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Maternal testosterone and placental function: Effect of electroacupuncture on placental expression of angiogenic markers and fetal growth

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ABSTRACT

Women with polycystic ovary syndrome (PCOS) have elevated circulating androgens during pregnancy and are at an increased risk of adverse pregnancy outcomes. Here we tested the hypotheses that maternal androgen excess decrease placental and fetal growth, and placental expression of markers of steroidogenesis, angiogenesis and sympathetic activity, and that acupuncture with low-frequency electrical stimulation prevents these changes. Pregnant rats were exposed to vehicle or testosterone on gestational day (GD)15-19. Low-frequency electroacupuncture (EA) or handling, as a control for the EA procedure, was given to control or testosterone exposed dams on GD16-20. On GD21, blood pressure was measured and maternal blood, fetuses and placentas collected. Placental steroid receptor expression and proteins involved in angiogenic, neurotrophic and adrenergic signaling were analyzed.

EA did not affect any variables in control rats except maternal serum corticosterone, which was reduced. EA in testosterone exposed dams compared with controls increased systolic pressure by 30%, decreased circulating norepinephrine and corticosterone, fetal and placental weight and placental VEGFR1 and proNGF protein expression, and increased the VEGFA/VEGFR1 ratio, mature NGF (mNGF) and the mNGF/proNGF ratio.

In conclusion, low-frequency EA in control animals did not have any negative influence on any of the studied variables. In contrast, EA in pregnant dams exposed to testosterone increased blood pressure and impaired placental growth and function, leading to decreased fetal growth.

1. Introduction

Women with polycystic ovary syndrome (PCOS) have elevated circulating androgens during pregnancy and are at an increased risk of adverse pregnancy outcomes such as gestational diabetes, preterm birth and preeclampsia (Boomsma et al., 2006; Falbo et al., 2010; Qin et al., 2013; Roos et al., 2011). During pregnancy, women with PCOS are more likely to have elevated blood pressure independent of body mass index (BMI) (Hu et al., 2007). They also have impaired uterine artery blood flow, which is related to poor pregnancy outcomes (Falbo et al., 2010; Palomba et al., 2010). This may in turn affect placental development including angiogenesis and steroidogenesis. Women with PCOS are more likely to give birth to small for gestational age (SGA) infants (Boomsma et al., 2006; Falbo et al., 2010; Qin et al., 2013; Mehrabian and Kelishadi, 2012), or large for gestational age (LGA) infants (Roos et al., 2011; Anderson et al., 2010), and infants born to mothers with PCOS have a higher incidence of meconium aspiration, admission to neonatal intensive care unit, and perinatal mortality (Roos et al., 2011). Daughters of women with PCOS have an increased risk of developing a PCOS related phenotype including excessive hair growth, reproductive

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and metabolic derangements in adult life (Sir-Petermann et al., 2007, 2009).

It is unclear whether these alterations are consequences of maternal androgen excess or maternal hyperinsulinemia, which alters the intrauterine milieu and directly affects the fetus, or whether maternal androgen excess alters the placental function and indirectly the fetus. Pregnant women with PCOS have higher circulating androstenedione, testosterone and dehydroepiandrosterone sulfate (DHEAS) levels than pregnant women without PCOS independent of the sex of the fetus (Sir-Petermann et al., 2002). Furthermore, placental 3β-hydroxysteroid dehydrogenase (3β-HSD) type 1 activity is increased and P450 aromatase activity is decreased in mothers with PCOS, suggesting an androgenized intrauterine environment (Maliqueo et al., 2013). In support of this hypothesis, higher umbilical androgen levels have been found in PCOS pregnancies at term compared with controls (Mehrabian and Kelishadi, 2012; Barry et al., 2010), although these differences were not observed in all studies (Hickey et al., 2009; Caanen et al., 2016).

Maternal (prenatal) androgen (PNA) exposure is a well-established model in rodents, sheep, and non-human primates, and their offspring are usually born growth restricted and develop a PCOS-like phenotype including reproductive and metabolic alterations in adult age (Abbott et al., 2008, 2009; Witham et al., 2012; Sullivan and Moenter, 2004; Wu et al., 2010; Birch et al., 2003). In PNA rats, fetal and placental weights are lower (Hu et al., 2015) in both male and females (Sathishkumar et al., 2011). Sathishkumar and coworkers reported that maternal androgen excess caused down-regulation of placental aminoacid transporter expression, decreased placental amino acid transfer (Sathishkumar et al., 2011), reduces the uterine blood flow and affects placental angiogenesis (Gopalakrishnan et al., 2016) which resulted in fetal growth restriction (FGR), without affecting fetal testosterone levels, suggesting that androgen excess have adverse effects on fetal development mediated by changes in placental function (Sathishkumar et al., 2011). Interestingly, gestational hypertension and preeclampsia, conditions related to FGR, may be mediated by increased sympathetic activity and by elevated androgen levels (Carter, 2012). In fact, placental androgen receptor (AR) expression and testosterone concentrations are increased in preeclamptic patients (Sathishkumar et al., 2012), and aromatase expression is decreased (Perez-Sepulveda et al., 2015), suggesting a link between androgens, arterial pressure and FGR. However, if FGR is related to sympathetic activation remains to be elucidated.

There is currently no treatment for pregnancies complicated by FGR. Metformin (Salvesen et al., 2007) and aspirin (Jamal et al., 2012) have been shown to improve uterine artery blood flow from the first to second trimester in women with PCOS without complications (Salvesen et al., 2007), however, without reduction of adverse pregnancy outcomes. Acupuncture with low-frequency electrical stimulation (EA) of the needles activates somatic afferent nerve fibers in skin and muscle (predominantly Aδ and C fibres) and has been shown to modulate efferent autonomic nerve activity to various internal organs through somato-autonomic reflexes (Kimura and Sato, 1997; Uchida et al., 2003). Repeated EA has been shown to decrease high muscle sympathetic nerve activity in women with PCOS (Stener-Victorin et al., 2009), and increase ovarian and uterine artery blood flow in rats and non-pregnant women undergoing *in vitro* fertilization, an effect that was mediated by the sympathetic nerve fibers innervating the ovary (Stener-Victorin et al., 1996, 2003a, 2003b, 2006). Low-frequency EA also restore the protein expression of increased ovarian nerve growth factor (NGF), a modulator of sympathetic nerve activity, and its low-affinity receptor p75NTR, in an estradiol valerate induced PCOS model (Bai et al., 2004; Manni et al., 2005; Stener-Victorin et al., 2000). In normal pregnancies, acupuncture decreases third trimester umbilical artery systolic/diastolic ratio, as measured using Doppler ultrasound, consistent with a decreased vascular resistance (Zeisler et al., 2001).

As maternal androgen excess has a negative impact on the maternal vascular system, placental function and fetal growth and because there is no treatment available for FGR, herein we tested the hypotheses that maternal androgen excess decrease placental and fetal growth, and placental expression of markers of steroidogenesis, angiogenesis and sympathetic activity, and that acupuncture with low-frequency electrical stimulation prevents these changes.

2. Materials and methods

2.1. Animals

Time pregnant Wistar rats (Charles River, Germany) arrived on gestational day (GD) 7. Animals were housed two per cage under a 12 h light/dark cycle, at a temperature of 21–22 °C and 55–65% humidity. They were fed with standard chow (no. 2016; Harlan Winkelmann, Harlan, Germany) and provided with tap water *ad libitum*. All animal procedures were approved by the Animal Ethics Committee of the University of Gothenburg (reference number: 53-2013), in accordance with the legal requirements of the European Community (Decree 86/609/EEC).

2.2. Experimental design and methods

The experimental design including the timing of injections, treatment and measurements is presented in Fig. 1. From GD15 to 19, animals were injected with either testosterone propionate or vehicle. Rats were randomly divided into four groups: control with handling (Ctrl, n = 10), control with low-frequency EA treatment (Ctrl + EA, n = 10), testosterone with handling (Testo, n = 10) and testosterone with acupuncture treatment (Testo + EA, n = 10).

Rats in the Testo and Testo + EA groups received subcutaneous injections (in the interscapular area) of 0.5 mg/kg of testosterone propionate (T1875, Sigma) in a mixture 1:1 of sesame oil (S3547, Sigma) and benzil benzoate (B6630, Sigma) in a total volume of 0.5 ml. Control dams received 0.5 ml of the oil mixture without testosterone.

Low-frequency EA was given daily from GD16 to 20 to conscious rats. Two acupuncture needles, 0.20 mm in diameter and 15 mm in length (HEGU Svenska, Landsbro, Sweden) were inserted into rectus abdominis and two needles were placed perpendicularly into the triceps surae muscle in each leg. Needles in the abdominal and hindlimb muscles on each side were connected to electrodes attached to an electrical stimulator (CEFAR ACU II; Cefar-Compex Scandinavia, Malmo, Sweden) and stimulated at 2 Hz frequency with 0.1-sec, 80-Hz burst pulses to induce visible muscle contraction for 15 min on the first day and 20 min the following days. The intensity varied from 0.7 to 1.4 mA during stimulation and was adjusted to produce local muscle contractions. The locations of the acupuncture needles are in somatic segments corresponding to the ovaries and uterus (i.e., from spinal levels T10 to L2 and at the sacral level). This acupuncture protocol and stimulation paradigm has been used in previous experimental studies without causing and unfavorable effects including stress (Maliqueo et al., 2015; Manneras et al., 2008; Johansson et al., 2013; Feng et al., 2012).

