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Innervation of the chinchilla testis, epididymis, and vas deferens

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Abstract

The study was performed on six male chinchillas. The animals were anaesthetised with ether and the anaesthesia was deepened with nembuthal injected intraperitoneally. The chinchillas were then transcardially perfused with 0.4 L of 4% buffered paraformaldehyde, and testes, epididymides, and vasa deferentia were collected. The tunica albuginea from one testis from each chinchilla was stained as whole-mount preparation. The tissues were cut into 12 μm-thick cryostat sections, and processed for double-immunofluorescence method. In all organs studied, the most abundant nerve fibres were dopamine β hydroxylase positive (DβH$^+$). Some of them contained neuropeptide Y (NPY). Sporadically NPY-positive-only nerve fibres were found. Single DβH$^+$ nerve terminals contained also galanine. Small numbers of the nerve fibres supplying studied organs were stained for substance P (SP) and calitonin gene related peptide (CGRP). Almost all SP$^+$ fibres were also CGRP$^+$, whereas single CGRP$^+$ nerves were SP-immunonegative. Some nerve terminals were immunoreactive to vesicular acetylcholine transporter and vasoactive intestinal peptide. The organs studied were innervated unevenly. The highest density of the nerves was found in the areas of the tunica albuginea adjacent to the mesorchial border of the testis and their number gradually decreased towards the free border of the gonad. None of the vascular tissue of the testicular parenchyma was free of the nerve fibres, except sporadically encountered DβH$^+$ nerves which supply seminiferous tubules. Within the head of the epididymis a moderate number of nerve terminals were found, but in the body and tail of the organ the number of nerves gradually increased. The vas deferens was supplied with very numerous nerve fibres. There were no differences in the density of the innervation between the funicular and abdominal part of the vas deferens.

Keywords: chinchilla, male genital organs, innervation.

Introduction

Studies dealing with the distribution and chemical coding of nerve fibres supplying male genital organs are one of the main directions of research on the innervation of the reproductive tract in many animal species. Innervation of the testes was already studied in the frog (1), rat (39), ruminants (33), donkey (36), cat (35), dog (29), pig (34), and human (27). The innervation of the epididymis was also comprehensively investigated in the rat (17) human (27), and many other mammals (7, 24, 36). The innervation of the vas deferens was also the subject of intense investigations. Among many species studied the largest body of evidence involves rodents. This issue was studied in the rat (9), guinea pig (31), and mouse (13). As clearly seen from the above-mentioned papers, there are many reports dealing with the innervation of the testis, epididymis, and vas deferens in some mammalian species, but there is no data on the innervation of these organs in chinchilla. Therefore, we decided to study this topic using double immuno-histochemical staining method, to disclose the pattern of innervation of the chinchilla testis, epididymis, and vas deferens.

Material and Methods

The study was performed on six sexually mature male chinchillas weighing about 400 g each. They were

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anaesthetised with ether-anaesthesia and then injected intraperitoneally with 30 mg/kg of pentobarbital sodium (Morbital, Biowet Puławy, Poland) to deepen the anaesthesia. The anaesthetised chinchillas were transcardially perfused with 0.4 L of 4% ice-cold buffered paraformaldehyde (pH 7.4), and the testes with epididymides and vasa deferentia were collected. The tissues were then postfixed by immersion in the same fixative for 2 h, rinsed with phosphate buffer (pH 7.4), and transferred to and stored in 30% buffered sucrose solution (pH 7.4) until further processing. The tunica albuginea obtained from one testis from each chinchilla was stained as whole-mount preparation. One testis from each animal was encapsulated and the tunica albuginea was stained for one combination of antibodies (Table 1). The remaining gonads and other organs studied were cut into 12 μm-thick cryostat sections, which were processed for the double-immunofluorescence method on slide-mounted sections. The sections were washed 3 × 10 min in phosphate buffer (PB), incubated for 45 min in 10% normal goat serum (NGS, Cappel, Poland) in PB containing 0.25% Triton X-100 (Sigma, USA) and then incubated overnight at room temperature (RT) with antibodies (Table 1) diluted in PB containing 0.25% Triton X-100.

