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ORIGINAL PAPER

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Distribution and regional variation of pituitary adenylate cyclase activating polypeptide and other neuropeptides in the rat urinary bladder and ureter: effects of age

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Abstract The distribution and regional variation of nerves immunoreactive for the neuropeptides pituitary adenylate cyclase activating polypeptide (PACAP), calcitonin gene-related peptide (CGRP), substance P (SP) and vasoactive intestinal polypeptide were investigated in the urinary bladder and distal ureter of young adult (3 months) and aged (24 months) male Wistar rats by indirect immunohistochemistry. Semi-quantitative estimations of nerve densities were made of peptidergic fibres innervating the dome, body and base of the urinary bladder and distal ureter. Sensory innervation of the dome was very sparse and the overall density of innervation increased progressively towards the base of the bladder. The density of innervation in the aged rats was closely comparable to that in the young adults, with the exception of slight reductions in CGRP and SP innervation of the muscle layer. Moreover, there was a marked reduction in the density of PACAP innervation of the subepithelial plexus and of the muscle layer of the bladder base. However, radioimmunoassay showed no significant difference ($P > 0.05$) in PACAP contents between young and aged rat urinary bladder. In the distal ureter of aged rats the densities of innervation by fibres immunoreactive for SP and PACAP but not CGRP were reduced. These findings suggest that the level of sensory innervation of the bladder and distal ureter are reduced in old age and that the afferent limb of voiding reflexes may in consequence be perturbed.

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Introduction

Afferent innervation of the pelvic viscera such as the urinary tract plays a vital role in the neural regulation of different physiological functions, including micturition [5]. Indeed, interruption of bladder afferents makes it impossible for natural voiding of the bladder to occur. Bladder afferents are mainly responsible for monitoring the distension of the bladder as it fills and also for the mediation of pain. In the rat, it has been estimated that approximately 50% of the nerve axons supplying the bladder are sensory in function [12], this proportion of the total innervation underlining its significance for normal functioning. By means of immunohistochemistry, various neuropeptides including pituitary adenylate cyclase activating polypeptide (PACAP) [8, 23, 24], calcitonin gene-related peptide (CGRP) [5, 12, 18, 26, 27, 28, 30] and substance P (SP) [1, 4, 20] have been demonstrated in putative sensory nerve fibres in the rat urogenital tract and in their somata in dorsal root ganglia. In addition, vasoactive intestinal polypeptide (VIP) has also been observed in nerves in the rat urinary bladder [7, 9, 14] but these may well originate from postganglionic parasympathetic neurons in the major pelvic ganglion. The ureter also possesses a dense sensory innervation, which is also responsive to distension and to irritant stimuli.

Studies on the effects of ageing on sensory peptidergic neurons have been limited [13]; for example, increases in vasodilatory and decreases in vasoconstrictor peptides have been reported in cerebral arteries [22] and a down-regulation of CGRP has been reported in aged rat lumbar dorsal root ganglia [3]. Electrophysiological studies of the effects of age on the sensory innervation of the urinary tract have shown that distension-sensitive afferents are less able to monitor bladder volume in the aged rat [16]. Decreases have been reported in the number of

unmyelinated fibres of the pelvic nerve, especially fibres of small diameter [25] but whether these fibres were unmyelinated preganglionic or C-fibre afferents was not determined. In the human bladder, there is a linear reduction in the number of cholinergic nerves (per unit area) with age [10]. In the rat, attrition of sympathetic neurons in the major pelvic ganglion [29] and sympathetic innervation of the lower urinary tract [30] takes place with increasing age. These changes in sympathetic innervation imply that a decrease in neuronal control of the urinary tract may occur in old age. In order to further our understanding of age-associated changes in the neural circuitry controlling micturition, it is important to investigate whether the distribution of the sensory innervation, as revealed by immunohistochemistry of putative peptidergic-positive nerves, is also affected by age.

Materials and methods

Sixteen white male Wistar rats aged 3 (young adult) and 24–30 months (aged) ($n = 8$ for each age group), maintained under conditions of constant temperature, humidity and lighting and allowed access to food and water ad libitum [21], were used in this study.

Tissue preparation

Rats were deeply anaesthetised on a Halothane Anaesthetising Unit (Fluorovac 240 V) by inhalation of 5% halothane (Rhône Mérirux, Dublin) in oxygen administered at 3 l/min. Animals were killed by injecting 2 ml of Euthatal (20 mg/ml pentobarbitone sodium; Rhône Mérirux, Dublin) into the liver. A 2 ml syringe was used to inject 1 ml heparin (Multiheparin, 5,000 units/ml; CP Pharmaceuticals) and 1 ml of 1% sodium nitrite (Fisons) into the left ventricle. This was followed by perfusion of fixative solution (4% paraformaldehyde + 100 mM phosphate-buffered saline; PBS) intracardially at a pressure of 116 ml/min by using a Watson-Marlow 20F/RL peristaltic pump. The bladder and the distal ureters were removed, pinned down on Sylgard and fixed in 4% paraformaldehyde in PBS (pH 7.4) at 4°C for a minimum of 2 h. After rinsing in PBS (three changes of 5 min each), the tissue was stored overnight at 4°C in PBS containing 20% sucrose. The bladder tissues from both age groups, 3 and 24 month-old rats, were divided into three anatomical regions (the dome, the body and the base which contained the trigone).

Immunohistochemistry

Serial, 10 μm, transverse cryosections of the bladder and ureters were then cut. Sections were collected onto slides coated with poly-L-lysine (Sigma, England) and allowed to dry for 30 min.