The control for EA treatment was a handling procedure without needle insertion. All rats, with or without testosterone treatment and with or without acupuncture treatment were handled in the same way to control for environmental factors. Rats were conscious and secured in a fabric harness and suspended above the desk. This control situation was selected because there are no existing inert

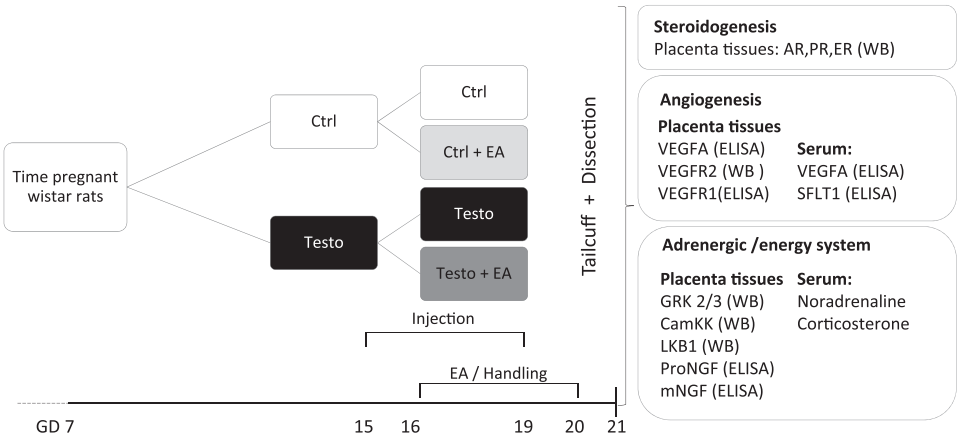

Fig. 1. Experimental design, time line and outcome measures. Time pregnant Wistar rats arrived at day 7 of pregnancy. At gestational day (GD) 15, they were randomized into 2 groups and received a daily subcutaneous injection of 0.5 mg/kg testosterone propionate or vehicle (control) until GD 19. At GD 16 dams were assigned to receive daily acupuncture treatment or handling until GD 20. Arterial blood pressure (tail cuff) was measured before dissection at GD 21. Blood was collected and tissues dissected and snap frozen in liquid nitrogen and stored in −80C until analyses by ELISA or western blot (WB) related to steroidogenesis, angiogenesis and adrenergic system.

sham acupuncture techniques (MacPherson et al., 2016). As soon as a needle is inserted (e.g. superficial, in wrong site) it activates sensory afferent nerve fibers and is therefore not an adequate control.

At GD21, dams were anesthetized with isoflurane at 2–4% and blood pressure was measured using tail-cuff plethysmography (Harvard Apparatus, Holliston, MA, USA). Subsequently, laparotomy was performed and maternal blood was collected by cardiac puncture, centrifuged and stored as serum and plasma at −80 for later analyses.

2.3. Fetal, placental and maternal tissue collection

Each fetus with its placenta was dissected, separated from the fetal membranes and umbilical cord, and weighed. The tail of each fetus was collected for sex determination by PCR. One third of each placenta from each dam was pooled and immediately washed in ice cold Tris-Saline buffer (10 mM Tris-HEPES, 154 mM NaCl) and homogenized on ice in buffer D (10 mM Tris-HEPES), 250 mM sucrose, 1 mM EDTA, and protease and phosphatase cocktail inhibitors (cat P8340, P5276 and P0044, Sigma) using a polytron. Aliquots were snap frozen and stored to −80 °C. Maternal adrenal glands, kidneys and liver were dissected, weighed, and snap frozen in liquid nitrogen and stored at −80 °C.

2.4. Analytical methods

Norepinephrine was measured by high pressure liquid chromatography (HPLC) with electrochemical (EC) detection, reference interval 0.18–2.36 nmol/L. Corticosterone (Cat, 80554, Crystal Chem) and sFLT1 (Cat, RF0110, Neobiolab) were measured in serum with intra-assay coefficients of variability (CV) of 7.1 and 12.8%, respectively. VEGFA (cat, RAB0511-1 KT, sigma Aldrich) and VEGF receptor 1 (VEGFR1, cat RV0026, Neobiolab) were measured in placental homogenates by enzyme-linked immunosorbent assay (ELISA) with CV of 7.4 and 8.9%. Moreover, VEGFA was measured in placenta homogenates with a CV of 7.4%, while Testosterone (Crystal Chem, Cat #80550) had a CV 8.4%. Mature NGF (mNGF) and proNGF were detected and quantified using MAB5260Z clone 27/21 and EP1318Y antibodies (Merck Millipore) respectively, using a homemade sandwich ELISA as previously described (Soligo et al., 2015). The CV for mNGF was 15.8% and for proNGF was 15.7%.

2.5. Sex genotyping

Each fetus and its corresponding placenta were weighed and data recorded. Because the placenta, as a product of conception, has the same sex as the fetus, a piece of the tail from each fetus was collected and DNA extracted (Promega, cat A2361), and conventional PCR using HotStartTaq® Master Mix Kit (Qiagen, 203445) was performed to amplify the sex-determining region Y (SRY) gene to identify the sex of each fetus and placenta. The PCR primers used for amplification were: forward 5′-CACAAGTTGGCTCAACAGAATC-3′ and reverse 5′-AGCTCTACTCCAGTCTTGTCCG-3′.

2.6. Western blot

Briefly, placental homogenates were centrifuged at 13,000 g for 10 min at 4 °C, the supernatant was collected and protein concentration was measured with a spectrometer (Direct detect, Millipore, USA). 30 μg of total protein was loaded in Criterion™ TGX (Tris-Glycine eXtended) Stain-Free™ precast gels (Biorad). The gel was activated with the ChemiDoc MP imager (Biorad) and transferred to polyvinylidene difluoride membranes (PVDF) in the turbo system Trans-Blot turbo transfer (Biorad). Thereafter, membranes were blocked and incubated with primary antibody (Table 1). The protein expression in each sample was normalized to total amount of loaded protein in each sample using Image Lab 5.0 (Biorad).

Western blot analysis for proNGF, $p75^{NTR}$, phospho-TrkA, phospho-Akt (Ser473) and phospho-JNK was performed on 6 tissue samples from each group, as follows: samples (30 μg of total protein) were separated by 8% or 12% SDS-PAGE, and electrophoretically transferred to PVDF membrane overnight. Thereafter, membranes were blocked and incubated with specific antibodies (Table 1). After washing with TBS-T, membranes were incubated 1 h with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody (Cell Signaling Technology, MA, USA) at room temperature. The blots were developed with an ECL chemiluminescent as the chromophore (Millipore, MA, USA). The GAPDH bands were used as a control for equal protein loading. The public domain Image J software (http://rsb.info.nih.gov/ij/) was used for gel densitometry and protein quantification following the method described at http://lukemiller.org/index.php/2010/11/analyzing-gels-and-western-blots-with-image-j/.The integrated density of GAPDH bands was used as normalizing factor.

Table 1
Antibodies and dilutions used in western blot.

	Manufacturer	Catalog number	Dilution
LKB1	Abcam	Ab50183	1:1500
CamkkIIα	R&D System	AF4536	1:800
GRK2/3	Santa Cruz Biotechnology	sc8329	1:500
VEGFR2	Cell Signaling Technology	2479	1:500
Progesterone receptor A-B	Santa Cruz Biotechnology	sc539	1:500
Estrogen receptor α	Santa Cruz Biotechnology	sc542	1:500
Estrogen receptor β	Santa Cruz Biotechnology	sc8974	1:1000
Androgen receptor	Santa Cruz Biotechnology	sc816	1:200
proNGF/mNGF	Santa Cruz Biotechnology	sc548	1:500
Phosphorylated TrkA	Santa Cruz Biotechnology	sc8058	1:1000
p75 neurotrophin receptor	Santa Cruz Biotechnology	sc53631	1:500
Phospho-Akt (Ser473)	Cell Signaling Technology	9271	1:1000
Phospho-JNK	Cell Signaling Technology	9255	1:500

LKB1: Liver kinase B1; GRK: G protein-coupled receptor kinases 2/3; CaMKKIIα: Calcium/calmodulin-dependent protein kinase kinase; VEGFR2: Vascular endothelial growth factor receptor 2; TrkA: Tyrosine-kinase receptor A; JNK: c-jun N-terminal kinase.

2.7. Statistical analysis

Data is presented as mean ± SEM and was analyzed with SPSS version 21.0 (SPSS, Chicago, IL, USA) and Prism GraphPad version 6.0 (GraphPad Software, La Jolla, CA, USA). Distribution of data was tested by Shapiro-Wilk test. As most variables were not normally distributed we used the Kruskal Wallis test followed by the Mann Whitney *U* test for group comparisons. Correlation was assessed using the Spearman's rank correlation test. *P*-values < 0.05 were considered statistically significant.

3. Results

3.1. Maternal and fetal variables

Maternal body weight gain did not differ between the groups at any time point (Table 2). There were no differences in organ weights between testosterone-treated dams and controls. Adrenal weight was greater in the testosterone group receiving EA compared with control dams, with no effect of EA in control dams (Table 2).

Systolic blood pressure did not differ between testosterone-treated dams and controls (Fig. 2A). EA in testosterone-treated dams resulted in higher systolic blood pressure compared with controls at GD21 with no effect of EA in control dams (Fig. 2A). Circulating level of testosterone was higher in testosterone injected dams treated with or without EA compared with animals injected with vehicle (Fig. 2B). Plasma norepinephrine did not differ between testosterone-treated dams and controls (Fig. 2C). EA in the testosterone-treated dams resulted in a lower circulating norepinephrine compared with controls and testosterone treated dams ($P < 0.05$). EA significantly decreased corticosterone levels in control animals and in those injected with testosterone compared with control dams ($P < 0.05$) (Fig. 2D).

