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Neurotransmitters in the human urethral sphincter in the absence of voiding dysfunction

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Abstract The purpose of this study was to elucidate the neuroregulation of sphincteric relaxation by investigating the density of nerves containing acetylcholine, noradrenaline, neuropeptide Y (NPY), galanin, vasoactive intestinal polypeptide (VIP) and calcitonin gene-related peptide (CGRP) in the urethral sphincter in patients without a voiding disorder. The complete urethral sphincter (from the bladder neck to beyond the striated external sphincter) was excised from four male and four female adult cadavers and one male and one female fetus. In transverse paraffin or cryostat sections, the above transmitters were identified by histochemical methods. The striated sphincter was densely innervated by cholinergic nerves. Adrenergic nerves next to striated fibers were rare, but were present in all patients. NPY was seen rarely along striated fibers. In the smooth sphincteric component, noradrenaline-, acetylcholine-, NPY- and galanin-reactive nerves were observed frequently. Only functional studies can clarify the clinical implications of these results. Judging from NPY's scarcity in the striated sphincter no efferent function is anticipated. In the smooth component the frequent appearance of NPY, galanin and noradrenaline suggests a regulatory role for these transmitters.

Key words Urethra · Sphincter · Neuropeptides

Introduction

In 1974, the triple innervation of the mammalian urethral sphincter was established: sympathetic (adrenergic), parasympathetic (cholinergic) and somatic [9]. Since then, cotransmitters to the classic transmitters noradrenaline and acetylcholine have been identified. It is now widely accepted that most autonomic nerves contain two or more neuropeptides modulating the release of noradrenaline and acetylcholine at the pre- and postjunctional levels [3].

In patients with neurogenic voiding disorders (e.g. spinal cord injury, meningomyelocele), the spasticity of the striated urethral sphincter endangers the upper urinary tract. Non-surgical measures such as the administration of baclofen [11] or botulinum toxin [8] have not gained wide acceptance because of their side effects and merely temporary efficacy. Thus far, no medical treatment specifically inhibiting the urethral sphincter exists. Therefore, transurethral sphincterotomy, irreversibly damaging urinary continence in most cases, is performed in many patients [7, 17, 22, 31].

A better understanding of the neuroregulation of sphincteric relaxation might eventually lead to the development of drugs affecting the striated sphincter specifically and reversibly, thus protecting the upper urinary tract and maintaining continence without the need for surgery.

Possible candidates with relaxant effects on the urinary sphincter among the neuropeptides are neuropeptide Y (NPY) [29, 32], galanin [16, 19, 21], vasoactive intestinal polypeptide (VIP) [3, 10] and calcitonin gene-related peptide (CGRP) [24]. This prompted us to investigate the distribution of the above peptides and classic transmitters (noradrenaline, acetylcholine) in the human urethral sphincter, with special reference to the striated sphincter. The available studies on this topic investigate sphincteric specimens from patients with neurogenic voiding disorders or bladder cancer [6, 7, 17, 22]. We looked at the complete urethral sphincter from patients who died of diseases not related to the urinary tract.

Materials and methods

Four male and four female adult cadavers and a male and a female foetus were used in this study. The corresponding clinical data are given in Table 1. In none was the pathologic condition related to

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Table 1 Characteristics of human cadavers

No./Sex	Age	Cause of death	Time to fixation (h)	Length of striated sphincter (cm)
Tissue embedded in paraffin				
1/male	57 years	Basalioma, cerebral Bleeding	48	2.4
2/male	74 years	Hodgkin's lymphoma	48	2.4
3/female	44 years	Metastatic carcinoma (unknown primary tumor)	24	2.0
4/female	71 years	Heart failure, coronary sclerosis	24	2.0
5/male[a]	31 weeks	Stillborn	24	1.2
6/female[a]	41 weeks	Placental insufficiency	8	1.2
Tissue frozen in liquid nitrogen				
7/male	48 years	Pancreatic carcinoma	68	2.0
8/female	74 years	Heart failure	36	2.0
9/male	61years	Meningitis, pneumonia	28	2.8
10/female	69 years	Intoxication	101	1.6

[a]Cadevers 5 and 6 were foetuses

the urinary sphincter. Cadavers with a history of an operation on the bladder or prostate were excluded.

The complete urethral sphincter was harvested. In the male, the urinary bladder, the sphincter and the proximal half of the penis were dissected out; in the female, the bladder and complete urethra. To obtain positive controls for the various peptides, adjacent tissue from the cadaver (ureter, ductus deferens, seminal vesicle, corpus cavernosum, vaginal tissue) and fresh tissue from patients undergoing prostatectomy or cystoprostatectomy were harvested. In the cadavers the time between death and tissue fixation did not exceed 4 days, with the cadavers cooled after death (Table 1). The urethral sphincter was dissected in transverse 4-mm blocks, beginning at the bladder neck and extending beyond the striated urethral sphincter.

The harvesting of specimens from patients as well as cadavers was approved by the ethics committe of the Westfälische Wilhelms-Universität Münster and in accordance with the standards of the 1964 Declaration of Helsinki.

Paraffin sections

The specimens were fixed in 8% formalin for 24–48 h and embedded in paraffin. Sections of 1-μm thickness were cut, mounted on gelatinized slides, and dried overnight at 37°C. The sections were deparaffinized in xylene, followed by rehydration in decreasing concentrations of ethanol (down to distilled water) and placed in 0.05 M TRIS-buffered saline (TBS) at pH 7.6.

Cryostat sections

The tissue samples were placed on cork platelets covered with GSV1 tissue-embedding medium (Slee Technik, Mainz, Germany), frozen in liquid nitrogen, and stored at −70°C. Care was taken to avoid air bubbles in the tissue-embedding medium. Sections of 5-μm thickness were cut, mounted on poly-L-lysine-coated (Sigma, St. Louis, Mo., P8920) slides, and air-dried for 1–2 h. If the slides were not stained immediately, they were stored at −20°C wrapped in aluminium foil. Before staining, the cryostat sections were warmed for 1–2 h at room temperature. Next the sections were fixed in ice-cold acetone for 90 s, washed in distilled water, and placed in TBS.