After incubation with primary antibody, the sections were washed 3 × 10 min in PB and further incubated with secondary antibodies for 1 h in RT. After incubation, the sections were washed 3 × 10 min in PB, coverslipped with buffered glycerol and examined under a confocal microscope (Zeiss LSM 700, Zeiss, Germany). The control of specificity of antisera was performed by preabsorbtion of a diluted antibody with 20 μg/mL of an appropriate antigen, which completely abolished the specific immunoreaction. In addition, the primary antibody was replaced by non-immune serum or by PB in order to verify the specificity of particular immunoreactions.

Results

The highest density of the nerve fibres was found in the areas of the tunica albuginea adjacent to the mesorchial border of the testis and their number gradually decreased towards the free border of the gonad. In parts of the tunica albuginea covering both extremities (superior and inferior) of the testis, the density of the nerve fibres was also higher than that found in the remaining areas of the testis capsule. Within the tunica albuginea, a moderate number of dopamine β hydroxylase positive (DβH$^+$) nerve fibres were observed (Fig. 1, Table 2). Some of them simultaneously contained neuropeptide Y (NPY) (Fig. 2). Most of the double-stained nerves were found to supply blood vessels. Sporadically NPY-positive-only nerve terminals were encountered (Fig. 2). Single DβH$^+$ nerve fibres expressed galanine (GAL) as well. Some nerve terminals expressed immunoreactivity to substance P (SP) and calitonin gene related peptide (CGRP) (Fig. 3). Virtually all SP-positive nerves were also CGRP-positive (Figs 3, 4), whereas single CGRP-positive nerve fibres showed no immunoreactivity to SP (Fig. 4). Some nerve fibres of the tunica albuginea were immunoreactive to vesicular acetylcholine transporter (VACHT) and vasoactive intestinal peptide (VIP). Nerve fibres penetrated from the tunica albuginea into the interior region of the testis and were observed in the septa of the gonad to supply the blood vessels (Fig. 4). Non-vascular tissue of the parenchyma did not contain the nerve fibres, except sporadically encountered DβH$^+$ nerve terminals, which supply the seminiferous tubules (Fig. 2).

Nerves running *via* the septa of the testis finally reached the mediastinum of the gonad. Within this part of the testis all previously described subpopulations of the nerve fibres were found (except VACHT- and VIP-immunoreactive nerve fibres), but they were less numerous than those observed in the tunica albuginea.

The epididymis was found to be richly but unevenly innervated. In the head of the epididymis, a moderate number of the DβH-immunoreactive nerve fibres was found (Fig. 5). Some of them simultaneously contained NPY (Fig. 5), but NPY-positive-only nerve fibres were sporadically observed (Fig. 5). In this part of the organ, GAL-positive nerve fibres were single and some of them were DβH-negative (Fig. 6). The head of the epididymis was supplied by moderate in number CGRP+ nerve fibres. Some of them were also immunoreactive to SP (Fig. 7), but SP-positive-only nerves were sporadically encountered (Fig. 7). Single VACHT- and VIP-containing nerve fibres were found within the head of the epididymis. The number of nerve fibres containing DβH (Figs 8, 11, and 13) VACHT (Figs 9 and 12), and VIP gradually increased from the body towards the tail of the epididymis, whereas the number of NPY-positive nerve fibres decreased (Figs 8 and 11). No clear-cut differences in the number of the nerve fibres immunoreactive for GAL (Fig. 13), SP, and CGRP (Figs 10 and 14) were determined. The vas deferens was supplied by very numerous DβH$^+$ nerve fibres (Fig. 15). Neuropeptide Y-positive nerves were single and were always DβH-immunoreactive. These fibres innervated blood vessels, which supply the organ studied. GAL-positive nerve fibres were slightly more numerous than those containing NPY. They were found within the wall of the vas deferens, closely to the lumen of the urethra, and most of them were also DβH-immunoreactive (Fig. 16). SP-positive nerve fibres, especially those located on the surface of the vas deferens, were moderate in number and some of them contained CGRP as well (Fig. 17). Many SP-immunoreactive nerve terminals, especially those distributed within the wall of the organ, were CGRP-immunonegative (Fig. 17). Some CGRP-positive nerves did not stain for SP (Fig. 17). SP/CGRP-immunoreactive nerve fibres were found in close vicinity to the blood vessels. VACHT-immunoreactivity was observed in numerous nerve fibres evenly distributed within the wall of the vas deferens (Fig. 18).

