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Monoaminergic and Peptidergic Contributions of the Superior and the Inferior Spermatic Nerves to the Innervation of the Testis in the Rat

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ABSTRACT: Sections of the rat testis and whole-mounts of the testicular capsule were studied microscopically using the glyoxylic acid-induced fluorescence method, to detect monoamines, and immunohistochemical procedures for the detection of immunoreactivities to protein gene-product 9.5 (PGP 9.5), the C-terminal accompanying peptide of neuropeptide Y (CPON), and vasoactive intestinal polypeptide (VIP). Monoaminergic nerves were only observed around the intracapsular blood vessels: the initial segment of the testicular artery and the superior venous plexus, and in the anterior aspect of the upper and lower testicular poles. These capsular nerve networks were associated with the superior and inferior ligaments of the testis. Nerves displaying PGP 9.5 and CPON immunoreactivity appeared in the same sites and followed the same distribution as monoaminergic nerves. By contrast, VIP-immunoreactive fibers were only found in the nerve network of the lower pole. Observations done after different surgical denervation procedures demonstrated that the superior spermatic nerve was the source of fibers for testicular vessels and for the nerve network of the upper pole. On the other hand, fibers from the inferior spermatic nerve were restricted to the nerve network of the lower pole.

Key words: Testicular capsule, mesorchium, protein gene product 9.5, vasoactive intestinal peptide, C-terminal peptide of neuropeptide Y, glyoxylic acid-induced fluorescence.

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The role played by innervation in testicular function has not yet been elucidated. This probably reflects the anatomical complexity of testicular nerves. The male gonad receives nerve fibers from both sensory and autonomic ganglia. Fibers converge to the testis along two major pathways, the superior and inferior spermatic nerves (SSN and ISN). The SSN runs from the mesenteric and renal plexuses alongside the testicular artery, whereas the ISN, originating in the pelvic and inferior mesenteric plexuses, accompanies the vas deferens and penetrates the epididymis. Classical studies assumed that the testis is mainly innervated by the SSN (Kuntz and Morris, 1946). However, the ISN would also supply the male gonad (Hodson, 1970; Santamaría et al, 1990).

In some mammalian species, autonomic nerves are both associated with the testicular parenchyma and capsule (Prince, 1992; Setchell et al, 1994). According to Bell (1972), the parenchyma is sparsely innervated in the rat testis, with nerves only associated with blood vessels. A recent immunohistochemical study using protein gene product 9.5 (PGP 9.5), a general structural neuronal marker (Lundberg et al, 1988), could not detect any immunostaining in the rat testicular parenchyma (Properzi et al, 1992).

Both monoaminergic and acetylcholinesterase-containing fibers have been found in the testicular capsule of several mammalian species, including rat, rabbit, ram, and monkey (Bell and MacLean, 1973; Langford and Silver, 1974; Campos et al, 1990a,b; Santamaría et al, 1990). In the rat, adrenergic nerves, revealed by dopamine-β-hydroxylase (DBH) or tyrosine hydroxylase (TH) immunoreactivity, are occasionally found within the testicular capsule and surrounding capsular vessels (Lamano Carvalho et al, 1986). Vasoactive intestinal polypeptide (VIP)-containing terminals have been reported in the capsule and around the blood vessels in cats and guinea pigs, but not in rats (Larsson et al, 1977; Alm et al, 1980). Nerve fibers displaying immunoreactivity for calcitonin gene-related peptide (CGRP) and neuropeptide Y (NPY) are also found in long varicose nerve fibers of the rat subcapsular insterstitium and around capsular blood vessels (Properzi et al, 1992). The source of these fibers has yet to be described.

Understanding the role of innervation on male gonad

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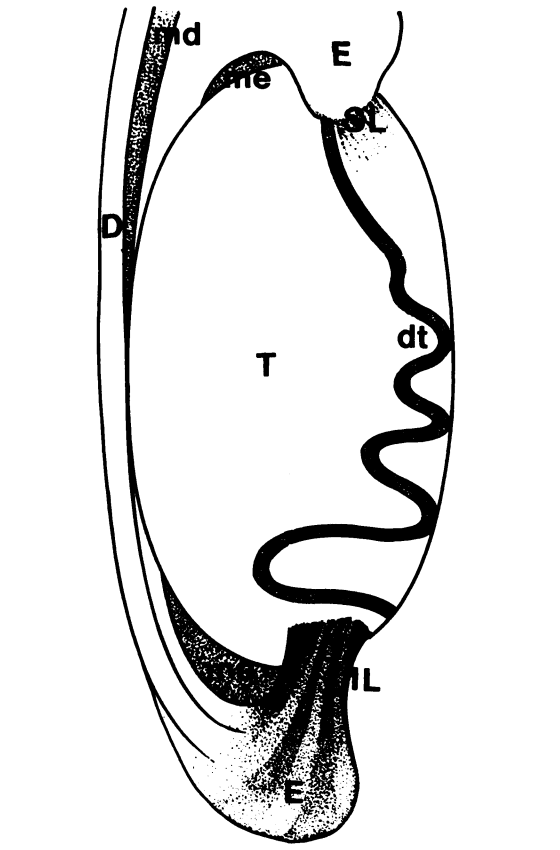

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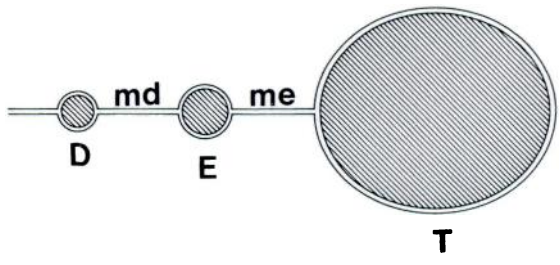

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FIG. 1. (A), A schematic view of the ventromedial aspect of a left rat testis (T) and its relationships with the epididymis (E) and vas deferens (D). The distal portion of the testicular artery (dt) coils beneath the ventral aspect of the testicular capsule (see Fig. 2). The visceral layer of the tunica vaginalis is closely wrapped about the testis, epididymis, and vas deferens, forming the mesorchium. The portion of the mesorchium between the dorsal aspect of the testis and the epididymis is the mesoepididymis (me), whereas the portion between the epididymis and the vas deferens is the mesodeferens (md). The testis is tightly bound to the ventral end of the caput epididymis, at the region of the superior ligament of the testis (SL), where the tunica vaginalis is thickened by numerous connective fibers. The inferior ligament (IL) connects the cauda epididymis and the lower testicular pole and is characterized by thick connective

function requires more data about nerves associated with different testicular structures. The specific aims of this investigation were: 1) to find out the sites of nerve entry to the testis, 2) to describe the distribution of monoaminergic and peptidergic nerves in different regions of the testicular parenchyma and capsule, and 3) to compare the contribution of the SSN and ISN to different areas of the capsule. Therefore, a histochemical and immunohistochemical study of the testis was made using intact rats and rats subjected to different surgical denervation procedures.

Materials and Methods

Animals

Adult male Sprague-Dawley rats were maintained under controlled lighting conditions (lights on from 0600 to 2000 hours) and given free access to food and water. Studies were done in 90-day-old animals weighing about 250–300 g.