Staining of cholinergic nerves

Cryostat sections were prepared as described without fixation in acetone. Acetylcholinesterase was localized with the copper-thiocholine method [15], using iso-OMPA (tetraisopropyl-pyrophosphoramide, Sigma T 1505) as pseudocholinesterase inhibitor [25]. The incubation medium was made fresh from stock solutions. The final concentrations of the medium (pH 5.5) were: acetylthiocholine iodide (Sigma A5751) 1.7×10^{-3} M; acetate buffer 65×10^{-3} M; sodium citrate 5×10^{-3} M; cupric sulfate 3×10^{-3} M; potassium ferricyanide 0.5×10^{-3} M; iso-OMPA 10^{-5} M. The sections were incubated for 1.5 h at 37°C, rinsed in distilled water, and counterstained in hematoxylin.

To establish the incubation time and concentration of acetylthiocholine iodide, ductus deferens was used as a positive control. As expected, we found cholinergic nerves in abundance in the urethral sphincter. Adjacent sections were stained with a polyclonal primary antibody to S100 (Sigma S2644, dilution 1:100 in RPMI) with the alkaline phosphatase/anti-alkaline phosphatase (APAAP) [5, 20] technique to ensure that we were looking at nerves [4] and not unspecific background colouring.

Staining of adrenergic nerves

We stained for adrenergic nerves in paraffin sections, prepared as described above, with the APAAP technique. We used a polyclonal primary antibody to arterenol (norepinephrine) raised in rabbit (Biotrend, Cologne, Germany, No 0730-3004, dilution 1:100 in RPMI). To demonstrate the density of nerves [4] and to exclude non-specific staining, adjacent sections were stained with a polyclonal primary antibody to S100 (Sigma S2644, dilution 1:100 in RPMI) with the APAAP technique.

Staining of neuropeptides

NPY-, galanin-, VIP- and CGRP-immunoreactive nerves were stained with the APAAP technique. The dilution of the primary antibodies was as follows: to NPY (Sigma 9528), 1:4000 in paraffin sections and 1:2000 in cryostat sections; to VIP (Peninsula IHC 7161), 1:2000 in paraffin sections and 1:500 in cryostat sections; to galanin (Peninsula IHC 7100), 1:4000 in paraffin sections; to CGRP (Peninsula IHC 6009), 1:1000 in cryostat sections. All primary antibodies were polyclonal and raised in rabbit.

The primary antibodies were diluted in RPMI 1640 (Gibco Life Technologies, Eggenstein, Germany, No. 72400) [23] containing inactivated bovine serum and sodium azide, pH 7.5. The sections were incubated with the primary antibodies for 45 min. The negative controls were incubated with buffer solution only. Tissue known to contain the antigen was used as positive control.

The sections were incubated with mouse anti-rabbit immunoglobulin (bridging antibody, Dako M 737; dilution 1:125 in RPMI) for 30 min. Next, the sections were incubated with rabbit anti-mouse immunoglobulin (bridging antibody, Dako Z 259; dilution 1:30 in RPMI containing normal human serum, Jackson Immuno Research, Dianova Hamburg, Germany, code 009000–121) for 30 min. Finally, the slides were incubated with APAAP (mouse monoclonal, Dako 651; dilution 1:100 in RPMI) for 60 min. All incubation procedures were done at room temperature in a humid chamber.

For each of the above steps, the slides were covered with a volume of 100 µl of primary antibody, bridging antibodies, and APAAP complex. After each staining procedure, the slides were washed with fresh TBS solution three times.

Substrate color reaction was developed with Fast Red TR (Sigma F 2768)/Naphtol AS-MX phosphat, Sigma N5000) solution containing Levamisol (Sigma L9756) to inhibit endogenous alkaline phosphatase. This solution was prepared immediately before use. Sections were cleared in water, counterstained in hematoxylin to identify the tissue around the nerves in question, and mounted with Kaiser's glycerol gelatin (Merck 9242). When it was not possible to identify the tissue architecture with hematoxylin counterstaining in the same section, the adjacent sections of the urethra were stained in hematoxylin and eosin or trichrome [12].

The sections were viewed with a Zeiss inverse camera microscope (ICM 405) and photographed on Kodak Ektachrome 64 T film. For quantification, two separate investigators (UJ, BvH) judged the nerve density as rare (+), numerous (++), and abundant (+++).

Results

Acetylcholine-containing nerves were numerous in the smooth muscle of the urethral sphincter and abundant in the striated sphincter (Table 2). This could not be demonstrated in formalin-fixed, paraffin-embedded sections. Stained for acetylcholine, the striated sphincter could be identified with the naked eye as brown muscle bundles in circular orientation inserting at the raphe of connective tissue at the 6 o'clock position (Fig. 1a). Microscopically, all striated fibers were supplied with cholinergic nerves in the male and female (Fig. 1b) urethra.

Noradrenaline-containing nerves were abundant in the proximal urethra (Fig. 2a) and along smooth sphincteric fibers. Along striated sphincteric fibers, noradrenaline-reactive nerves were seen in rare numbers in all but one cadaver (a female), in which they were numerous (Fig. 2b, c, Table 3). In the proximal urethra, groups of thick nerve bundles running longitudinally were detected (Fig. 2a). Along striated sphincteric fibers, two populations of nerves were seen: thin nerves, supplying single striated fibers, running in circular orientation (Fig. 2b); and thicker fibers, in circular and longitudinal orientations [the latter seen in cross-section (Fig. 2c)].

Neuropeptide Y was abundant in cavernosal vessels, the seminal vesicle, and ductus deferens in the male adult and fetus. It was also abundant in fetal detrusor and in the cervix and urethral vessels of the adult.

In the urethral sphincter NPY was numerous in the smooth muscle. NPY-positive nerves innervating a striated sphincteric fiber were the exception, but could be detected rarely in both male and female tissue. This was true for tissue embedded in paraffin (Fig. 3a) as well as for frozen tissue (Fig. 3b): i.e., in Fig. 3a NPY-positive nerves are visible in the wall of a vessel and next to a striated sphincteric fiber. NPY was seen along striated fibers in both sexes, in 5 of the 10 subjects (for more detail, see Table 4).

To determine the effect of the different tissue preparations (paraffin-embedded versus frozen tissue) on the quality of the NPY stain, we compared both techniques in fresh ductus deferens (near the seminal vesicle) of the same patient (Fig. 3c, d). The tissue preparation had little effect on the staining quality: in frozen tissue, the staining of NPY-positive nerves was not as clear (slightly washed out, Fig. 3b, d) as in paraffin-embedded tissue (Fig. 3c); in no instance did frozen tissue give results superior to those of paraffin sections.