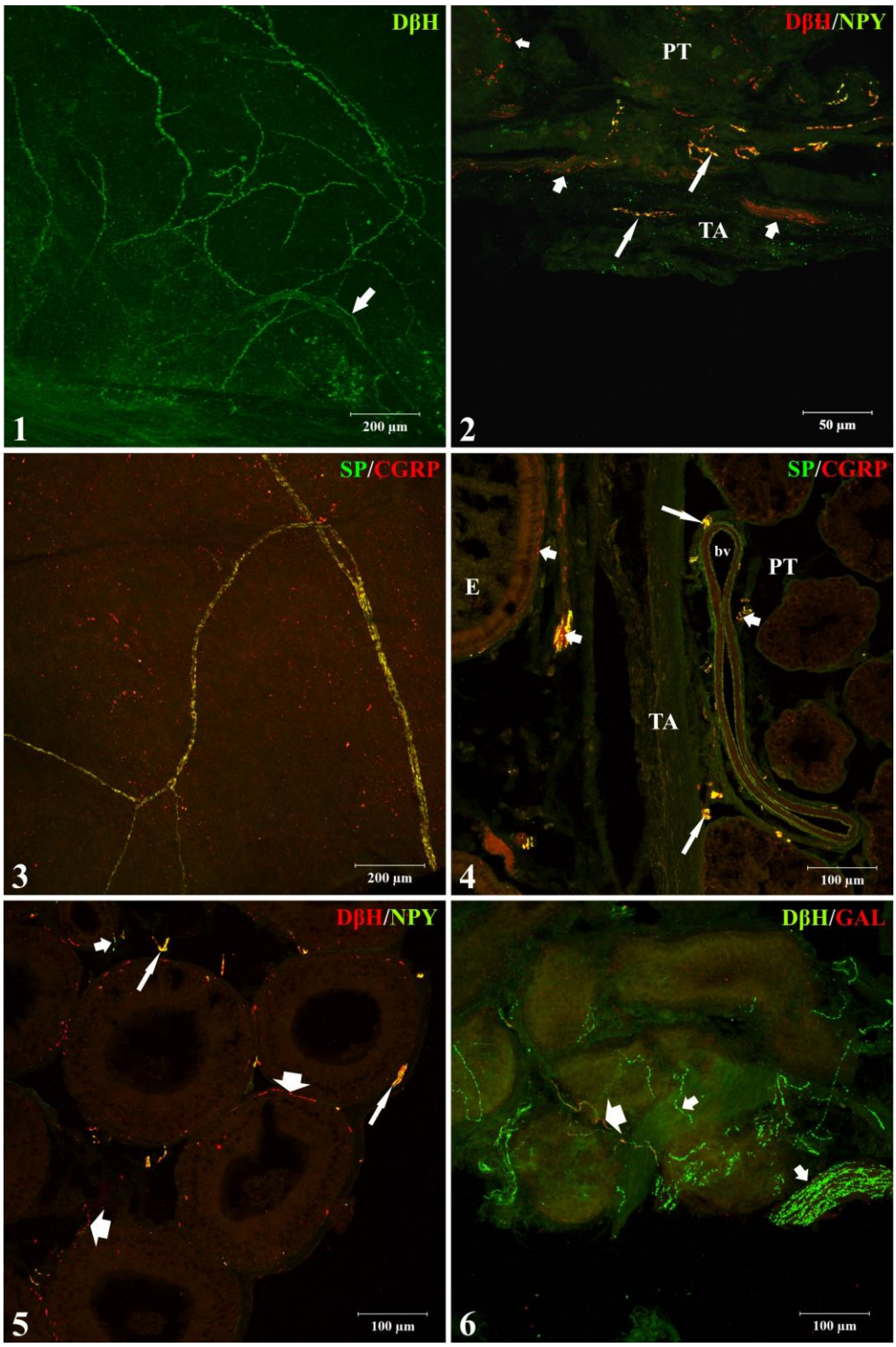


Fig. 1. The tunica albuginea of the testis. Whole-mount preparation stained with antibody against DβH. Tailed scan connected with 3D reconstruction of a large area of the tunica albuginea. Arrow shows nerves supplying blood vessel
Fig. 2. DβH- (short arrows) and DβH/NPY- (long arrows) positive nerve fibres within tunica albuginea (TA) and parenchyma of the testis (PT)
Fig. 3. Tunica albuginea of the testis. Whole-mount preparation stained with antibodies against SP and CGRP. Tailed scan connected with 3D reconstruction of a large area of the tunica albuginea
Fig. 4. CGRP- (short arrows) and SP/CGRP- (long arrows) positive nerve fibres within the parenchyma of the testis (PT) and epididymis (E). All nerve fibres distributed within the PT are located in close vicinity to a blood vessel (bv)
Fig. 5. DβH- (wide arrows), NPY- (small arrow) and DβH-NPY-positive (long arrows) nerve fibres within the head of the epididymis
Fig. 6. GAL- (wide arrow), DβH- (small arrow) positive nerve fibres within the head of epididymis. Section tangential to the surface of the duct of the epididymis

