—Original Article—

Long-term Estradiol-17 β Administration Decreases the Number of Neurons in the Caudal Mesenteric Ganglion Innervating the Ovary in Sexually Mature Gilts

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Abstract. The effect of estradiol-17 β (E₂) on the number and distribution of neurons in the caudal mesenteric ganglion (CaMG) supplying the ovary of adult pigs was investigated. Also, the numbers of ovarian dopamine- β -hydroxylase (D β H-), neuropeptide Y (NPY-), somatostatin (SOM-), galanin (GAL-) and estrogen receptor (ER)-immunoreactive perikarya as well as the density of the intraganglionic nerve fibers containing D β H and/or NPY, SOM, GAL were determined. E₂ was administered i.m. from day 4 of the first studied estrous cycle to the expected day 20 of the second studied cycle. Injections of E₂ (1) increased the E₂ level in the peripheral blood approximately 4–5 fold, (2) decreased the number of small-sized Fast Blue-positive postganglionic neurons in the CaMG, (3) decreased the number of small perikarya in the ventral, dorsal and central regions of the CaMG, (4) decreased the number of large perikarya in the dorsal and central regions, (5) decreased the number of small and large perikarya in the CaMG that were D β H⁺/NPY⁺, (6) decreased the number of small D β H⁺ but NPY⁻ perikarya, (7) decreased the number of small perikarya coded D β H⁺/SOM⁺ and D β H⁺/SOM⁻, (8) decreased the number of small D β H⁺/GAL⁻ perikarya, (9) decreased the number of small and large perikarya expressing ER subtypes α and β and (10) decreased the total number of nerve fibers in the CaMG containing D β H and/or NPY and D β H and/or GAL. These results show that long-term E₂ treatment of adult gilts downregulates the populations of both noradrenergic and ERs expressing ovarian neurons in the CaMG. Our findings suggest also that elevated E₂ levels that occur during pathological states may regulate gonadal function(s) by affecting ovary supplying neurons.

Key words: Caudal mesenteric ganglion, Estradiol-17 β , Ovarian neuron, Pig

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In pigs, the caudal mesenteric ganglion (CaMG) is virtually the sole source of sympathetic prevertebral neuronal input to the neural circuits controlling the large intestine [1, 2], ovary [3, 4], oviduct [5] and uterus [6]. Virtually all ovary-projecting postganglionic CaMG neurons in immature and adult pigs are noradrenergic; that is, they express enzymes participating in the synthesis of noradrenaline-tyrosine hydroxylase (TH) and dopamine- β -hydroxylase (D β H) [3, 4]. Moreover, sympathetic perikarya and nerve fibers within this ganglion have also been found to be immunoreactive to other active substances in a number of mammals, including adult cats [7], immature rats [8], fetal and adult guinea-pigs [9] and prepubertal pigs [3, 4]. The reactive substances include neuropeptide Y (NPY), somatostatin (SOM), galanin (GAL), calcitonin gene-related peptide (CGRP), cholecystokinin, bombesin/gastrin-releasing peptide, substance P (SP), vasoactive intestinal polypeptide (VIP), opioid peptides and enkephalins. The abundance of bioactive substances expressed in postganglionic sympathetic CaMG perikarya innervating the ovary correlates with a variety of the ovarian sympathetic actions including regulation of steroidogenesis, blood supply as well as development and ovulation of follicles (as reviewed by Kotwica *et al.* [10] and Jana *et al.* [11]).

In rats, a close relationship was found between estrogens and sympathetic and sensory neurons supplying the genitourinary system. Estrogen receptor (ER) subtypes α and β are localized in pre- and paravertebral (at thoracolumbar neuromeres) sympathetic neurons projecting to the ovary and uterus [12, 13] and proximal urethra [14]. The number of celiac ganglion neurons innervating the rat ovary and the content of ERs in these cells decreased after prenatal diethylstilbestrol exposure [15]. In turn, prepubertal β -estradiol-17-cypionate treatment increased the neuronal size in the rat celiac ganglion [12]. In female rats, DRG sensory neurons in L₆-S₁ segments that innervate the uterus [16, 17] and urinary bladder [18] also express ER subtypes α and β . In rats, an increase in the content of ER α in L₆-S₁ DRG neurons projecting to the uterine cervix near term is thought to occur in parallel with augmentation of plasma estrogens. The expressions of ERs, SP [19], CGRP [20] and pituitary adenylate cyclase-activating polypeptide (PACAP) [21] in these ganglia may be important for cervical ripening and consequently for initiation of the birth process. Moreover, estradiol-17 β (E₂) dose-dependently increased the synthesis of the above-mentioned peptides in the DRGs of ovariectomized rats [19–21]. In addition, long-term treatment with E₂ downregulated the expression of β -preprotachykinin mRNA encoding SP in the DRG [22].