Galanin-positive nerves (see Table 5) were abundant in the ductus deferens, seminal vesicle, corpus cavernosum, and urethral vessels of the male adult and foetus. The same was true for the cervix, ureter, urethral vessels and urethral smooth muscle in the female.

In the urethral sphincter, the striated fibers were devoid of galanin, while the smooth sphincteric fibers were frequently galanin-positive (Fig. 4a). It was the exception to find a galanin-positive nerve next to a striated fiber (Fig. 4b), and it could not be ruled out that the nerve supplied a vessel rather than a striated sphincteric fiber.

When frozen tissue was stained for galanin, we found either no galanin or so much background staining that a valid interpretation was not possible.

VIP-positive nerves were numerous to abundant in the ductus deferens, the seminal vesicle, the corpus cavernosum and urethral vessels of the adult male and the urethral vessels of the female adult. In fetal tissue the ductus deferens was devoid of VIP, while in the male cadavers the ductus deferens contained VIP-reactive nerves. This discrepancy – indicating a post-fetal development of VIP-positive nerves in ductus deferens – was not found for NPY and galanin.

Along the smooth and striated fibers of the urethral sphincter, no VIP staining was found, while vessels in the sphincter were supplied with VIP-positive nerves (Fig. 5). These observations were made in paraffin sections.

Table 2 Presence of acetylcholine (tissue frozen in liquid nitrogen)

Cadaver no.	Positive control[a] (S100)	Urethral sphincter	
		Smooth	Striated
7	Urethral sphincter, adjacent section	++	+++
8	Urethral sphincter, urethral vessels	++	+++
9	Urethral sphincter, adjacent section	+	+++
10	Urethral sphincter	++	+++

+++ abundant, ++ numerous, + rare

[a] See Methods

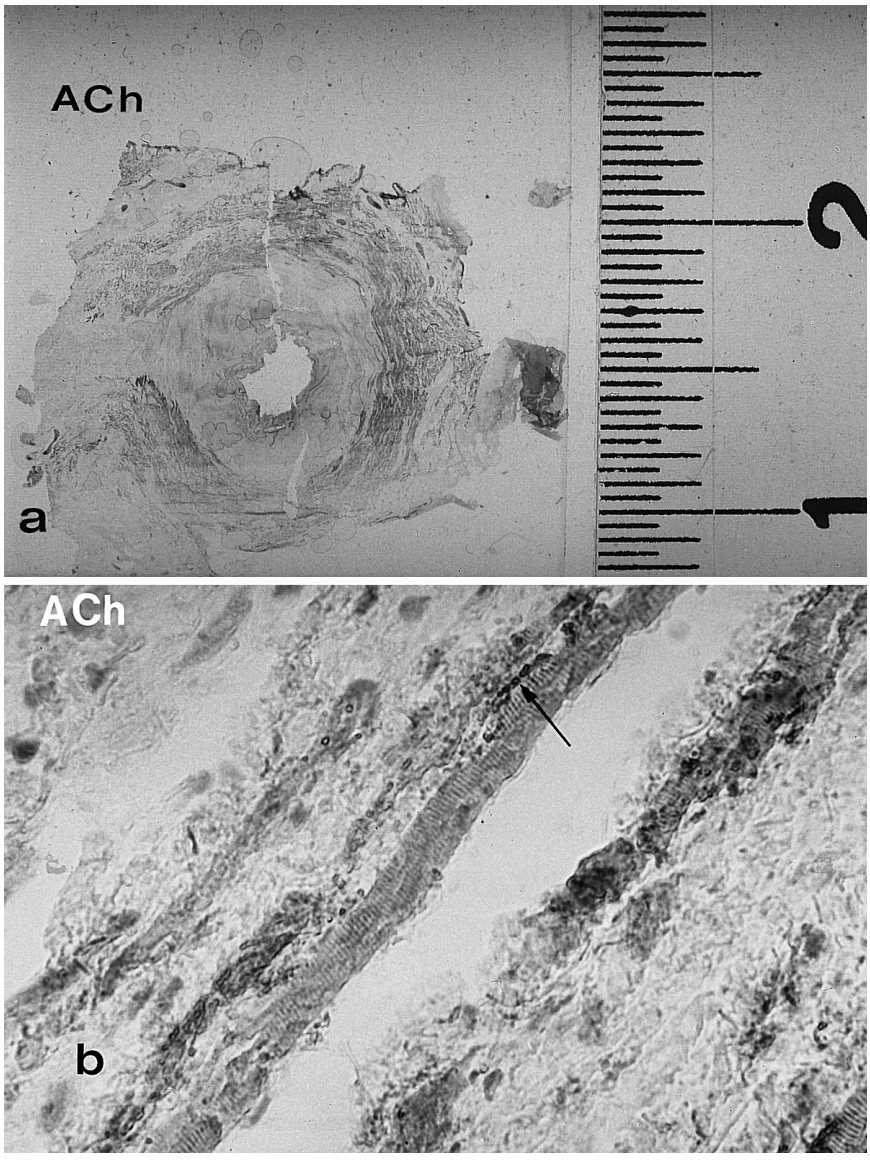


Fig. 1a, b Acetylcholine staining (cryostat section, acetylcholinesterase localized with copper-thiocholine, counterstained in hematoxylin). (**a**) In this urethral sphincter from a 61-year-old male cadaver (No. 9 in the tables), the striated sphincter can be identified with the naked eye. Its brown muscle bundles, circularly oriented, insert at the raphe of connective tissue at the 6 o'clock position (on the left). Scale to the right in centimeters. (**b**) In this urethral sphincter from a 69-year-old female cadaver (No. 10 in the tables), the *arrow* shows beaded nerve next to striated fiber. ×400

In cryostat sections, abundant nerves were found in the seminal vesicle associated with considerable background staining. (A concentration of 1:500 to 1:1000 of antibody was necessary.) Glandular tissue in the proximal urethra, next to the colliculus seminalis, and the intima of small vessels showed VIP-positive staining. The smooth muscle in the urethral sphincter was devoid of VIP, as were most striated fibers. Some striated fibers were supplied by nerves of black and washed-out appearance. Because these nerves were not reddish/bright pink as expected, we regarded their coloring as non-specific.

CGRP-positive nerves were not detected in paraffin-embedded and formalin-fixed sections. When frozen tissue was stained, CGRP-positive nerves were found in the corpus cavernosum and spongiosum and along urethral vessels (Table 6).