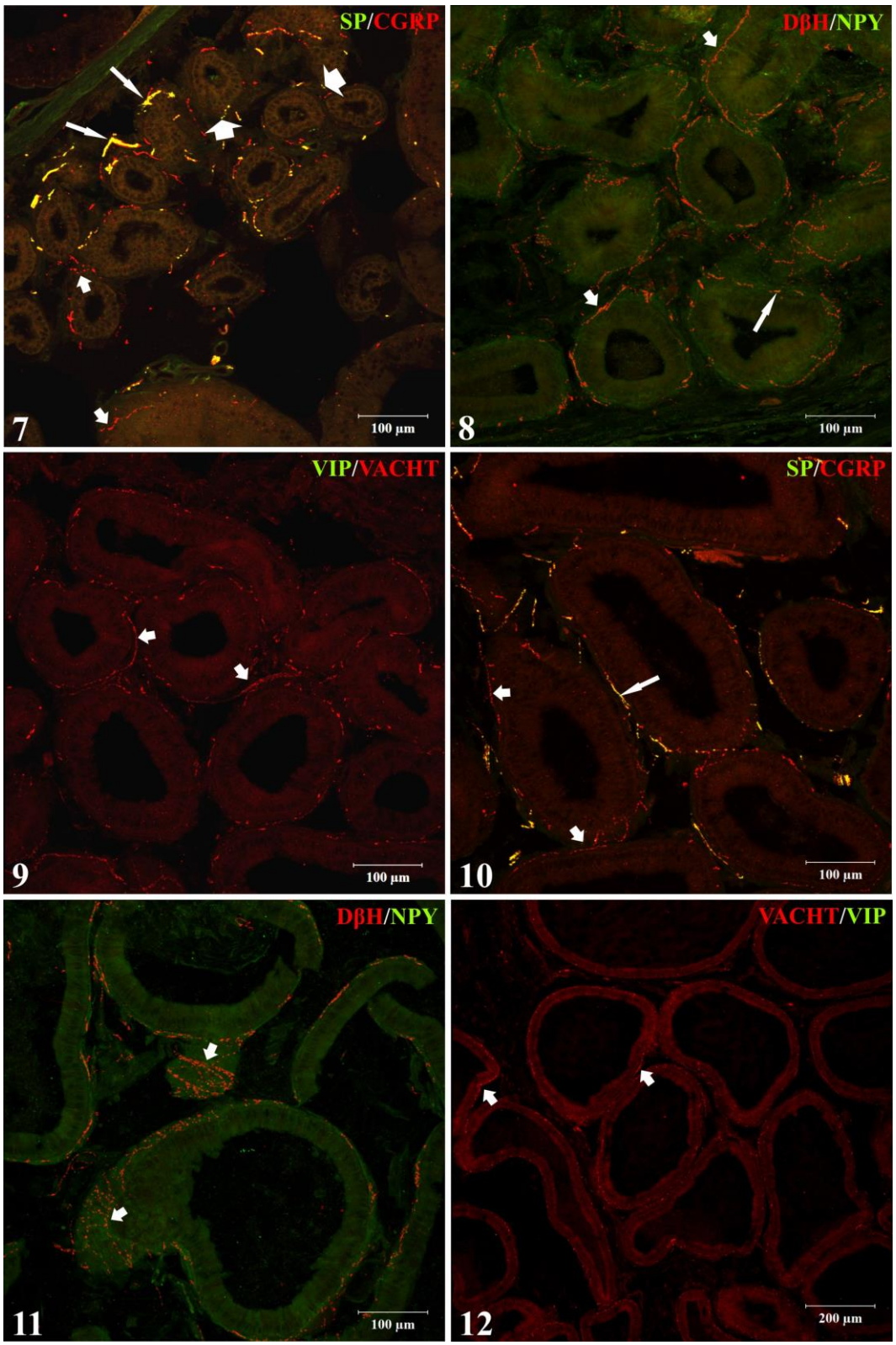


Fig. 7. SP- (wide arrow), CGRP- (small arrow) and SP/CGRP-positive nerve fibres (long arrows) within the head of the epididymis
Fig. 8. DβH- (small arrow) and DβH/NPY- (long arrow) positive nerve fibres within the body of the epididymis
Fig. 9. VACHT- (small arrow) positive nerve fibres within the body of the epididymis
Fig. 10. CGRP- (small arrow) and SP/CGRP-positive nerve fibres (long arrows) within the body of the epididymis
Fig. 11. DβH- (small arrow) positive nerve fibres within the tail of the epididymis
Fig. 12. VACHT- (small arrow) positive nerve fibres within the tail of the epididymis

