

Porcine Dorsal Root Ganglia Ovarian Neurons Are Affected by Long Lasting Testosterone Treatment

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Summary

We studied the effect of testosterone overdose on the number, distribution and chemical coding of ovarian neurons in the dorsal root ganglia (DRGs) in pigs. On day 3 of the estrous cycle, the ovaries of both the control and experimental gilts were injected with retrograde tracer Fast Blue. From day 4 of the estrous cycle to the expected day 20 of the second studied cycle, the experimental gilts were injected with testosterone, while the control gilts received oil. After the completion of the protocol the Th16-L5 DRGs were collected. Injections of testosterone increased the testosterone (~3.5 fold) and estradiol-17 β (~1.6 fold) levels in the peripheral blood, and reduced the following in the DRGs: the total number of the Fast Blue-positive perikarya, the population of perikarya in the L2-L4 ganglia, and the numbers of SP⁺/CGRP⁺, SP⁺/PACAP⁺, SP⁺/nNOS⁺ and SP/nNOS⁺ perikarya. In the testosterone-injected gilts, the populations of SP⁺/CGRP⁺, small and large androgen receptors-expressing perikarya were increased. These results suggest that elevated androgen levels during pathological states may regulate the transmission of sensory modalities from the ovary to the spinal cord, and antidromic regulation of the ovarian functions.

Key words

Testosterone • Dorsal root ganglia • Ovarian neurons • Gilts

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Introduction

It is well recognized that the mammalian ovary receives neuronal inputs from the sympathetic, parasympathetic and sensory components of the peripheral nervous system (PNS). In pigs, the sensory ovary-projecting neurons (referred to further as ovarian perikarya or ovarian neurons) occur in the dorsal root ganglia (DRGs) neuromers from Th10 to L5. Studies on the chemical nature of DRGs ovarian neurons in pigs revealed that they are immunoreactive (IR) to substance P (SP), calcitonin gene-related peptide (CGRP), galanin (GAL), pituitary adenylate cyclase-activating polypeptide (PACAP), neuronal isoform of nitric oxide synthase (nNOS), somatostatin (SOM), vasoactive intestinal peptide (VIP) and tyrosine hydroxylase (TH) (Jana *et al.* 2012, Majewski *et al.* 1997, Majewski *et al.* 2002). Previously, it was indicated that ovarian-related afferent nerves, besides their basic function of the transmission of sensory modalities from the ovary to the spinal cord, also play an essential role in local mechanisms of folliculogenesis and vascular tone regulation in the gonad (based on the phenomenon of antidromic nervous stimulation) (Majewski 1997).

There are data concerning the influence of sex steroids on the morphological and chemical plasticity of PNS neurons supplying the urogenital targets. Regarding the androgens, it has been shown that androgen receptors (AR) are expressed in perikarya of the pelvic ganglion (PG) (Keast and Saunders 1998, Purves-Tyson *et al.* 2007) and DRGs (Keast and Gleeson 1998) in male rats. Moreover, the AR-positive PG neurons contained VIP,

TH or nNOS (Keast and Saunders 1998, Purves-Tyson *et al.* 2007, Schirar *et al.* 1997), and viscera-supplying lumbar and sacral afferent neurons simultaneously expressed AR and CGRP (Keast and Gleeson 1998). Androgens changed the morphology of viscera supplying PG neurons (Keast and Saunders 1998) and the chemical phenotype of neurons in the hypogastric ganglion (Hamill and Schroeder 1990) and in the PG (Löffler *et al.* 2004). These effects are believed to be important for many reproductive behaviors in males, as well as in urinary bladder and lower bowel functions (Kanjhan *et al.* 2003). It is also known that AR occur in the developing DRGs of sheep fetuses of both genders (Luo *et al.* 2008), and also that androstenedione acting by these receptors localized in the celiac ganglion neurons may mediate a luteotropic effect in rats during late pregnancy (Vallcaneras *et al.* 2009) and after parturition (Vallcaneras *et al.* 2011).

Hyperandrogenism is one of the most common endocrine disorders of adult women, and can result from many pathological states. The ovary is the main source of androgens in women with polycystic ovary syndrome (PCOS) (Panidis *et al.* 2005) and idiopathic hyperandrogenism, while increased adrenal androgen secretion is prevalent in non classic 21-hydroxylase deficiency (Carmina 2006). Augmentation in peripheral blood androgen levels is found in patients suffering from adrenal hyperplasia (Goodarzi *et al.* 2003) and ovarian (Singh *et al.* 2012) and adrenal androgen-secreting tumors (Varma *et al.* 2013). Extensive production of androgens takes place in pigs with uterine inflammation (Jana *et al.* 2004) and polycystic ovaries (Szulańczyk-Mencel *et al.* 2010), and in dogs with adrenal dysfunction (Hill *et al.* 2005).

Studies on the influence of androgens on the PNS neurons supplying the reproductive organs of domestic animals are very limited. In fact, the pig, due to its embryological, anatomical and physiological similarity to humans, constitutes an especially valuable species for bio-medical research, including that of ovary functions (Swindle *et al.* 2012, Szulańczyk-Mencel *et al.* 2010). We previously presented that long-term testosterone treatment altered the morphological and immunochemical organization of the caudal mesenteric ganglion (CaMG) (Jana *et al.* 2013b), the sympathetic chain ganglia (SChGs) (Jana *et al.* 2015) and the paracervical ganglion (PCG) (Jana *et al.* 2014) ovarian neurons in adult gilts. We hypothesize that augmented concentrations of androgens during pathological states

can also change the number, distribution and chemical coding of ovarian neurons in the DRGs. In support of this, we studied the total number and distribution of ovarian perikarya, visualized by intraovarian injection of the neuronal retrograde tracer Fast Blue (FB), and the numbers of FB-labeled ovarian perikarya expressing SP and/or CGRP, GAL, PACAP, nNOS, as well as AR in the DRGs of long-term testosterone-treated adult gilts.

Materials and Methods

Animals and experimental procedures

Six crossbred (Large White x Landrace) gilts, aged 7-8 months and weighing 90-110 kg, with at last two regular estrous cycles were used in this experiment. The estrous-showing behavior was detected using a boar-tester. Three days before surgical operations the gilts were transported from a farm to a local animal house and kept in individual stalls under natural light and temperature (April, May). They were fed a commercial grain mixture and tap water *ad libitum*. The study procedures were approved by the Local Ethics Committee, University of Warmia and Mazury in Olsztyn (Agreement no 21/N).