Within the urethral sphincter CGRP-positive nerves were detected rarely along smooth fibers, while striated fibers were devoid of CGRP (Fig. 6, Table 6). Where smooth and striated fibers ran parallel in the circular layer, CGRP-containing nerves were identified parallel with and close to striated fibers. These most likely supplied smooth muscle fibers or vessels, as CGRP-containing nerves in direct contact with a striated fiber were not seen.

A summary of the above results is given in Table 7.

Discussion

Acetylcholine-reactive nerves were numerous in non-vascular smooth sphincteric fibers, and all striated fibers of the urethral sphincter were supplied with cholinergic nerves in both sexes. This is in agreement with previous investigations [7, 17].

Of the investigated transmitters only acetylcholine (as classical transmitter on nicotinic receptors) was found in abundance along striated sphincteric fibers. This has

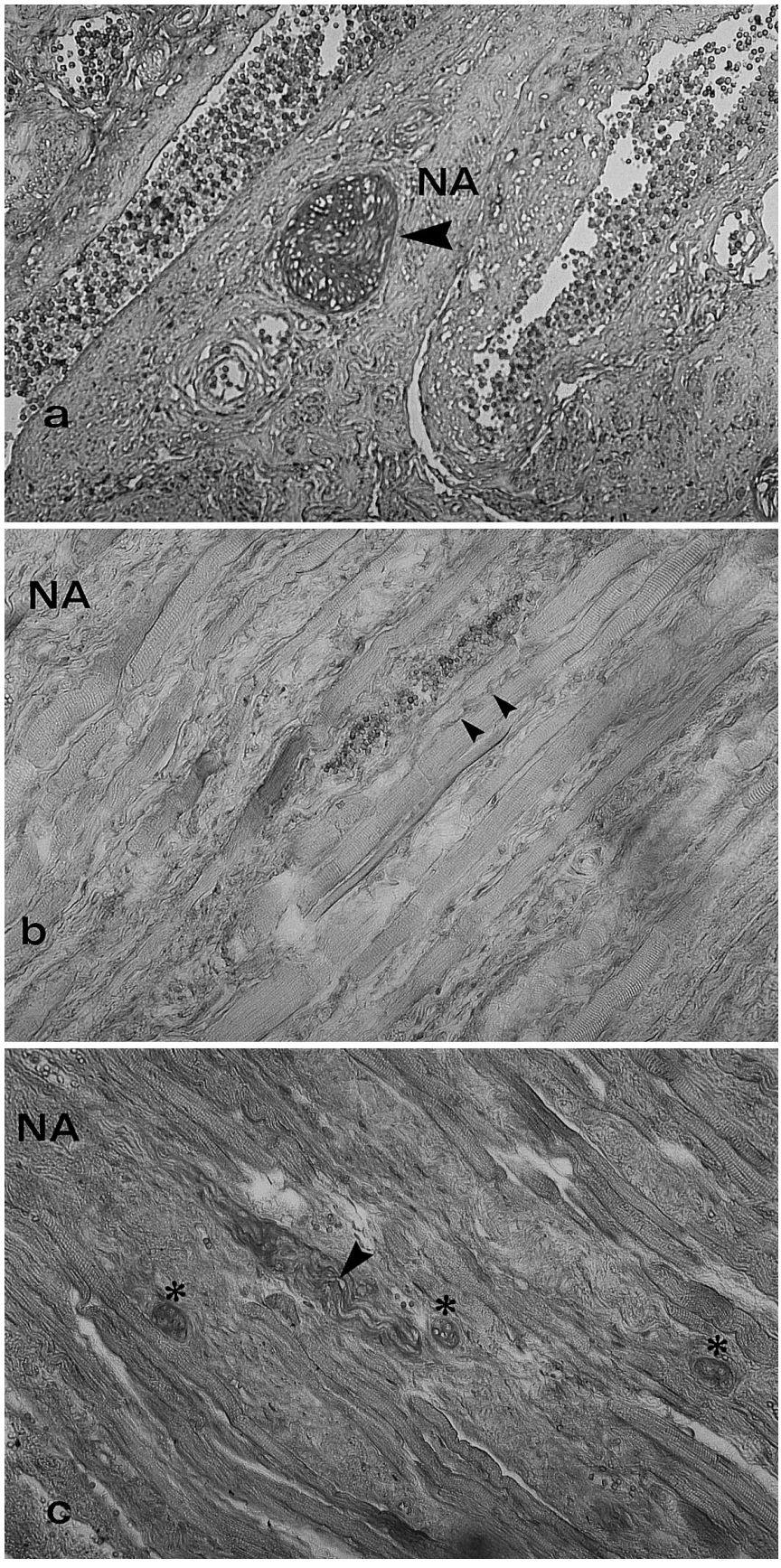


Fig. 2a–c Noradrenaline staining (female cadaver, 44 years, no. 3 in the tables, paraffin section, × 160 magnification). (**a**) In the proximal urethra, thick noradrenergic nerve bundles running longitudinally are visible in cross-section (*arrow*). Adrenergic nerves in the vessel wall (upper and lower right) are much smaller. (**b**) In this view of the striated urethral sphincter, striated fibers are supplied frequently by thin branches of nerves containing noradrenaline (*arrow*), while in (**c**) thick bundles of noradrenergic nerves run next to striated fibers in circular (*arrow*) and longitudinal (*asterisks*) orientations (the latter seen in cross-section)