Litter size did not differ between the groups. Although lower, maternal testosterone exposure did not significantly affect fetal and placental weights compared with controls (Table 3). In contrast, placental and fetal weights were decreased in androgenized dams treated with EA as compared with the control group ($P < 0.05$), with no effect of EA in control dams (Table 3). There were no significant differences in fetal or placental weight between testosterone-treated dams and controls when male and female fetuses were studied separately (Table 3). The weight of female fetuses from testosterone-treated dams receiving EA was lower compared with the controls, while EA did not affect the weight of female fetuses in controls ($P < 0.05$). EA did not affect male fetal and placental weight in control or testosterone-treated dams (Table 3). The ratio of fetal to placental weight (a crude proxy for placental efficiency) did not differ between the groups.

3.2. Markers of placental angiogenesis

Maternal testosterone exposure with or without EA treatment did not affect circulating levels of VEGFA, sFLT1 or VEGF/sFLT1 ratio (Fig. 3A-C). Maternal testosterone exposure did not affect placental expression of VEGFA, VEGFR1 or VEGFA/VEGFR1 ratio (Fig. 3D-F). VEGFR1 expression was lower in placentas from dams injected with testosterone and treated with EA compared with controls (Fig. 3E). VEGFA/VEGFR1 ratio in the placenta was higher in testosterone-treated dams receiving EA than in controls and in testosterone

Table 2
Maternal body and tissue weights.

	Control (n = 9)	Testosterone (n = 9)	Ctrl + EA (n = 9)	Testo + EA (n = 8)	Ctrl vs testo	Ctrl vs ctrl + EA	Testo vs testo + EA	Testo + EA vs Ctrl + EA	Ctrl vs testo + EA
Increment of Weight (%)	24.33 ± 1.63	24.88 ± 1.08	26.21 ± 1.57	23.82 ± 1.01	ns	ns	ns	ns	ns
Ovaries (mg)	131.20 ± 16.34	125.41 ± 8.77	132.63 ± 22.10	161.15 ± 18.54	ns	ns	ns	ns	ns
Adrenal (mg)	70.10 ± 5.03	78.29 ± 6.73	72.62 ± 4.52	87.71 ± 6.05	ns	ns	ns	<0.05	<0.05
Liver (gr)	14.18 ± 0.92	15.12 ± 0.60	14.33 ± 0.65	14.85 ± 0.70	ns	ns	ns	ns	ns
Kidneys (gr)	1.91 ± 0.25	1.88 ± 0.08	1.75 ± 0.09	1.97 ± 0.05	ns	ns	ns	ns	ns
Mesenteric (gr)	1.97 ± 0.15	1.99 ± 0.15	2.08 ± 0.22	1.96 ± 0.28	ns	ns	ns	ns	ns
Inguinal (gr)	2.38 ± 0.31	2.35 ± 0.29	1.69 ± 0.23	2.08 ± 0.27	ns	ns	ns	ns	ns
Retroperitoneal (gr)	1.68 ± 0.19	2.23 ± 0.41	1.96 ± 0.34	2.37 ± 0.64	ns	ns	ns	ns	ns

Values are shown as mean ± SEM.
BW, body weight; Ctrl, Control group; Testo, Testosterone group; EA, electroacupuncture; Mann Whitney test; ns: non-significant.

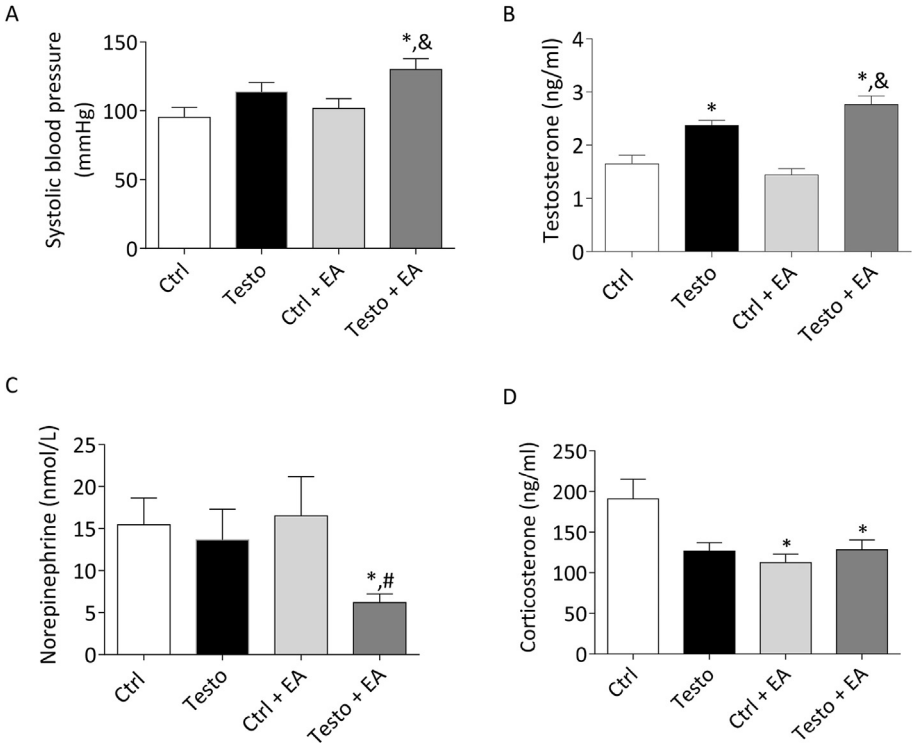

Fig. 2. Systolic blood pressure, circulating norepinephrine and serum corticosterone. (A) Systolic blood pressure measured by tail cuff at GD 21 (Ctrl, n = 9; Testo, n = 9; Ctrl + EA, n = 9; Testo + EA, n = 8.). (B) Serum testosterone (Ctrl, n = 5; Testo, n = 6; Ctrl + EA, n = 7; Testo + EA, n = 8). (C) Plasma norepinephrine (Ctrl, n = 9; Testo, n = 9; Ctrl + EA, n = 9; Testo + EA, n = 8). (D) Serum corticosterone (Ctrl, n = 7; Testo, n = 8; Ctrl + EA, n = 8; Testo + EA, n = 8). Values are means ± SEM. *$P < 0.05$ vs Ctrl, #$P < 0.05$, vs Testo, & $P < 0.05$, vs Ctrl + EA group analyzed by Kruskal Wallis followed by Mann-Whitney *U* test.

Table 3
Fetal and placental data.

	Ctrl (n = 9)	Testo (n = 9)	Ctrl + EA (n = 9)	Testo + EA (n = 8)	Ctrl vs testo	Ctrl vs Ctrl + EA	Testo vs testo + EA	Testo + EA vs Ctrl + EA	Ctrl vs Testo + EA
Whole litter									
Female/male ratio n, (% Females)	39/46 (45)	57/43 (57)	54/43 (46)	50/52 (49)	ns	ns	ns	ns	ns
Litter size (mean)	11.2 ± 1.0	11.3 ± 0.7	13.1 ± 0.5	13.0 ± 0.7	ns	ns	ns	ns	ns
Weight fetus (gr)	4.26 ± 0.15	4.09 ± 0.16	4.23 ± 0.16	3.87 ± 0.12	ns	ns	ns	ns	< 0.05
Weight placenta (gr)	0.56 ± 0.02	0.54 ± 0.02	0.54 ± 0.02	0.51 ± 0.01	ns	ns	ns	ns	< 0.05
Weight fetus/placenta (AU)	8.04 ± 0.55	7.76 ± 0.41	8.08 ± 0.46	7.70 ± 0.22	ns	ns	ns	ns	ns
Female fetus and placenta weight									
Weight fetus (gr)	4.26 ± 0.16	3.97 ± 0.17	4.09 ± 0.15	3.78 ± 0.14	ns	ns	ns	ns	< 0.05
Weight placenta (gr)	0.56 ± 0.02	0.54 ± 0.02	0.53 ± 0.03	0.51 ± 0.02	ns	ns	ns	ns	< 0.05
Weight fetus/placenta (AU)	7.69 ± 0.47	7.51 ± 0.44	8.00 ± 0.50	7.58 ± 0.30	ns	ns	ns	ns	ns
Male fetus and placenta weight									
Weight fetus (gr)	4.30 ± 0.18	4.23 ± 0.14	4.32 ± 0.16	3.98 ± 0.10	ns	ns	ns	ns	ns
Weight placenta (gr)	0.56 ± 0.02	0.54 ± 0.02	0.54 ± 0.02	0.51 ± 0.00	ns	ns	ns	ns	ns
Weight fetus/placenta (AU)	7.78 ± 0.51	7.98 ± 0.40	8.13 ± 0.44	7.84 ± 0.17	ns	ns	ns	ns	ns

Values are shown as mean ± SEM. Ctrl, Control group; Testo, Testosterone group; EA, Electroacupuncture treatment; Mann Whitney test; ns: non-significant.

injected animals not receiving EA (Fig. 3F). Neither placental VEGFR2 expression (Fig. 3G) nor VEGFA/VEGFR2 ratio (Fig. 3H) differed between the groups.

3.3. Steroidogenesis

Placental androgen receptor (AR) expression tended ($P = 0.07$) to be lower in testosterone-treated dams than in controls (Fig. 4A), with no effect of EA. The placental protein expression of progesterone receptors (PR) A and B and estrogen receptor (ER) α and β did not differ between the groups (Fig. 4B-E).

