Adrenergic, nitrergic and peptidergic innervation of the urethral muscle in the boar

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Abstract: In this study, the innervation of the urethral muscle in adult male pigs was investigated using combined NADPH-diaphorase (NADPH-d) histochemistry and immunocytochemistry. Nerve fibres supplying the urethral muscle were found to show NADPH-d activity and they also expressed immunoreactivity to catecholamine synthesising enzymes including tyrosine hydoxylase (TH) and dopamine-β-hydroxylase (DβH) as well as to: vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY). Different subpopulations of the nerve fibres (NADPH-d positive, TH-, DβH-, VIP- and NPY-immunoreactive (IR), but also NADPH-d/VIP- and NADPH-d/NPY-IR) were disclosed. These nerve fibres were observed not only to run among muscle fibres of the urethral muscle, but also within extrinsic nerve trunks. Moreover, in the organ studied, numerous ganglia were found. The intramural ganglia, composed of a few to 30 neurons were located in the proximal, middle and distal regions of the pelvic urethra. In the vicinity of the urethral muscle, there were mainly small ganglia containing two to several neurons, but also larger ganglia consisting of up to tens neurons were encountered in the connective tissue surrounding the pelvic urethra. In the ganglia observed in the neighbourhood of the urethral muscle, different subpopulations of nerve cells were found, namely: catecholaminergic, nitrergic, VIP-IR, NPY-IR and also NADPH-d/DβH-, NADPH-d/VIP- and NADPH-d/NPY-positive. Possible sources of the innervation for this muscle were also discussed.

Key words: Urethral muscle - Innervation - Catecholamine synthesising enzymes - NADPH-diaphorase - Neuropeptides - Pig, male

Introduction

The innervation of the lower urinary tract has been studied in some mammalian species including rat [30, 31], cat [3, 24, 29], dog [6, 8, 27], guinea-pig [3, 17, 18, 32, 35], female pig [11] and humans [10]. In general, the greatest density of the nerve fibres has been observed in the smooth muscle coat of the distal urethra, followed by the bladder neck, middle urethra, and proximal urethra. These nerve fibres have been found to contain different biologically active substances. The urethral muscle is supplied by nerve fibres immunoreactive to catecholamine synthesising enzymes [2, 3, 6, 8, 9, 21, 24, 25, 29], NPY [5, 19, 26, 31, 33, 34], VIP [1, 5, 15, 16, 26, 33, 35], enkephalins [4, 5], somatostatin [5, 16], galanin [33, 34] and also by nerve fibres showing acetylcholinesterase and acetylcholintransferase activity [28, 30, 34].

Studies performed in the pig focused on the innervation of the female lower urinary tract revealed that these organs are innervated by acetylcholinesterase-positive as well as catecholamine- and peptide-containing nerve fibres. The peptides examined included: VIP, substance P, somatostatin, Met-enkephalin and gastrin [11]. Crowe and Burnstock [11] described the innervation of the lower urinary tract in female pigs. In their paper, the innervation of the lower urinary tract in these animals was compared to that of the human bladder and urethra described previously and the authors concluded that the lower urinary tract in the pig is a good model for some studies of innervation of the lower urinary tract in humans. It is very important to find a good model for the human bladder and urethra innervation because this parameter often changes after injures or other pathological processes [13, 14, 16].

As already mentioned, innervation of the urethral muscle, a striated muscle surrounding the pelvic urethra, was studied only in female pigs and nothing is known about this problem in male pigs. Thus it seems to be very interesting to know features of the innervation of urethral muscle also in the male animals, because the pig is

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considered as a good model to study experimentally evoked pathological changes in the lower urinary tract and methods of their treatment. These results can be extrapolated to human patients with autonomic dysreflexia and detrusor-sphincter dyssynergia, which is often observed after operations of the urinary bladder or in patients with spinal cord injuries.

Materials and methods

This study was performed on four adult male pigs. Thirty minutes before the main anaesthetic, pentobarbital (Vetbutal, Biowet, Poland; 25 mg/kg b.w.) was given intravenously, all the animals were pretreated with propionylpromasine (Combelen, Bayer, Germany; 0.4 mg/kg of b.w., i.m). The animals were perfused transcardially with 4% paraformalehyde dissolved in 0.1 M phospate buffer, pH 7.4 (PB). Then, the tissues (pelvic part of the urethra with the urinary bladder neck) were removed and postfixed by immersion for 4 h in the same fixative. The tissues were rinsed in PB and transferred into 30% sucrose solution in PB (4˚C for 72 h). Serial cryostat sections 12 μm thick were put on chrome alum-coated slides and stored in a freezer (-30˚C) until further processing.

