOP Institute of Isotopes Co., Ltd, Budapest, Hungary). Rat LH standards were used in the range between 0.8 and 50 ng ml^{-1}. The sensitivity of the assay was 0.9 ng ml^{-1}, and the intra- and interassay coefficients of variation were 6.5 and 7.7–10.9% ($n = 14$), respectively.

Laser microdissection and pressure catapulting

Brains were extracted, snap frozen in liquid nitrogen, and stored at –80°C until analyses. The frozen brain was placed in a cell culture well with a thin layer of TissueTec (enough to cover the cerebellum), and sectioned at 20 μm with a cryostat. Selected sections were adhered one by one on 1 mm polyethylene naphthalate (PEN)-membrane slides (Carl Zeiss MicroImaging, Munich, Germany) that had been exposed to ultraviolet light for at least 30 min before use. The sections were then placed in 50% EtOH for 90 s and 70% EtOH for 90 s, and stained with 1% cresyl violet diluted in 99% EtOH for 30 s on ice. Thereafter, the slides were dipped twice in 70% EtOH and once in 95% EtOH to remove excess colour, dried, placed in 50 ml Falcon tubes, and stored at –80°C.

For LMPC, the slides were thawed at room temperature and placed on a Zeiss inverted microscope fitted with a PALM MicroBeam Laser System (Zeiss, Munich, Germany) and ×10 objectives. Specific hypothalamic nuclei (Avpv, Arc and MPOA) were identified at ×5 magnification and manually delineated on the computer screen with PALM Robo Pro software. The microscope was instructed to collect delineated regions. Nuclei of interest were dissected with a fine-tuned 377 nm pulsed nitrogen laser and retrieved along with the underlying PEN membrane in a non-contact fashion by catapulting into a Zeiss AdhesiveCap (500 μl; Carl Zeiss MicroImaging).

The collected areas of the different brain nuclei were 40–50 μm^2. Images of tissue sections before and after section capture and of captured nuclei attached to the lid of the caps were taken with a Nikon OPTIPHOT-2 (Nikon, Tokyo, Japan) and a Zeiss Axiocam (Zeiss). Dissected tissue from 12–16 slices in one cap was added to 350 μl of RLT buffer (Qiagen, Hilden, Germany), placed upside down, incubated for 30 min, vortexed and centrifuged for 5 min, and stored at –80°C. The entire procedure was completed within 30 min.

Extraction and quantification of RNA

Total RNA from laser-catapulted tissues was extracted with RNeasy MiniElute Cleanup Kits (Qiagen) according to the manufacturer's protocol, including DNase treatment (Qiagen). The RNA concentrations were measured with a spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, Denmark), and RNA integrity was checked by 260/280 ratio of RNA, which was 1.8–2.0. Reverse transcription was performed with SuperScript III (Invitrogen, Lidingö, Sweden) using 1 μl dNTP and 1 μl random hexamers (Invitrogen) as primers, according to the manufacturer's instructions. Complementary DNA was preamplified with TaqMan preAmp Master Mix and TaqMan (Applied Biosystems, Foster City, CA, USA). The temperature profile was 25°C for 5 min, 55°C for 45 min and 70°C for 15 min for reverse transcription, and 95°C for 10 min, 95°C 15 s and 60°C 4 min for 14 cycles and 99°C for 10 min for complementary DNA preamplification.

Real-time RT-PCR

Real-time RT-PCR analysis was performed with custom TaqMan low-density arrays (Applied Biosystems) with primers and probes for 24 selected rat genes, which are listed along with the corresponding TaqMan gene expression assay numbers and GenBank accession numbers in Table 1. Eight samples were randomly analysed in duplicate per card in one run; 25 μl of complementary DNA mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) and RNase-free water in a total volume of 100 μl was loaded into each sample loading port. Thermal cycling and florescence detection were performed on an ABI Prism 7900HT Sequence Detection System with SDS software (version 2.1; Applied Biosystems). Thermal cycling was carried out for 2 min at 50°C and 10 min at 94.5°C, followed by 40 cycles of 30 s at 97°C and 1 min at 59.7°C.