On day 3 of the first studied estrous cycle (day 0 of the experiment) median laparotomies were performed under general anesthesia induced by azaperone (2 mg/1 kg of body mass, Stresnil, Janssen Pharmaceutica N.V., Belgium) and sodium pentobarbital (30 mg/1 kg of body mass, Vetbutal, Biowet, Puławy, Poland) in all gilts to expose the ovaries. After an abdominal incision, the right and left ovaries were exposed after gentle removal of the surrounding tissues, and a 5% solution of fluorescent retrograde neuronal tracer FB (EMS-CHEMIE GmbH, Germany) was applied to each ovary. The right and left sides of the organ were injected 5 times (10 µl of the dye solution *per* 1 injection, with a total volume of 100 µl *per* ovary) using a Hamilton syringe equipped with a 26-gauge needle, keeping a similar distance between the sites of the injections. To minimize the diffusion of the tracer into surrounding tissues (e.g. bursa ovary, mesosalpinx, oviductal infundibulum) the needle was left *in situ* for at least 4 min after each injection, and there after the injection area was subsequently rinsed with isotonic saline and gently wiped with gauze. In all gilts a polyvinyl cannula (outer diameter 2.2 mm, inner diameter 1.8 mm, Tomel, Tomaszów Maz., Poland) was also inserted into the jugular vein in order to collect blood samples.

The gilts were randomly assigned to one of two groups: a control group (group I, n=3) and the experimental group (group II, n=3). In the gilts of group I, from day 4 of the first studied estrous cycle (day 1 of the study) to the expected day 20 of the second studied cycle (i.e. for 38 consecutive days), 2 ml of oil were injected i.m. every 12 h (at 07:00 and 19:00 h). In the gilts of group II, at the same time and in the same manner 1000 µg of testosterone (Cat. # 35800, Serva Electrophoresis GmbH, Germany) in 2 ml of corn oil were injected. The applied dose of testosterone was determined based on our preliminary experiment showing that its application increases the peripheral blood testosterone concentration about 3.5 fold. To estimate the levels of testosterone, androstenedione, estradiol-17β and estrone, blood samples were collected from gilts of both groups 1 h before the first testosterone/oil injection (at 6:00 h, day 1 of the study) and throughout the whole period of testosterone/oil injection (twice a day at 09:00 and 21:00 h, days 1-38 of the study). The samples were then immediately placed in an ice bath, where they were kept until centrifugation (10 min, 1 500 × g, at 4 °C). The plasma was decanted and stored at -20 °C until further processing. The analysis of daily concentrations of androgens and estrogens in the peripheral blood of gilts was described earlier (Jana *et al.* 2013b). The concentrations of these hormones before testosterone/oil injection on day 1 of the study and on day 38 are presented in Table 1. After the last testosterone/oil injection the gilts were deeply re-anesthetized (following the same procedure as applied prior to the laparotomy) and perfused transcardially *via* the ascending aorta with 4 % paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). After perfusion the bilateral CaMG, SChGs from Th16 to S2, the uterine cervix with PCG, and the bilateral DRGs (along with spinal cord segments Th16 to L5) were removed, postfixed by immersion in the same fixative for 10 min, then washed with 0.1 M PB (pH 7.4) over two days and finally transferred to and stored at 4 °C in 18 % buffered sucrose solution (pH 7.4) containing 0.01 % natriumazide. The tissues were then kept at -80 °C until further processing. Data concerning the morphology and immunochemical structural organization of the CaMG (Jana *et al.* 2013b), SChGs (Jana *et al.* 2015) and PCG (Jana *et al.* 2014) in testosterone-injected gilts were published earlier.

Immunohistological procedures

Frozen ganglia were cut in a cryostat (Frigocut, Reichert-Jung, Nussloch, Germany) into 10-µm thick

Table 1. Mean (\pm SD) levels of testosterone, estradiol-17β, estrone and androstenedione in the peripheral blood (pg/ml) on day 1 of the study before first injection of oil in the control gilts and testosterone injection in the experimental gilts, and daily levels of these hormones on day 38 of the study after oil/testosterone injection.

	Day 1 before oil/testosterone injection	Day 38 after oil/testosterone injection
Testosterone		
<i>Control</i>	35.2±6.46	24.8±12.6
<i>Testosterone -treatment</i>	29.7±8.33	89.1±33.15***
Estradiol-17β		
<i>Control</i>	7.17±1.3	17.2±2.35
<i>Testosterone -treatment</i>	8.5±2.04	14.5±4.99
Estrone		
<i>Control</i>	17.1±5.07	15.66±1.29
<i>Testosterone -treatment</i>	2.8±1.3	5.5±0.85*
Androstenedione		
<i>Control</i>	45.13±8.3	40.2±18.7
<i>Testosterone -treatment</i>	38.6±11.9	1.36±21.8

* P<0.05, *** P<0.001 – indicate differences between both groups for the same hormone/time sample collection.

sections and mounted on chrome alum-coated slides. With the use of an Olympus BX51 microscope (Olympus, Poland), equipped in epi-illumination fluorescence microscopy (V1 module: excitation filter 330-385 nm, barrier filter 420 nm), the occurrence of retrogradely FB-labeled neurons was checked in serial sections of the bilateral ganglia. Sections with FB-labeled perikarya were processed for a routine double-labeling immunofluorescence technique to estimate SP and/or CGRP, GAL, PACAP, nNOS immunoreactivity, and for a routine single-immunofluorescence technique to determine AR (Majewski and Heym 1991). After air-drying at room temperature (rt) for 45 min and rinsing in 0.1 M phosphate-buffered saline (PBS; pH 7.4; 3 x 10 min) the sections were incubated in a blocking buffer containing: 10 % of normal goat serum (MP Biomedicals, USA), in 0.1 M PBS, 0.1 % donkey serum (Abcam, UK), 1 % Triton X-100 (Sigma-Aldrich, USA),