After washing with PB (3×10 min), the sections were processed for single-labelling immunohistochemistry using antisera against TH, DβH, NPY and VIP (Tab. 1) The sections were incubated in a blocking mixture containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X100 in Tris buffered saline (TBS; 0.1M, pH 7.4). Then, they were rinsed in TBS (3×10 min) and incubated with the primary antiserum for 24 h at room temperature (RT). After rinsing in TBS (3×10 min), the sections were incubated with a secondary antiserum (1 h; RT; Tab. 1). Then they were rinsed in TBS (3×10 min) and next sections incubated with biotinylated secondary antiserum were incubated with avidin-peroxidase complex (1 h; RT). After incubation, they were subsequently rinsed in TBS (3×10 min), Tris-HCl buffer pH 7.4 (10 min) and in sodium acetate buffer, pH 5.0 (SAB) (10 min). Afterwards, the sections were incubated for 5 min with 3-amino-9-ethylcarbazole (AEC, reagent for peroxidase enzyme) solution (4 mg of AEC dissolved in 0.5 ml NN-dimethylformamide and added to 9.5 ml 0.05 M SAB ; before use 5 μl 30% H_2O_2 was added).

After the incubation, the sections were rinsed in SAB (10 min), Tris-HCl pH 7.4 (10 min.) and deionised water (10 min). Next, the sections were stained with NADPH-d method. They were washed with PB (0.1 M, pH 7.4, 3×10 min) and incubated (60 min, 38˚C) in a solution containing 5.0 mg β-NADPH, (Biomol, Hamburg, Germany), 0.5 mg nitroblue tetrazolium (NBT, Biomol, Hamburg, Germany), and 15 μl Triton X-100 (Sigma, Deisenhofen, Germany) in 5 ml PB (0.1 M pH 7.4). After washing with PB (3×10 min), the sections were mounted with Aquamonunt. The stained sections were studied using a light microscope (Nikon FXA Microphot).

Table 1. Antisera used in the study

| Antigen	Code/Lot	Species	Dilution	Supplier
Primary antibodies				
TH	2/40/15	mouse monoclonal	1:50	Boehringer
DβH	TE103DβH	rabbit polyclonal	1:1000	ETI
VIP	20077	rabbit polyclonal	1:7000	Incstar
NPY	NT 115 (NPY)	rat polyclonal	1:1000	ETI
Secondary reagents				
Goat anti rat IgG (H+L) HRP-conjugated			1:100	Zymed
Goat anti rabbit IgG biotinlylated			1:200	Vector
Goat anti mouse IgG HRP-conjugated			1:100	Dakopatts
Rabbit anti rat IgG (H+L) biotinlylated			1:100	Vector
Avidin-peroxidase complex			1:200	Vector

Neurons stained for NADPH-d displayed from blue to navy-blue colour and those immunoreactive for other substances investigated were red, and thus, the double-stained perikarya were purple. All the neuronal profiles including those stained for NADPH-d only, those immunoreactive for one of the neuropeptides or catecholamine synthesizing enzyme only, and those double-stained were counted in some sections from the cranial, middle and caudal part of the pelvic region studied. The distance between the sections was at least 60 μm to avoid the double analysis. Only neuronal profiles with clearly visible nuclei were considered. Finally, the percentages of the different neuronal subpopulations were calculated.

Control preabsorption of a diluted antiserum with 20 μg/ml of an appropriate antigen abolished the specific immunoreaction completely. In addition, experiments were carried out in which the primary antisera were replaced by non-immune sera, or by PBS, in order to indicate the specificity of particular immunoreactions. NADPH-d-staining was not observed when β-NADPH or NBT were absent from the incubation medium.

Results

In the organ studied, very numerous nerve fibres immunoreactive to DβH were found, whereas TH-IR nerve fibres were slightly less numerous. The catecholaminer-