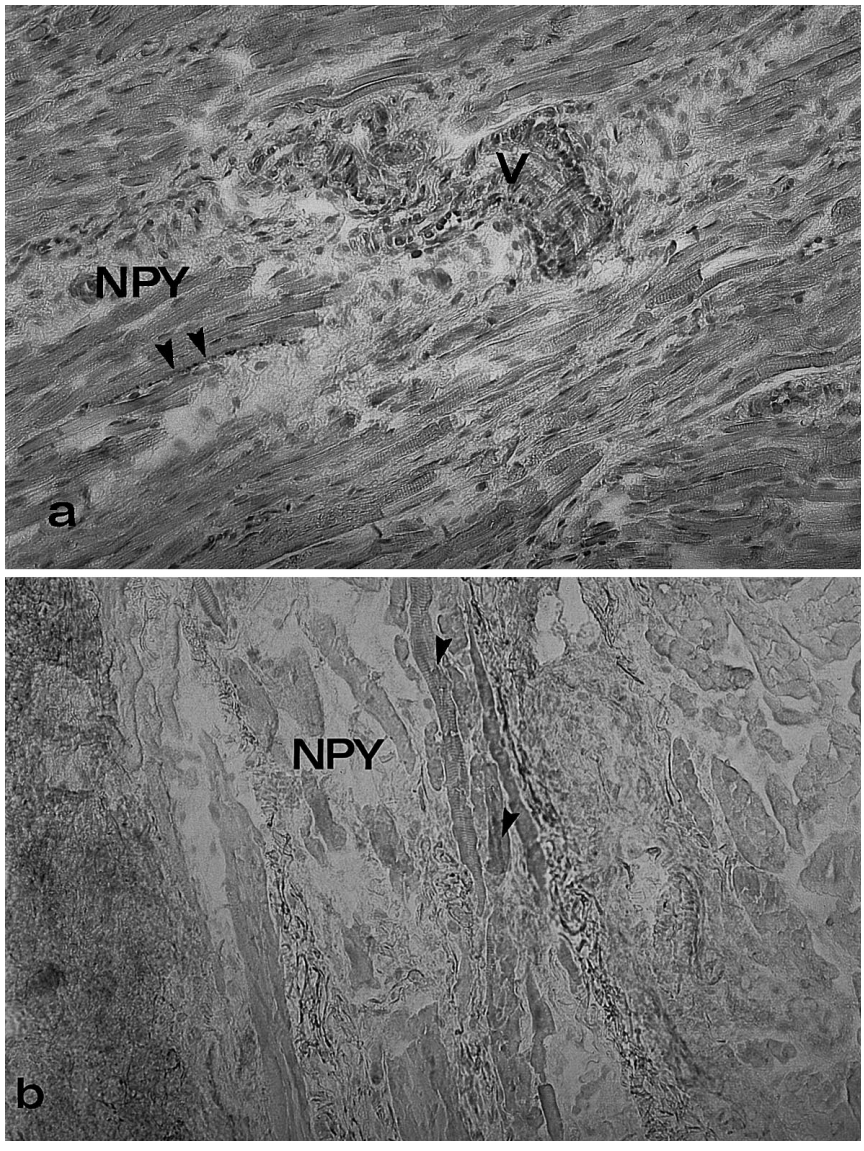


Fig. 3a–d Staining for NPY. (**a**) Striated urethral sphincter from a female foetus (41 weeks, no. 6 in tables, paraffin section, ×160 magnification). An NPY-positive nerve running along a striated fiber is seen (*arrow*). NPY-positive nerves are also seen in a vessel wall. (**b**) Striated urethral sphincter from a male cadaver (61 years, no. 9 in tables, cryostat section, ×160 magnification). *Arrows* mark striated fibers innervated by NPY-positive nerves. (**c, d**) Ductus deferens, next to seminal vesicle (male cadaver, 67 years). In (**d**), the cryostat section, the *arrow* marks one of the NPY-positive nerves that are not as clear as in **c**, the paraffin section (×160 magnification)

also been true in patients with neurogenic voiding disorders [7, 17, 22]. Acetylcholine and VIP are frequent cotransmitters in parasympathetic nerves [3]. Thus, we expected to find VIP-positive nerves along striated fibers, as these were densely acetylcholine-positive, but this was not the case.

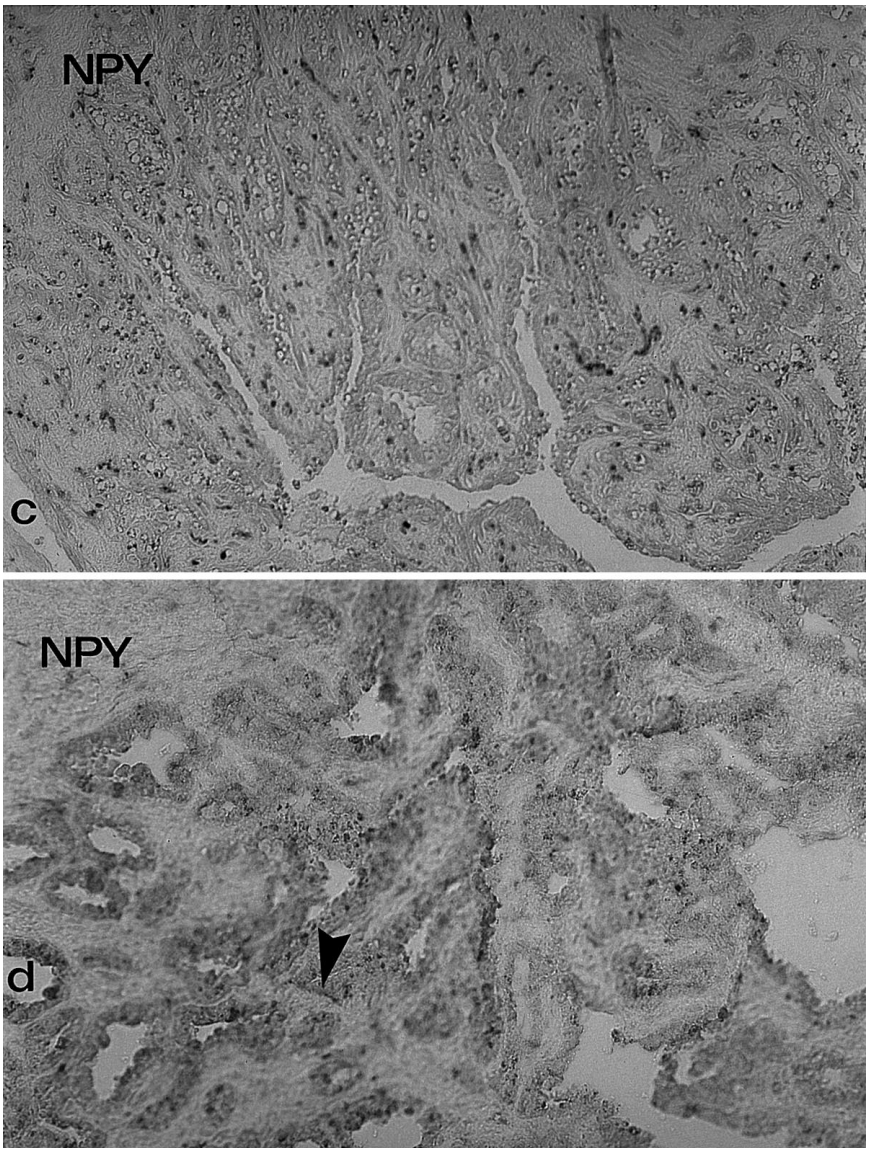
Table 3 Presence of noradrenaline (tissue embedded in paraffin)[a]

Cadaver/foetus no.	Urethral sphincter	
	Smooth	Striated
1	++	+
2	++	+
3	++	++
4	++	+
5	++	+
6	+	+

++ numerous, + rare
[a] For all samples, the positive control was the proximal urethra

Noradrenaline-reactive nerves were numerous in smooth sphincteric muscle. In contrast to the above neuropeptides, which were all visible (if at all) as small, thin fibers, thick adrenergic nerve bundles of circular and longitudinal orientation were found as well as thin branches. The thickness of some of these nerves suggested that they were passing through the sphincter rather than innervating it (Fig. 2a).