0.05 % Thimerosal (Sigma-Aldrich, USA) and 0.01 % NaN_3 for 1 h (rt) to reduce non-specific background staining. Subsequently, after another wash in PBS (3 x 10 min), the sections were incubated overnight (rt) with primary antisera raised against SP (rat monoclonal, AdD Serotec, Cat. # 8450-0505, UK, 1: 700) and/or CGRP (rabbit polyclonal, Millipore, Cat. # AB5920, USA, 1: 8 000), GAL (rabbit polyclonal, Peninsula Lab., Cat. # T-4326, USA, 1: 8 000), PACAP (rabbit polyclonal, Peninsula Lab., Cat. # T-4465, USA, 1: 15 000) and nNOS (rabbit polyclonal, Chemicon International a Serogicals Company, Cat. # AB5380, USA, 1: 15 000). Following subsequent rinsing in PBS (3 x 10 min), co-expression of SP/CGRP, SP/GAL, SP/PACAP and SP/nNOS was visualized by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Cat. # 112-035003, Jackson Immuno. Lab., USA, dilution 1: 800) and with biotinylated goat anti-rabbit IgG (Cat. # AP132B Chemicon, UK, diluted 1: 1 000) and then with CY3-conjugated streptavidin (Cat. # 016160084, Jackson Immuno. Lab., USA, dilution 1: 9 000) for 1 h (rt). The ganglionic sections with FB-labeled perikarya were also incubated with antisera against the AR (polyclonal rabbit anti-rat, N-terminus, Cat. # 1358-P1, NeoMarkers Fremont, CA, USA, dilution 1: 250). To visualize the primary antibody, sections were incubated with biotinylated goat anti-rabbit IgG (Cat. # AP132B Chemicon, UK, diluted 1: 1 000) and then with CY3-conjugated streptavidin (Cat. # 016160084, Jackson Immuno. Lab., USA, dilution 1: 9 000) for 1 h (rt). Next, the washed sections were cover-slipped in carbonate-buffered glycerol (pH 8.6). Control slides were processed as described, but without incubation with the primary antibody. Retrogradely labeled/immunostained neurons were analyzed and photographed with the appropriate filter sets for FITC (B1 module, excitation filter 450-480 nm, barrier filter 515 nm) and CY3 (G1 module excitation filter 510-550 nm, barrier filter 590 nm). All SP- and/or CGRP-, GAL-, PACAP-, nNOS-, AR-IR as well as all retrogradely labeled cell bodies were counted in every fourth section of the bilateral DRGs. Only neuronal profiles with a visible nucleus were scored to avoid double counting. Pictures were digitalized and analyzed with Analysis software (version 3.02, Soft Imaging System, Munich, Germany). The diameter of a particular neuron was determined by acquisition software, and the data were used to divide ovary-projecting neurons into three size-classes: small (average diameter up to 30 μm),

medium-sized (diameter 31-50 μm) and large (diameter >51 μm) cells (Bossowska *et al.* 2009). The distribution of all ovarian perikarya was evaluated in cranial, caudal, ventral, dorsal and central regions of the DRGs. During light microscopic analysis the area located in the middle part of each DRG was defined as the central region. Consequently, the dorsal, ventral, cranial and caudal regions were defined respectively. Particular regions displayed similar areas.

Statistical procedures

To calculate the mean (\pm SD) total numbers of ovarian perikarya, the numbers of different size-classes of perikarya and total numbers of AR-expressing cells, data gained from six control and six hyperandrogenized DRGs, were averaged *per* one DRG for each group, and then the mean (\pm SD) total numbers between both groups were compared by Student's *t*-test. For calculation of the statistical significance in mean (\pm SD) numbers of the neuronal sets (in particular ganglia and regions, with different chemical coding, expressing AR) a one-way analysis of variance (ANOVA) followed by a Newman-Keuls test was chosen. Differences with a probability of $P < 0.05$ were considered significant.

Results

The number and distribution of ovarian perikarya in the DRGs

After treatment with testosterone the total number of ovarian perikarya in the DRGs Th16-L5 was lower ($P < 0.01$) than in the control ganglia (75 \pm 17.3 vs. 152.3 \pm 41.1, respectively). Testosterone application led to a decrease ($P < 0.05$) in the number of medium perikarya as compared to the control group (24.7 \pm 16.7 vs. 55.7 \pm 32.7, respectively). The populations of small and large perikarya were similar in the control and testosterone-treated gilts (21.7 \pm 11 vs. 19.1 \pm 13.4, 33.75 \pm 22 vs. 28.6 \pm 13.5, respectively). After testosterone-treatment a reduction in the population of ovarian perikarya in the L2 ($P < 0.001$), L3 ($P < 0.01$) and L4 ($P < 0.05$) ganglia, as well as a lack of perikarya in the Th16 ganglion, were revealed. The number of ovarian perikarya in the central region of the DRGs Th16-L5 was lower ($P < 0.05$) in the testosterone-treated gilts than in the control ones. The populations of perikarya in particular ganglia and ganglionic regions of the DRGs of both groups are depicted in Figures 1 and 2, respectively.

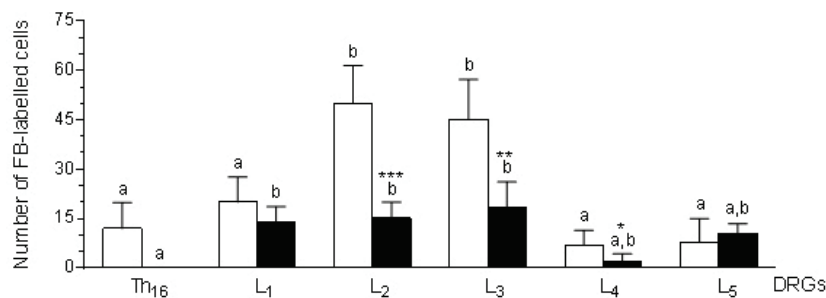


Fig. 1. Mean (\pm SD) numbers of ovarian perikarya in particular ganglia of the DRGs Th16-L5 in the control (open bars) and testosterone-injected (solid bars) gilts. Different letters (a, b) indicate differences ($P < 0.05-0.001$) among the particular ganglia within the control or experimental groups; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ – indicate differences between both groups for the same ganglion.

