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Long-term testosterone administration affects the number of paracervical ganglion ovary-projecting neurons in sexually mature gilts

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ABSTRACT

The influence of testosterone (T) overdose on the number and distribution of ovarian neurons in the paracervical ganglion (PCG) in pigs was examined. To identify the ovarian neurons, on day 3 of the estrous cycle, the ovaries of both the control and experimental gilts were injected with retrograde neuronal tracer Fast Blue. From next day to the expected day 20 of the second studied cycle, experimental gilts were injected with T, while control gilts received oil. The PCG was then collected and processed for double-labeling immunofluorescence. T injections increased the T (~3.5-fold) and estradiol-17β (~1.6-fold) levels in the peripheral blood, and reduced the following in the PCG: the total number of Fast Blue-positive neurons, the number of perikarya in the lateral part of the PCG, the numbers of VAChT$^+$/SOM$^+$, VAChT$^+$/VIP$^+$, VAChT$^+$/nNOS$^+$, VAChT$^+$/VIP$^-$, VAChT$^+$/DβH$^-$, VAChT$^-$/SOM$^-$, VAChT$^-$/VIP$^-$, VAChT$^-$/nNOS$^-$ and VAChT$^-$/DβH$^-$ perikarya, In the T-affected PCG, the populations of ovarian perikarya coded VAChT$^-$/SOM$^+$, VAChT$^-$/VIP$^+$ and VAChT$^-$/DβH$^+$, and expressing androgen receptor were increased. After T treatment within the PCG dropped the density of nerve fibers expressing VAChT and/or SOM, VIP, DβH. Obtained data suggest that elevated androgen levels occurring during pathological processes may regulate ovary function(s) by affecting the PCG gonad-supplying neurons.

1. Introduction

Many pathological processes occurring in the endocrine glands of both women and animal females, may lead to significant disturbances in their steroidogenic activity which, in turn, leads to alterations in the patterns of circulating steroid hormones. A significant augmentation in the peripheral blood androgen concentrations takes place in patients suffering from polycystic ovary syndrome (PCOS), (Panidis et al., 2005; Choi et al., 2011), adrenal hyperplasia (Goodarzi et al., 2003) as well as by ovarian (Singh et al., 2012) and adrenal (Marcondes et al., 2011) androgen-secreting tumors. Raised blood androgen levels are also found in the course of uterine inflammation in gilts (Jana et al., 2004).

In pigs, the paracervical ganglion (PCG) is one of the sources for neuronal inputs to the neural circuits controlling the reproductive tract, including the ovary. The post-ganglionic PCG neurons which supply the ovary are cholinergic (constitute the most numerous population, express choline acetyltransferase (ChAT), vesicular acetylcholine transporter – VAChT), noradrenergic (express

Abbreviations: ACh, acetylcholine; A_4, androstenedione; AR, androgen receptors; CaMG, caudal mesenteric ganglion; CG, celiac ganglion; ChAT, choline acetyltransferase; CGRP, calcitonin gene-related peptide; DβH, dopamine-β-hydroxylase; DRGs, dorsal root ganglia; E_1, estron; E_2, estradiol-17β; ERs, estrogen receptors; FB, Fast Blue; GAL, galanin; IGF-1, insulin-like growth factor-1; NA, noradrenaline; NGF, nerve growth factor; nNOS, neuronal isoform of nitric oxide synthase; NPY, neuropeptide Y; NANC, non-adrenergic-noncholinergic; PACAP, pituitary adenylate cyclase-activaiting polypeptide; PCG, paracervical ganglion; PCOS, polycystic ovary syndrome; PGs, pelvic ganglia; PNS, peripheral nervous system; VAChT, vesicular acetylocholine transporter; VIP, vasoactive intestinal polypeptide; SChGs, sympathetic chain ganglia; SOM, somatostatin; SP, substance P; T, testosterone.

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noradrenaline – NA, tyrosine hydroxylase – TH, dopamine-β-hydroxylase – DβH) and non-adrenergic–noncholinergic (NANC) (Majewski, 1997). Apart from catecholamines and acetylcholine (ACh), the pelvic ganglia (PGs), including female PCG and its male counterpart in laboratory animals and pig, were also found to express the neuronal isoform of nitric oxide synthase (nNOS), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), substance P (SP), somatostatin (SOM), galanin (GAL) and enkephalins (Papka et al., 1987; Mitchell, 1993; Keast, 1995a,b; Majewski, 1997; Jobling and Lim, 2008; Podlasz and Wasowicz, 2008; Burliński et al., 2012). Moreover, in the gilts, the above-mentioned factors were found in the nerve fibers in the area of PCG (Majewski, 1997; Podlasz and Wasowicz, 2008). It was indicated that the PCG cholinergic and NANC ovary-projecting neurons in gilts participate in neuronal control of blood vessels and follicles, while noradrenergic neurons may control only part of the cortical blood vessels (Majewski, 1997).