3.4. Nerve growth factor, a marker of sympathetic activity

Placental proNGF expression, measured by ELISA, did not differ in testosterone-treated dams compared with controls (Fig. 5A). EA did not change proNGF expression in controls but lowered proNGF expression in the placenta of testosterone-treated dams compared with testosterone-treated control dams (Fig. 5A). Mature nerve growth factor (mNGF) expression in placenta did not differ between testosterone-treated dams and controls (Fig. 5B). While EA did not affect placental expression of mNGF in controls, EA increased mNGF in testosterone-treated dams compared with controls ($P < 0.05$). The ratio of placental mNGF/proNGF expression

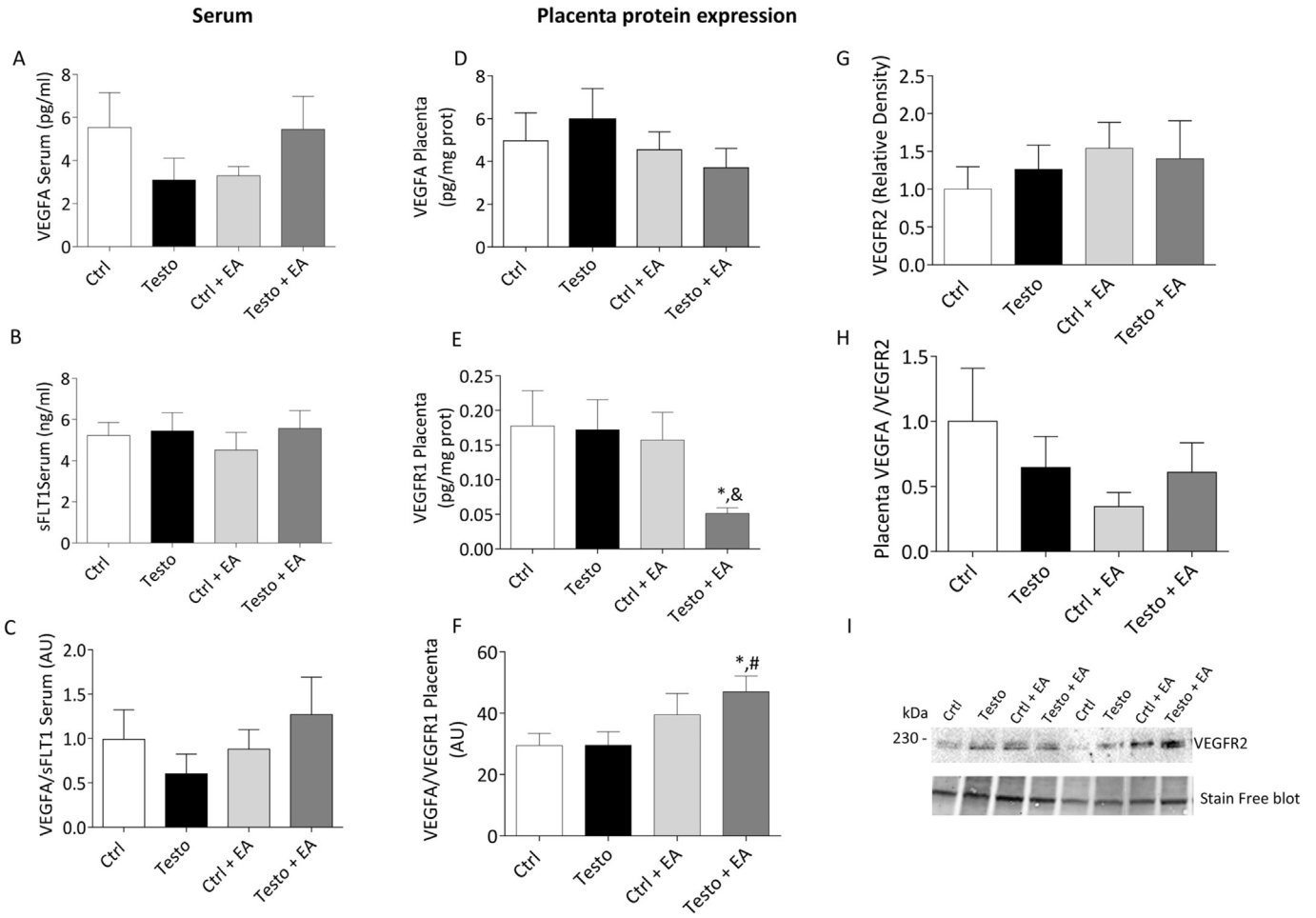

Fig. 3. Angiogenic markers in serum and placenta homogenates at GD 21. Serum VEGFA (A) and sFLt1 (B) and ratio of VEGFA/sFLT1 (C) in Ctrl (n = 7–8), Testo (n = 8), Ctrl + EA (n = 6–8) and Testo + EA (n = 5–8) group. Placenta protein expression of VEGFA (D), VEGFR1 (E) and ratio of VEFGA/VEGFR1 (F), VEGFR2 (G), and ratio VEGFA/VEGFR2 (H) in Ctrl (n = 7–9), Testo (n = 7–9), Ctrl + EA (n = 6–9) and Testo + EA (n = 5–8). Representative blot of VEGFR2 (I). Values are means ± SEM. *$P < 0.05$ vs Ctrl, #$P = 0.05$ vs Testo, & $P < 0.05$ vs Ctrl + EA group analyzed by Kruskal Wallis followed by Mann-Whitney test.

was not affected in androgenized dams compared with controls (Fig. 5C). EA increased the mNGF/proNGF ratio in testosterone-treated dams compared with the control and testosterone groups ($P < 0.05$), with no effects in controls dams (Fig. 5C). Next we determined the expression of different proNGF isoforms and found that they are differently modulated in response to experimental treatments (Fig. 5D-E). The proNGF-A splice variant (34 kDa) and proNGF-B splice variant (25 kDa) were not affected, neither in testosterone-treated dams compared with controls, nor EA or EA in combination with testosterone (Fig. 5D-E). The activated (phosphorylated) form of the ProNGF/mNGF receptor TrkA did not change by either testosterone or acupuncture (Fig. 5F). The proNGF receptor $p75^{NTR}$ was not changed in testosterone-treated dams compared with controls (Fig. 5G). Neither testosterone nor EA changed $p75^{NTR}$ protein levels in the placenta. The serine-473 phosphorylation of the intracellular signaling factor Akt, activated after TrkA phosphorylation, was not affected by testosterone or EA (Fig. 5H). The phosphorylation of the JNK signaling molecule, activated by the challenge of $p75^{NTR}$ by proNGF (Al-Gayyar et al., 2013), was increased in testosterone-treated dams compared with controls (Fig. 5I). EA lowered JNK phosporylation in the placenta of testosterone-treated dams compared with testosterone-treated dams (Fig. 5I). Protein expression of the energy sensor, liver kinase B1 (LKB1), the G protein-coupled receptor kinases (GRK)2/3 and Calcium/calmodulin-dependent protein kinase kinase (CaMKK)IIα (Fig. 6A-C) was not affected by testosterone or EA treatment compared with controls.

4. Discussion

The novel finding of this study is that low-frequency EA in pregnant rats exposed to testosterone, contrary to our hypothesis, resulted in lower fetal and placental weight and elevated maternal blood pressure. This effect may be mediated via activation of the sympathetic nervous system, as indicated by changes in the expression of proteins that are well-established readouts of adrenergic activity. Of note, EA did not affect any variables in pregnant control rats.

Women with PCOS commonly give birth to either small (Boomsma et al., 2006; Qin et al., 2013; Mehrabian and Kelishadi, 2012; Doherty et al., 2015) or large (Roos et al., 2011; Anderson et al., 2010) babies for gestational age. Daughters of mothers with PCOS are at increased risk of developing reproductive and metabolic features of PCOS in later life (Pandolfi et al., 2008). Also, sons of mothers with PCOS are more prone to develop metabolic alterations in adulthood (Recabarren et al., 2008). Therefore it is of importance to investigate the underlying mechanism and the effect of new treatment strategies.