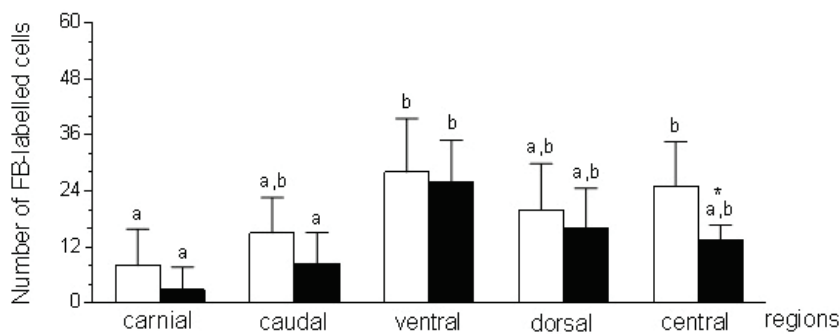


Fig. 2. Mean (\pm SD) numbers of ovarian perikarya in particular regions of the DRGs Th16-L5 in the control (open bars) and testosterone-injected (solid bars) gilts. Different letters (a, b) indicate differences ($P < 0.05-0.001$) among the particular regions within the control or experimental groups; * $P < 0.05$ – indicates differences between both groups for the same ganglion.

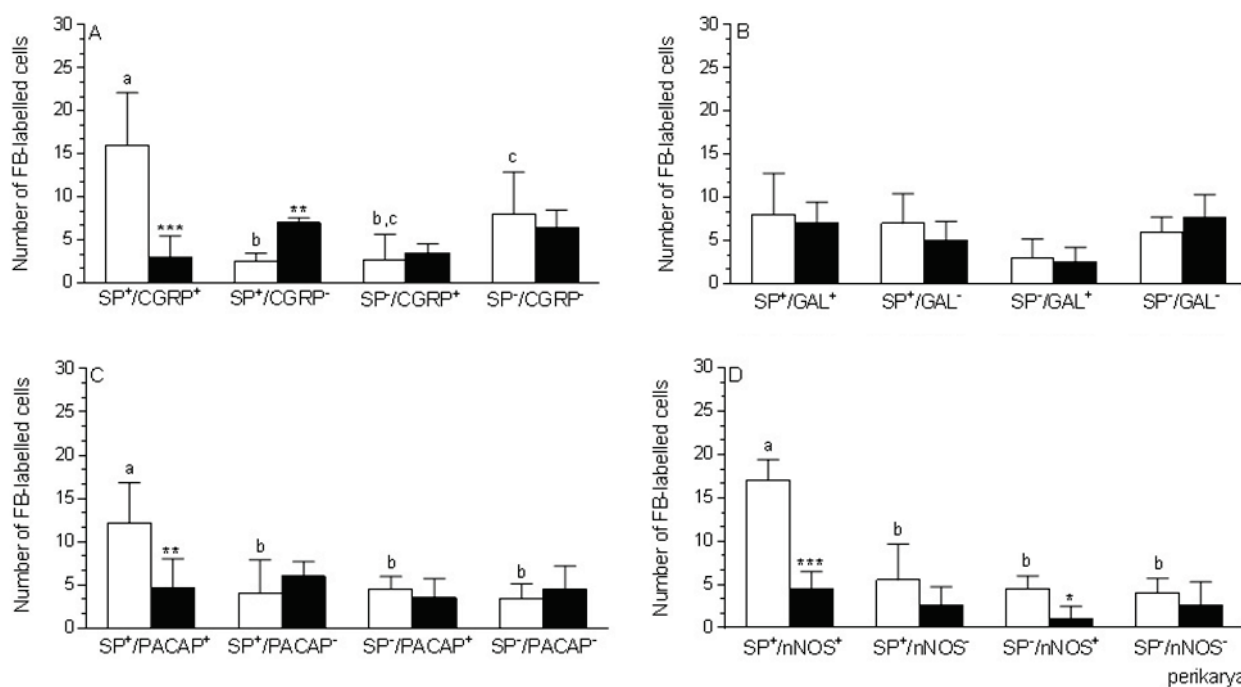


Fig. 3. Mean (\pm SD) numbers of ovarian perikarya immunoreactive to SP and/or CGRP (A), SP and/or GAL (B), SP and/or PACAP (C) and SP and/or nNOS (D) as well as immunonegative to the above-mentioned substances in the DRGs Th16-L5 of the control (open bars) and testosterone-injected (solid bars) gilts. Different letters (a, b, c) indicate differences ($P < 0.05-0.001$) among the particular neuronal populations within the control group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ – indicate differences between both groups for the same neuronal population.

The number of ovarian perikarya containing SP, CGRP, GAL, PACAP and nNOS in the DRGs

In all studied DRGs, injections of testosterone led to a drop ($P < 0.001$) in the number of SP⁺/CGRP⁺ perikarya, and to an increase ($P < 0.01$) in the number of

SP⁺/CGRP⁻ perikarya, while the numbers of SP⁻/CGRP⁺ and SP⁻/CGRP⁻ perikarya did not statistically differ in either group (Figs 3A and 4A-H). Following testosterone administration the populations of SP⁺/GAL⁺, SP⁺/GAL⁻, SP⁻/GAL⁺ and SP⁻/GAL⁻ coded perikarya were not

significantly changed (Figs 3B and 4I-P). In the DRGs of testosterone-injected gilts the number of SP⁺/PACAP⁺ perikarya was lower ($P < 0.01$) than in the control group, and SP⁺/PACAP⁻, SP⁻/PACAP⁺ and SP⁻/PACAP⁻ populations did not statistically differ in either group (Figs 3C and 5A-H). Treatment with testosterone also resulted in a decrease in the populations of SP⁺/nNOS⁺ ($P < 0.001$) and SP⁻/nNOS⁺ ($P < 0.05$) perikarya, while numbers of SP⁺/nNOS⁻ and SP⁻/nNOS⁻ perikarya were similar in both groups (Figs 3D and 5I-P). The numbers of ovarian perikarya containing SP and/or CGRP, GAL, PACAP, nNOS as well as those devoid of the above-mentioned substances within the DRGs of the control and testosterone-treated gilts, are presented in Figures 3A-D.

The number of ovarian perikarya expressing AR in the DRGs

The total number of perikarya containing AR in the DRGs Th16-L5 of testosterone-injected gilts was higher ($P < 0.05$) than in the control ganglia (28.38 ± 12.7 vs. 18.2 ± 3.6 , respectively). The populations of small ($P < 0.001$) and large ($P < 0.05$) perikarya expressing AR were increased in testosterone-affected ganglia, while populations of AR-positive medium perikarya did not statistically differ in either group (Fig. 6 and Figs 7A-F). No labeling of AR was observed when the primary antibodies were omitted (Figs 7G-I). The numbers of ovarian perikarya expressing AR, as well as those devoid of this receptor within the ganglia of both groups, are given in Figure 6.