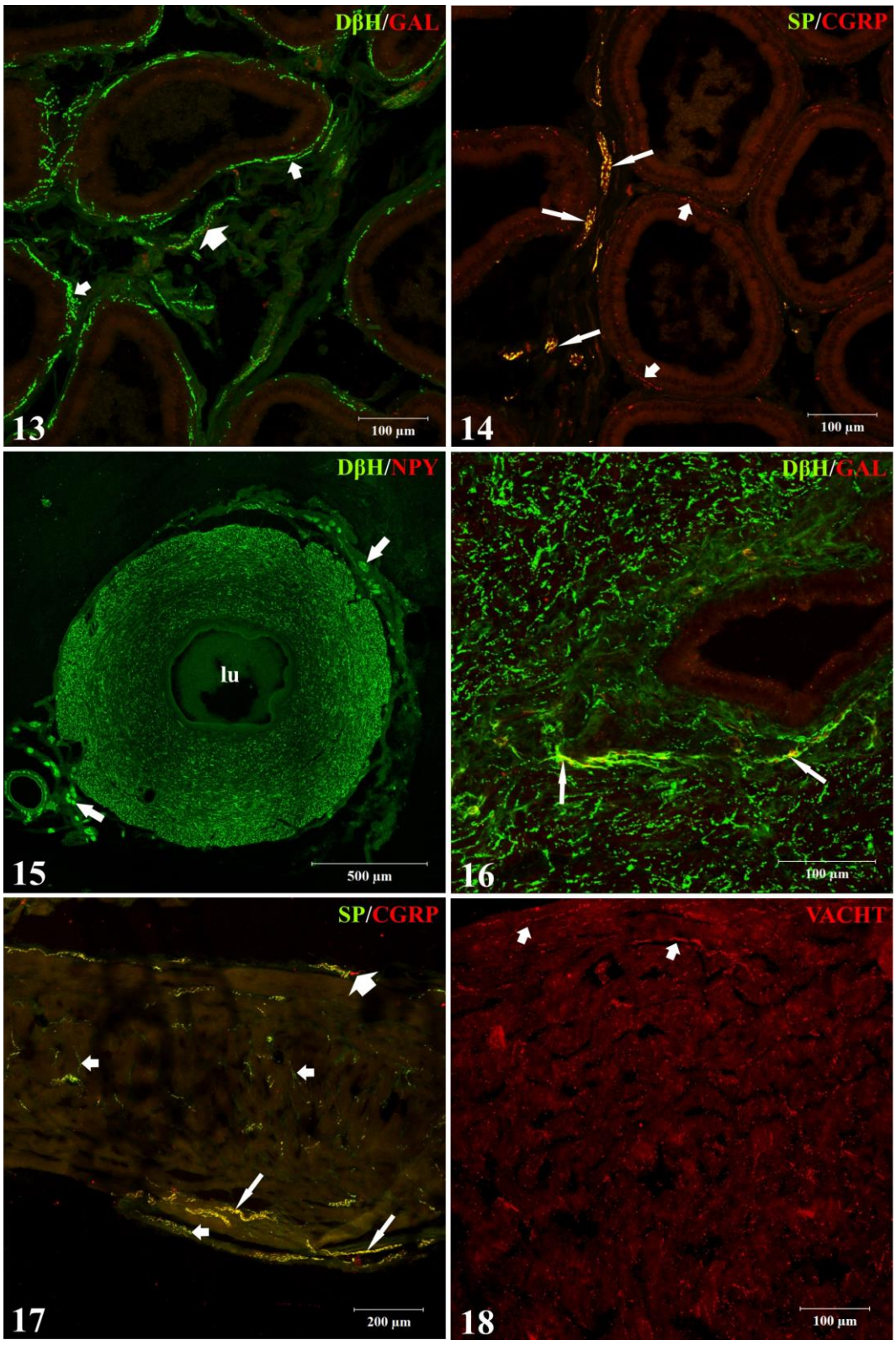


Fig. 13. GAL- (wide arrow), DβH- (small arrow) positive nerve fibres within the tail of the epididymis
Fig. 14. CGRP- (small arrow) and SP/CGRP-positive nerve fibres (long arrows) within the tail of epididymis
Fig. 15. Transverse section from the abdominal part of the vas deferens stained with antibody against DβH. Tailed scan connected with 3D reconstruction showing the whole area of the organ. Arrows show thick bundles of the nerves running along the vas deferens. lu - lumen of the vas deferens
Fig. 16. Longitudinal section from the abdominal part of the vas deferens stained with antibody against DβH and GAL. Long arrows show DβH/GAL-positive nerve fibres
Fig. 17. Longitudinal section through the abdominal part of vas deferens stained with antibody against SP and CGRP. Long arrows show the SP/CGRP-positive nerve fibres, small arrows show SP-positive nerves, large arrow points the bundle of CGRP-positive fibres
Fig. 18. Longitudinal section from the abdominal part of the vas deferens stained with antibody against VACHT. Arrows show nerve fibres running along the smooth muscle fibre

Table 1. Antibody used in the study

Antigen	Host	Type	Dilution	Cat. No.	Supplier
DβH	mouse	monoclonal	1:500	MAB308	Milipore
NPY	rabbit	polyclonal	1:400	NA1233	Biomol
GAL	rabbit	polyclonal	1:2000	RIN 7153	Peninsula Lab.
SP	rat	monoclonal	1:150	8450-0505	ABD Serotec UK
CGRP	rabbit	polyclonal	1:2000	11535	Cappel
VACHT	rabbit	polyclonal	1:5000	V5387	Sigma
VIP	mouse	monoclonal	1:500	MaVIP	East Acres Biologicals