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Many pathological processes occurring in the ovaries and other organs (e.g., pituitary and uterus) of both women and animal females may cause significant disturbances in the steroidogenic activity of the ovaries, resulting in changes in the patterns of circulating steroid hormones. Ovarian follicular cysts cause marked increases in the E_2 and/or estrone concentrations in blood plasma in cows [23–25] and pigs [26, 27]. Hyperestrogenism is also caused by ovarian tumors such as *folliculoma* and *thecoma* [28]. In addition, many foods contain xenoestrogens, such as pesticides, mycotoxins (zearalenone and its derivatives) and phytoestrogens (substances naturally occurring in plants), acting mainly *via* ERs to disturb many endocrine functions and reproductive processes [29]. Although neuroendocrine interactions have been studied in rodents, there is lack of data concerning the effect of sex steroids on the peripheral nervous system of females of domestic animals. In fact, the pig, due to its embryological, anatomical and physiological similarities to humans, constitutes an especially valuable species for biomedical research [30]. Based on these findings, we hypothesize that elevated levels of endogenous estrogens during pathological states (also xenoestrogens) can change the number and distribution of sympathetic ovarian neurons in the CaMG and consequently affect gonadal functions. To test this hypothesis, we examined the CaMGs of mature, cycling gilts to estimate the effect of long-term E_2 administration (simulation of pathological states occurring with elevated estrogen levels) on 1) the overall number and distribution of ovarian neuronal perikarya, 2) the numbers of ovarian perikarya containing D β H and/or NPY, SOM, GAL as well as ER subtypes α and/or β and 3) the density of nerve fibers containing D β H and/or NPY, SOM, GAL.

Materials and Methods

The experiment was performed on six female crossbred pigs (Large White $\times$ Landrace), aged 7–8 months and weighting 90–110 kg, over the course of two controlled estrous cycles. The estrous-showing behavior was detected using a boar. Three days before surgical operations, the animals were transported from a farm to the local animal house and kept in 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).

On day 3 of the first studied estrous cycle (day 0 of the study), all gilts were subjected to median laparotomy under general anaesthesia induced by azaperone (1 ml/10 kg of body mass, Stresnil, Janssen Pharmaceutica N.V., Belgium) and sodium pentobarbital (30 mg/1 kg of body mass, Vetbutal, Biovet, Poland) to expose the ovaries. After an abdominal incision, the right and left ovaries were gently removed from the surrounding tissues, and a 5% solution of fluorescent retrograde neuronal tracer Fast Blue (FB; Ems-Chemie GmbH, Germany) was applied to each ovary. The right and left sides of the organs were injected 5 times (10 μ l of the dye solution *per* 1 injection for a total volume of 100 μ l *per* ovary) using a Hamilton syringe equipped with a 26-gauge needle, keeping a similar distance between the places of the injections. To

minimize diffusion of the tracer into surrounding tissues (e.g., bursa ovary, mesosalpinx and oviduct 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 addition, the polyvinyl cannula (outer diameter 2.2 mm, inner diameter 1.8, Tomel Tomaszów Maz., Poland) was inserted into the jugular vein of each gilt in order to collect blood samples.