The NormFinder algorithm (http://www.mdl.dk/publicationsnormfinder.htm) was used to calculate the expression stability of five putative reference genes (18S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase, β-actin, peptidyl-prolyl isomerase A and hydroxymethylbilane synthase) for normalization. A combination of the latter two genes had the lowest intragroup and intergroup variability in dissected hypothalamic nuclei, and they were used as endogenous controls. Gene expression values were calculated with the $2^{-\Delta\Delta Ct}$ method as previously described (Mannerås *et al.* 2008, 2009). The cycle threshold (ΔCt) value of each sample was determined by subtracting the average Ct value of the reference genes from the average Ct value of the target gene. The $\Delta\Delta Ct$ value was then calculated by subtracting the ΔCt of the sample with highest expression (i.e. the lowest ΔCt value) from the ΔCt value of the sample. The target gene expression level relative to the sample with highest expression was then estimated as $2^{-\Delta\Delta Ct}$.

Statistical analyses

All statistical evaluations were performed with SPSS software (version 18.0; SPSS, Chicago, IL, USA). The effect of electrical or manual stimulation on changes in oestrous cyclicity was analysed with the χ^2 test. Values for gene expression ($2^{-\Delta\Delta Ct}$) are reported as means ± SEM. All variables displayed normal distribution, except mRNA expression of *Oprm*, *Tac2*, *Th*, *Gnrh1* and *Gnrhr*. These genes underwent logarithmic transformation before statistical analysis. The effect of electrical or manual stimulation on circulating sex steroids and mRNA expression in specific hypothalamic nuclei was analysed with one-way ANOVA and Bonferroni *post hoc* test. A value of $P < 0.05$ was set as the limit of statistical significance.

Results

Low-frequency EA and manual stimulation improve oestrous cyclicity in DHT-induced PCOS rats

Using daily vaginal smears, oestrous cyclicity was analysed during the last 2 weeks of the experiment. Untreated rats with DHT-induced PCOS (control animals) were constantly in a 'pseudo-dioestrus' stage, exhibiting predominantly leukocytes (Fig. 1*A*). During the same period, seven of eight (88%) rats in the EA group had epithelial keratinocytes, demonstrating oestrous cycle changes ($P = 0.034$ *versus* control animals); the cycle changes occurred three times in two rats, twice in three rats, once in two rats and not at all in one rat; 1.75 ± 0.36 times (mean ± SEM) during the last 14 days (Fig. 1*C*). In the manual stimulation group, five of eight (62%) rats had oestrous cycle changes (P = n.s. *versus* control animals), which occurred twice in four rats, once in one rat and not at all in three rats; 1.12 ± 0.35 times during the last 14 days (Fig. 1*B*). There was no difference in the number of cycle changes between the electrical and manual stimulation groups.

Table 1. Genes on the TaqMan low-density arrays, TaqMan gene expression assay number and GenBank accession number

Gene symbol	Gene description	TaqMan assay no.	GenBank accession no.
Target genes			
Gnrh1	Gonadotrophin-releasing hormone 1	Rn00562754_m1	NM_012767.1
Gnrhr	Gonadotrophin-releasing hormone receptor	Rn00578981_m1	NM_031038.3
Kiss1	Kiss-1 metastasis suppressor	Rn00710914_m1	NM_181692.1
Kiss1r	KISS1 receptor	Rn00576940_m1	NM_023992.1
Pomc	Pro-opiomelanocortin	Rn00595020_m1	NM_139326.2
Pdyn	Prodynorphin	Rn00571351_m1	NM_019374.2
Oprm1	Opioid receptor, μ1	Rn00565144_m1	NM_013071.1
Oprk1	Opioid receptor, κ1	Rn00567737_m1	NM_017167.1
Npy	Neuropeptide Y	Rn01410146_m1	NM_012614.1
Tac1	Tachykinin 1	Rn01500392_m1	NM_012666.1
Tac2	Tachykinin 2	Rn00569758_m1	NM_019162.1
Dbh	Dopamine β-hydroxylase	Rn00565819_m1	NM_013158.1
Lepr	Leptin receptor	Rn00561465_m1	NM_012596.1
Ghrl	Ghrelin/obestatin prepropeptide	Rn01425835_m1	NM_021669.1
Ar	Androgen receptor	Rn00560747_m1	NM_012502.1
Esr1	Oestrogen receptor 1	Rn01430445_m1	NM_012689.1
Esr2	Oestrogen receptor 2	Rn00562610_m1	NM_012754.1
Pgr	Progesterone receptor	Rn00674394_m1	NM_022847.1
Th	Tyrosine hydroxylase	Rn00562500_m1	NM_012740.2
Putative reference genes			
18S	18S ribosomal RNA	Hs99999901_s1	X03205
Actb	β-Actin	Rn00667869_m1	NM_012583.2
Hmbs	Hydroxymethylbilane synthase	Rn01527840_m1	NM_013168.2
Ppia	Peptidylprolyl isomerase A	Rn00690933_m1	NM_017101.1
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase	Rn99999916_s1	NM_017008.2