The connections between androgens and autonomic and sensory peripheral nervous system (PNS) neurons projecting to the reproductive organs and/or urinary system were examined predominantly in rats (Koszykowska et al., 2008). Neurochemical studies in male rats have identified the androgen receptors (AR) in neurons of the PG (Keast and Saunders, 1998; Purves-Tyson et al., 2007) and the dorsal root ganglia (DRGs) at lumbo-sacral neuromeres (Keast and Gleeson, 1998). In these animals, androgens significantly affected the morphology of viscera supplying autonomic PG neurons (Keast and Saunders, 1998). It is known that the AR-positive PG neurons express VIP, TH and nNOS (Schirar et al., 1997; Keast and Saunders, 1998; Purves-Tyson et al., 2007) and that lumbar and sacral DRG perikarya possessed AR and calcitonin gene-related peptide (CGRP), (Keast and Gleeson, 1998). Furthermore, in response to testosterone (T), essential changes in the chemical coding of sympathetic neurons in the hypogastric ganglion (Hamill and Schroeder, 1990) and in cholinergic neurons in the PG (Meusburger and Keast, 2001) were determined. It is generally accepted that androgen steroids affecting the morphology, chemistry and functions of pelvic autonomic neurons regulate many reproductive behaviors in males as well as urinary, bladder and lower bowel functions (Keast, 1999). There are also data showing that AR are localized in the sympathetic neurons of coeliac ganglion (CG) in late pregnant rats and that androstenedione (A_4) in these animals (Vallcaneras et al., 2009) and after parturition (Vallcaneras et al., 2011) may mediate a luteotropic effect acting at the CG and transmitting to the ovary a signal via a neural pathway. In both female and male rats, the AR location was also detected in the sheep fetus DRGs (Luo et al., 2008) and in Schwann cells of the sciatic nerve (Magnaghi et al., 1999).

There is little information on the androgen influence on the PNS neurons supplying the genito-urinary system in females of domestic animals. 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 the ovary functions (Verma et al., 2011; Swindle et al., 2012). Previously, we reported that long-term T administration changed the specific morphological and immunochemical structural organization of the caudal mesenteric ganglion (CaMG) ovary-projecting neurons (referred to further as ovarian perikarya or ovarian neurons) in adult gilts (Jana et al., 2013b). In relation to these findings, we suppose that elevated levels of endogenous androgenic steroids during pathological states can also affect the morphological and chemical plasticity of ovarian neurons in the PCG and, consequently, change gonadal functions. Therefore, the PCG of long-term testosterone-treated adult gilts, as an animal model for human hyperandrogenic pathology, were evaluated to determine: (1) the overall number and distribution of ovarian neuronal perikarya, (2) the numbers of ovarian perikarya expressing VAChT and/or SOM, VIP, nNOS, DβH as well as AR, and (3) the density of nerve fibers expressing VAChT and/or SOM, VIP, nNOS, DβH.

2. Materials and methods

2.1. Animals

The study was performed on female crossbred pigs (Large White × Landrace), aged 7–8 months and weighing 90–110 kg, with two controlled subsequent estrous cycles. The estrous-showing behavior was detected using a boar-tester. Three days before surgical operations, the animals were transported from a farm to a local animal house and kept in the individual stalls under natural light and temperature. They were fed a commercial grain mixture and tap water ad libitum. We followed the principles of animal care (NIH publication No. 86-23, revised in 1985), which were approved by the Local Ethics Commission for Animal Experiments (Agreement no 21/N).

2.2. Surgeries, treatments and material collection

On day 3 of the first studied estrous cycle (designed as day 0 of the study), median laparotomies were performed under general anesthesia induced by azaperone (1 ml/10 kg of body mass Stresnil, Jansen Pharmaceutica N.V., Belgium) and sodium pentobarbital (30 mg/1 kg of body mass, Vetbutal, Biovet, Poland) on all gilts to expose the ovaries. After an abdominal incision, the right and left ovary was gently removed from surrounding tissues and 5% solution of fluorescent retrograde neuronal tracer Fast Blue (FB, EMS-CHEMIE GmbH, Germany) was applied into the each ovary. The right and left side of the organ was injected 5 times (10 μl of the dye solution *per* 1 injection, in a total volume of 100 μl *per* ovary) using a Hamilton syringe equipped with a 26-gauge needle, keeping a similar distance between the places 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 thereafter the injection area was subsequently rinsed with isotonic saline and gently wiped with gauze. In all gilts, the polyvinyl cannula (outer diameter 2.2 mm, inner diameter 1.8, Tomel Tomaszów Mazowiecki, Poland) was also inserted into the jugular vein in order to collect blood samples.

Next, the gilts were randomly assigned to one of two groups: a control group (group I, $n = 3$) and an 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. on 38 consecutive days, 2 ml of oil was injected *i.m.* every 12 h (at 07:00 and 19:00 h). In turn, in the gilts of group II, at the same time and in the same manner, 1000 μg of T (cat. nr 35800, Serva Electrophoresis GmbH, Germany) in 2 ml of corn oil was injected. The applied dose of T was determined based on our preliminary experiment showing that its application increases the peripheral blood T concentration about 3.5-fold. According to available reports, about 3- and 5-fold increase in the total T and bioavailable T, respectively, in blood concentrations accompanies adrenal hyperplasia (Goodarzi et al., 2003), while the free androgen index is about 5-fold higher in women with PCOS than in controls (Panidis et al., 2005). For estimation of T, A_4, estradiol-17β (E_2) and estrone (E_1) levels, blood samples were collected from gilts of both groups throughout the period of T/oil injection (twice a day – 09:00 and 21:00 h). The samples were then immediately placed in an ice bath, where they were kept until centrifugation (10 min, 1500 × *g*, at 4 °C). The plasma was decanted and stored at −20 °C until further processing. The analysis of androgen and estrogen concentrations in the peripheral blood of gilts was described