Secondary antibodies					
Host		fluorochrome	Dilution	Code	Supplier
Goat-anti-rabbit IgG (H+L)		Alexa Flour 488	1:500	A11008	Invitrogen
Goat-anti-mouse IgG (H+L)		Alexa Flour 488	1:500	A11001	Invitrogen
Goat-anti-rabbit IgG (H+L)		Alexa Flour 568	1:500	A11011	Invitrogen
Goat-anti-mouse IgG (H+L)		Alexa Flour 568	1:500	A11004	Invitrogen
Goat-anti-rat IgG (H+L)		Alexa-Fluor 488	1:500	A11006	Invitrogen

Table 2. Relative density and distribution of nerve fibres supplying some male reproductive organs in the chinchilla

Organ	Part of organ	Substance studied						
		DβH	NPY	GAL	SP	CGRP	VIP	VACHT
Testis	Tunica albuginea	+ + +	+	+	+ +	+ +	+	+
	Testis parenchyma – blood vessels	+ + +	+	-	+	+ +	+	+
	Testis parenchyma – nonvascular tissue	+	-	-	-	-	-	-
	Testis mediastinum	+ +	+	+	+	+	-	-
Epididymis	Head	+ + +	+ +	+	+ +	+ + +	+	+
	Body	+ + + +	+	+	+ +	+ + +	+	+ + +
	Tail	+ + + +	+	+	++	+ + +	+ +	+ + +
Vas deferens	Funicular part	+ + + +	+	+ +	+ + +	+ + +	+ +	+ + + +
	Abdominal part	+ + + +	+	+ +	+ + +	+ + +	+ +	+ + + +

Arbitrary evaluation of the number of nerve fibres:
— - nerve fibres not observed, + - single nerve fibres, ++ - moderate number of nerve fibres, +++ - numerous nerve fibres, ++++ - very numerous nerve fibres

VIP-positive nerve fibres were less numerous and the vast majority of them were also immunoreactive to VACHT. VIP-positive-only nerves were only occasionally found. No clear-cut differences in the innervation between the funicular and abdominal parts of vas deferens were determined.