Next, the gilts were randomly assigned to one of the following two groups: the control group (group I, $n=3$) and 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., within 38 consecutive days, 2 ml of oil was injected i.m. every 12 h (at 0700 and 1900 h). In the gilts of group II, 1000 μ g of E_2 (Sigma) in 2 ml of corn oil was injected at the same time and in the same manner as for group I. The applied dose of E_2 was determined based on our preliminary experiment showing that its application increases the peripheral blood E_2 concentration by about 4–5 fold. According to the available reports, this E_2 blood concentration accompanies pathological states, such as ovarian cysts [24, 27] and tumors [31]. It has also been found that estrogen application results in significant decreases in ERs peripheral sympathetic [15] and sensory [32] neurons. Moreover, for determination of the E_2 level, blood samples were collected from gilts of both groups through out the whole period of E_2 /oil administration (twice a day—0900 and 2100 h). Immediately afterwards, the samples were placed on ice, where they were kept until centrifugation (1500 $\times$ g, at 4 C). After the last E_2 /oil injection, the gilts were deeply reanesthetized (following the same procedure as applied before 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 CaMGs were removed, postfixed by immersion in the same fixative for 10 min, 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% sodium azide (NaN_3) until further processing.

Serial 10- μ m cryostat sections (Frigocut, Reichert-Jung, Nussloch, Germany) of the ganglion were mounted on chrome alum-coated slides. By the use of an Olympus BX51 microscope (Olympus Polska Sp. z o.o., Warsaw, Poland), equipped for epillumination fluorescence microscopy (V1 module: excitation filter 330–385 nm, barrier filter 420 nm), the occurrence of retrogradely FB-labelled neurons was checked in serial sections of the bilateral ganglia. Sections with FB-labelled perikarya were processed for immunocytochemistry, applying a routine double-labelling immunofluorescence technique. After air-drying at room temperature for 45 min and rinsing in 0.1 M phosphate-buffered saline (PBS; pH 7.4; 3 $\times$ 10 min), the sections were incubated in a blocking buffer containing 10% normal goat serum (MP Biomedicals, Solon, OH, USA), 0.1 M PBS, 0.1% donkey serum (Abcam, Cambridge, UK), 1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA), 0.05% Thimerosal (Sigma-Aldrich) and 0.01% NaN_3 for 1 h at room temperature to reduce non-specific background staining. Subsequently, after another wash in PBS (3 $\times$ 10 min), the sections were incubated overnight at room temperature with primary anti-

era raised against the D β H (mouse, MAB308, Chemicon, Harrow, UK, dilution 1:1500) and/or NPY (rabbit, NA1233, Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA, dilution 1:1500), SOM (rabbit, 11180, MP Biomedicals, LLC, Solon, USA, dilution 1:1500), GAL (rabbit, T-4326, Peninsula Laboratories, San Carlos, CA, USA, dilution 1:8000). Also, the ganglionic sections with FB-labelled perikarya were incubated with antisera against ER subtype α (polyclonal rabbit against the C-terminus of ER α of mouse origin, sc-542, Santa Cruz Biotechnology, Santa Cruz, CA, USA, dilution 1:1000) and antisera against ER subtype β (monoclonal mouse against the human C-terminus region of the ER β 1 isoform, MCA1974, AbD Serotec, Raleigh, NC, USA, dilution 1:20). According to previous reports, immunocytochemical detection revealed the nuclear as well as cytoplasmic localization of ERs in the studied tissues [12, 14, 17, 18].

Following subsequent rinsing in PBS (3×10 min), the sections were incubated with biotinylated donkey anti-rabbit IgG (AP132B, Chemicon, Harrow, UK, dilution 1:1000) for 1 h at room temperature and subsequently with FITC-conjugated donkey anti-mouse IgG (715095150, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, dilution 1:800) and CY3-conjugated streptavidin (016160084, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA, dilution 1:9000) for 1 h at room temperature to visualize the antibody combinations: D β H/NPY, D β H/SOM and D β H/GAL. Next, the washed sections were coverslipped in carbonate-buffered glycerol (pH 8.6). Standard tests (preabsorption for the used antisera with the respective antigen at a concentration of 20–50 μ g antigen/ml diluted antiserum, omission of primary or secondary antisera and replacement by non-immune sera of all the primary antisera used) were employed to control the specificity of immunofluorescence. Specificity of the retrograde tracing was checked using various tests described previously [3].