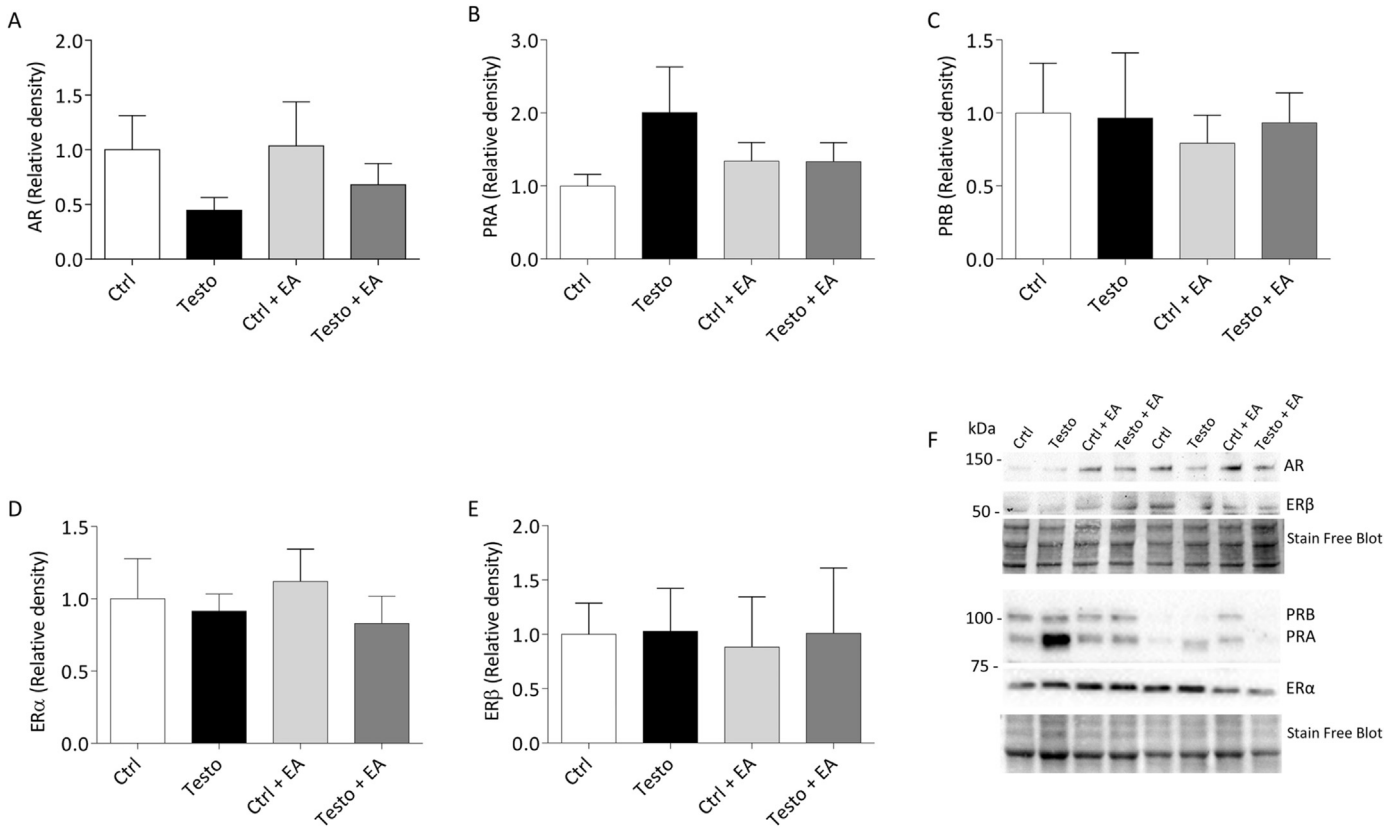

Fig. 4. Protein expression of sex steroid receptors in placenta homogenates at GD 21. Androgen receptor (AR) (A), Progesterone receptor A (PRA) (B), Progesterone receptor B (PRB) (C), Estrogen receptor (ER)α (D), and ERβ (E) in placenta homogenates from pregnant rats. Representative blot of each protein (F). Ctrl (n = 8); Testo (n = 7–9); Ctrl + EA (n = 8–9); Testo + EA (n = 7–8) at day 21. Protein expression was quantified by western blot and normalized by total protein loaded in each line in the stain free blot. AR and ERβ were probed in the same blot. PRA, PRB and ERα were probed in a second blot. Both Stain free blot are shown. Values are means ± SEM analyzed by Kruskal Wallis and Mann-Whitney test.

Fetal and placental weight in this study was not significantly affected by maternal testosterone exposure. The lack of effect on placenta weight is unexpected as we recently have demonstrated lower placental weights in the same model (Hu et al., 2015). On the other hand, we demonstrate that female fetuses seem to be more vulnerable for androgen exposure as demonstrated by lower (non-significant) fetal weight in testosterone-treated dams. A previous study using a dose ten times higher (5 mg/kg) resulted in lower birth weight and smaller placental size (Sun et al., 2012). In order to model the two-fold increased level of circulating testosterone in pregnant women with PCOS, we used a ten times lower dose of testosterone in the present study (0.5 mg/kg). This dose has been used in previous studies and resulted in fetal growth restriction (Sathishkumar et al., 2011; Chinnathambi et al., 2014). Despite this we did not find any significant effect of maternal androgen exposure on fetal and placental growth. One plausible explanation may be that the number of animals was to low or that inbred Sprague Dawley rats were used previously (Sathishkumar et al., 2011; Chinnathambi et al., 2014), whereas we here used outbred Wistar rats.

As there are no effective treatments for FGR and because low-frequency EA has been shown to increase uterine and ovarian blood flow, we therefore tested the hypothesis that EA improves fetal and placental growth in pregnancies with maternal androgen excess, which has been associated with FGR. Of note is the finding that EA in control animals did not affect any variables. On the other hand, EA given to pregnant dams exposed to testosterone resulted in decreased fetal and placental weights, an effect that was more pronounced among female fetuses and their placentas than in male fetuses. This suggests that there is a negative interaction, probably sex dependent, between maternal testosterone exposure and acupuncture on fetal growth. In this regard, it has previously been suggested that female fetuses may be more sensitive to an adverse intrauterine environment (Frank et al., 2014).

In both controls and testosterone exposed dams EA resulted in lower circulating corticosterone concentrations. These findings suggest that, the adverse effect of low-frequency EA in testosterone exposed pregnant dams is not mediated by maternal stress, which is in line with previous acupuncture studies in non-pregnant female rats (Tripathi et al., 2008; Jarvis et al., 2012). EA have been used in a large number of experimental and clinical settings without any negative side effects (Usselman et al., 2015; Brauer, 2008; Toti et al., 2006; Tometten et al., 2004; MacGrogan et al., 1992).

Maternal testosterone exposure alone did not affect systolic arterial blood pressure. However, EA increased systolic arterial blood pressure in testosterone exposed dams, despite having no impact on controls. This effect seemed to be independent of chronic stress as demonstrated by the low, rather than high, levels of plasma corticosterone. It is well known that there is increased sympathetic activity during EA stimulation, but this is followed by a depression of sympathetic activity post-treatment (Manneras et al., 2008; Johansson et al., 2013; Lobos et al., 2005). Blood pressure was measured one day after the last EA and a potential increase in sympathetic activity due to stimulation seems unlikely. Further, circulating norepinephrine was significantly lower in EA-treated

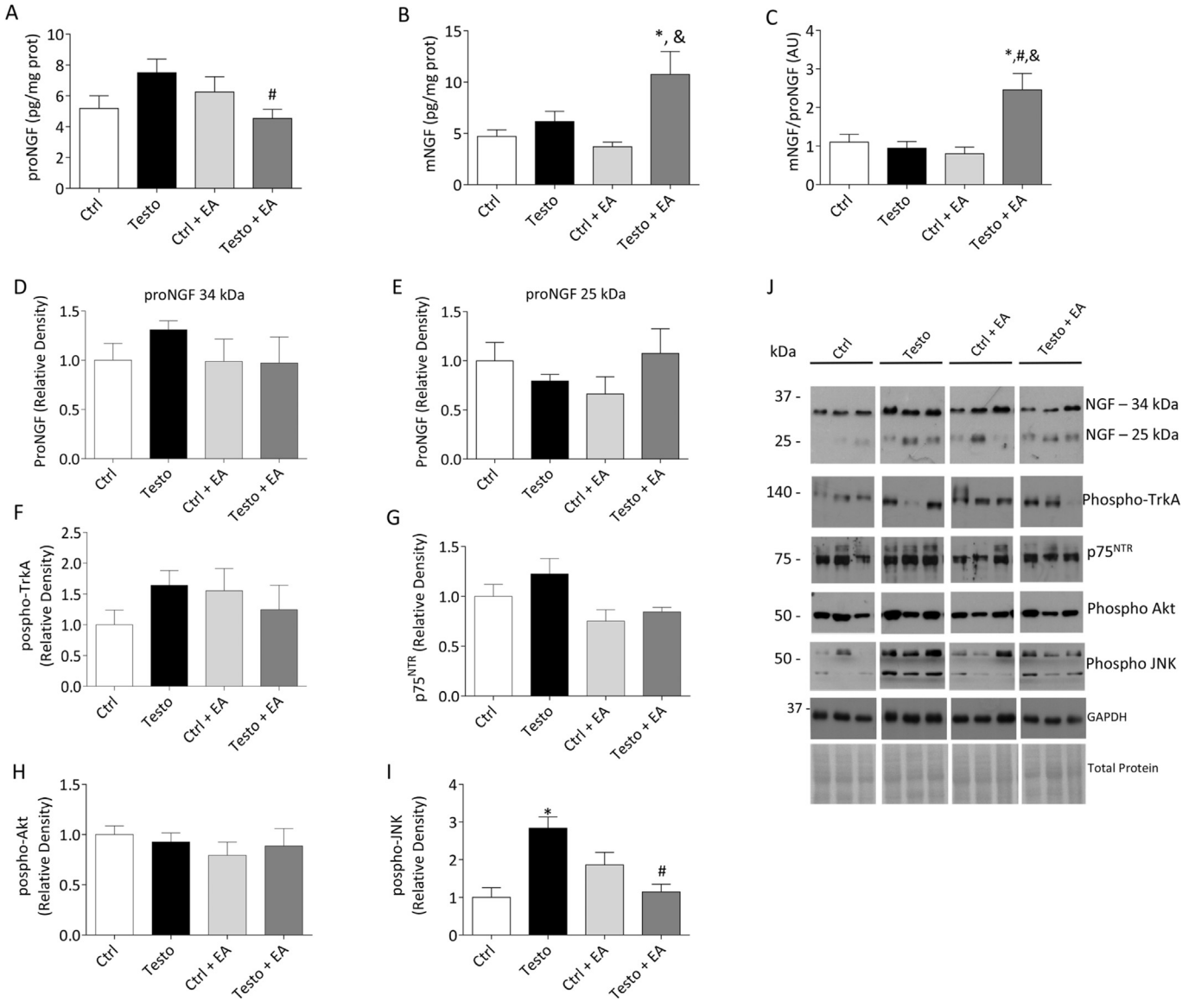

Fig. 5. ProNGF, mature NGF, and proNGF splicing variants and downstream signaling in placenta homogenates at GD 21. Protein expression of pro nerve growth factor (NGF) (A), matureNGF (B), and ratio mNGF/proNGF (C) in Ctrl (n = 9), Testo (n = 8–9), Ctrl + EA (n = 8–9) and Testo + EA (n = 8). Protein expression of splicing variant of proNGF-A (34 kDa) (D), and proNGF-B (25 kDa) (E), phosphorylated NGF receptor TrkA (phospho-TrkA) (F), phosphorylated Akt (Ser473) (downstream TrkA) (G), neurotrophin receptor p75 ($p75^{NTR}$) (H), and phosphorylated-JNK (downstream $p75^{NT}$) (I) in Ctrl (n = 6), Testo (n = 6), Ctrl + EA (n = 6) and Testo + EA (n = 6). (J) Representative blot of each protein. All the proteins were probed in the same membranes which were stripped and reprobed for analyzing different proteins. Protein expression was quantified by western blot and normalized by GAPDH. The red ponceau stain for the blot is shown. Values are means ± SEM, *$P < 0.05$ vs Ctrl, #$P < 0.05$ vs Testo, & $P < 0.05$ vs Ctrl + EA group analyzed by Kruskal Wallis and Mann-Whitney test.

testosterone-exposed rats during pregnancy.