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Fig. 1. DβH-IR nerve fibres forming dense networks around an extrinsic blood vessel. $\times 480$. **Fig. 2.** DβH-IR nerve fibres forming dense networks around an intrinsic blood vessel. $\times 480$. **Fig. 3.** DβH-IR nerve fibres runing among muscle fibres of the porcine urethral muscle. $\times 480$. **Fig. 4.** NPY-IR nerve fibres innervating an intrinsic blood vessel. $\times 480$. **Fig. 5.** NPY-IR nerve fibre supplying muscle fibres of the urethral muscle. Inset shows NPY/NADPH-d positive nerve fibre . $\times 480$. **Fig. 6.** VIP-IR nerve fibre supplying the urethral muscle. $\times 480$. **Fig. 7.** NADPH-d positive nerve fibres supplying blood vessel within the urethral muscle. High activity of NADPH-d is also visible in the endothelium of blood vessel. $\times 280$. **Fig. 8.** NADPH-d positive nerve trunks located in the tissue surrounding the urethral muscle. $\times 480$. **Fig. 9.** NADPH-d positive nerve fibres running among muscle fibres. $\times 480$. **Fig. 10.** A small intramural ganglion containing only NADPH-d positive neurons. $\times 480$. **Fig. 11.** Double stained ganglion (DβH/NADPH-d). The main subpopulation are neurons containing immunoreactivity to DβH (red cells). Some cells are only NADPH-d-positive (small arrow) and some neurons show simultaneously immunoreactivity for DβH and NADPH-d-activity (big arrows). $\times 480$. **Fig. 12.** Double stained ganglion (NPY/NADPH-d). Numerous cells are NADPH-d-positive (one of them is indicated with a small arrow), some of them contain simultaneously NPY (big arrow). $\times 480$. Some cells are only NPY-IR (inset, the smallest arrows, $\times 280$). **Fig. 13.** Double stained ganglion (VIP/NADPH-d). NADPH-d-positive cells are blue, some cells are simultaneously immunoreactive to VIP (big arrow). Some other cells are VIP-IR only (small arrows). $\times 480$.
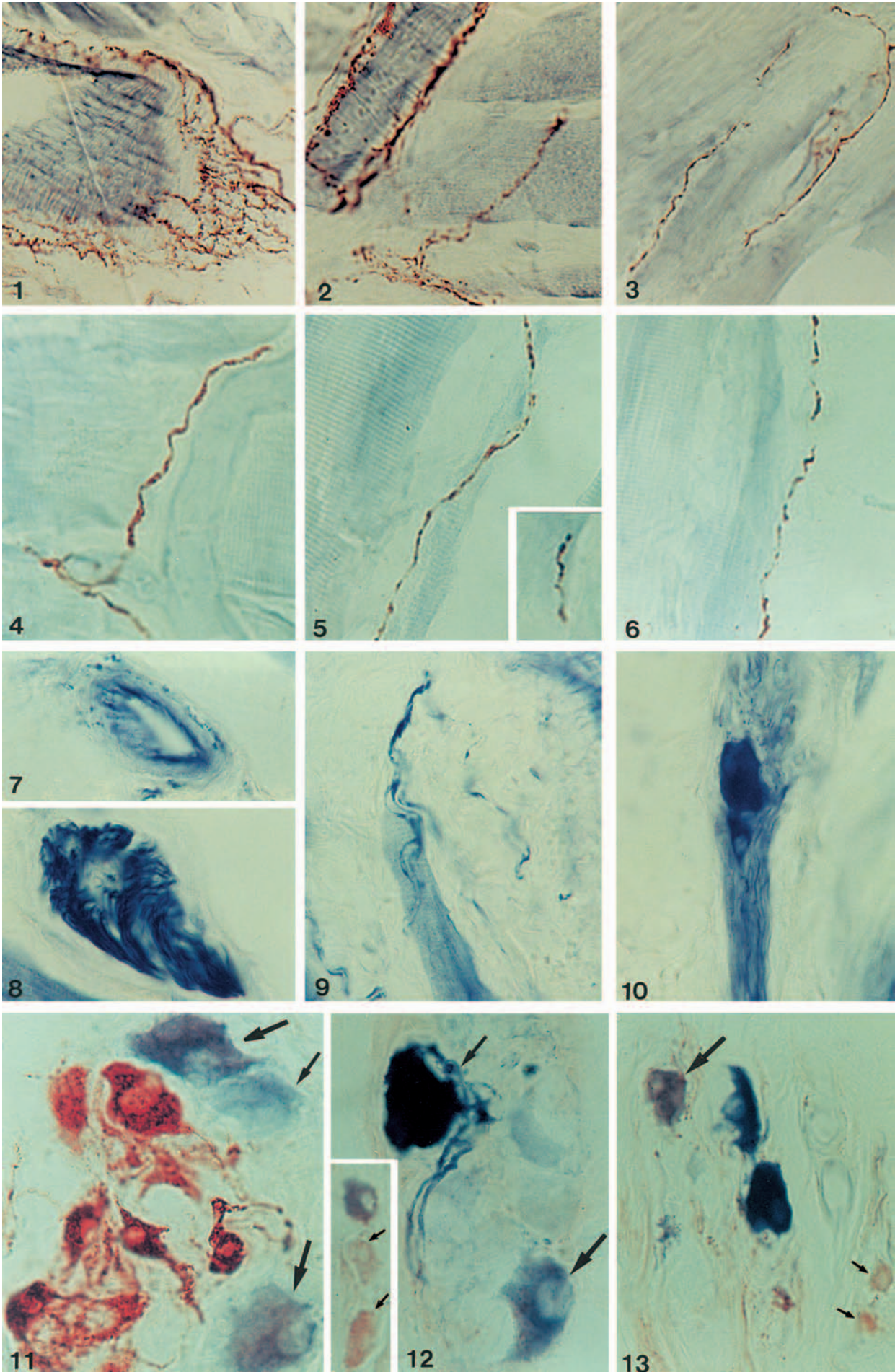
gic nerves formed dense networks especially around the extrinsic (Fig. 1) and intrinsic (Fig. 2) blood vessels. They were very often found to form thick trunks, observed in the tissue surrounding the pelvic urethra. Numerous nerve fibres were also found to run among muscle fibres of the muscle studied (Fig. 3). The number of these fibres gradually decreased towards the caudal part of the urethral muscle. NPY-IR nerve fibres (Figs. 4, 5) were less numerous than those containing immunoreactivity to TH and DβH, while VIP-IR nerve fibres (Fig. 6) were even less numerous than NPY-IR axons. Morphological features of the peptidergic nerves were similar to those of the catecholaminergic nerve fibres. In the sections studied, some NADPH-d positive structures were found. The high activity of this enzyme was observed not only in sarcolemma of the muscle fibres but also in the endothelium of blood vessels and in numerous nerve fibres associated with the vessels (Fig. 7). Some NADPH-d positive nerve fibres formed nerve trunks (Fig. 8). NADPH-d positive nerve fibres were also found to run among muscle fibres (Fig. 9). Their localisation was similar to that of the catecholaminergic nerve fibres. Some nerve fibres displayed NADPH-d activity and simultaneously immunoreactivity to VIP- or NPY (Fig. 5).