Discussion

As already mentioned, the innervation of the male gonad was investigated in many animal species. These studies have revealed some interspecies similarities dealing with the general distribution pattern of nerve fibres within the gonad. The highest number of the nerve terminals was observed along the epididymal border of the testis and its extremities (superior and inferior). The density of the nerves gradually decreased towards the free border. Considering the parenchyma, the highest density of the nerve fibres was found in its peripheral areas and again gradually decreased towards the mediastinum. Such distribution of nerve fibres supplying the testis was reported in papers describing the innervation of the gonad in the cat and large domestic and wild animals (34, 35). Similar arrangement of the nerve fibres has been found within the chinchilla's testes. The associated nerves reach the gonad by three different routes. Firstly, they follow the extrinsic testicular blood vessels and penetrate the testis *via* the head extremity (funicular route). Secondly, they reach the tail of the epididymis through the ligamentous connection between the head of the epididymis and the inferior extremity of the testis (caudal route), and,

finally, they pass through the mesentery connecting the epididymis with the epididymal border of the testis (mesorchial route) (24, 34). Similarities in the distribution of the nerve fibres in the testis between previously studied animals and the chinchilla allow to conclude that the nerves reach the chinchilla gonad by the same routes as previously described in pigs (24, 34) and bulls (33). Nerves supplying male gonads express many biologically active substances. Histochemically, the presence of cholinergic nerve fibres was revealed within the rat testis (21). These fibres were observed to penetrate into the testicular capsule with the testicular artery and form well developed network in the mediastinum. Many fibres are distributed beneath the rete testis epithelium. These observations are in agreement with our findings except the presence of the dense network of the nerve terminals within the mediastinum of the gonad. The presence of catecholamines or their synthesising enzymes in nerve fibres supplying the testis has been reported in many previous contributions (3, 33, 34, 39). Generally, the features of the catechoalminergic innervation of the male chinchilla gonad resemble those described in other species. The distribution of NPY-immunoreactivity in the chinchilla testis is also similar to that previously reported (1, 27, 33, 34). However, no NPY-immunoreactivity was found within spermatogenic elements of the seminiferous tubules as previously described in the human testis (32). The presence of SP- and CGPR-immunoreactivity within the testicular nerves was already reported in several papers (1, 27, 33, 34). The localisation and distribution of CGRP-immunoreactive nerves in the chinchilla gonad are similar to those previously described, whereas SP-immunoreactive nerve fibres are more abundant in the chinchilla than in other species. The presence of VIP-immunoreactivity within the rat testis (39) as well as within the testis of large domestic and wild animals was mentioned in several papers (34). VIP-positive nerves are very sparse in chinchilla's gonad. They are distributed only in the tunica albuginea and associated with the testicular vasculature, like in other species investigated, but contrary to the rat gonad (18) no VIP-immunoreactivity within the testis was found.