Surgical Procedures

The testis, epididymis, and vas deferens are enclosed within a peritoneal fold that determines complex anatomical relationships between these structures (Fig. 1). Surgical denervations were done bilaterally in animals anesthetized with ether.

Section of the superior spermatic nerves (SSN-X) was carried out by a mid-abdominal incision. These nerves were isolated in the testicular pedicle, along the proximal third of the spermatic artery, where about 1 cm was removed from each nerve (Campos et al, 1990a).

Section or ligation of the superior or inferior testicular ligaments (Fig. 1) was also done through a mid-abdominal incision. Both testes and accompanying structures were gently pulled out from the scrotum by handling fatty pads. Fine scissors were used for section of the ligaments (superior and inferior ligament, SL-X and IL-X). In sham-operated animals, the testis and epididymis were exposed and manipulated as before but the ligaments were not cut. Ligation (superior and inferior, SL-L and IL-L) was done with polypropylene suture (Prolene 6-0, Ethicon). A loose loop around the ligaments was made in sham-operated animals.

A mid-scrotal incision was used for vasectomy. After placing ligatures at each end of the vas deferens, the duct was dissected and severed together with its accompanying nerves and vessels. Sham-vasectomized animals were treated similarly but the vas deferens was not manipulated.

Penicillin (4,500 IU/rat) was administered intramuscularly immediately after surgery. The animals were kept warm until recovery and were sacrificed 2 weeks after denervation.

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strands. The inferior ligament is also continuous with the caudal end of the mesorchium extending to the scrotal wall (not shown). **(B),** A cross-section through the mesorchium showing its different portions: the mesoepididymis between the testis and the epididymis, the mesodeferens between the epididymis and the vas deferens, and the peripheral region between the vas deferens and the scrotal wall.

Histological Procedures

For immunohistochemistry, intact rats were anesthetized with sodium pentobarbital (40 mg/kg body weight) and perfused transcardially with a mixture containing 4 g/100 ml paraformaldehyde and 14 ml/100 ml saturated picric acid in phosphate buffer, pH 7.2. Testes were excised and immersed in the same fixative overnight at 4°C. Specimens were stored in 15% sucrose in phosphate-buffered saline solution (PBS) for at least 24 hours. Sections (16 μm) were obtained with a cryostat and mounted on gelatin-coated slides.

Cryostat sections were dehydrated in graded ethanol solutions and defatted in xylene. After incubation in 0.03% hydrogen peroxide in methanol for 20 minutes, all sections were rehydrated, washed in PBS, and incubated in a primary antiserum. Rabbit antisera against the following antigens were tested: human PGP 9.5 (Ultraclone, Isle of Wight, UK), C-terminal peptide of neuro peptide Y (CPON; Peninsula Labs, Belmont, CA, USA), and VIP (Peninsula Labs, Belmont, CA, USA). Bound antibodies were detected with biotinylated goat anti-rabbit serum and avidin–biotin–peroxidase complex (Vectastain Elite, Vector Labs, Burlingame, CA, USA). All antibodies were diluted in PBS containing 0.01% bovine seroalbumin, 0.03% Triton X-100, and 0.01% sodium azide. Peroxidase activity was revealed using diaminobenzidine with nickel enhancement (Shu et al, 1988), and sections were dehydrated, cleared, and mounted with Permount.

Capsules submitted to immunohistochemistry were dissected out in PBS and stretched over a wax plate using entomology pins. After overnight fixation at 4°C in the paraformaldehyde–picric acid mixture, they were transferred to clean dishes and further processed as free-floating specimens. They were repeatedly washed in PBS (six changes in at least 2 days), dehydrated in graded ethanol solutions, and defatted in xylene. Immunohistochemical procedures were similar to those used for sections. However, to allow for good penetration of antibodies, the primary incubation was made for 7 days or more, and the antibody diluent contained a higher Triton X-100 concentration (0.3%) than the one used for sections. After dehydration and clearing, capsules were flat-mounted inner side up.

The glyoxylic acid method (De la Torre, 1980), as modified by Santamaría et al (1990), was used for the histochemical detection of monoamines. Cryostat sections (16 μm), obtained from fresh-frozen specimens, were incubated for 1.5 hours at room temperature in a freshly prepared solution containing 0.2 M sucrose, 0.236 M KH_2PO_4, and 1% glyoxylic acid at pH 7.4. Glyoxylic acid was omitted in negative controls. For histochemistry of capsule flat-mounts, the whole testis was immersed in this solution immediately after excision. The capsule was dissected out from the parenchyma during the incubation. After the incubation, the capsule was flat-mounted on a clean slide, inner side up, with the help of a brush and a few radial cuts. Specimens were dried out under the cool air of a hair dryer, heated at 100°C for exactly 5 minutes, and coverslipped using mineral oil.

Results

Intact Animals

For the initial screening of nerve distribution, serial sections were made through transverse, dorso-ventral, and latero-medial planes of the testis and studied either with immunohistochemistry or glyoxylic acid-induced fluorescence. Both PGP 9.5 immunohistochemistry and glyoxylic acid-induced fluorescence gave essentially the same results and showed that nerves were only present in the following locations: the testicular artery, the superior venous plexus, and the upper and lower poles of the testicular capsule.

The testicular artery makes a long unbranching course (Figs. 1 and 2A). At the testicular hilum it runs dorsally and becomes embedded within the testicular capsule. This intracapsular segment runs straight down to the lower pole of the testis and then passes to the subcapsular space, where it winds along the ventral surface. After four or five coils, the artery turns into the parenchyma at a point close to the superior ligament. The artery then makes a short course between the seminiferous tubules and subdivides into several smaller branches.

Along its intracapsular course (i.e., the straight dorsal segment; see Fig. 2A), the testicular artery showed a very rich innervation revealed by PGP 9.5 (Fig. 3A) and CPON immunohistochemistry, and by glyoxylic acid-induced fluorescence (Fig. 3B). One or two fine nerve trunks ran along this arterial segment, and their branches penetrated the arterial wall. No VIP-immunoreactive fibers were detected around this or subsequent segments of the testicular artery.

The subcapsular segment of the testicular artery lay embedded within the seminiferous tubules and was not anatomically connected with the capsule. Fibers displaying glyoxylic acid-induced fluorescence were not found in this arterial segment. Proximal coils, close to the lower pole, still contained some fibers displaying PGP 9.5 and CPON immunoreactivity (Fig. 3C), but no nerve fibers were detected in the most distal coils, close to the upper pole. No nerves were detected in the intratesticular arterial branches.

Parenchymatous veins were not innervated. Nerve fibers only appeared around intracapsular veins of the superior venous plexus, a venous network carved within the albuginea (Fig. 2A). These nerves displayed both PGP 9.5 and CPON immunoreactivities, but they were not stained by VIP antiserum. Flat-mounts showed that a fine nerve trunk was placed along each side of the larger venous spaces (Fig. 3D). Each trunk produced numerous perpendicular branches that penetrated the venous walls (Fig. 3E).

Although every region of the testis was checked with serial sections, no nerves were detected within the testicular parenchyma, in association with blood vessels, interstitial cells, or seminiferous tubules. They were also absent in the intratesticular portion of the rete testis.