The density of adrenergic nerves along smooth sphincteric fibers is in agreement with the observations of Lincoln et al. [17] in male patients with spinal cord injury and those with bladder cancer whose sphincter was partly resected transurethrally. Throughout the length of the urethral sphincter, adrenergic nerves were found on smooth muscle bundles in all, although the density varied considerably in the group with spinal injury [17]. The same authors detected no adrenergic nerves in the striated sphincteric fibers [17]. Only vessels supplying the striated muscle were catecholamine-posi-



tive in both patients with neurogenic and those with non-neurogenic disorders. In contrast, we found thin nerve fibers next to and parallel with striated fibers as a rare occurrence in all but one woman, in whom they were numerous (Fig. 2b).

The adrenergic innervation of striated sphincteric fibers is controversial: Crowe et al. [6] claim a substantial invasion of adrenergic nerves in smooth and striated sphincteric fibers in patients with lower motor neuron lesions, but Wein et al. [30] dispute any adrenergic innervation in the human striated sphincter. With our staining technique, the nerves appeared slightly washed out, which made it difficult at times to differentiate adrenergic nerves from background staining (Fig. 2b). Thus, we cannot say with certainty that junctions between nerve and muscle were present. Still, the arrangement of nerve and muscle suggests an adrenergic nerve supply of striated sphincteric fibers in our patients, who were without urologic or neurourologic disease (Fig. 2b, c). NPY-reactive nerves were detected rarely along striated fibers in half of our patients and adrenergic nerves rarely in all our patients. This finding could indicate an interplay of both transmitters, as NPY is known to coexist with noradrenaline at pre- and postjunctional levels in the cardiovascular system [18]. (Because we did no double-labeling to show co-localization, this must remain speculative.) That both noradrenaline and NPY were found next to striated fibers could also have functional significance. In patients with areflexic bladders, Milner et al. [22] found 60%–85% of the individual striated fibers to be NPY-reactive. Unfortunately, this group did not investigate patients without a voiding disoder. Our patients – without spinal injury – showed NPY-positive striated fibers only rarely. The high NPY density in spinal-cord-injured patients could indicate a plasticity of autonomic innervation, initiated by trauma or surgery [3].

Table 4 Presence of neuropeptide Y

Cadaver/foetus no.	Positive control	Urethral sphincter	
		Smooth	Striated
Tissue embedded in paraffin			
1	Arteries in corpus cavernosum	++	Negative
2	Seminal vesicle, ductus deferens	++	Negative
3	Artery, vein	+	+
4	Cervix artery, vein	++	Negative
5	Prostatic glandular tissue, ductus deferens	++	+
6	Detrusor	++	+
Tissue frozen in liquid nitrogen			
7	Urethral artery, vein	++	Negative
8	Urethral artery, vein	++	Negative
9	Seminal vesicle	++	+
10	Urethral artery, vein	++	+