Numerous ganglia were found in the organ studied. The intramural ganglia, composed of a few to 30 neurons (Fig. 10), were located in the proximal, middle and distal regions of the pelvic urethra. In the vicinity of the urethral muscle, just between muscle and surrounding tissue, there were mainly small ganglia containing from two to several neurons, but also larger ganglia containing up to tens neurons were encountered in the connective tissue surrounding the urethra. Within these ganglia, the main subpopulation of neurons were those containing catecholamine-synthesising enzymes. Double-staining for these enzymes and NADPH-d revealed mostly the neurons stained for one studied substance only, but occasional neurons showed simultaneously immunoreactivity for DβH and NADPH-d (Fig. 11). In the ganglia studied, numerous cells exhibited NADPH-d activity. Some of them were simultaneously immunoreactive to NPY (Fig. 12) and, especially, VIP (Fig. 13), but also solely NPY- or VIP-positive neurons were found (Figs. 12, 13). Among VIP-IR nerve cell bodies, about 50% displayed simultaneously NADPH-d activity, while only approximately 20% of NPY-IR neurons were stained for NADPH-d.

Discussion

This paper describes for the first time the innervation of the urethral muscle in the male pig. As already mentioned, the innervation of this organ was studied in many mammalian species including female pigs. Crowe and Burnstock [11] reported the presence and localisation of different biologically active substances in the lower urinary tract of sows. The greatest number of the nerve fibres was found among muscle fibres of the distal urethra, followed by the bladder neck, middle urethra, and proximal urethra, with the smallest one in the detrusor muscle. The greatest number nerve fibres stained for acetylcholinesterase (AChE) followed by those containing vasoactive intestinal polypeptide- and catecholamine-containing fibres. The present study revealed that the nerve fibres immunoreactive to catecholamine-synthesising enzymes, especially to DβH, were the most numerous, while VIP-IR nerve fibres were much less abundant. In male guinea-pigs, catecholamine-containing nerves were observed among striated muscle fibres only in the junctional zone between the inner layer of the muscle and an outer layer of the striated muscle cells [18]. In this study, catecholaminergic nerve fibres were found not only in the junctional zones but also between the striated muscle fibres. Similar distribution pattern of the catecholaminergic nerves was described previously in male humans [20]. In women, noradrenergic nerve fibres supplying the bladder neck and proximal urethra are rarely observed. In contrast, the male human proximal urethra is richly supplied with noradrenergic nerves, what indicates that this region is under direct sympathetic control and its muscle coat contracts to prevent reflux of semen to the urinary bladder [22]. The features of the catecholaminergic innervation of the male porcine pelvic urethra described in this paper are very similar to those of the human male urethral muscle.