Both fluorescent and immunoreactive nerves were identified in cryostat sections of the capsule. However, their density was very low, and their distribution could only be studied in flat-mounts. The testicular capsule was innervated by two different fiber networks occupying the

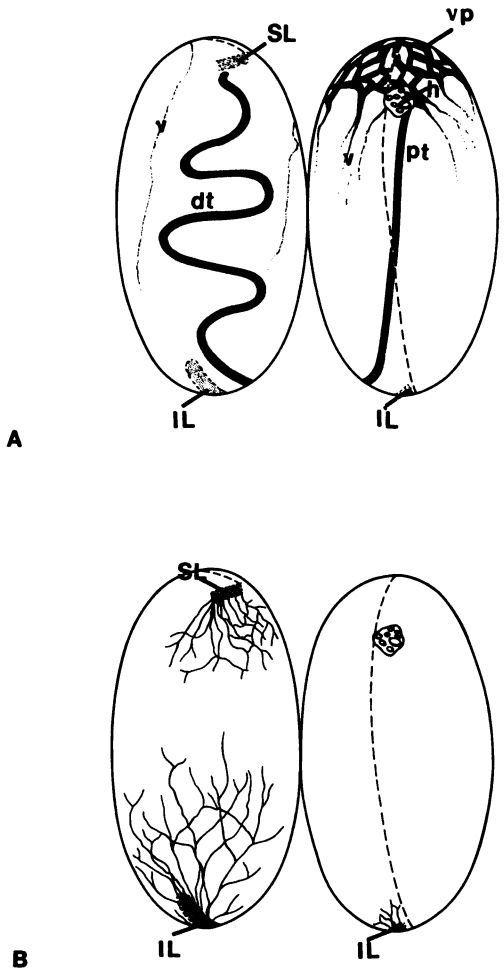


FIG. 2. (A), Drawing of a left rat testicular capsule opened through a longitudinal plane. The ventral half is shown at the left and the dorsal half is at the right. Both halves are joined by their medial region. At the dorsal region of the upper pole (right), the superior venous plexus (vp) occupies an intracapsular position (shown in black). Subcapsular veins (v, shown as dotted lines) join the superior venous plexus. The proximal portion of testicular artery (pt) penetrates through the testicular hilum (h) and follows a straight intracapsular (black segment) course close to the insertion of the mesoepididymis (shown as a dashed line). After passing to the subcapsular space (dotted segment), its distal portion (dt) arches around the lower pole and follows a winding course beneath the ventral aspect of the capsule. It finally penetrates the testicular parenchyma at a point close to the SL. Notice that both the SL and IL are located on the ventral aspect of the capsule and that the SL occupies a more lateral position than the IL. They are continuous with the mesoepididymis on the dorsal aspect of the testis. (B), A similar drawing showing the course of the upper capsular nerve network in relationship to the SL and the lower nerve network in relationship to the IL.

anterior aspect of the upper and lower testicular poles, respectively (Fig. 2B). The upper nerve network was associated with the superior ligament of the testis, whereas the lower nerve network was associated with the inferior ligament. All capsules showed the same innervation pattern (Table 1), in spite of slight individual differences.

Several thin nerve trunks entered the capsule through the superior ligament (Figs. 2B, 4A,C). Glyoxylic acid-induced fluorescence and immunostaining with PGP 9.5 or CPON showed a similar distribution of fibers on the

Table 1. *Regional innervation of the testicular capsule and its connecting structures*

	Mono-amines	PGP 9.5	CPON	VIP
Superior ligament	++*	++	++	0
Upper nerve network	++	++	++	0
Cephalic mesorchium	+	++	++	0
Venous plexus	+++	+++	+++	0
Testicular artery	+++	++	++	0
Medial mesorchium	+	+++	+++	0
Caudal mesorchium	+	+++	+++	++
Inferior ligament	+++	+++	+++	++
Lower nerve network	+++	+++	+++	++

* Innervation density was scored as 0, no fibers; +, very few fibers; ++, moderate amount of fibers; or +++, very large number of fibers. Values represent the average of 10, 2, and 5 specimens immunostained with PGP 9.5, CPON, and VIP antisera, respectively.

ventral aspect of the capsule. Branching apparently followed a random pattern (Figs. 4B,D), occasionally showing right-angle bifurcations.

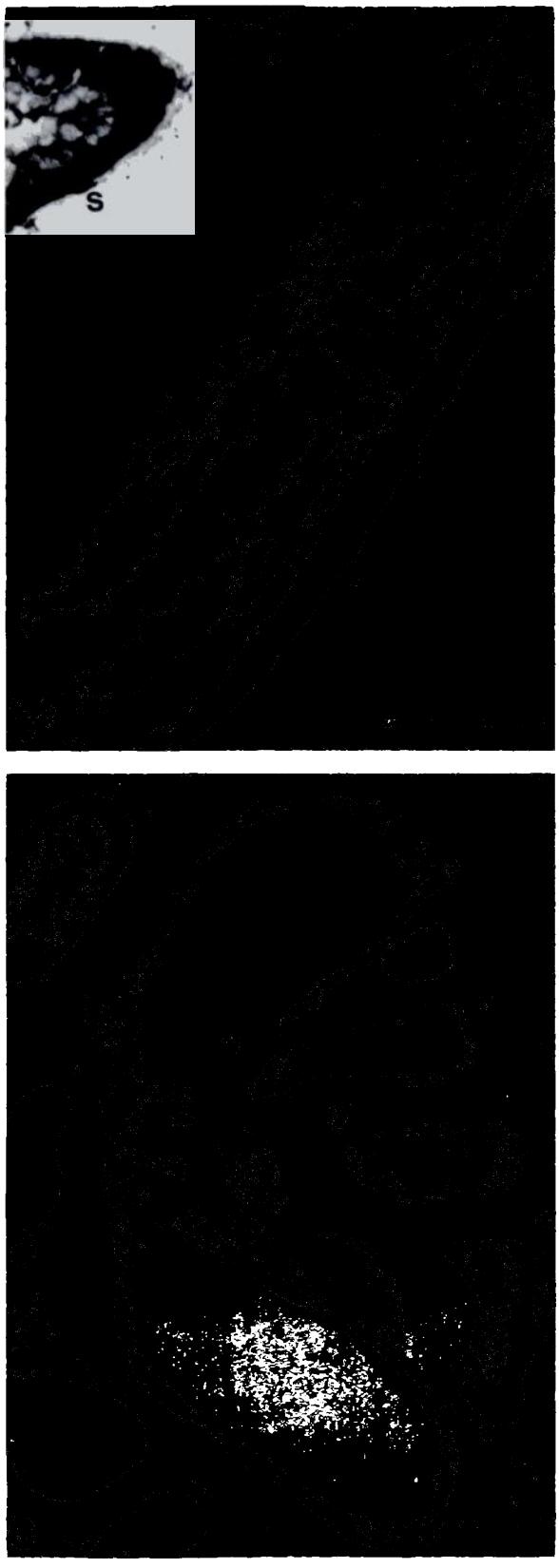
A large nerve network was associated with the inferior testicular ligament (Fig. 2B). Within the ligament, thick nerve trunks extended along three or more connective strands between the cauda epididymis and the testicular lower pole (Figs. 1, 5A). The main branches were thicker than those from the superior ligament. They ramified extensively over the ventral aspect of the lower testicular pole, but a few shorter branches also appeared in the lateral and dorsal aspects. The lower nerve network extended on the capsular area, overlaying the first coils of the subcapsular artery. Its fibers were revealed both by glyoxylic acid-induced fluorescence (Fig. 5B) and by immunohistochemistry. Those nerves displaying PGP 9.5 or CPON immunoreactivity were as abundant as those showing induced fluorescence. In contrast difference with the upper network, the lower one contained a significant amount of VIP-immunoreactive fibers (Fig. 5C).