Retrogradely labelled/double-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 D β H-, NPY-, SOM- and GAL-immunoreactive and/or all retrogradely labelled cell bodies were counted in every fourth section of the bilateral CaMGs. Only neuronal profiles with a visible nucleus were scored to avoid double counting. The localization of perikarya was estimated for particular regions of the CaMG, according to the method described previously [3]. Briefly, the cranial region of the ganglion constitutes the area corresponding to the first lumbar splanchnic nerve and intermesenteric nerve; the dorsal region constitutes the area corresponding to the second and third splanchnic nerves; the caudal region constitutes the area corresponding to the fourth lumbar splanchnic nerve and hypogastric nerve; and finally, the ventral region constitutes the area corresponding to the caudal colonic nerves. Also, perikarya were divided into two subclasses, small (23 ± 10 μ m in diameter) and large (51 ± 17 μ m in diameter) [3]. Their sizes were measured by using the Cell-F imaging software Olympus Polska Sp. z o.o., Warsaw, Poland. Sizes of neurons were calculated by measurements of the long and short axes of the perikaryon. The average value of the diameter of the perikaryon was assigned using the equation $d = \sqrt{l \times k}$, where d equals the diameter of a circle with a surface

area that is the most similar to the surface area of an ellipsoidal figure with long axis l and short one k [33]. In addition, the intraganglionic numbers of nerve fibers containing D β H and/or NPY, SOM or GAL found near ovarian perikarya were counted. Pictures were captured by a digital camera connected to a PC and analyzed with the AnalySIS software (version 3.02, Olympus Soft Imaging Solutions, Muenster, Germany).

The level of E₂ in blood plasma was measured by the radioimmunoassay procedure described by Hotchkiss *et al.* [34]. The antibodies (code BS/88/754) were obtained from the Department of Animal Physiology, University of Warmia and Mazury in Olsztyn, Poland, and characterized by Szafrńska *et al.* [35]. The sensitivity of the assay was 5 pg/ml. The intra- and interassay coefficients of variation were 7.1 and 12.2%, respectively.

All data concerning ganglia were calculated *per* one ganglion for the control and experimental groups. The mean ($\pm$ SEM) total number of perikarya and numbers of nerve fibers were compared between the groups by the Student's *t*-test. To calculate the statistical significance of the mean ($\pm$ SEM) numbers of particular neuronal sets (small, large, with different chemical coding, expressing ERs) in the ganglion, one-way analysis of variance (ANOVA) followed by the Newman-Keuls test was performed. The mean ($\pm$ SEM) E₂ levels were calculated for all samples taken each day for all control and E₂-treated gilts. The collected data were statistically assayed by two-factor analysis of variance (group $\times$ time) with repeated measures. The Neuman-Keuls test was applied for calculation of the statistical significance of mean differences (InStat, GraphPad, San Diego, CA, USA). Differences with a probability of $P < 0.05$ were considered significant.

Results

Concentrations of E₂ in peripheral blood

The mean daily E₂ concentrations in the peripheral blood of the gilts receiving this hormone were higher ($P < 0.05$ – 0.001 , about 4–5-fold) on all studied days than those in the control gilts.

The number and distribution of ovarian (FB⁺) neurons in the CaMG

In the experimental group, the total number of FB-positive perikarya in the CaMGs decreased ($P < 0.05$) compared with the control ganglia (95.03 ± 49.44 vs. 342.1 ± 98.41 , respectively). The number of small cells in the control group was greater ($P < 0.01$) than that of large ones (262.5 ± 79.13 vs. 80.33 ± 24.56 , respectively), while the proportions of the two size classes of cells in the experimental group were similar (54.33 ± 28.72 and 41.1 ± 21.13 , respectively). Compared with the control group, the E₂ treatment led to decrease ($P < 0.05$) only in the number of small perikarya (262.5 ± 79.13 vs. 54.33 ± 28.72 , respectively), while the numbers of large perikarya did not significantly differ in both groups.

Compared with the control group, the experimental group had fewer ($P < 0.001$) small cells in the ventral, dorsal and central regions (Fig. 1A). Also, the numbers of large cells in the dorsal ($P < 0.05$) and central ($P < 0.001$) areas of the CaMGs in the experimental group were lower than those in the control ganglia (Fig.