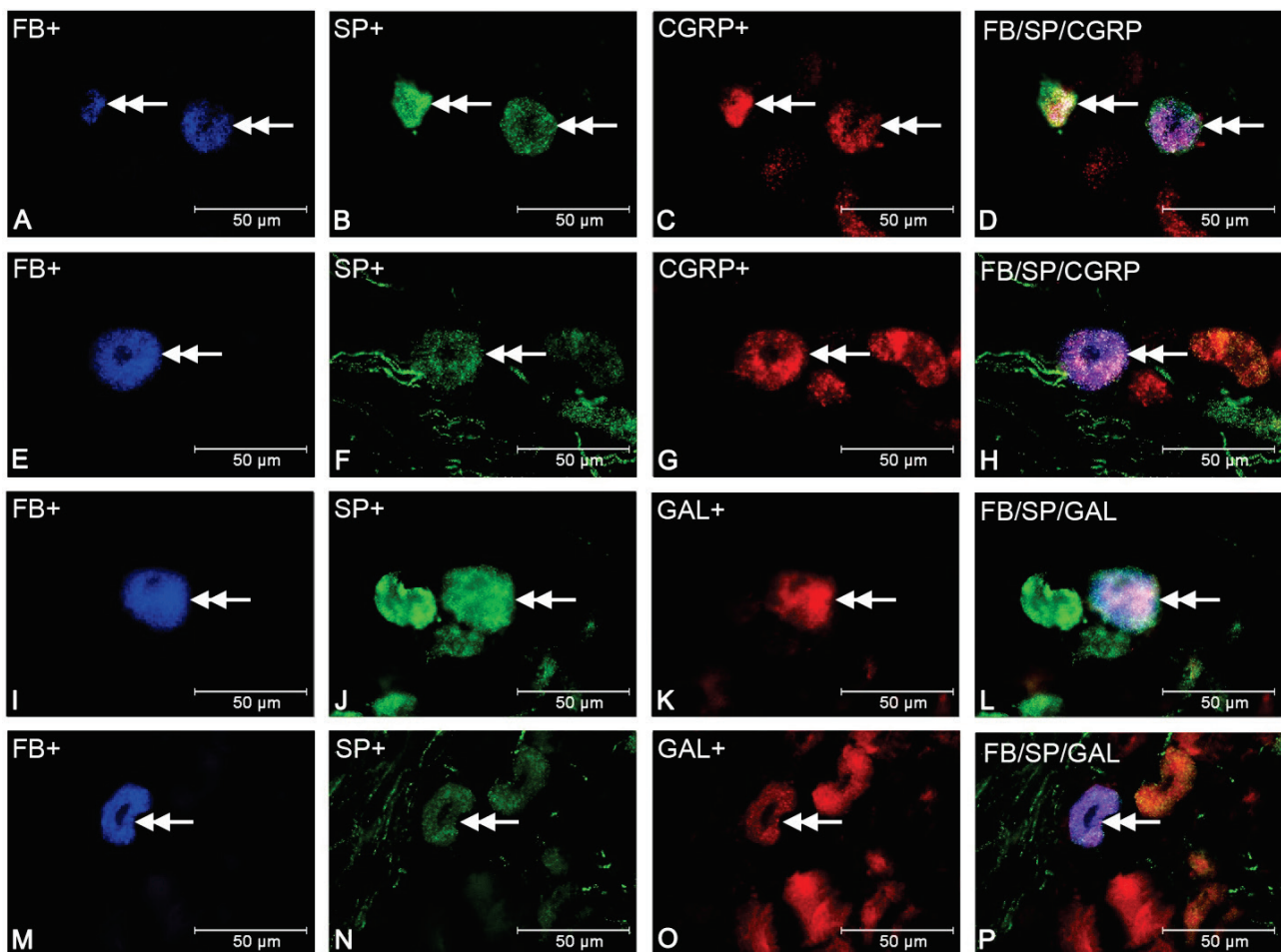


Fig. 4. Fluorescent micrographs showing SP- (B, F, J, N), CGRP- (C, G) and GAL- (K, O) immunoreactivity in the DRGs of the control (A-D; I-L) and testosterone-injected (E-H; M-P) gilts after injections of FB into the ovaries. The double arrow indicates FB-positive ovarian perikaryon containing the two examined substances. Photographs D, H, L and P have been created by digital superimposition of three color channels; FB-positive (blue), SP-positive (green), and CGRP- or GAL-positive (red). Two perikarya immunoreactive to SP and CGRP visible in the L3 ganglion of the control gilt (A-D). Note in the testosterone-injected gilt, only one perikaryon immunoreactive to these substances is present in L3 ganglion (E-H). In the DRG L2 of the control (I-L) and testosterone-injected (M-P) gilts is present one perikaryon expressing SP and GAL.