Both nerve networks showed the same branching pattern. Fibers were at different levels of the albuginea, some of them close to its inner aspect in the immediate vicinity of the interstitium (Fig. 6A). The smallest branches ended as free thin fibers (Fig. 6B), or as a bulbous terminal (Fig. 6C). Some fibers were associated with mast-like cells, but most of them appeared isolated.

In the mesoepididymis (Fig. 7), immunostaining with PGP 9.5 and CPON antisera showed numerous nerve bundles, whereas immunostaining with VIP antiserum only showed positive fibers in its caudal portion. By contrast, glyoxylic acid-induced fluorescence only showed a very small number of fibers. Nerve bundles extended in a cephalo-caudal direction. Most fibers remained within the mesoepididymis, but some fibers extended into the capsule in about half of the specimens.

Denervated Animals

The glyoxylic acid-induced fluorescence procedure was used for comparison of different denervation protocols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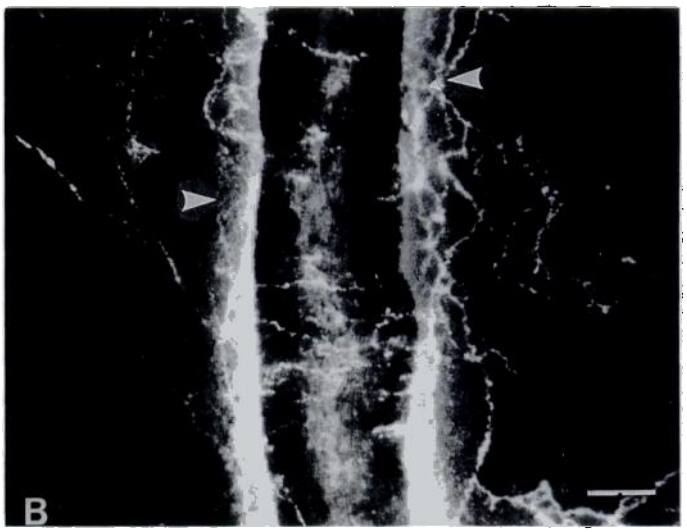
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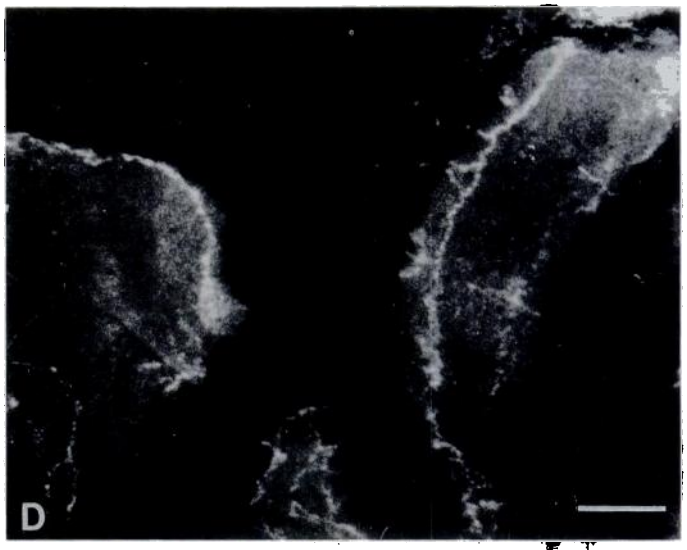
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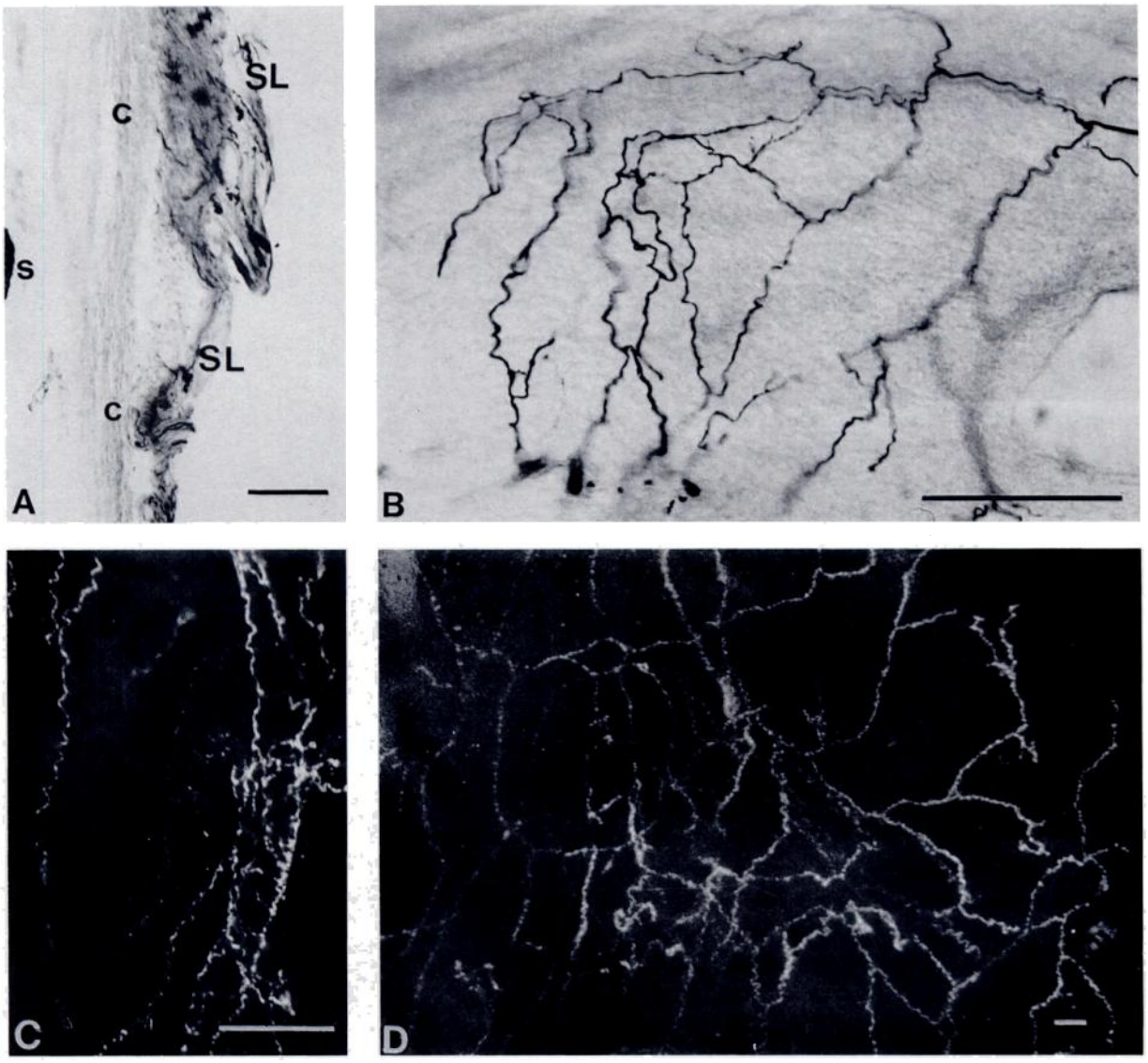