Table 2. Serum concentrations of 17β-oestradiol, progesterone, testosterone and luteinizing hormone in untreated control rats (PCOS), in the electro-acupuncture (EA) group (PCOS EA) and in the manual stimulation group (PCOS manual), and in rats responding with oestrous cycle change during treatment

All	PCOS $n = 8$	PCOS EA $n = 8$	PCOS manual $n = 8$	ANOVA *P* value
17β-Oestradiol (nmol l^{-1})	0.09 ± 0.007	0.31 ± 0.13	0.12 ± 0.18	0.103
Progesterone (nmol l^{-1})	29.10 ± 5.64	63.11 ± 14.76	61.37 ± 10.97	0.073
Testosterone (nmol l^{-1})	0.86 ± 0.13	0.45 ± 0.08	0.79 ± 0.14	0.053
Luteinizing hormone (ng ml^{-1})	15.56 ± 3.76	14.25 ± 1.60	15.56 ± 4.74	0.707
Responders	$n = 8$	$n = 7$	$n = 5$	
17β-Oestradiol (nmol l^{-1})	0.09 ± 0.007	0.12 ± 0.20	0.28 ± 0.17	0.214
Progesterone (nmol l^{-1})	29.10 ± 5.64	67.35 ± 16.33*	75.62 ± 8.98*	0.020
Testosterone (nmol l^{-1})	0.86 ± 0.13	0.41 ± 0.08*	0.94 ± 0.17	0.019
Luteinizing hormone (ng ml^{-1})	15.56 ± 3.76	14.14 ± 1.70	16.20 ± 5.35	0.609

Values are means ± SEM. *$P < 0.05$ *versus* PCOS (one-way ANOVA followed by Bonferroni *post hoc* test).

Manual and electrical stimulation increase serum progesterone, while electrical stimulation decreases testosterone in DHT-induced PCOS rats defined as responders

Circulating levels of progesterone, oestradiol, testosterone and LH did not differ between the groups when all rats were included in the analysis (Table 2). When non-responders (those with no oestrous cycle change) were excluded, the mean serum progesterone level was higher in both treated groups compared with control animals ($P = 0.024$ and $P = 0.041$, respectively), but did not differ from the manual stimulation group. The mean serum testosterone level was lower in the electrical stimulation group compared with control animals ($P = 0.05$) in responders, and differed from the manual stimulation group ($P = 0.037$). Circulating oestradiol and LH were not affected when non-responders were excluded (Table 2).

Gene expression in hypothalamic nuclei

The mRNA expression of 19 target genes and five putative reference genes was measured in tissue obtained by LMPC

from three hypothalamic nuclei (Arc, Avpv and MPOA; Table 1). Residual sections from a typical microdissection are shown in Fig. 2. In the following subsections, genes regulated by electrical and or manual stimulation are presented. Data from genes that were not regulated by stimulation are not presented.

Electrical stimulation decreases opioid receptor mRNA expression in hypothalamic Arc, while manual stimulation decreases oestrogen, progesterone and kisspeptin receptor mRNA expression

Expression of κ-opioid receptor 1 (*Oprk1*) and μ-opioid receptor 1 (*Oprm1*) mRNA was lower in the EA group than in control animals ($P = 0.008$ and $P = 0.031$, respectively); no significant changes were detected in the manual stimulation group (Fig. 3). The mRNA expression of the oestrogen receptor β (*Esr2*), progesterone receptor (*Pgr*) and kisspeptin receptor (*Kiss1r*) was lower in the manual group than in control animals ($P = 0.022$, $P = 0.033$ and $P = 0.033$, respectively); no changes in expression were detected in the EA group (Fig. 3). When non-responders were excluded, the mRNA expression of *Oprk1* was lower in the EA group than in control animals ($P = 0.005$), and *Oprm1* tended to be decreased ($P = 0.065$). None of the genes regulated by manual stimulation, i.e. *Esr2*, *Pgr* or *Kiss1r*, remained downregulated when non-responders were excluded. There were no differences between the electrical and manual stimulation groups.