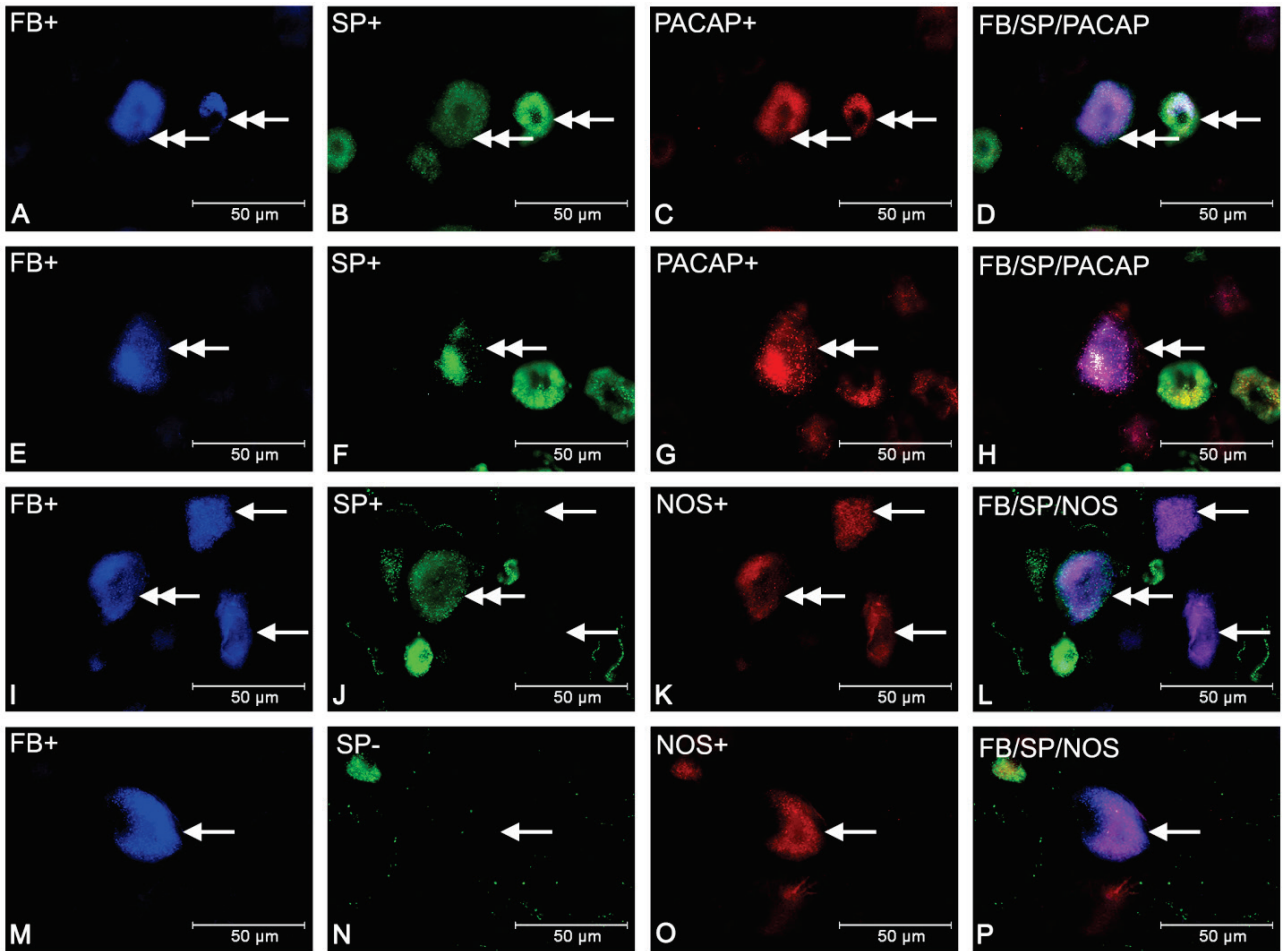


Fig. 5. Fluorescent micrographs showing SP- (**B, F, J, N**), PACAP- (**C, G**) and nNOS- (**K, O**) immunoreactivity in the DRGs of the control (**A-D; I-L**) and testosterone-injected (**E-H; M-P**) gilts after injections of FB into the ovaries. The double arrow indicates FB-positive ovarian perikaryon containing two examined substances. The arrow shows FB-positive ovarian perikaryon containing one of the two examined substances. Photographs D, H, L and P have been created by digital superimposition of three color channels; FB-positive (blue), SP-positive (green), and PACAP or nNOS-positive (red). In the L3 ganglion of the control gilt visible two perikarya positive to SP and PACAP (A-D). Note in the testosterone-injected gilt, the drop in the number of perikarya containing SP and PACAP in L3 ganglion (E-H). In the control DRG L2 are present two perikarya immunoreactive to NOS but immunonegative to SP and one perikaryon immunoreactive to these substances (I-L). In the testosterone-affected DRG L2 only one perikaryon immunoreactive to NOS but immunonegative to SP is visible (M-P).

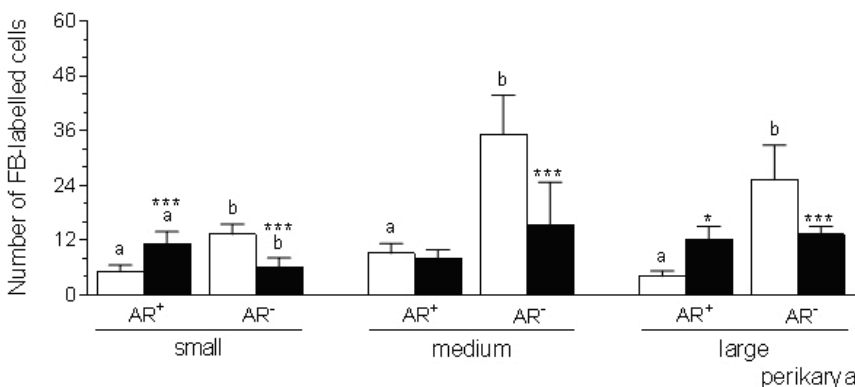


Fig. 6. Mean (\pm SD) numbers of small, medium and large ovarian perikarya immunoreactive to AR as well as immunonegative to AR in the DRGs Th16-L5 of the control (open bars) and testosterone-injected (solid bars) gilts. Different letters (a, b) indicate differences ($P < 0.05-0.001$) among the particular neuronal populations within the control or experimental groups; * $P < 0.05$, *** $P < 0.001$ – indicate difference between both groups for the same neuronal population.

Discussion

We show that long-term testosterone treatment of adult gilts results in alterations in the morphological and immunochemical organization of the DRGs

ovarian neurons.

Our earlier studies demonstrate that long-term testosterone administration in gilts led to a decrease in the total population of ovarian neurons in the CaMG (Jana *et al.* 2013b), PCG (Jana *et al.* 2014) and SchGs (Jana *et al.*