Fig. 1. Mean ($\pm$ SEM) numbers of small and large ovarian perikarya in particular regions of the CaMGs in the control (open bars) and experimental (solid bars) groups. a, b, c, d, e: different letters indicate differences ($P < 0.05$ – 0.001) among the particular regions within the control and experimental groups. * $P < 0.05$, ** $P < 0.001$: asterisks indicate differences between the groups for the same region.

1B). The numbers of ovarian perikarya populations in particular regions of the CaMGs of both groups are depicted in Fig. 1A and B.

The number of ovarian (FB⁺) neurons containing D β H, NPY, SOM and GAL in the CaMG

E₂ injections led to a drop ($P < 0.05$) in the numbers of D β H⁺/NPY⁺ and D β H⁺/NPY⁻ small perikarya. Also, this steroid application reduced ($P < 0.05$) the population of large neurons coded D β H⁺/NPY⁺ (Fig. 2A, Fig. 3A–H). In the CaMGs of E₂-treated gilts, the numbers of D β H⁺/SOM⁺ ($P < 0.05$) and D β H⁺/SOM⁻ ($P < 0.001$) small cells were lower than in the control group. Comparative analysis of the control and experimental groups revealed that the numbers of D β H⁺/SOM⁺, D β H⁺/SOM⁻, D β H⁻/SOM⁺ and D β H⁻/SOM⁻ large cells were similar in both groups (Fig. 2B, Fig. 3I–P). Following E₂ administration, the number of D β H⁺/GAL⁻ small perikarya was lower ($P < 0.001$) than that in the control group. In all studied gilts, the numbers of D β H⁺/GAL⁺, D β H⁺/GAL⁻, D β H⁻/GAL⁺ and D β H⁻/GAL⁻ large perikarya were similar (Fig. 2C, Fig. 3Q–X). The numbers of ovarian perikarya sets containing D β H and/or NPY, SOM, GAL as well as not expressing of the

above-mentioned substances within the CaMG of the control and E₂-injected gilts are presented in Fig. 2A–C.

The number of ovarian (FB⁺) neurons expressing ERs in the CaMG

In the CaMGs of the gilts receiving E₂, the total number of perikarya containing ER subtypes α and/or β decreased ($P < 0.001$) in comparison with the control ganglia (32.04 ± 6.79 vs. 118.8 ± 15.17 , respectively). This decline refers to the populations of small ($P < 0.001$) and large ($P < 0.05$) perikarya expressing ER subtypes α and β (Fig. 4 and 5A–P). The numbers of ovarian perikarya expressing ER subtypes α and/or β as well as not expressing these receptors within the ganglia of the control and experimental groups are presented in Fig. 4.

The density of nerve fibers containing D β H, NPY, SOM and GAL in the CaMG

Compared with the control group, application of E₂ resulted in a drop ($P < 0.001$) of the total number of the intraganglionic nerve fibers containing D β H and/or NPY (119.3 ± 1.85 vs. 59.1 ± 2.05 , respectively) and D β H and/or GAL (134.7 ± 2.18 vs. 43.3 ± 4.8 , respectively). The populations of fibers containing D β H and/or SOM differed insignificantly in the experimental and control groups (70.02 ± 1.52 vs. 62.1 ± 2.88 , respectively). E₂ treatment reduced ($P < 0.05$ – 0.001), as compared with the control group, the numbers of several types of nerve fibers, including D β H⁺/NPY⁺ (7.6 ± 1.45 vs. 11.67 ± 1.2 , respectively), D β H⁺/NPY⁻ (28.03 ± 1.5 vs. 37.33 ± 1.7 , respectively), D β H⁻/NPY⁺ (23.3 ± 2.01 vs. 70.3 ± 2.4 , respectively), D β H⁺/GAL⁺ (6.33 ± 1.45 vs. 11.6 ± 1.2 , respectively), D β H⁻/GAL⁺ (22.7 ± 1.8 vs. 36.6 ± 1.85 , respectively) and D β H⁻/GAL⁻ (13.3 ± 2.3 vs. 86.3 ± 1.45 , respectively). The population of D β H⁺/SOM⁻ fibers in the experimental group was found to be higher ($P < 0.01$) than that in the control group (33.67 ± 1.2 vs. 23.6 ± 2.02 , respectively; Fig. 6A–L).