Electrical and manual stimulation do not affect mRNA expression of selected genes in the hypothalamic MPOA or Avpv nucleus

Previously, we found that androgen receptor immunoreactivity and GnRH immunoreactivity were increased

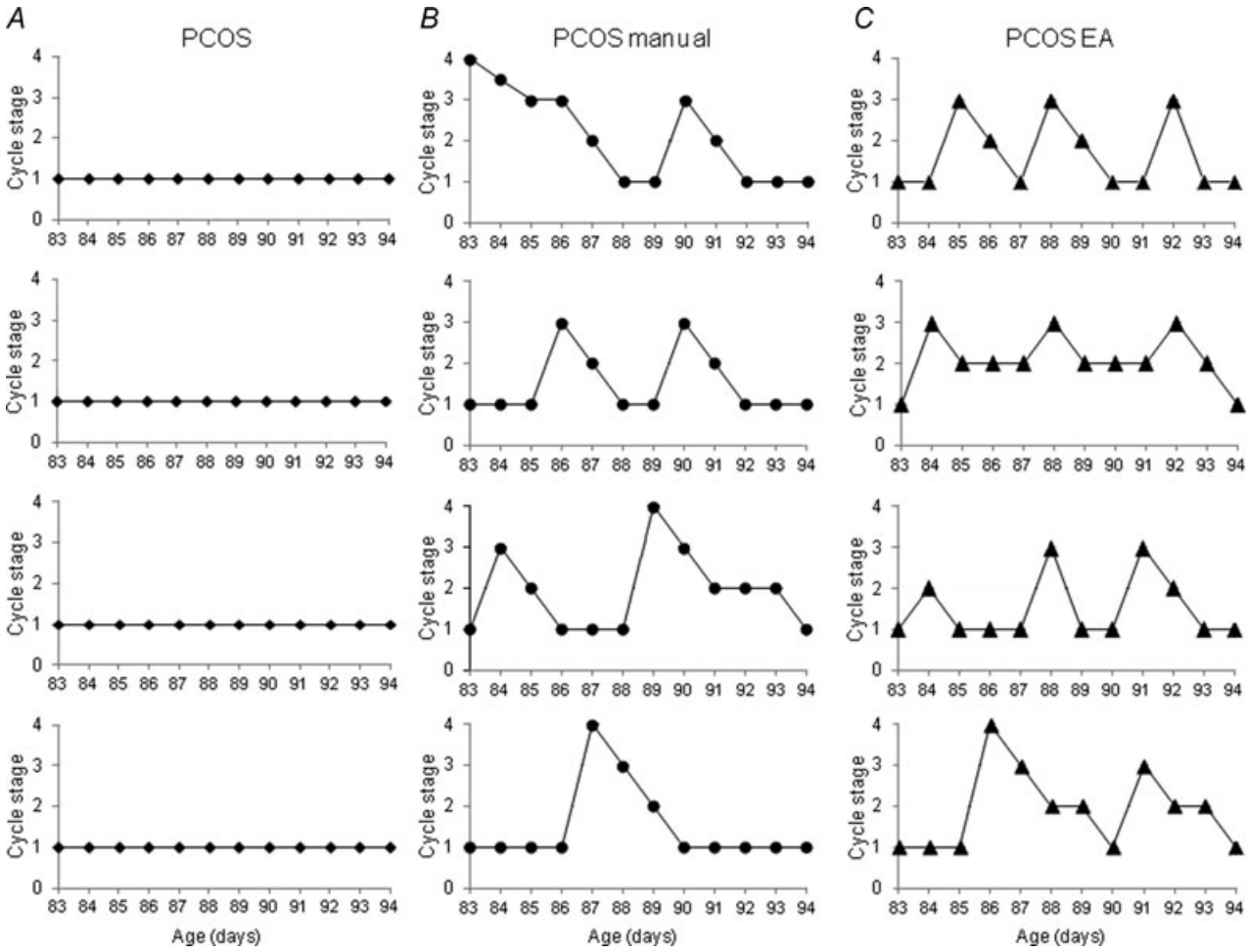

Figure 1. Oestrous cycle changes in four representative rats from each group
Cycle stages are as follows: 1, dioestrus; 2, pro-oestrus; 3, oestrus; and 4, metoestrus. Groups are as follows: PCOS, 5α-dihydrotestosterone-treated control rats; PCOS EA, electro-acupuncture group; and PCOS manual, manual stimulation group.

in the MPOA nucleus after intense low-frequency electrical stimulation (Feng *et al.* 2009). In the present study, however, neither electrical nor manual stimulation affected the mRNA expression of the selected genes in the hypothalamic MPOA or Avpv nucleus (data not shown).