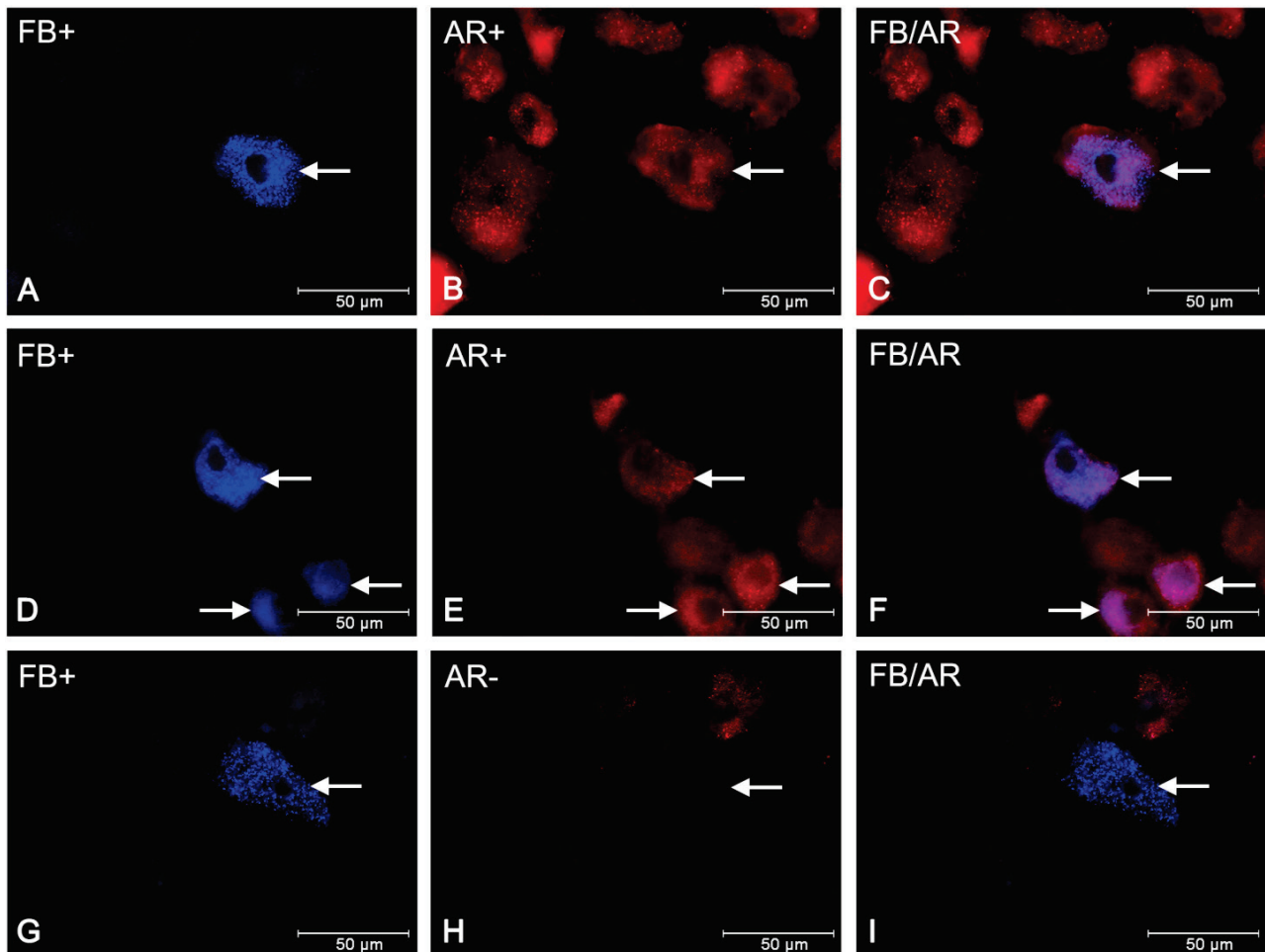


Fig. 7. Fluorescent micrographs showing AR- (**B, C, E, F**) immunoreactivity in the DRGs of the control (**A-C**) and testosterone-injected (**D-F**) gilts after injections of Fast Blue (FB) into the ovaries. The arrow indicates FB-positive ovarian perikaryon containing AR. Photographs C and F have been created by digital superimposition of two color channels; FB-positive (blue) and AR-positive (red). Visible one perikaryon expressing AR in the L3 DRG of the control gilt (A-C). In the testosterone-affected DRG L3 three AR-positive perikarya occur (D-F). Negative control for AR (G-I).

2015). The present findings also show the reduction in the total number of ovarian perikarya, including medium ones, in the DRGs of testosterone-injected gilts. We suppose that this situation was not directly caused by the elevated testosterone level. The above statement may partly result from the present study, in which the rise in the total set of AR-positive ovarian perikarya in the DRGs of testosterone-treated gilts was revealed. Moreover, in the testosterone group a decrease in the number of medium-sized perikarya was convergent with the unchanged number of these perikarya positive to AR. We also revealed the greater populations of small and large ovarian perikarya positive to AR, and a lack of changes in the numbers of these structures. Literature data also show that androgens maintain and stimulate somata size and growth, and that castration or androgens did not affect the vitality of TH-, nNOS- and VIP-IR

perikarya in male rat PG (Keast and Saunders 1998, Melvin *et al.* 1988, Melvin *et al.* 1989, Meusburger and Keast 2001, Purves-Tyson *et al.* 2007). Thus, the drop in the population of the ovarian perikarya in the DRGs of testosterone-administered gilts (present study) could be caused by increased estradiol-17 β level in the peripheral blood, which resulted from aromatization of the injected testosterone. The sets of ovarian perikarya lowered in the DRGs (Jana *et al.* 2012), as well as the SChGs (Koszykowska *et al.* 2011a), CaMG (Koszykowska *et al.* 2011b) and PCG (Jana *et al.* 2013a) of long-term estradiol-17 β -treated gilts. The increased estradiol-17 β level in the gilts used in the present study might be accompanied by down-regulated estrogen receptors (ERs) expression in the DRGs ovarian perikarya, but this is not yet known. Therefore, the diminution in the ERs content in the perikarya could markedly limit the direct

estradiol-17 β effects promoting the survival of these structures, leading to their loss. The decreased sets of ovarian perikarya expressing ERs were revealed in the DRGs (Jana *et al.* 2012, Sohrabji *et al.* 1994), SChGs (Koszykowska *et al.* 2011a) and PCG (Jana *et al.* 2013a) exposed to estrogens. The decreased set of ovarian perikarya in the testosterone-receiving gilts (present study) may also result from the reduced expression of nerve growth factor (Kaur *et al.* 2007, Pieretti *et al.* 2013, Sohrabji *et al.* 1994) and insulin-like growth factor-1 and/or its receptor (Böttner and Wuttke 2006) under the influence of the elevated testosterone and estradiol-17 β levels.