++ numerous, + rare

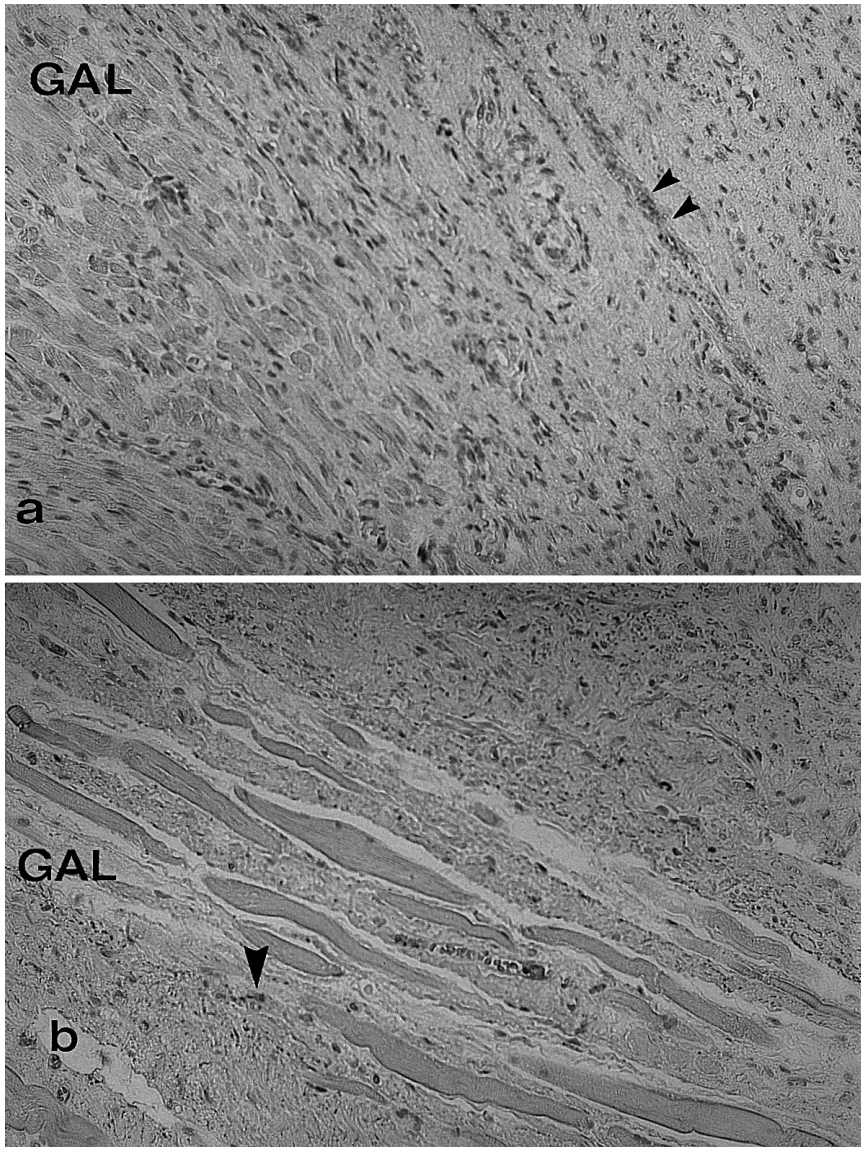


Fig. 4a, b Galanin staining. (**a**) Striated urethral sphincter from a female foetus (41 weeks no. 6 in tables, paraffin section, ×160 magnification). The striated fibers (on the left side) are devoid of galanin, while the smooth fibers (on the right side, *arrow*) show frequent galanin-positive staining. (**b**) In this striated urethral sphincter from a female cadaver (71 years, no. 4 in the tables, paraffin section, ×160 magnification), a galanin-positive nerve is seen close to a striated fiber (*arrow*)

Table 5 Presence of galanin (tissue embedded in paraffin)

Cadaver /foetus no.	Positive control	Urethral sphincter	
		Smooth	Striated
1	Seminal vesicle, corpus cavernosum	++	Negative
2	Seminal vesicle, ductus deferens	++	Negative
3	Urethral artery, vein	+	Negative
4	Cervix, ureter	++	+
5	Urethral artery, vein, ductus deferens	+	+
6	Urethral smooth muscle	++	Negative

++ numerous, + rare

Neuropeptide Y immunoreactivity in the human urogenital tract suggests that it is a major peptide in the sympathetic nervous system, as it is present in noradrenaline-containing neurons [10]. In the human fallopian tube, NPY inhibits noradrenaline release, suggesting a prejunctional inhibitory action on adrenergic transmission [26]. In the urethral muscle of male rats, Zoubek et al. [32] found NPY to inhibit muscle contraction in response to electrical stimulation (1–20 Hz) by 90%–100%. The same group demonstrated that NPY has a direct inhibitory effect on adrenergic terminals (a decrease in noradrenaline release) and that it decreases the heterosynaptic, cholinergic inhibition of noradrenaline release in male rats [29]. These in vitro experiments were done on spiral cuts of the urethra, however. Thus, whether NPY induced these effects in the smooth or striated sphincteric component could not be determined [29, 32].

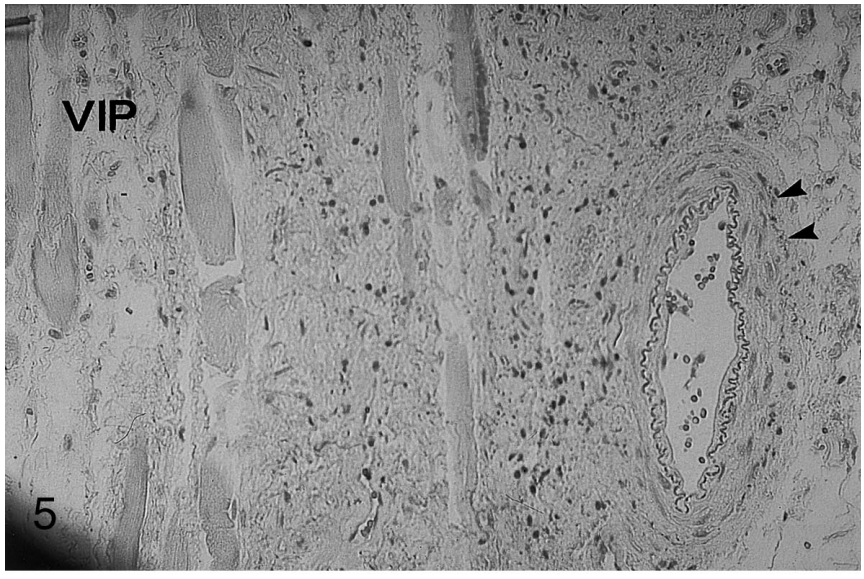


Fig. 5 Staining for VIP: Striated urethral sphincter (female cadaver, 71 years, no. 4 in the tables, paraffin section, ×160 magnification). The striated fibers (left side) are devoid of VIP, but an artery (on the right) is supplied with VIP-positive nerves (*arrows*)

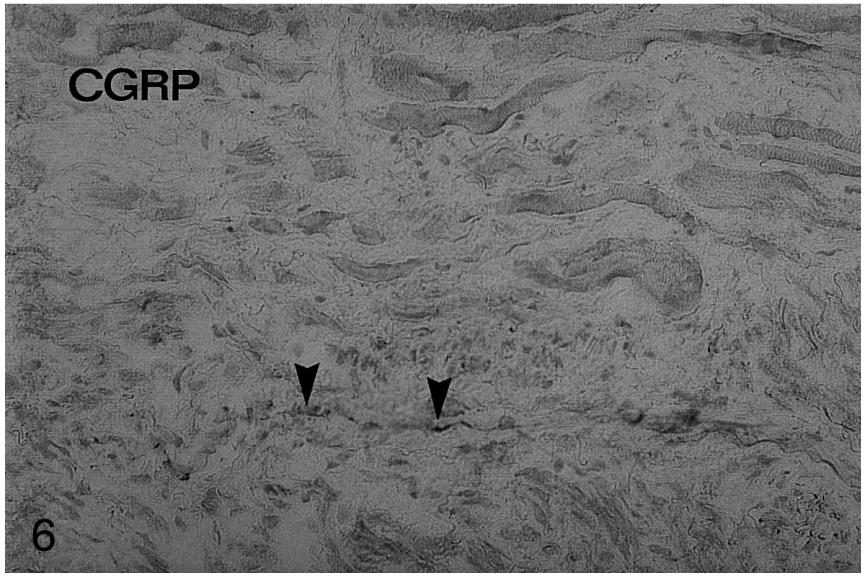


Fig. 6 Staining for CGRP. Striated and smooth urethral sphincter (male cadaver, 61 years, no. 9 in the tables, cryostat section, ×160 magnification). The striated fibers (upper half) are devoid of CGRP; a CGRP-containing nerve (*arrows*) is found parallel to smooth muscle fibers (lower half)

Table 6 Presence of calcitonin gene-related peptide (tissue embedded in lequid nitrogen)