Discussion

This study demonstrates that both low-frequency EA and manual acupuncture affect oestrous cyclicity in non-cycling rats with DHT-induced PCOS. Although these changes were more pronounced in the EA group, we cannot conclude that electrical stimulation is superior to manual stimulation. Our findings suggest that electrical and manual stimulation affect neuroendocrine and reproductive function through different mechanisms, EA by regulating the endogenous opioid receptor system, and manual stimulation by regulating steroid hormone receptors (see Fig. 4 for summary); however, the definitive mechanisms for the effect on oestrous cycle remain to be elucidated.

Several factors may influence the outcome of clinical and basic studies of acupuncture, including the number and placement of needles, the depth of needle insertion, the type of stimulation (electrical and/or manual), the frequency of stimulation (high or low frequency, number of times needles are manipulated or no stimulation at

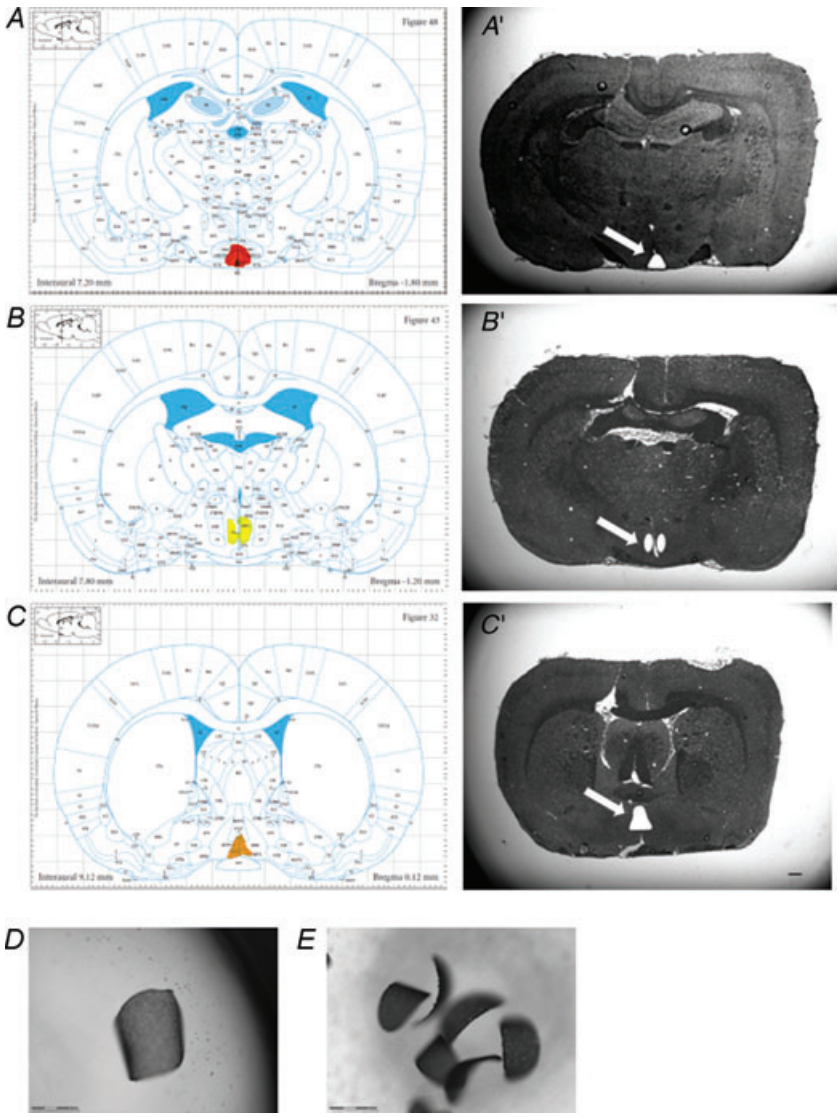

Figure 2. Laser microdissection of rat hypothalamic nuclei according to *The Rat Brain in Stereotaxic Coordinates* (Paxinos & Watson, 2009)
A, arcuate nucleus (Arc). *B*, medial preoptic area (MPOA). *C*, anteroventral periventricular (Avpv). White arrows in right-hand panels indicate nuclei after sequential dissection. Scale bar represents 500 μm. *D* and *E*, dissected tissue catapulted into the AdhesiveCaps. Scale bars represent 300 μm.

all; White *et al.* 2008). Each stimulation parameter can affect the response and needs to be tested step by step to find the optimal dose of acupuncture for a specific disorder. In experimental studies, electrical (Kaufman *et al.* 1984) and manual stimulation (Kagitani *et al.* 2005) differentially activate high-threshold afferents (Aδ and C fibres; Higashimura *et al.* 2009). To our knowledge, electrical and manual acupuncture stimulation have not been compared directly in women with PCOS or in any rat model of PCOS.