FIG. 4. Superior ligament and upper nerve network: **(A)**, A cryostat section immunostained with PGP 9.5 antiserum illustrates the testicular capsule (c) at the point of attachment of the superior ligament. Notice the presence of immunoreactive nerve fibers and bundles within the ligament. **(B)**, A flat-mount of the testicular capsule immunostained with CPON antiserum shows the nerve fibers of the upper nerve network. Notice the presence of fibers in different focal planes. **(C)**, Monoaminergic fibers of the superior ligament in a flat-mount preparation incubated with glyoxylic acid. **(D)**, A panoramic view of the upper nerve network showing the extensive branching of monoaminergic nerve fibers. Calibration bars, 100 μm.

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FIG. 3. The intracapsular segments of testicular blood vessels. **A–C** are different views of the testicular artery, whereas **D** and **E** illustrate the superior venous plexus. **(A)**, A tangential section along the intracapsular segment of the testicular artery immunostained with PGP 9.5 antiserum. Longitudinal and circumferential nerve fibers are observed. s, seminiferous tubules. **(B)**, A flat-mount incubated with glyoxylic acid demonstrating monoaminergic nerves in the same arterial region as in Figure 1. Although the flat-mount included the complete artery, microscopic focusing only shows the sagittal plane of the blood vessel. Arrows indicate the arterial wall. Notice the presence of numerous fluorescent fibers, both as longitudinal trunks parallel to the arterial wall and thinner radial branches around the artery. **(C)**, Cryostat section of the subcapsular arterial coils illustrating the presence of CPON-immunoreactive fibers. **(D)**, This micrograph of the superior venous plexus is focused on the longitudinal nerve trunks running along its branches. **(E)**, The perpendicular nerve branches penetrating the venous walls are shown at a higher magnification. Calibration bars, 100 μm. (The actual size of the specimens is modified by dehydration and desiccation during processing of sections and flat-mounts.)

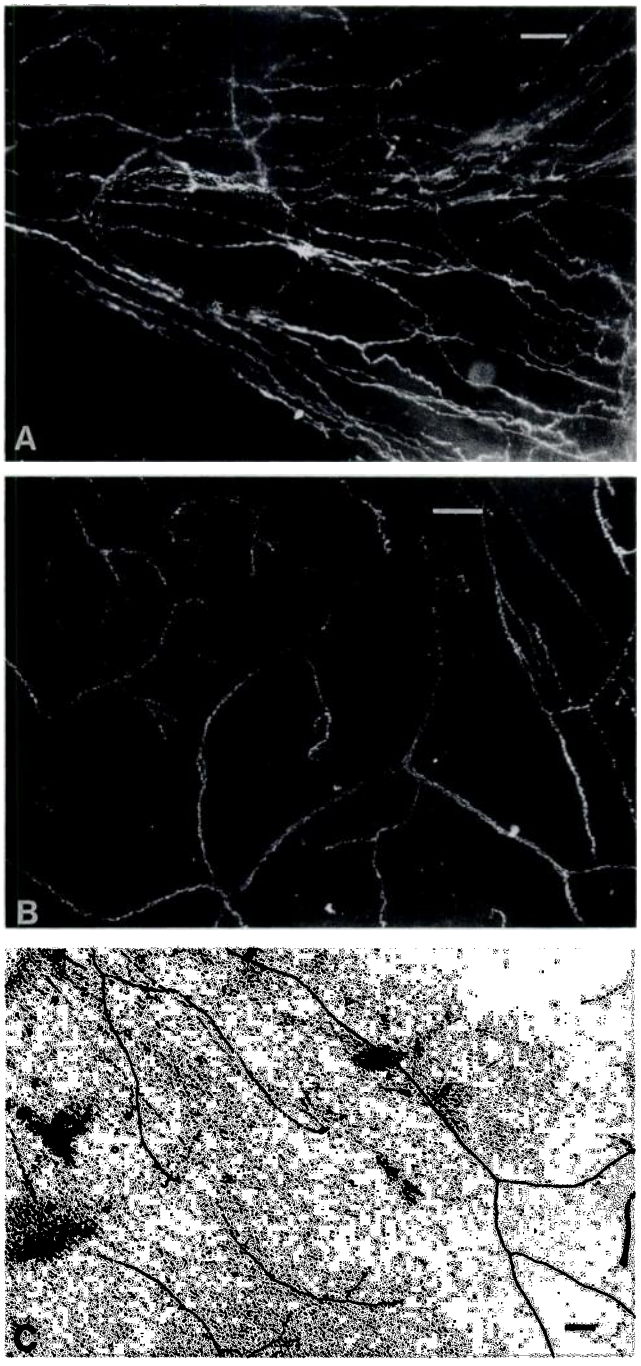

FIG. 5. Interior ligament and lower nerve network: (A), Glyoxylic acid-induced fluorescence demonstrates the presence of numerous nerve fibers and bundles in a flat-mount of the inferior ligament. (B), A similar preparation shows a partial view of the lower nerve network of the testicular capsule. (C), The same region of the capsule is shown in a flat-mount immunostained with VIP antiserum. The density of VIP-immunoreactive fibers is smaller than the density of monoaminergic nerves. Calibration bars, 100 μm.