The populations of ovarian perikarya in the gilt DRGs L2-L4 were decreased after testosterone administration, and these structures were not present in Th16. As mentioned earlier, we also found a drop in the number of medium, but not small and large ovarian perikarya. In response to testosterone injections a lower population of ovarian perikarya was found in the central regions of DRGs. These changes are partly in line with findings revealed in the DRGs of estradiol-17 β -treated pigs (Jana *et al.* 2013a), as well as in the porcine CaMG after testosterone (Jana *et al.* 2013b) and estradiol-17 β (Koszykowska *et al.* 2011b) injections. Taking into consideration literature data (Jana *et al.* 2013b, Koszykowska *et al.* 2011b, Majewski *et al.* 2002, Taleghany *et al.* 1999) we suppose that changes in the distribution of ovarian perikarya in the DRGs of testosterone-treated gilts might result from elevated testosterone and estradiol-17 β concentrations, and differentiated AR and/or ERs expression in different size classes of neurons.

In the DRGs Th16-L5 of testosterone-administered gilts we determined the reduction in the populations of ovarian perikarya coded SP/CGRP, SP/PACAP and SP/nNOS, and perikarya negative to SP but reactive to nNOS, as well as an increase in the population of perikarya reactive to SP but negative to CGRP. These findings are in agreement with studies showing a drop in the numbers of ovarian perikarya expressing the above-given substances in the gilt DRGs (Jana *et al.* 2012) and in the population of CGRP-IR neurons in the rat female lumbar DRGs (Yang *et al.* 1998) exposed to estradiol-17 β . In turn, in the PCG of testosterone-treated gilts the number of ovarian perikarya coding vesicular acetylcholine transporter (VAChT)/nNOS was reduced, while the populations of ovarian perikarya expressing SOM, VIP or dopamine

β -hydroxylase but not VAChT were upregulated (Jana *et al.* 2014). In contrast to the present study, the administration of testosterone in gilts led to a decrease in the numbers of ovarian perikarya containing GAL in the CaMG (Jana *et al.* 2013b) and SChGs (Jana *et al.* 2015). It may be assumable that the effects of testosterone and estradiol-17 β on sensory neurons are partly dependent on their chemical coding (present study), and may result from varied density and the cellular location of AR and ERs in the particular sets. The co-expression of these receptors and particular substances in the neurons of DRGs was not estimated in our study. As mentioned earlier, the male rat DRGs neurons possess AR and CGRP (Keast and Gleeson 1998), and PG neurons are simultaneously positive to AR and VIP, TH or nNOS (Keast and Saunders 1998, Purves-Tyson *et al.* 2007, Schirar *et al.* 1997). ERs are present in CGRP-, SP- and PACAP-IR neurons of the DRGs projecting to the uterine cervix in rats (Mowa and Papka 2004, Papka and Mowa 2003) and in nNOS-positive parasympathetic PG neurons of male rats (Purves-Tyson *et al.* 2007). Exposition of rats to estradiol-17 for 4 days up-regulated the synthesis rate of SP and CGRP (Mowa and Papka 2004, Papka and Mowa 2003), while long-term treatment with estradiol-17 β down-regulated the expression of β -preprotachykinin mRNA encoding SP in the DRGs (Liuzzi *et al.* 1999). Data explaining the molecular mechanism of changes in the chemical coding of neurons in response to steroid hormones are very limited. It was only suggested by Meusburger and Keast (2001) that testosterone, through AR, may affect neuronal gene expression in cholinergic neurons. Existing data show that the exposure of rat DRG neurons to estradiol-17 β led to the phosphorylation of cyclic AMP response-element binding protein (CREB), which mediates transcriptional regulation in sensory neurons (Purves-Tyson and Keast 2004).

As found in the present study, the treatment of gilts with testosterone led mainly to a down-regulation in the numbers of ovarian perikarya expressing SP and/or CGRP, PACAP, nNOS in the DRGs Th16-L5. Moreover, according to our unpublished observations these changes were accompanied with decreased populations of nerve fibers immunoreactive to all above-mentioned substances in ovaries of gilts from which the DRGs were obtained to the present study. Reduction of the fibers has been found within ground plexus and around blood vessels, while CGRP-IR fibers were not present in vicinity of the follicles. All the data may suggest that these changes may

underlie disturbances in neuronal transmission from the ovary to the spinal cord and in antidromic regulation of the ovarian cells. Earlier reports show the involvement of SP, CGRP and nNOS in the transmission of nociceptive information induced by noxious stimuli (Meller and Gebhardt 1993, Yang *et al.* 1998). In addition, under physiological conditions PACAP and GAL regulate follicular growth (Majewski *et al.* 2002, Morelli *et al.* 2008), and these peptides, as well as SP, CGRP and nNOS, modulate the ovarian blood flow and steroidogenesis (Debeljuk 2006, Majewski 1997). It is worth mentioning that receptors for SP (Löffler *et al.* 2004), GAL (Pang *et al.* 1999) and PACAP (Morelli *et al.* 2008) are expressed in the ovarian cells.

It is worth adding that injections of 1000 µg of testosterone twice a day in gilts led to the following changes in the concentrations of steroid hormone in the peripheral blood as compared to the controls: testosterone levels were increased on all days of the study (approximately 4-5 fold), except for days 2, 12 and 35-37; estradiol-17β levels were increased on days 4-14 and 21-29 (approximately 1.6 fold); estrone levels were decreased on days 8-11, 15, 16, 18, 19, 29-32, 37 and 38; androstendione levels did not markedly differ between either group (Jana *et al.* 2013b). The level of testosterone revealed in gilts after the application of the above dose of testosterone was similar to values of the total and bioavailable testosterone appearing in the peripheral blood of women with bilateral mononuclear adrenal

hyperplasia (Goodarzi *et al.* 2003). Moreover, the free androgen index is about 5-fold higher in women with PCOS than in controls (Panidis *et al.* 2005). The levels of testosterone in testosterone-injected gilts (our study) are also in agreement with those determined in the blood of gilts with ovarian cysts (Fitko *et al.* 1998).

In conclusion, our study shows that long-term testosterone treatment of adult gilts results in alterations in both the spatial and neurochemical organization pattern of ovary sensory innervation. The data suggest that hyperandrogenism may regulate the transmission of sensory modalities from the ovary to the spinal cord, and antidromic regulation of the ovarian structure functions by affecting the ovary supplying DRGs neurons. Although our study concentrated only on ovarian DRG Th16-L5 perikarya, testosterone treatment could affect also the DRG neurons supplying other urogenital organs and intestine, due to the fact that those DRG neurons may express receptors for steroid hormones. This question needs further investigations.

Conflict of Interest

There is no conflict of interest.

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