Discussion

The present study shows, for the first time, that long-term E₂ treatment of adult gilts results in a decreased number of CaMG neurons projecting to the ovary. Moreover, reduction of both ovarian noradrenergic and ER-positive perikarya as well as the density of the nerve fibers containing D β H and/or NPY and D β H and/or GAL was observed.

We revealed that in E₂-injected gilts the total number of ovarian postganglionic neurons in the CaMG was reduced. A similar phenomenon was observed previously in the celiac ganglion projecting to the ovary in rats after prenatal diethylstilbestrol exposure [15]. Repeated administration of estradiol benzoate in female rats also decreased the survival of the hippocampal cells [36]. In turn, Chávez-Genaro *et al.* [37] described a reduction of the neuronal soma size during late pregnancy and parallel degeneration of intrauterine sympathetic nerves when the level of E₂ increased. As demonstrated here, the loss of the ovarian perikarya in the CaMGs of E₂-injected gilts may be the consequence of long-term enhancement of the E₂ level. Although the mechanism for this situation is unclear, we speculate that the E₂ level increase might be accompa-

Fig. 2. Mean ($\pm$ SEM) numbers of small and large ovarian perikarya immunoreactive to D β H and/or NPY (A), D β H and/or SOM (B) and D β H and/or GAL (C) as well as immunonegative to the above-mentioned substances in the CaMGs of the control (open bars) and experimental (solid bars) groups. a, b: different letters indicate differences ($P < 0.05$ – 0.001) among the particular neuronal populations within the control and experimental groups. * $P < 0.05$, ** $P < 0.001$: asterisks indicate differences between the groups for the same neuronal population.

Fig. 3. Fluorescent micrographs showing D β H- (B, F, J, N, R, V), NPY- (C, G), SOM- (K, O) and GAL- (S, W) immunoreactivity in the CaMGs of the control (A–D; I–L; Q–T) and E₂-treated (E–H; M–P; U–X) gilts after injections of FB into the ovaries. The double arrows indicate an FB-positive ovarian perikaryon containing two examined substances. The arrow shows an FB-positive ovarian perikaryon containing one of the two examined substances. The photographs D, H, L, P, T and X were created by digital superimposition of three color channels, FB-positive (blue), D β H-positive (green), and NPY-, SOM- or GAL-positive (red). A–D) In the control ganglion, two small ovarian perikarya immunoreactive to D β H and NPY as well as one perikaryon positive to D β H but negative to NPY are visible. E–H) Note in the E₂-affected CaMG, there is a drop in the number of small perikarya containing D β H and NPY and a lack of perikarya expressing only D β H. I–L) In the control CaMG there are two small perikarya immunoreactive to D β H and SOM as well as one perikaryon positive to D β H but negative to SOM. M–P) In the CaMG after E₂ injections, there is a decrease in the population of small perikarya immunoreactive to D β H and SOM and a lack of perikarya immunoreactive only to D β H. Q–T) Small perikarya positive to D β H but negative to GAL and a single perikaryon containing these two substances can be seen in the control CaMG. U–X) A noticeable drop in the number of small perikarya containing only D β H as well as a lack of perikarya expressing D β H and GAL can be seen in the E₂-affected CaMG. $\times 200$.

Fig. 4. Mean ($\pm$ SEM) numbers of small and large ovarian perikarya immunoreactive to ER subtypes α and/or β as well as those immunonegative to ERs in the CaMGs of the control (open bars) and experimental (solid bars) groups. a, b: different letters indicate differences ($P < 0.05$ – 0.001) among the particular neuronal populations within the control group. * $P < 0.05$, ** $P < 0.001$; asterisks indicate differences between the groups for the same neuronal population.