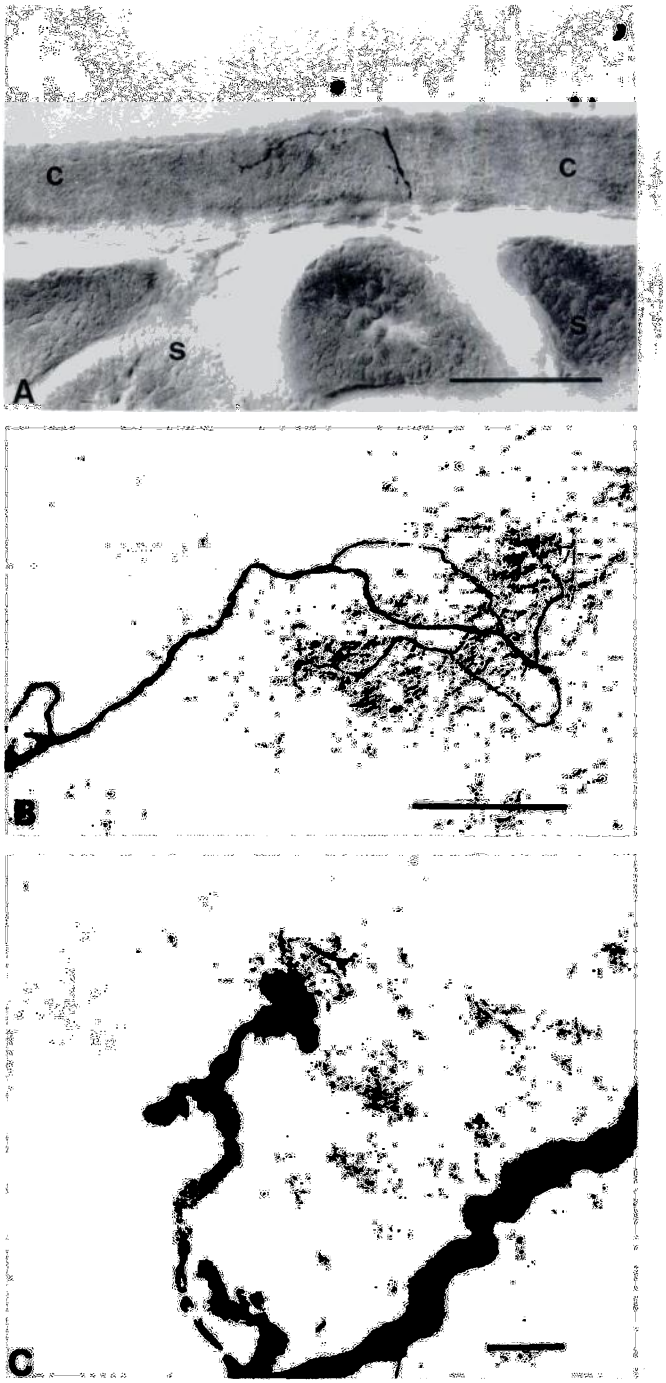

FIG. 6. Nerve endings in the testicular capsule. (A), A cryostat section showing the course of a CPON-immunoreactive fiber within the testicular capsule. Notice that this fiber is in contact with the interstitial space of the testis. (B), This flat-mount, immunostained with PGP 9.5 antiserum, illustrates a typical pattern of terminal branching in the capsular nerve network. (C), A higher magnification shows the bulbous dilatations of nerve endings. Calibration bars, A and B, 100 μm; C, 10 μm.

(Table 2). After section of the SSN in the testicular pedicle fluorescent fibers disappeared from the superior venous plexus, the intracapsular segment of the testicular artery and the nerve network associated with the superior testicular ligament. Thus, section of the SSN not only completely abolished all fluorescent nerve fibers in the upper pole, but also those in the lower portion of the intracapsular testicular artery. No changes were detected in the

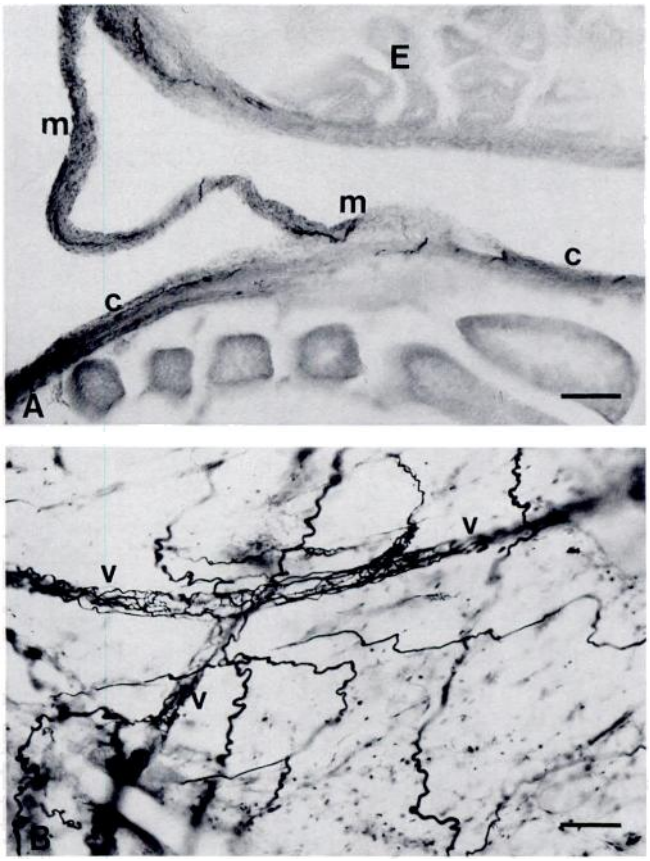

FIG. 7. Different views of the mesoepididymis. **(A)**, A cryostat section through the upper pole illustrating the presence of CPON-immunoreactive fibers in the capsule of the caput epididymis (E), the cephalic mesoepididymis (m), and the testicular capsule (c). **(B)**, A flat-mount of the caudal mesoepididymis illustrating a well-innervated blood vessel (v) and several nerve bundles not associated with blood vessels. Calibration bar, 100 μm.

fiber density or fluorescent intensity of the lower capsular nerve network.

After ligation or section of the superior testicular ligament, fluorescent nerve fibers disappeared in the ventral aspect of the superior pole. However, the fluorescent nerves associated with the testicular artery and the venous plexus remained intact.

By contrast, vasectomy (VAS-X) or blockage of the inferior ligament (IL-X or IL-L) caused complete disappearance of fluorescent nerves in the lower pole. These manipulations, however, did not induce changes in the innervation pattern of the intracapsular testicular artery, even in its lowermost portion.

Complete monoaminergic denervation was obtained either by section of the SSN plus vasectomy (SSN-X plus VAS-X) or by section of the SSN plus section or ligation of the inferior ligament (SSN-X plus IL-X/L).

Complete disappearance of PGP 9.5- and VIP-immunoreactive fibers in the intracapsular vessels and capsular nerve networks was also observed after combined section of both superior and inferior fibers (SSN-X plus VAS-X). Section of the superior spermatic nerves (SSN-X) only induced the disappearance of PGP 9.5-immunoreactive fibers in the cephalic pole. By contrast, after caudal denervations (IL-L), both PGP 9.5- and VIP-immunoreactive fibers were lost from the caudal nerve network (Table 3).

Discussion

Our results show that nerves reached the testis through several different routes. Fibers entered together with the testicular blood vessels at the testicular hilum or through the mesorchial ligaments, i.e., via the superior and inferior ligaments of the testis. A few fibers also reached the testis through the middle and caudal portions of the mesoepididymis.

All these fibers innervated capsular structures, because no nerves associated with parenchymatous structures were detected, and vascular nerves were only associated with the largest blood vessels. Innervation of the testicular ar-

Table 2. *Density of monoaminergic fibers in the rat testicular capsule after various denervation procedures*

Surgical procedures	(*n*)	Upper nerve plexus	Venous plexus	Testicular artery (intracapsular segment) Upper	Testicular artery (intracapsular segment) Lower	Lower nerve plexus
Sham-operated	36	+++*	+++	+++	+++	+++
SSN-X†	6	0	0	0	0	+++
SL-X/L	6	0	+++	+++	+++	+++
IL-X/L	5	+++	+++	+++	+++	0
VAS-X	3	+++	+++	+++	+++	0
SSN-X plus IL-X/L	5	0	0	0	0	0
SSN-X plus VAS-X	6	0	0	0	0	0
SL-L plus IL-L	5	0	+++	+++	+++	0

* Innervation density is scored as 0, no fibers; +++, very high number of fibers.