FGR is a common consequence of the hypertensive disorders of pregnancy, including pre-eclampsia. Of note, elevated circulating testosterone and increased placental AR expression have been described in pre-eclamptic women irrespective of fetal sex (Stener-Victorin et al., 2009), suggesting a link between androgens, high arterial pressure and poor pregnancy outcomes (Sharifzadeh et al., 2012). Interestingly, PCOS is associated with elevated blood pressure independent of BMI during pregnancy (Hu et al., 2007). Uterine artery blood flow is commonly compromised in these patients and has been related to adverse perinatal outcomes (Falbo et al., 2010). FGR, altered placentation (Palomba et al., 2014) and lower placental aromatase expression in preeclampsia (Perez-Sepulveda et al., 2015) suggest that it is characterized by local hyperandrogenism at a placental level that may alter placental function.

Poor placentation and altered angiogenesis in preeclamptic patients (Cerdeira and Karumanchi, 2012) are related to decreased circulating VEGF (Hentges et al., 2015) and a concomitant increase in sFlt-1 (Tripathi et al., 2008), which indicates a lower availability of angiogenic factors. VEGFA is the most recognized regulator of vasculogenesis and angiogenesis in the placenta and binds to VEGFR1 or VEGFR2 as well as s-Flt1, the truncated and soluble form of VEGFR1, which has an antagonistic effect on VEGF (Cerdeira and Karumanchi, 2012). In this study neither maternal testosterone exposure nor EA in control animals affected serum or placental VEGFA expression or serum sFlt1. The fact that EA in testosterone exposed dams resulted in lower placental VEGFR1 expression and a higher VEGFA/VEFGR1 ratio is consistent with impaired angiogenesis, which may contribute to FGR.

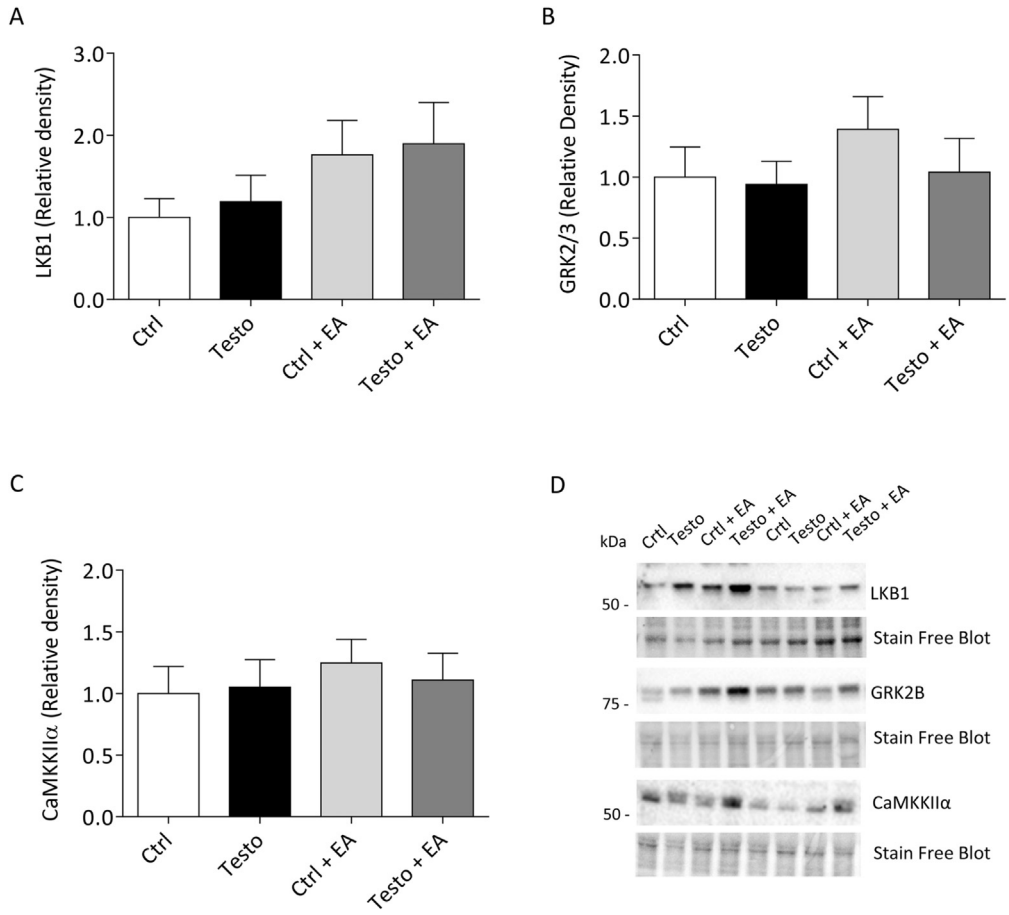

Fig. 6. Markers of adrenergic signaling pathways molecules in placenta homogenates at GD 21. Protein expression of expression of energy cell sensor LKB1 (A) and downstream adrenergic receptor molecules GRK 2/3 (B) and CaMKKIIα (C). Representative blot of each protein (D). In Ctrl (n = 9), Testo (n = 9), Ctrl + EA (n = 9) and Testo + EA (n = 8). Protein expression was quantified by western blot and normalized by total protein loaded in each line in the stain free blot. Values are means ± SEM analyzed by Kruskal Wallis and Mann-Whitney test.

There is evidence of sympathetic activation in early pregnancy (Jarvis et al., 2012). Furthermore, normal pregnant women in the third trimester display exacerbated sympathoexcitation compared with non-pregnant women in response to a cold pressor test (Usselman et al., 2015). Despite increased sympathetic discharge ability, nerve fibers in the pregnant uterus degenerates during pregnancy and are partially regenerated after delivery, concomitant with a decreased level of norepinephrine in the uterus (Brauer, 2008). In the present study we found that EA decreased circulating norepinephrine in testosterone-exposed dams with no effect in controls. However, how catecholamines affect placental function is largely unknown. Here we studied GRK2/3 and CamKKII α, which acts downstream of adrenergic receptors. Neither maternal testosterone exposure nor EA affected the expression of these proteins, indicating that norepinephrine does not exert its action directly in the placenta.

As androgen excess has been shown to increase sympathetic nerve activity and because the effect of EA is, at least in part, mediated via the sympathetic nervous system, we next investigated the expression of placental NGF, a marker of sympathetic activity. In the placenta, NGF is present in cytotrophoblast and syncytiotrophoblast cells as well as the extravillous trophoblast cells in the inner layer of the maternal vessels within the decidual component, but not in fetal vessels in the first and third trimesters (Toti et al., 2006). NGF receptors TrkA and $p75^{NTR}$ have also been identified in placental cells (Tometten et al., 2004; MacGrogan et al., 1992) and a role for the NGF system in placentation and the regulation of the fetal-maternal interface has been postulated (Frank et al., 2014). The precursor of NGF, proNGF, represents the most highly expressed form of NGF in peripheral tissues (Bierl et al., 2005). It could exert either a pro-survival, trophic action or a pro-apoptotic effect, depending on the relative levels of its receptor systems, TrkA/$p75^{NTR}$ or $p75^{NTR}$-sortilin complex respectively (Hempstead, 2014). On the other hand, mNGF regulates survival of nerve cells and acts via TrkA/$p75^{NTR}$ receptors (Levi-Montalcini, 1987). The specific action of proNGF and mNGF in the placenta has not previously been reported. Nevertheless, in the rat uterus, proNGF is augmented from GD7 and decreased after day 1 post-partum, suggesting that its activity is related to the presence and activity of nerves in the uterus (Lobos et al., 2005). Here we demonstrate a decrease in proNGF, an increase in mNGF, and an increased mNGF/proNGF ratio in the placenta of dams injected with testosterone and treated with EA. Moreover, our data suggest the ability of EA in reducing the JNK signaling that is possibly activated by proNGF, but also in increasing the ratio of mNGF/proNGF after testosterone. This suggest a possible modulation of both placental cell functions and uterine innervation by EA which in turn could modulate utero-placental blood.

A large number of comparisons has been done without corrections for multiple comparisons with a risk of rendering some findings to be not significant. However, many of the independent analyses points towards the same direction that EA activate

autonomic tone and that this is only evident in testosterone exposed dams.

In conclusion, low-frequency EA in control animals did not have any negative influence on any of the studied variables. In contrast, EA in pregnant dams exposed to testosterone increased blood pressure and impaired placental growth and function, leading to decreased fetal growth.

Competing financial interest

The authors declare no competing financial interest.

Author contributions

R.F., M.H., M.M., A.B., D.C., H.B., T.J., and E.SV conceived and designed the study. R.F., M.H., M.M., T.J., L.M., and M.K. performed experiments and statistical analyses. R.F., M.H., M.M., A.B., D.C., H.B., T.J., L.M., and E.SV. contributed to the analysis and interpretation of data. R.F. and E.S.-V. wrote the manuscript. All of the authors revised critically and approved the manuscript.

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References

Abbott, D.H., Barnett, D.K., Levine, J.E., Padmanabhan, V., Dumesic, D.A., Jacoris, S., Tarantal, A.F., 2008. Endocrine antecedents of polycystic ovary syndrome in fetal and infant prenatally androgenized female rhesus monkeys. Biol. Reprod. 79 (1), 154–163.

Abbott, D.H., Tarantal, A.F., Dumesic, D.A., 2009. Fetal, infant, adolescent and adult phenotypes of polycystic ovary syndrome in prenatally androgenized female rhesus monkeys. Am. J. Primatol. 71 (9), 776–784.