The epididymis in the chinchilla was found to be richly but unevenly supplied with the nerve fibres. The head of the gonad is relatively weakly innervated and the density of the nerve terminals increases towards the tail of the epididymis, forming a dense network of the nerves within this part of the organ. Such distribution of the nerve fibres within the epididymis seems to be a general feature of their localisation, because very similar findings were described in the rat (17), pig (7), guinea pig (4), and human (27). The dense network of adrenergic nerves supplying the epididymal duct was reported previously in human (23), rodents (15), and other animal species (7). These reports confirm the results of the present investigation, although in marsupials (14), adrenergic fibres are present only around blood vessels. Cholinergic component of the epididymal innervation was described in laboratory animals (15, 25) camel (20), and human (23). The localisation and distribution of the cholinergic nerve fibres reported in these studies are similar to those found in the chinchilla's epididymis. VIP-immunoreactivity was reported in some previously published papers (17, 27) and the most abundant nerve fibres were found within the tail of epididymis, whereas the head of the organ is supplied by very scarce fibres (12), or this part of the epididymis was even reported to be free of VIP-immunoreactivity (17). In the chinchilla's epididymis, VIP-immunoreactive fibres are single in the head and their number increases towards the tail of the organ, meaning that our observations are in accordance with those reported by Lakomy *et al.* (12). Immunoreactivity to CGRP was found within the epididymis of the human (27), rat (17, 38), and in large domestic and wild animals (12, 37). Most of CGRP-immunoreactive nerve fibres are simultaneously SP-positive. In the rat epididymis (38), marked regional differences in the number of CGRP-immunoreactive nerve terminals was observed. The nerve fibres are particularly numerous in the tail of the epididymis. In the remaining parts of the organ, CGRP-immunoreactive nerve fibres are scarce, or they are even absent from the caput of the epididymis (17), although a small number of the CGRP-immunoreactive nerve fibres was constantly found to be associated with small blood vessels (38). Similar findings were obtained in pigs (12), in which the most abundant innervation was observed in the tail of epididymis, and the density of the nerves distinctly decreased towards the head of the organ. In the camel epididymis, CGRP-positive axons are moderate in number, but they were never associated with arteries (20). Contrary to all the above-mentioned reports, in the chinchilla's epididymis, no regional differences in the density of the SP- and CGRP-positive nerve fibres were found. Such observations are in agreement with the findings regarding SP-IR innervation of the guinea pig epididymis (4). In the chinchilla, some nerves were also observed to supply epididymal blood vessels as previously described in the rat (38). Histochemical studies revealed a dense network of adrenergic nerves innervating the vas deferens in many mammalian species including rat (10), guinea pig (26), rabbit (22), opossum (14), bull (11), and pig (7). These observations are in accordance with the present results, whereas in human (6) as well as in the dog and fox (16), the vas deferens is relatively weakly supplied with adrenergic nerves. Adrenergic nerve fibres innervating the muscular coat in chinchilla vas deferens contain no immunoreactivity to NPY; however, DβH-IR nerves innervating deferential blood vessels simultaneously express NPY, which is in agreement with the results of previous studies

regarding the innervation of the porcine vas deferens (7). This suggests that only a small proportion of "muscular" adrenergic fibres contain NPY-IR. In other species studied the majority of the adrenergic "muscular" axons in the vas deferens contain NPY-IR (6). Cholinergic nerve fibres innervating the vas deferens were visualised by histochemistry using acetylcholinesterase-activity method (2, 19). In most cases, cholinergic nerves were found subepithelially in the connective tissue of the mucosa, whereas in the guinea pig, acetylcholinesterase-positive axons were distributed within the longitudinal smooth muscle layer of vas deferens (19), which is in agreement with the present observations. The vast majority of VIP-positive nerves supplying the vas deferens are cholinergic in nature which was confirmed by previous investigations (2, 30). The mammalian vas deferens is supplied by SP- and/or CGRP-positive nerve fibres (7). CGRP-positive nerves innervating the vas deferens were found in the human, rat, and guinea pig (7). The presence of the nerves containing SP was described in the mouse, guinea pig, cat, and rabbit (7) but there are some discrepancies regarding the innervation of the human vas deferens. Some studies revealed the presence of solitary SP-positive nerves within the human organ (5). Moreover, some authors did not encounter any SP-immunoreactive nerves (28). The most comprehensive studies were performed in pigs (8). They revealed almost complete colocalisation of SP and CGRP in nerve fibres supplying the vas deferens, but solitary fibres stained for either substance were found. These observations do not correspond with the results of the present study because in the chinchilla vas deferens a large portion of the SP-immunoreactive fibres does not express CGRP.

The present study revealed three major populations of the nerve fibres supplying the chinchilla vas deferens: adrenergic nerves innervating seminiferous elements within the testis, smooth musculature of the genital organs, and their vasculature; cholinergic nerves innervating smooth muscles, lamina propria, and blood vessels (especially those containing VIP); non-adrenergic sensory nerves containing SP and/or CGRP.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Animal Rights Statement: The animals were housed and treated in accordance with the rules approved by the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education, protocol 3/2011 of the 1st Local Ethics Committee in Kraków, Poland).

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