In the present trial, electrical stimulation of the needles significantly improved oestrous cyclicity in comparison to untreated PCOS control rats. These results are in line with findings in DHT-induced PCOS rats (Feng *et al.* 2009; Mannerås *et al.* 2009) and in a randomized controlled trial in women with PCOS (Jedel *et al.* 2011). Manual stimulation also improved oestrous cyclicity in PCOS rats, but the improvement was smaller than in the electrical stimulation group and not statistically significant *versus* untreated PCOS control animals. Serum progesterone levels were increased in both treatment groups, consistent with the results of vaginal smears, which showed improved oestrous cyclicity after both electrical and manual stimulation. In addition, electrical stimulation decreased circulating testosterone after electrical stimulation, while no change was observed after manual stimulation in rats defined as responders. These results are in line with our clinical findings, where we demonstrated that low-frequency EA decreased circulating testosterone both directly after a treatment period and at follow up 16 weeks after the last treatment (Jedel *et al.* 2011). These findings were in line with improved menstrual bleeding pattern.

Previously, we demonstrated that the effect of low-frequency EA on ovarian function is mediated as a reflex response via the ovarian sympathetic nerves, and the response was controlled via supraspinal pathways (Stener-Victorin *et al.* 2006). To compare possible central effects of electrical and manual stimulation on reproductive function, we assessed the hypothalamic mRNA expression of key regulatory genes encoding ligands and receptors involved in reproductive and neuroendocrine function. We hypothesized that the effect of electrical and/or manual acupuncture stimulation is mediated, at least in part, by these pathways (see Fig. 4 for summary).

The hypothalamic Arc nucleus is the site of the GnRH pulse generator and participates in the regulation of female reproduction (Quiñones-Jenab *et al.* 1997). The Arc nucleus has three distinct neuronal populations, β-endorphin, tyrosine hydroxylase and neuropeptide Y (Magoul *et al.* 1993; Magoul & Tramu, 1997). In particular, β-endorphin controls secretion of GnRH, *Gnrh1* gene expression and LH. Furthermore, *Pomc* mRNA expression in the Arc nucleus is regulated by gonadal steroids (Wilcox & Roberts, 1985). Neurons in the Arc nucleus also produce neurokinin B and dynorphin, which also express oestrogen receptor α and the progesterone receptor (Goodman *et al.* 2007; Smith, 2008). β-Endorphin acts through *Oprm1* and dynorphin through *Oprk1*. After ovariectomy in rats, short-term exposure to oestrogen and progesterone increases MPOA *Oprm1* labelling, and oestrogen increases *Oprm1*mRNA expression in the Arc nucleus (Joshi *et al.* 1993).

In the present study, low-frequency EA-induced improvement in oestrus cyclicity was accompanied by decreased mRNA expression of *Oprm 1* and *Oprk1*