Al-Gayyar, M.M., Mysona, B.A., Matragoon, S., Abdelsaid, M.A., El-Azab, M.F., Shanab, A.Y., Ha, Y., Smith, S.B., Bollinger, K.E., El-Remessy, A.B., 2013. Diabetes and overexpression of proNGF cause retinal neurodegeneration via activation of RhoA pathway. PLoS One. 8 (1), e54692.

Anderson, H., Fogel, N., Grebe, S.K., Singh, R.J., Taylor, R.L., Dunaif, A., 2010. Infants of women with polycystic ovary syndrome have lower cord blood androstenedione and estradiol levels. J. Clin. Endocrinol. Metab. 95 (5), 2180–2186.

Bai, Y.H., Lim, S.C., Song, C.H., Bae, C.S., Jin, C.S., Choi, B.C., Jang, C.H., Lee, S.H., Pak, S.C., 2004. Electro-acupuncture reverses nerve growth factor abundance in experimental polycystic ovaries in the rat. Gynecol. Obstet. Invest. 57 (2), 80–85.

Barry, J.A., Kay, A.R., Navaratnarajah, R., Iqbal, S., Bamfo, J.E., David, A.L., Hines, M., Hardiman, P.J., 2010. Umbilical vein testosterone in female infants born to mothers with polycystic ovary syndrome is elevated to male levels. J. Obstet. Gynaecol. 30 (5), 444–446.

Bierl, M.A., Jones, E.E., Crutcher, K.A., Isaacson, L.G., 2005. 'Mature' nerve growth factor is a minor species in most peripheral tissues. Neurosci. Lett. 380 (1–2), 133–137.

Birch, R.A., Padmanabhan, V., Foster, D.L., Unsworth, W.P., Robinson, J.E., 2003. Prenatal programming of reproductive neuroendocrine function: fetal androgen exposure produces progressive disruption of reproductive cycles in sheep. Endocrinology 144 (4), 1426–1434.

Boomsma, C.M., Eijkemans, M.J., Hughes, E.G., Visser, G.H., Fauser, B.C., Macklon, N.S., 2006. A meta-analysis of pregnancy outcomes in women with polycystic ovary syndrome. Hum. Reprod. Update. 12 (6), 673–683.

Brauer, M.M., 2008. Cellular and molecular mechanisms underlying plasticity in uterine sympathetic nerves. Auton. Neurosci. 140 (1–2), 1–16.

Caanen, M.R., Kuijper, E.A., Hompes, P.G., Kushnir, M.M., Rockwood, A.L., Meikle, W.A., Homburg, R., Lambalk, C.B., 2016. Mass spectrometry methods measured androgen and estrogen concentrations during pregnancy and in newborns of mothers with polycystic ovary syndrome. Eur. J. Endocrinol. 174 (1), 25–32.

Carter, J.R., 2012. Sympathetic neural tone: large and in charge throughout pregnancy. J. Physiol. 590 (Pt 15), 3411–3412.

Cerdeira, A.S., Karumanchi, S.A., 2012. Angiogenic factors in preeclampsia and related disorders. Cold Spring Harb. Perspect. Med. 2 (11).

Chinnathambi, V., Blesson, C.S., Vincent, K.L., Saade, G.R., Hankins, G.D., Yallampalli, C., Sathishkumar, K., 2014. Elevated testosterone levels during rat pregnancy cause hypersensitivity to angiotensin II and attenuation of endothelium-dependent vasodilation in uterine arteries. Hypertension 64 (2), 405–414.

Doherty, D.A., Newnham, J.P., Bower, C., Hart, R., 2015. Implications of polycystic ovary syndrome for pregnancy and for the health of offspring. Obstet. Gynecol. 125 (6), 1397–1406.

Falbo, A., Rocca, M., Russo, T., D'Ettore, A., Tolino, A., Zullo, F., Orio, F., Palomba, S., 2010. Changes in androgens and insulin sensitivity indexes throughout pregnancy in women with polycystic ovary syndrome (PCOS): relationships with adverse outcomes. J. Ovarian Res. 3, 23.

Feng, Y., Johansson, J., Shao, R., Manneras-Holm, L., Billig, H., Stener-Victorin, E., 2012. Electrical and manual acupuncture stimulation affect oestrous cyclicity and neuroendocrine function in an 5alpha-dihydrotestosterone-induced rat polycystic ovary syndrome model. Exp. Physiol. 97 (5), 651–662.

Frank, P., Barrientos, G., Tirado-Gonzalez, I., Cohen, M., Moschansky, P., Peters, E.M., Klapp, B.F., Rose, M., Tometten, M., Blois, S.M., 2014. Balanced levels of nerve growth factor are required for normal pregnancy progression. Reproduction 148 (2), 179–189.

Gopalakrishnan, K., Mishra, J.S., Chinnathambi, V., Vincent, K.L., Patrikeev, I., Motamedi, M., Saade, G.R., Hankins, G.D., Sathishkumar, K., 2016. Elevated testosterone reduces uterine blood flow, spiral artery elongation, and placental oxygenation in pregnant rats. Hypertension 67 (3), 630–639.

Hempstead, B.L., 2014. Deciphering proneurotrophin actions. Handb. Exp. Pharmacol. 220, 17–32.

Hentges, C.R., Silveira, R.C., Procianoy, R.S., 2015. Angiogenic and antiangiogenic factors in preterm neonates born to mothers with and without preeclampsia. Am. J. Perinatol. 32 (12), 1185–1190. http://dx.doi.org/10.1055/s-0035-1552932.

Hickey, M., Sloboda, D.M., Atkinson, H.C., Doherty, D.A., Franks, S., Norman, R.J., Newnham, J.P., Hart, R., 2009. The relationship between maternal and umbilical cord androgen levels and polycystic ovary syndrome in adolescence: a prospective cohort study. J. Clin. Endocrinol. Metab. 94 (10), 3714–3720.

Hu, S., Leonard, A., Seifalian, A., Hardiman, P., 2007. Vascular dysfunction during pregnancy in women with polycystic ovary syndrome. Hum. Reprod. 22 (6), 1532–1539.

Hu, M., Richard, J.E., Maliqueo, M., Kokosar, M., Fornes, R., Benrick, A., Jansson, T., Ohlsson, C., Wu, X., Skibicka, K.P., Stener-Victorin, E., 2015. Maternal testosterone exposure increases anxiety-like behavior and impacts the limbic system in the offspring. Proc. Natl. Acad. Sci. U. S. A. 112 (46), 14348–14353.

Jamal, A., Milani, F., Al-Yasin, A., 2012. Evaluation of the effect of metformin and aspirin on utero placental circulation of pregnant women with PCOS. Iran. J. Reprod. Med. 10 (3), 265–270.

Jarvis, S.S., Shibata, S., Bivens, T.B., Okada, Y., Casey, B.M., Levine, B.D., Fu, Q., 2012. Sympathetic activation during early pregnancy in humans. J. Physiol. 590 (Pt 15), 3535–3543.

Johansson, J., Manneras-Holm, L., Shao, R., Olsson, A., Lonn, M., Billig, H., Stener-Victorin, E., 2013. Electrical vs manual acupuncture stimulation in a rat model of polycystic ovary syndrome: different effects on muscle and fat tissue insulin signaling. PLoS One. 8 (1), e54357.

Kimura, A., Sato, A., 1997. Somatic regulation of autonomic functions in anesthetized animals—neural mechanisms of physical therapy including acupuncture. Jpn. J. Vet. Res. 45 (3), 137–145.

Levi-Montalcini, R., 1987. The nerve growth factor 35 years later. Science. 237 (4819), 1154–1162.

Lobos, E., Gebhardt, C., Kluge, A., Spanel-Borowski, K., 2005. Expression of nerve growth factor (NGF) isoforms in the rat uterus during pregnancy: accumulation of precursor proNGF. Endocrinology 146 (4), 1922–1929.

MacGrogan, D., Saint-Andre, J.P., Dicou, E., 1992. Expression of nerve growth factor and nerve growth factor receptor genes in human tissues and in prostatic adenocarcinoma cell lines. J. Neurochem. 59 (4), 1381–1391.

MacPherson, H., Hammerschlag, R., Coeytaux, R.R., Davis, R.T., Harris, R.E., Kong, J.T., Langevin, H.M., Lao, L., Milley, R.J., Napadow, V., Schnyer, R.N., Stener-Victorin, E., Witt, C.M., Wayne, P.M., 2016. Unanticipated insights into biomedicine from the study of acupuncture. J. Altern. Complement. Med. 22 (2), 101.

Maliqueo, M., Lara, H.E., Sanchez, F., Echiburu, B., Crisosto, N., Sir-Petermann, T., 2013. Placental steroidogenesis in pregnant women with polycystic ovary syndrome. Eur. J. Obstet. Gynecol. Reprod. Biol. 166 (2), 151–155.