Fig. 5. Fluorescent micrographs showing ER subtypes β (B, F, J, N) and α (C, G, K, O) immunoreactivity in the CaMGs of the control (A–D; I–L) and E_2 -treated gilts (E–H; M–P) after injections of FB into the ovaries. The double arrows indicate an FB-positive ovarian perikaryon containing two subtypes of ERs. Asterisks indicate an FB-positive perikaryon devoid of ERs. The photographs D, H, L and P were created by digital superimposition of three color channels, FB-positive (blue), ER β -positive (green) and ER α -positive (red). Two small (A–D) and two large (I–L) perikarya expressing simultaneously ER subtypes α and β in the control CaMG. Note that individual small (E–H) and large (M–P) perikarya containing ERs are visible in the E_2 -affected ganglion. M–P) A small ovarian perikaryon negative to ERs was also encountered. $\times 200$.

Fig. 6. Fluorescent micrographs showing the nerve fibers containing D β H (B, F, H, K) and/or NPY (C, F) or GAL (I, L) around ovarian perikarya (A, D, G, J) in the CaMGs of the control (A–C; G–I) and E_2 -treated gilts (D–F; J–L). The double arrows indicate an FB-positive perikaryon. The arrowheads show nerve fibers containing examined substances. Around small perikarya, numerous nerve fibers positive to D β H and/or NPY (A–C) as well as D β H and/or GAL (G–I) can be seen in the control ganglion. In the E_2 -affected CaMG, there is visible reduction in the number of nerve fibers containing simultaneously D β H and NPY around a large perikaryon (D–F) and nerve fibers that are D β H-positive and GAL-negative around small perikarya (J–L). $\times 200$.

nied by downregulated ER expression in the studied perikarya. It is possible that the diminution in the content of the ERs in the studied perikarya could markedly limit the direct E₂ effects promoting survival of these perikarya, leading to their loss. This can be partly confirmed by the decrease in the number of perikarya positive for ER subtypes α and β in the CaMGs of E₂-injected gilts in the present study. A similar phenomenon was observed previously in the celiac ganglion of rats prenatally exposed to diethylstilbestrol [15] and in the DRG of estradiol benzoate-treated rats [38]. On the fundamental importance of ERs in survival of neurons points out the complete blockade of survival of DRG neurons after application of E₂ together with ER antagonists [39]. However, it has also been suggested that estrogens may have neuroprotective effects that are not mediated by the ER signaling pathways [40]. On the other hand, the suppression of the number of ovarian perikarya found by us in the E₂-treated gilts may also be due to reduced production of NGF, which, as is generally known, plays a crucial role for development and survival of neurons. This supposition is based on the finding that E₂ treatment decreased NGF protein content in the sympathetic cervical ganglion of rats [41]. It seems that E₂ could also contribute to the loss of neurons in the porcine CaMG by desensitization of these cells to NGF, which has previously been observed in rat DRG neurons following a single injection [38] and long-term [22] replacement of estrogen. Also, significant reduction in the number of neurons displaying p75 protein has been observed at the end of pregnancy, when estrogen levels are increased [42]. Moreover, considering the expression of ERs by neurons in autonomic and sensory areas of the lumbosacral spinal cord that have connections with female reproductive system [43], it is possible that the reduction in the number of postganglionic neurons found in the present study may partly result from changes of the density of ERs receptors on preganglionic neurons in response to E₂ treatment, which could ultimately affect both the morphology and activity of these cells and consequently disturb the neuronal network function.

It is important to underline that a significant decrease in the number of small but not large ovarian perikarya was observed in the CaMGs of E₂-treated gilts in the present study. Simultaneous, reductions in the numbers of small and large perikarya expressing ER subtypes α and β were also found in these animals. However, the decreasing effect was more visible in relation to small neurons. The above findings suggest a differential effect of long-term E₂ administration on the neuronal perikarya depending on their size and ER expression. This supposition is supported by our results showing that the control gilts had a greater number of small ovarian perikarya than large ovarian perikarya expressing ERs in the CaMG as well as by the study of Taleghany *et al.* [32], which shows that, in the neurons of L₁–S₁ DRGs of female and male rats, ER mRNA and protein were localized especially in small neurons.