† SSN-X, section of the spermatic superior nerve; SL-X/L, section or ligation of the superior testicular ligament; IL-X/L, section or ligation of the inferior testicular ligament; VAS-X, vasectomy; SL-L, ligation of the superior testicular ligament; IL-L, ligation of the inferior testicular ligament.

tery decreased in its subcapsular portion and disappeared completely before this vessel penetrated the parenchyma. Two nerve networks innervated the capsule itself.

The capsular nerve networks associated with the mesorchial ligaments have not been described previously. The distribution and branching pattern of their fibers suggests that they ended freely within the albuginea. Although both networks overlaid the subcapsular coils of the testicular artery, their topographical distribution does not necessarily imply a specific relationship with this vessel. Moreover, no anatomical connection could be detected between the subcapsular artery and the capsule.

Denervation results demonstrate that fibers from the SSN innervated the large intracapsular blood vessels and the ventral aspect of the upper pole. Ligation or section of the superior ligament induced the disappearance of fibers in the upper capsular nerve network but not in the intracapsular testicular artery and venous plexus. It follows that these structures must be innervated by different branches of the SSN. Fibers for the large intracapsular blood vessels probably entered together with them through the testicular hilum. The distribution of vascular nerve fibers seen in flat-mounts also suggests that they were continuous with fibers in the testicular pedicle. Results of blockage of the superior ligament indicate that fibers connecting the SSN and the upper nerve network probably separated from the main nerve trunk at some distance from the hilum. Most likely, these fibers accompanied the superior epididymal artery and surrounded the epididymal capsule before entering the superior ligament of the testis.

The origin of fibers from the lower capsular network is completely different. They remained after section of the SSN and disappeared after vasectomy or blockage of the inferior ligament of the testis. Thus, they most probably derive from fibers of the ISN. These fibers, accompanying the vas deferens, would enter the capsule of the cauda epididymis and then run through the inferior ligament.

Each segment of the spermatic artery featured a characteristic innervation pattern, probably reflecting functional differences in the control of blood flow. The intracapsular segment, i.e., the proximal portion along the dorsal aspect of the testis, contained numerous monoaminergic fibers as well as nerves displaying PGP 9.5 and CPON immunoreactivities. By contrast, the subcapsular segment only exhibited a small amount of nerve fibers. This innervation pattern is consistent with physiological observations in the ram and rabbit, where sensitivity to norepinephrine decreases along the course of the testicular artery (Waites et al, 1975). The rich innervation of the venous plexus, also containing many monoaminergic fibers, may be involved in the regulation of venous blood flow.

Several functions for the nerve fibers in the upper and lower capsular networks can be suggested. They could regulate contraction of the testicular capsule, a phenomenon that has been associated with extrusion of spermatozoa, maintenance of interstitial pressure, and control of blood flow (Setchell et al, 1994). However, capsular movement in the rat testis would not be as important as in other species (Hargrove and Legrande, 1976). Smooth muscle cells are lacking, and the rat capsule is only provided with actin-containing cells (Santamaría et al, 1990; Maekawa et al, 1991). However, their distribution is not the same as the capsular nerve networks.

Table 3. *Distribution of immunoreactive fibers in testicular capsules after section of the vas deferens*

Surgical procedures	*n*	Innervation density* PGP 9.5 Upper plexus	PGP 9.5 Lower plexus	VIP Upper plexus	VIP Lower plexus
Sham-operated	10	++	+++	0	++
SSN-X plus VAS-X	4	0	0	0	0
SSN-X	3	0	+++	0	++
IL-L	3	++	0	0	0

* In these experiments, one of the capsules was immunostained for PGP 9.5 and the other for VIP. Innervation density is scored as 0, no fibers, ++, moderate amount of fibers; +++, very high number of fibers.

Recent evidence suggests that autonomic and sensory testicular nerves have a role in modulating the hypothalamo–pituitary–testicular axis in the rat (Frankel and Ryan, 1981; Mizunuma et al, 1983; Frankel et al, 1984; Preslock and McCann, 1985; Damber, 1990; Campos et al, 1993). Bilateral excision of the SSNs blocks the ether stress-induced increase of plasma testosterone in adult rats (Frankel and Ryan, 1981) and results in a significant inhibition of human chorionic gonadotropin (hCG)-stimulated androgen production and a decrease in the number of testicular luteinizing hormone (LH) receptors (Campos et al, 1993). Involvement of the ISN in the control of testosterone secretion has also been proposed (Frankel et al, 1984). Pharmacological experiments *in vivo* and *in vitro* suggest that adrenergic nerves may be involved in these responses (Cooke et al, 1982; Moger et al, 1982; Anakwe et al, 1985; Mayerhofer et al, 1989, 1992). Because the rat parenchyma is poorly innervated, the effects described above can only be mediated by capsular nerves or by fibers associated with capsular blood vessels.

As suggested by Campos et al (1990a), capsular nerves could contribute various factors, including neurotransmitters, to the underlying subcapsular artery and interstitium. Nerve fibers in close association with the interstitial aspect of the capsule support this view. Besides, some capsular nerves apparently ended in relationship with mast-like cells. These could also be under neural control.

Our results demonstrate that both the SSN and the ISN contribute monoaminergic and peptidergic fibers to the rat testis. The presence of monoaminergic fibers has been previously reported, both in the SSN (Lamano Carvalho, 1986; Campos et al, 1990a) and the ISN (Santamaría et al, 1990).

Comparison of the amount and distribution of capsular nerve fibers revealed by glyoxylic acid-induced fluorescence and CPON immunostaining suggests that many of the latter probably are catecholaminergic fibers. This is to be expected because these peptides can be found in adrenergic nerves (Lundberg et al, 1982; Gulbenkian et al, 1985). However, CPON immunoreactivity in the mesoepididymis was not paralleled by a similar distribution of glyoxylic acid-induced fluorescence, as has been shown in other tissues (Milner et al, 1991).

VIP-immunoreactive nerves have not been previously reported in the rat testicular capsule. Denervation experiments demonstrated that capsular VIP-immunoreactive fibers were associated with the ISN but not with the SSN. Thus, VIP immunoreactivity was only detected in lower nerve network. By contrast, CPON immunoreactivity was present in both nerve networks. Therefore, our observations demonstrate that the ISN and the SSN contribute different kinds of peptidergic fibers to the rat testicular capsule.

Conclusions

In summary, both glyoxylic acid-induced fluorescence and immunohistochemistry showed that the rat testis receives nerve fibers from the SSN and the ISN and that these fibers are distributed to intra- or subcapsular blood vessels and to capsular nerve networks. The latter, providing intrinsic innervation to the testicular capsule, are restricted to specific regions in the anterior aspect of the upper and lower poles. Both the SSN and ISN contained monoamines and CPON. The ISN also contained VIP-immunoreactive nerves that selectively innervated the lower pole of the capsule. Further physiological studies are required to understand the role of the different nerve groups.