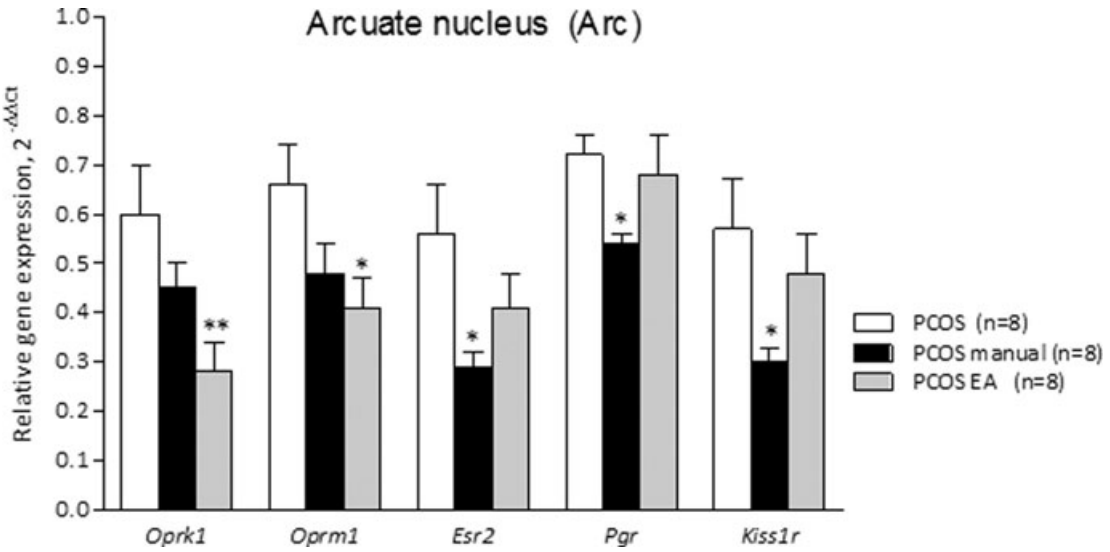

Figure 3. Regulated genes in the hypothalamic arcuate nucleus (Arc) in untreated control animals (PCOS, $n = 8$) the EA group (PCOS EA, $n = 8$) and the manual stimulation group (PCOS manual, $n = 8$)
Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* PCOS.

in the hypothalamic Arc in DHT-induced PCOS rats, consistent with reports that low-frequency EA affects the regulation of opioid peptides or their receptors (Han, 2004; Liang *et al.* 2010). We measured opioids and their receptors because there is evidence that the central β-endorphin system exerts tonic inhibitory control on the GnRH pulse generator and on pituitary LH release (Genazzani *et al.* 1993) and modulates sympathetic tone (Cumming *et al.* 1984), all of which are dysregulated in women with PCOS (Blank *et al.* 2007). Furthermore, in anovulatory women with PCOS, the μ-opioid receptor antagonist naltrexone induces ovulation and decreases LH concentration, the ratio of LH to follicle-stimulating hormone, and testosterone levels; these findings support the hypothesis that PCOS is associated with elevated β-endorphin secretion (Ahmed *et al.* 2008). Low-frequency EA also decreases circulating β-endorphin levels (Chen & Yu, 1991; Stener-Victorin *et al.* 2000), improves menstrual dysfunction and regulates circulating sex steroids in women with PCOS (Chen & Yu, 1991; Stener-Victorin *et al.* 2000; Jedel *et al.* 2011). Surprisingly, the relative mRNA expression of androgen receptor was not regulated by electrical stimulation in the present study. With the same electrical stimulation protocol, we have previously demonstrated that protein expression of AR in whole hypothalamus is decreased by electrical stimulation (Feng *et al.* 2009). One reasonable explanation for the divergent results is that regulation of AR is a post-translational event.

Manual stimulation of acupuncture needles, in contrast, also improves menstrual function and has regulatory effects on LH, follicle-stimulating hormone and oestradiol in women with undefined ovulatory dysfunction and PCOS (Xiaoming *et al.* 1993; Lai *et al.* 2010). In patients with fibromyalgia, measurement of μ-opioid receptor binding potential revealed regulatory effects of manual acupuncture stimulation (Harris *et al.* 2009). In rats with DHT-induced PCOS, manual stimulation did not affect mRNA expression of opioids or their receptors. Instead, unlike electrical stimulation, it decreased mRNA expression of *Esr2*, *Pgr* and *Kiss1r* in the Arc nucleus. Thus, manual stimulation directly affects steroid hormone receptors. Kisspeptin, which acts through its G protein-coupled receptor, is produced and located in Arc and Avpv nuclei; it is a potent stimulator of GnRH and LH secretion and is involved in the feedback actions of ovarian steroids (Li *et al.* 2009). Mice lacking functional *Kiss1* are infertile, with no oestrous cycle, small ovaries and decreased gonadotrophin secretion (d'Anglemont de Tassigny *et al.* 2007). Most kisspeptin neurons also express *Esr* and *Pgr*, consistent with the hypothetical role of these genes as mediators of steroid feedback (Smith *et al.* 2005, 2006; Franceschini *et al.* 2006), and both are essential for normal oestrous cyclicity (Krege *et al.* 1998). Our results suggest that the effect of manual acupuncture stimulation is mediated through direct regulation of *Esr2*, *Pgr* and *Kiss1r*. To our knowledge, this is the first study to investigate potential mechanisms of manual acupuncture stimulation on reproductive function.