The present study revealed that the porcine male urethra is also supplied by VIP-IR nerve fibres. In female pigs, these nerves were numerous [11], whereas in the males, VIP-positive nerve fibres were sparsely distributed in the muscle. In humans, immunoreactivity to VIP was observed to occur only occasionally in fine nerve fibres [33]. Similar results were obtained in the bovine male, female guinea-pig and rat muscular tissue of the urogenital apparatus [5, 31, 35].

Moderate in number NPY-IR nerve fibres were observed in the muscle studied and their distribution pattern was similar to that of catecholaminergic and VIP-positive nerve fibres. In the human [26, 33] and rat [31] urethral muscle, NPY-IR nerves were found to run parallel to individual muscle fibres. They were quite numerous between the striated muscle bundles, especially in the intrinsic external urethral sphincter. Our observations in the male were in accordance with these findings.

NADPH-d positive nerve fibres formed thick bundles found in a close vicinity of the urethral muscle. Some of such trunks and sparsely distributed nerve fibres were also observed among the muscle fibres. In the female rabbit urethra, numerous NADPH-d-positive, fine varicose nerve fibres were observed both around arteries and in association with muscle bundles [36]. In the human urethral muscle, immunoreactivity to NOS and

NADPH-d activity were detected in the sarcolemma of the intramural striated muscle fibres. NOS-IR nerve trunks and fine nerve fibres, few of which terminated on muscle fibres, were present in the striated sphincter [23]. In both humans and guinea pigs, varicose NOS-IR nerve fibres provided a moderate innervation to the detrusor muscle of the bladder body, and a denser innervation to the urethral muscle. The immunoreactive nerves also projected to the subepithelium and supplied blood vessels, but they were rarely observed encircling intramural ganglia [32]. Our results corroborate all these observations.

In the present study, autonomic ganglia were also observed. These ganglia were found close to the urethral muscle. Small ganglia containing a few to approximately 20 cells were located in a close vicinity to the muscle fibres. Larger ganglia, consisting of to tens of neurons were present mainly in the connective tissue surrounding the organ studied. These observations are in accordance with findings obtained by other authors in pigs and guinea-pigs [11, 35]. In the human urethra, autonomic ganglia, containing from two to 20 nerve cell bodies, were found in the smooth and striated muscle layers of the urethral sphincter; they were rarely observed in the distal urethra and were absent from the prostatic urethra [12].

The neurons observed in our study were mainly catecholaminergic. TH or DβH-IR neurons were found mostly in larger ganglia distributed in the connective tissue surrounding the urethral muscle. Single catecholaminergic neurons contained simultaneously NADPH-d activity. Only a few catecholaminergic nerve cells were observed in ganglia bordering the organ studied. The last observation is in agreement with previous findings reported by Werkstrom and co-workers [35]. Numerous ganglionic nerve cells displayed NADPH-d activity only. These cells were found mainly in ganglia located close to the organ studied but also in ganglia distributed in the connective tissue. Similar findings have been obtained in humans and guinea pigs [32, 35]. Some of NADPH-d-positive neurons displayed immunoreactivity to VIP, but also moderate in number, solely VIP-positive neurons were encountered. In guinea-pigs, about 70% of the ganglionic cell bodies were nitrergic, whereas a minor part was VIP-IR [35]. Neuropeptide Y immunoreactivity was observed in some nerve cell bodies dispersed throughout all the ganglia. Morphological features and localisation of these cells was similar to that described previously in humans [12].

The present study revealed different subpopulations of the nerve cells in ganglia distributed in the neighbourhood of urethral muscle, namely: catecholaminergic, nitrergic, VIP-IR, NPY-IR and also NADPH-d/DβH-, NADPH-d/VIP- and NADPH-d/NPY-positive. In the urethral muscle, corresponding subpopulations of nerve fibres were found which suggests that neurons located

in the ganglia are the most probable source of the innervation for this muscle.

Similarities in the pattern of the innervation between the porcine and human male urethra suggest that the pig can be a very good model to study experimentally evoked changes in this organ and methods of treatment of human lower urinary tract disorders.

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