Cadaver no.	Positive control	Urethral sphincter	
		Smooth	Striated
7	Urethral artery, vein	+	Negative
8	Urethral artery, vein	+	Negative
9	Corpus cavernosum, corpus spongiosum	+	Negative
10	Urethral artery,vein	+	Negative

+ rare

Table 7 Overview

Transmitter	Urethral sphincter	
	Smooth	Striated
Acetylcholine	++	+++
Noradrenaline	++	+
NPY	++	Negative to rare
Galanin	++	Negative
VIP	Negative	Negative
CGRP	+	Negative

+++ abundant, ++ numerous, + rare

In patients undergoing transurethral sphincterotomy, Milner et al. [22] reported that those with areflexic bladders had more NPY-containing nerves in the striated sphincter than those suffering from detrusor-sphincter dyssynergia. Tainio [28] found NPY-positive nerves to be "quite numerous" between striated muscle bundles in men with bladder carcinoma. The patients we studied had neither a urologic nor a neurourologic disease, which may explain why we found a striated sphincteric fiber innervated by NPY-positive nerves only rarely.

One might argue that the different staining technique (immunofluorescence versus histochemical staining), tissue fixation (paraffin versus cryostat section) and time to fixation (immediate versus a delay of 1–3 days) might have compromised our results. We used histochemical stains because they were easier to handle (no fading), gave less background coloring, and provided a clear architecture of the tissue around the nerve. Further, when we compared the quality of NPY stains in paraffin sections and cryostat sections, paraffin/formalin sections worked as well as, if not better than, cryostat sections (Fig. 3c, d). Accordingly, the results for NPY in paraffin-embedded tissue did not differ from those of cryostat sections (see Table 4). Finally, the time to fixation did not influence our results. When we compared the NPY density in tissue fixed immediately (ductus deferens) with that in ductus obtained from cadavers (fixed after hours to days), no difference was found.

The above observations are strengthened by our findings for NPY in non-striated urethral tissue. In smooth sphincteric fibers, the urethral connective tissue, and blood vessels, we saw NPY-positive nerves frequently – in agreement with Milner et al. [22] and Tainio [28].

Galanin has been shown to be a potent inhibitor of cholinergic transmission in the human urinary bladder, producing a concentration-dependent inhibition of contraction to field stimulation in human detrusor strips [19]. High concentrations of galanin in human vas deferens, corpus cavernosum, and blood vessels suggest a regulatory role in smooth muscle tone and blood flow [2, 10]. In the rat central nervous system, the localization of galanin-like peptides in cholinergic and noradrenergic hippocampal afferents suggests a peptidergic modulation of these transmitters [21].

Galanin is found not only in smooth muscle and vessels: recently, Kuramoto and Endo [16] demonstrated that 88% of acetylcholinesterase-positive motor endplates of rat oesophageal striated muscle were co-innervated by galanin, suggesting that it may be an efferent cotransmitter in the vagus nerve.

Unlike these findings in the striated rat oesophagus, our findings in the human striated sphincter showed only rare innervation by galanin-reactive nerves (Fig. 4b), and most likely these nerves supplied a vessel rather than a striated sphincteric fiber. This contradicts Tainio [28], who found numerous galanin-reactive nerves between striated fibers of the human urethral sphincter. However, because our control tissue showed galanin in abundance and the tissues in which we identified galanin were in agreement with the literature [2] (see Table 5), we felt our results were not invalid.

Vasoactive intestinal polypeptide and acetylcholine are believed to be cotransmitters in parasympathetic nerves [3]. In the male urogenital tract the inhibitory and relaxant effects of VIP on non-vascular smooth muscle have been demonstrated in the human vas deferens and urethra and the urinary bladder of most mammals [10]. As human striated sphincteric fibers resemble slow-twitch fibers, able to maintain tone over a long time [13], Crowe et al. [7] suggested that VIP may have a stabilizing and inhibitory influence on these fibers or act as a prejunctional modulator of acetylcholine release from motor nerve endings. In the non-vascular smooth sphincteric fibers, VIP likely aids relaxation during micturition [7].

In patients with cervical spinal cord lesions and detrusor-sphincter dyssynergia and in bladder cancer, Crowe et al. [7] investigated the presence of VIP in the urethral sphincter [7] and found dense VIP-reactive innervation in vascular and non-vascular smooth muscle. In the striated sphincter VIP-reactive nerves (singly and in bundles) were found along striated fibers, with a density lower than in the non-vascular smooth muscle [7] and about the same as seen for NPY [22]. No difference in nerve density between bladder cancer patients and spinal-cord-injured patients was found for VIP [7].

We did not detect VIP-reactive nerves along the smooth and striated fibers of the urethral sphincter; only vessels and glandular tissue were VIP-positive. This contradicts both Crowe et al. [7] and Tainio [28]. (Tainio

[28] found a moderate density of small nerves in smooth sphincteric fibers in patients with bladder cancer.)

Because the vessels in the sphincter were VIP-reactive (Fig. 5), our staining technique cannot be the reason for the absence of VIP. Possibly the so-called "plasticity of autonomic innervation" [3], initiated by spinal trauma, is responsible for VIP-reactive nerves in neurogenic patients, although this argument would not explain VIP-positive nerves in bladder cancer patients.

Calcitonin gene-related peptide could be involved in reflexes associated with micturition, as it is abundant beneath the epithelium of the urinary bladder and ureter [10]. As capsaicin pretreatment results in a depletion of CGRP in the urogenital tract, a sensory role for CGRP is suggested [10]. The anatomy of CGRP-positive reticular nerve terminals in the male canine urethra led to the hypothesis of a combined afferent-efferent function [14]. In men, CGRP injected in the corpus cavernosum increased arterial flow and relaxed the cavernous smooth muscle, resulting in medium tumescence in 8 of 10 patients [27]. A relaxant role during micturition has been suggested by the administration of CGRP to noradrenaline-precontracted rat external urethral sphincter, which resulted in a relaxation of 20%–40% [24]. However, the data are as yet insufficient to support this thesis in man [1].

Within the urethral sphincter we detected CGRP-positive nerves rarely along smooth muscle fibers and never along striated fibers. From our observations we do not expect a functional role for CGRP in urethral sphincteric relaxation.

In conclusion, the striated sphincter in patients without a voiding disorder is densely innervated by cholinergic nerves as expected. NPY was seen rarely along striated fibers. Adrenergic nerves next to striated fibers were rare but present in all patients (Table 7). Only functional studies can clarify the clinical implications of these results.

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