References

Alm P, Alumets J, Häkanson R, Owman Ch, Sjöberg N-O, Sundler F, Walles B. Origin and distribution of VIP (vasoactive intestinal polypeptide) nerves in the genito-urinary tract. *Cell Tissue Res* 1980;205: 337–347.

Anakwe OO, Murphy PR, Moger WH. Characterization of β-adrenergic binding sites on rodent Leydig cells. *Biol Reprod* 1985;33:815–826.

Bell C. Autonomic nervous control of reproduction: circulatory and other factors. *Pharmacol Rev* 1972;24:657–736.

Bell C, McLean JR. The autonomic innervation of the rat testicular capsule. *J Reprod Fertil* 1973;32:253–258.

Campos MB, Chiocchio SR, Calandra RS, Ritta MN. Effect of bilateral denervation of the immature rat testis on testicular gonadotropin receptors and *in vitro* androgen production. *Neuroendocrinology* 1993;57:189–194.

Campos MB, Vitale ML, Calandra RS, Chiocchio SR. Serotonergic innervation of the rat testis. *J Reprod Fertil* 1990a;88:475–479.

Campos MB, Vitale ML, Ritta MN, Chiocchio SR, Calandra RS. Catecholamine distribution in adult rat testis. *Andrologia* 1990b;22:247–250.

Cooke BA, Golding M, Dix CJ, Hunter MG. Catecholamine stimulation of testosterone production via cAMP in mouse Leydig cells in monolayer culture. *Mol Cell Endocrinol* 1982;27:221–231.

Damber J-E. The effect of guanethidine treatment on testicular blood flow and testosterone production in rats. *Experientia* 1990;46:486–488.

De la Torre JC. An improved approach to histofluorescence using the SPG method for tissue monoamines. *J Neurosci Methods* 1980;3:1–5.

Frankel AI, Ryan EL. Testicular innervation is necessary for the response of plasma testosterone levels to acute stress. *Biol Reprod* 1981;24: 491–495.

Frankel AI, Mock EJ, Chapman JC. Hypophysectomy and hemivasectomy can inhibit the testicular hemicastration response of the mature rat. *Biol Reprod* 1984;30:804–808.

Gulbenkian S, Wharton J, Hacker GW, Varndell IM, Bloom SR, Polak JM. Co-localization of neuropeptide Y (NPY) and its C-terminal peptide (C-PON). *Peptides* 1985;6:1237–1243.

Hargrove JL, Legrande CE. Autonomic nerves versus prostaglandins in the control of rat and rabbit testicular capsular contractions *in vivo* and *in vitro*. *Biol Reprod* 1976;14:651–657.

Hodson NP. The nerves of the testis, epididymis and scrotum. In: Johnson AD, Gomes WR, Vandermark NL, eds. *The Testis*. Vol 1. New York: Academic Press, 1970.

Kuntz A, Morris RE. Components and distribution of the spermatic nerves and the nerves of the vas deferens. *J Comp Neurol* 1946;85: 33–44.

Lamano Carvalho TL, Hodson NP, Blank MA, Watson PF, Mulderry PK, Bishop AE, Gu J, Bloom SR, Polak JM. Occurrence, distribution and origin of peptide-containing nerves of guinea pig and rat male genitalia and the effects of denervation on sperm characteristics. *J Anat* 1986;149:121–141.

Langford GA, Silver A. Histochemical localization of acetylcholinesterase-containing nerve fibres in the testis. *J Physiol* 1974;242:9P–10P.

Larsson LI, Fahrenkrug J, Schaffalitzky de Muckadell OB. Occurrence of nerves containing vasoactive intestinal polypeptide immunoreactivity in the male genital tract. *Life Sci* 1977;21:503–508.

Lundberg JM, Terenius L, Hökfelt T, Martling CR, Tatemoto K, Mutt V, Polak JM, Bloom SR. Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and the effects of NPY on sympathetic function. *Acta Physiol* 1982;116:477–480.

Lundberg L-M, Alm P, Wharton J, Polak JM. Protein gene product 9.5 (PGP 9.5): a new neuronal marker visualizing the whole uterine innervation and pregnancy-induced and developmental changes in the guinea pig. *Histochemistry* 1988;90:9–17.

Maekawa M, Nagano T, Kamimura K, Murakami T, Ishikawa H, Dezawa M. Distribution of actin-filament bundles in myoid cells, Sertoli cells, and tunica albuginea of rat and mouse testes. *Cell Tissue Res* 1991;266:295–300.

Mayerhofer A, Bartke A, Steger RW. Catecholamine effects on testicular testosterone production in the gonadallay active and the gonadally regressed adult golden hamster. *Biol Reprod* 1989;40:752–761.

Mayerhofer A, Steger RW, Gow G, Bartke A. Catecholamines stimulate testicular testosterone release of the immature golden hamster via interaction with alpha-/and beta-adrenergic receptors. *Acta Endocr* 1992;127:526–530.

Milner P, Lincoln J, Corr LA, Aberdeen JA, Burnstock G. Neuropeptide

Y in non-sympathetic nerves of the rat: changes during maturation but not after guanethidine sympathectomy. *Neuroscience* 1991;43: 661–669.

Mizunuma H, Palatis L, McCann SM. Effect of unilateral orchidectomy on plasma FSH concentration: evidence for a direct neural connection between testes and CNS. *Neuroendocrinology* 1983;37:291–296.

Moger WH, Murphy PR, Casper RF. Catecholamine stimulation of androgen production by mouse interstitial cells in primary culture. *J Androl* 1982;3:227–231.

Preslock JP, McCann SM. Short-term effects of vasoligation upon plasma follicle-stimulating hormone, luteinizing hormone and prolactin in the adult rat: further evidence for a direct neural connection between the testes and central nervous system. *Biol Reprod* 1985;33:1120–1125.

Prince FP. Ultrastructural evidence of indirect and direct autonomic innervation of human Leydig cells: comparison of neonatal, childhood and pubertal ages. *Cell Tissue Res* 1992;269:383–390.

Properzi G, Cordeschi G, Francavilla S. Postnatal development and distribution of peptide-containing nerves in the genital system of the male rat: an immunohistochemical study. *Histochemistry* 1992;97: 61–68.

Santamaría L, Reoyo A, Regadera J, Paniagua R. Histochemistry and ultrastructure of nerve fibres and contractile cells in the tunica albuginea of the rat testis. *Acta Anat* 1990;139:126–133.

Setchell BP, Maddocks S, Brooks DE. Anatomy, vasculature, innervation, and fluids of the male reproductive tract. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction.* Vol 1, 2nd ed. New York: Raven Press; 1994:1063–1175.

Shu S, Ju G, Fan L. The glucose oxidase–DAB–nickel method in peroxidase histochemistry of the nervous system. *Neurosci Lett* 1988;85: 169–171.

Waites GMH, Archer V, Langford GA. Regional sensitivity of the testicular artery to noradrenaline in the ram, rabbit, rat and boar. *J Reprod Fertil* 1975;45:159–163.