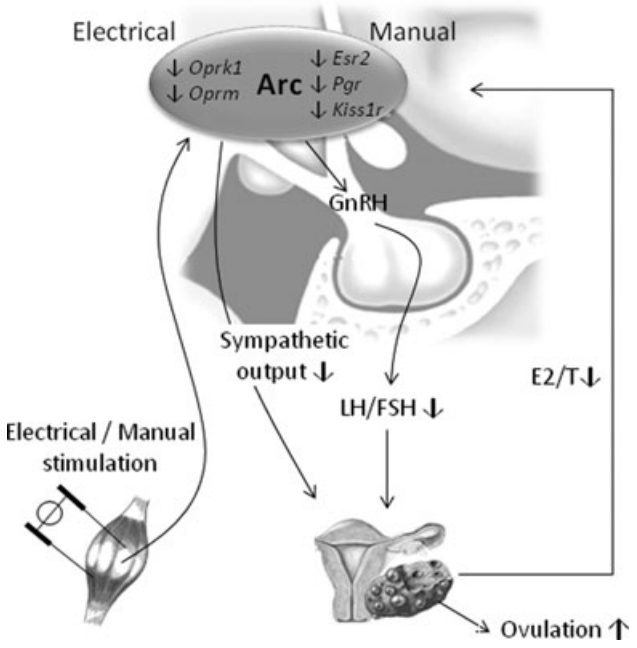

Figure 4. Hypothetical model of how manual and electrical stimulation differentially regulate opioid receptors and steroid hormone receptors and how these changes may affect sympathetic outflow as well as reproductive (oestrous cycle change/ovulation) and endocrine function (circulating gonadotrophins and sex steroids)
Abbreviations: Oprk 1, kappa-opioid receptor 1; Oprm 1, my-opioid receptor 1; Esr2, Estrogen receptor 2; Pgr, Progesterone receptor; Kiss1r, KISS1 receptor; GnRH, gonadotropin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; E2, estradiol; T, testosterone.

Divergent regulatory effects of electrical and manual acupuncture stimulation

In functional magnetic resonance imaging analysis, electrical stimulation produces a more widespread signal increase than manual stimulation (Napadow *et al.* 2005), and electrical and manual stimulation activated different regions of the brain (Napadow *et al.* 2005). Both EA and manual stimulation enhance cell proliferation and neurogenesis in rat hippocampus, although EA has a greater effect (Hwang *et al.* 2010). Thus, low-frequency EA and manual stimulation may induce different responses through different mechanisms. However, it seems that electrical stimulation induces stronger regulatory effects compared with manual stimulation, because when non-responders were excluded, the mRNA expression of *Oprk1* was lower and *Oprm1* tended to be decreased after electrical stimulation. None of the genes regulated by manual stimulation, i.e. *Esr2*, *Pgr* or *Kiss1r*, remained

downregulated when non-responders were excluded. Importantly, as both types of stimulation are frequently applied in the clinic, most often in combination, further investigation is needed.

Methodological considerations

The frequency of electrical stimulation is crucial to the effectiveness of EA treatment (Liang *et al.* 2010). In previous studies, we systematically tested the efficacy of different stimulation frequencies and intensities and needle placements in rats. The optimal ovarian response was received/produced by low-frequency EA at 2 Hz delivered as a 0.1 s, 80 Hz burst pulse (which evokes muscle twitches) with needles in abdominal and hindlimb muscles (Stener-Victorin *et al.* 2003, 2004, 2006). Therefore, we used this stimulation protocol in the present study. In the manual acupuncture group, needles were stimulated three times during each treatment. More frequent manual stimulation might have resulted in a different response on oestrous cyclicity and on hypothalamic mRNA expression of the selected genes. In clinical trials, we suggest a combination of manual and electrical stimulation to be applied, because both seem to have a beneficial effect in the regulation of oestrous cyclicity and thus may potentiate each other.

In conclusion, low-frequency EA restored disturbed oestrous cyclicity but did not differ from the manual stimulation group, although electrical stimulation lowered serum testosterone in responders, those with restored oestrous cyclicity, and differed from both control animals and the manual stimulation group. Thus, EA cannot in all aspects be considered superior to manual stimulation. Even though our findings suggest that the effects of low-frequency EA are mediated by central opioid receptors, whereas the effects of manual stimulation may involve regulation of steroid hormone and peptide receptors, the definitive mechanisms for the effect on the oestrous cycle remain to be elucidated.

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