Our results show the presence of small and large neuronal cells in all studied regions of the CaMGs in both the control and experimental groups. However, following E₂ application, the numbers of small perikarya decreased in the ventral, dorsal and central regions of the CaMG, while the numbers of large perikarya were lowered only in the dorsal and central regions. The consequence of the reduction in the neuronal populations in the central area as well as

along dorsal and ventral domains was substantial diminution of the “ovarian center” observed in the control CaMG, according to earlier findings in immature gilts [3].

We revealed that injections of E₂ in the gilts decreased the CaMG populations of small perikarya coded D β H/NPY (also large perikarya) and D β H/SOM as well as the numbers of perikarya immunoreactive to D β H but immunonegative to NPY, SOM and GAL. Similarly, the number of TH-positive neurons as well as the TH mRNA expression level diminished in the periventricular region of the preoptic area in developing female rats exposed to estrogen [44]. It should be also added that E₂ replacement in ovariectomized rats decreased the content of TH protein in the sympathetic cervical ganglion neurons [41] and the level in TH mRNA in the nucleus of solitary tract [45]. In contrast, upregulation of TH expression was detected in the rat sympathetic cervical ganglion perikarya during late pregnancy, which is when the estrogen levels are at their highest [46]. While the number of noradrenergic perikarya was downregulated in the present study, no significant changes in the numbers of non-noradrenergic perikarya were found. This is in line with the report of Toji *et al.* [47] demonstrating that long-term E₂ treatment caused no significant alteration in the amount of CGRP- and SP-IR sensory neurons of the urinary bladder in female rats. In turn, other data in the literature show enhancement in NPY and GAL mRNA expression in the trigeminal neurons of mice during proestrus and estrus, which is when E₂ level is at its highest [48], as well as in the GAL content in lumbosacral sympathetic cells innervating the avian uterine oviduct after E₂ injection [49]. Also, E₂ stimulated expression of SP [19], CGRP [20] and PACAP [21] in the DRG neurons projecting to the uterine cervix in adult rats. It is difficult to explain why the numbers of noradrenergic perikarya decreased in the CaMGs of the gilts receiving E₂, while the numbers of non-noradrenergic ones were not markedly changed. In spite of the fact that coexpression of ERs and particular substances has yet to be found in the neurons of the sympathetic ganglion, it seems possible that the differential effect of E₂ on neurons depending on their chemical coding observed by us in the present study could have resulted from a varied density and cellular location of ER α and/or ER β in the examined populations of neurons [12, 13, 16].

In the present study, nerve fibers containing D β H, NPY, SOM and/or GAL were present in the CaMGs of both the control and experimental groups. However, the number of nerve fibers immunoreactive to D β H and/or NPY and D β H and/or GAL in the CaMG of the gilts receiving E₂ was markedly lowered, while the population of D β H- and/or SOM-immunoreactive fibers was only slightly changed. There is a lack of data concerning the effect of steroids on the number of intraganglionic nerve fibers. However, it can be assumed that a drop in the density of noradrenergic- and/or peptide-immunoreactive fibers in the CaMG of E₂-injected gilts could partly result from, as revealed in this study, the loss of ovarian perikarya within the ganglion. This assumption is based on earlier data showing that the reduction in the set of the intraovarian sympathetic nerve terminals in rats prenatally exposed to diethylstilbestrol [50] was accompanied by a decrease in the number of sympathetic ovarian perikarya in the celiac ganglion [15]. The changes in the population of nerve fibers within the CaMGs of

E₂-treated gilts observed in our study can also result from the effect of E₂ on perikarya located in other ganglia, such as sympathetic chain ganglia and DRGs. It is known that nerve fibers present in the CaMG originate also from the area of these ganglia, as was shown in gilts [3] and other female species as reviewed by Mawe [51]. Moreover, ERs, as mentioned above, are present in the population of peripheral autonomic and sensory ganglia in female rats [12, 16, 17, 32].

Our data show that long-term E₂ treatment of adult gilts results in loss of a specific population of noradrenergic and ERs expressing ovarian neurons in the CaMG. Although further efforts are needed to recognize the mechanism of estrogen influence on these neurons, our findings suggest that an elevated E₂ level during pathological states may regulate gonadal function(s) by affecting the CaMG ovary supplying neurons. The present results further support the importance of estrogen as a modulator of neuronal plasticity.

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