Maliqueo, M., Benrick, A., Alvi, A., Johansson, J., Sun, M., Labrie, F., Ohlsson, C., Stener-Victorin, E., 2015. Circulating gonadotropins and ovarian adiponectin system are modulated by acupuncture independently of sex steroid or beta-adrenergic action in a female hyperandrogenic rat model of polycystic ovary

syndrome. Mol. Cell Endocrinol. 412, 159–169.
Manneras, L., Jonsdottir, I.H., Holmang, A., Lonn, M., Stener-Victorin, E., 2008. Low-frequency electro-acupuncture and physical exercise improve metabolic disturbances and modulate gene expression in adipose tissue in rats with dihydrotestosterone-induced polycystic ovary syndrome. Endocrinology 149 (7), 3559–3568.
Manni, L., Lundeberg, T., Holmang, A., Aloe, L., Stener-Victorin, E., 2005. Effect of electro-acupuncture on ovarian expression of alpha (1)- and beta (2)-adrenoceptors, and p75 neurotrophin receptors in rats with steroid-induced polycystic ovaries. Reprod. Biol. Endocrinol. 3 (1), 21.
Mehrabian, F., Kelishadi, R., 2012. Comparison of the metabolic parameters and androgen level of umbilical cord blood in newborns of mothers with polycystic ovary syndrome and controls. J. Res. Med. Sci. 17 (3), 207–211.
Palomba, S., Falbo, A., Russo, T., Battista, L., Tolino, A., Orio, F., Zullo, F., 2010. Uterine blood flow in pregnant patients with polycystic ovary syndrome: relationships with clinical outcomes. BJOG 117 (6), 711–721.
Palomba, S., Falbo, A., Chiossi, G., Tolino, A., Tucci, L., La Sala, G.B., Zullo, F., 2014. Early trophoblast invasion and placentation in women with different PCOS phenotypes. Reprod. Biomed. Online. 29 (3), 370–381.
Pandolfi, C., Zugaro, A., Lattanzio, F., Necozione, S., Barbonetti, A., Colangeli, M.S., Francavilla, S., Francavilla, F., 2008. Low birth weight and later development of insulin resistance and biochemical/clinical features of polycystic ovary syndrome. Metabolism 57 (7), 999–1004.
Perez-Sepulveda, A., Monteiro, L.J., Dobierzewska, A., Espana-Perrot, P.P., Venegas-Araneda, P., Guzman-Rojas, A.M., Gonzalez, M.I., Palominos-Rivera, M., Irarrazabal, C.E., Figueroa-Diesel, H., Varas-Godoy, M., Illanes, S.E., 2015. Placental aromatase is deficient in placental ischemia and preeclampsia. PLoS One. 10 (10), e0139682.
Qin, J.Z., Pang, L.H., Li, M.J., Fan, X.J., Huang, R.D., Chen, H.Y., 2013. Obstetric complications in women with polycystic ovary syndrome: a systematic review and meta-analysis. Reprod. Biol. Endocrinol. 11, 56.
Recabarren, S.E., Smith, R., Rios, R., Maliqueo, M., Echiburu, B., Codner, E., Cassorla, F., Rojas, P., Sir-Petermann, T., 2008. Metabolic profile in sons of women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 93 (5), 1820–1826.
Roos, N., Kieler, H., Sahlin, L., Ekman-Ordeberg, G., Falconer, H., Stephansson, O., 2011. Risk of adverse pregnancy outcomes in women with polycystic ovary syndrome: population based cohort study. BMJ 343, d6309.
Salvesen, K.A., Vanky, E., Carlsen, S.M., 2007. Metformin treatment in pregnant women with polycystic ovary syndrome–is reduced complication rate mediated by changes in the uteroplacental circulation? Ultrasound Obstet. Gynecol. 29 (4), 433–437.
Sathishkumar, K., Elkins, R., Chinnathambi, V., Gao, H., Hankins, G.D., Yallampalli, C., 2011. Prenatal testosterone-induced fetal growth restriction is associated with down-regulation of rat placental amino acid transport. Reprod. Biol. Endocrinol. 9, 110.
Sathishkumar, K., Balakrishnan, M., Chinnathambi, V., Chauhan, M., Hankins, G.D., Yallampalli, C., 2012. Fetal sex-related dysregulation in testosterone production and their receptor expression in the human placenta with preeclampsia. J. Perinatol. 32 (5), 328–335.
Sharifzadeh, F., Kashanian, M., Fatemi, F., 2012. A comparison of serum androgens in pre-eclamptic and normotensive pregnant women during the third trimester of pregnancy. Gynecol. Endocrinol. 28 (10), 834–836.
Sir-Petermann, T., Maliqueo, M., Angel, B., Lara, H.E., Perez-Bravo, F., Recabarren, S.E., 2002. Maternal serum androgens in pregnant women with polycystic ovarian syndrome: possible implications in prenatal androgenization. Hum. Reprod. 17 (10), 2573–2579.
Sir-Petermann, T., Maliqueo, M., Codner, E., Echiburu, B., Crisosto, N., Perez, V., Perez-Bravo, F., Cassorla, F., 2007. Early metabolic derangements in daughters of women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 92 (12), 4637–4642.
Sir-Petermann, T., Codner, E., Perez, V., Echiburu, B., Maliqueo, M., Ladron de Guevara, A., Preisler, J., Crisosto, N., Sanchez, F., Cassorla, F., Bhasin, S., 2009. Metabolic and reproductive features before and during puberty in daughters of women with polycystic ovary syndrome. J. Clin. Endocrinol. Metab. 94 (6), 1923–1930.
Soligo, M., Protto, V., Florenzano, F., Bracci-Laudiero, L., De Benedetti, F., Chiaretti, A., Manni, L., 2015. The mature/pro nerve growth factor ratio is decreased in the brain of diabetic rats: analysis by ELISA methods. Brain Res. 1624, 455–468. http://dx.doi.org/10.1016/j.brainres.2015.08.005.
Stener-Victorin, E., Waldenstrom, U., Andersson, S.A., Wikland, M., 1996. Reduction of blood flow impedance in the uterine arteries of infertile women with electro-acupuncture. Hum. Reprod. 11 (6), 1314–1317.
Stener-Victorin, E., Lundeberg, T., Waldenstrom, U., Manni, L., Aloe, L., Gunnarsson, S., Janson, P.O., 2000. Effects of electro-acupuncture on nerve growth factor and ovarian morphology in rats with experimentally induced polycystic ovaries. Biol. Reprod. 63 (5), 1497–1503.
Stener-Victorin, E., Kobayashi, R., Kurosawa, M., 2003. Ovarian blood flow responses to electro-acupuncture stimulation at different frequencies and intensities in anaesthetized rats. Aut. Neurosci. Basic Clin. 108 (1–2), 50–56.
Stener-Victorin, E., Lundeberg, T., Cajander, S., Aloe, L., Manni, L., Waldenstrom, U., Janson, P.O., 2003. Steroid-induced polycystic ovaries in rats: effect of electro-acupuncture on concentrations of endothelin-1 and nerve growth factor (NGF), and expression of NGF mRNA in the ovaries, the adrenal glands, and the central nervous system. Reprod. Biol. Endocrinol. 1 (1), 33.
Stener-Victorin, E., Fujisawa, S., Kurosawa, M., 2006. Ovarian blood flow responses to electroacupuncture stimulation depend on estrous cycle and on site and frequency of stimulation in anesthetized rats. J. Appl. Physiol. 101 (1), 84–91.
Stener-Victorin, E., Jedel, E., Janson, P.O., Sverrisdottir, Y.B., 2009. Low-frequency electroacupuncture and physical exercise decrease high muscle sympathetic nerve activity in polycystic ovary syndrome. Am. J. Physiol. Regul. Integr. Comp. Physiol. 297 (2), R387–R395.
Sullivan, S.D., Moenter, S.M., 2004. Prenatal androgens alter GABAergic drive to gonadotropin-releasing hormone neurons: implications for a common fertility disorder. Proc. Natl. Acad. Sci. U. S. A. 101 (18), 7129–7134.
Sun, M., Maliqueo, M., Benrick, A., Johansson, J., Shao, R., Hou, L., Jansson, T., Wu, X., Stener-Victorin, E., 2012. Maternal androgen excess reduces placental and fetal weights, increases placental steroidogenesis, and leads to long-term health effects in their female offspring. Am. J. Physiol. Endocrinol. Metab. 303 (11), E1373–E1385.
Tometten, M., Klapp, B.F., Joachim, R., Fest, S., Zenclussen, A.C., Peters, E.M., Hertwig, K., Arck, P.C., 2004. Nerve growth factor and its functional receptor TrkA are up-regulated in murine decidual tissue of stress-triggered and substance P-mediated abortion. Am. J. Reprod. Immunol. 51 (1), 86–93.
Toti, P., Ciarmela, P., Florio, P., Volpi, N., Occhini, R., Petraglia, F., 2006. Human placenta and fetal membranes express nerve growth factor mRNA and protein. J. Endocrinol. Invest. 29 (4), 337–341.
Tripathi, R., Rath, G., Jain, A., Salhan, S., 2008. Soluble and membranous vascular endothelial growth factor receptor-1 in pregnancies complicated by pre-eclampsia. Ann. Anat. 190 (5), 477–489.
Uchida, S., Hotta, H., Kagitani, F., Aikawa, Y., 2003. Ovarian blood flow is reflexively regulated by mechanical afferent stimulation of hindlimb in non-pregnant anesthetized rats. Aut. Neurosci. Basic Clin. 106, 91–97.
Usselman, C.W., Wakefield, P.K., Skow, R.J., Stickland, M.K., Chari, R.S., Julian, C.G., Steinback, C.D., Davenport, M.H., 2015. Regulation of sympathetic nerve activity during the cold pressor test in normotensive pregnant and nonpregnant women. Hypertension 66 (4), 858–864.
Witham, E.A., Meadows, J.D., Shojaei, S., Kauffman, A.S., Mellon, P.L., 2012. Prenatal exposure to low levels of androgen accelerates female puberty onset and reproductive senescence in mice. Endocrinology 153 (9), 4522–4532.
Wu, X.Y., Li, Z.L., Wu, C.Y., Liu, Y.M., Lin, H., Wang, S.H., Xiao, W.F., 2010. Endocrine traits of polycystic ovary syndrome in prenatally androgenized female Sprague-Dawley rats. Endocr. J. 57 (3), 201–209.
Zeisler, H., Eppel, W., Husslein, P., Bernaschek, G., Deutinger, J., 2001. Influence of acupuncture on Doppler ultrasound in pregnant women. Ultrasound Obstet. Gynecol. Off. J. Int. Soc. Ultrasound Obstet. Gynecol. 17 (3), 229–232.