CGRP, SP and VIP immunohistochemistry

The sections were then incubated in PBS containing 5% normal swine serum (1 h), rinsed in PBS + 0.5% bovine serum albumin (BSA). The sections were then incubated overnight at 4°C with one of the following primary antisera: (A) polyclonal rabbit anti-CGRP (dilution: 1:500; Affiniti Research Products, England), (B) polyclonal rabbit anti-SP (dilution: 1:2000; Affiniti Research Products) and (C) polyclonal rabbit anti-VIP (dilution: 500; Affiniti Research Products). After rinsing in PBS + 0.5% BSA (3×10 min), sections were further incubated in fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit immunoglobulins (Dakopatts) (dilution: 1:50) for 1 h at room temperature. This was followed by rinsing in PBS + 0.5% BSA and mounting in Vectashield antifade mountant.

PACAP immunohistochemistry

Sections of 10 μm thickness were processed for immunohistochemistry using monoclonal mouse anti-PACAP antibody [15]. The monoclonal PACAP antibody displays equal affinity for PACAP-38 and PACAP-27. Sections were incubated overnight with the primary antibody (anti-PACAP antibody, supernatant diluted 1:10) at 40°C. This was followed by washing in PBS + 0.25% BSA + 0.1% Triton X-100 and then by incubating in biotinylated rabbit anti-mouse antiserum (E464, Dako, Denmark; diluted 1:1600) for 1 h at room temperature. The sections were then washed in PBS-BT and incubated in avidin-biotin complex (ABC)-streptavidin horseradish peroxidase complex (Dako, Denmark) for 30 min at room temperature. After washing in PBS-BT, sections were incubated with biotinylated tyramide (Tyramide System Amplification, TSA-Indirect, Life Science NEN, Boston, Mass.; diluted 1:100 in amplification buffer) for 30 min. This was followed by washing in PBS-BT and then incubating in a streptavidin-FITC-conjugated complex (Vector Laboratories, USA; diluted 1:100) for 30 min, followed by washing in PBS-BT and mounted in Vectashield (Vector Laboratories, Calif., USA). Control slides (replacing the primary antibody with PBS or incubating with antibodies preabsorbed with the respective antigens) showed no immunostaining. Finally, the slides were examined with a "Leitz DMRD" microscope equiped with epifluorescence illumination and the appropriate filters (fluorescein channel: excitation wavelength 488 nm, emission wavelength 535 nm). Photomicrographs were taken with ×10, ×20, ×40 Fluotar lenses using an automatic camera system on ILFORD MP5 (black and white) films.

Quantitation

The density of the PACAP, CGRP, SP and VIP-immunofluorescent neural elements was assessed semiquantitatively by assigning values on the basis of a (–) to (+ + + +) scale, where (–) represents an undetectable level of innervation, (+) a very sparse innervation, (+ +) sparse to moderate innervation, (+ + +) moderate innervation and (+ + + +) a dense innervation.

PACAP radioimmunoassay

The urinary bladders from young and aged rats were dissected out, freed from the surrounding tissues, weighed and stored at –80°C for PACAP radioimmunoassay. Before peptide analysis, the frozen tissue specimens were extracted in boiling water/acetic acid. The extracted samples were then reconstituted and analysed by radioimmunoassay specific for PACAP-38 [8, 9]. The PACAP-38 antiserum (code no.: 733C) was used in a final titre of 1:180,000. Iodinated PACAP 28–38, labelled with ^{125}I by iodogen to a specific radioactivity of 31 Bq/fmol, was used as tracer. A total of 500 μl of reconstituted sample or standard in assay buffer containing 0.04 M phosphate buffer pH 7.4, 58 μM human serum albumin (HAS), 0.1 M NaCl, Trasylol (400,000 KIE/I) and 2.5 M $CaCl_2$ was incubated with 200 μl antiserum in 0.04 M phosphate buffer pH 7.4 containing 58 μM HAS for 48 h at 4°C. After the addition of 100 μl radiolabelled peptide was diluted in 0.04 M PBS with 58 μM HAS for another 48 h. Bound and free peptides were separated by absorption to plasma-coated activated charcoal at 4°C. After centrifugation, the supernatant and charcoal precipitate were counted. The detection limit of this assay was 5 pM and the working range was 5–50 pM. Synthetic PACAP-38 (Peninsula Laboratories, St Helens, U.K) were used as standards. The radioimmunoassay was specific for the respective peptide and did not show any cross-reactivity with structurally related peptides. The within- and between-assay coefficients of variation were below 10%. All tissue extracts were assayed in duplicate in at least two different dilutions.

Results

In all sections examined, there was a gradual transition in the pattern of innervation from the dome to the base of the bladder. The base of the urinary bladder in all rats had the greatest density of innervation compared to the body, which had more innervation than the dome. Serial sections from the bladder base showed no difference in immunostaining between the trigone and other parts of the base. It is noteworthy that the peri- and para-vascular nerves in all regions showed a variable degree of immunoreactivity to all antibodies. Nerve fibres were distinguishable from background autofluorescence by the bead-like appearance of their varicosities and their bright fluorescence. The densities of immunoreaction of different parts of the urinary bladder and ureter, in both age groups, were estimated semi-quantitatively (Tables 1, 2).

Urinary bladder

PACAP

Numerous subepithelial varicosities (running just under the epithelium) occurred mainly in the base of urinary bladder (Fig. 1). The majority of axons were not straight and their size was variable. The lamina propria showed similar axons with varicosities, but fewer in number. In the muscle layers, most of the smooth muscle bundles were innervated by axons. There were many varicosities which ran parallel to and inside the muscle bundles. These varicosities were also observed along most of the blood vessels (arteries and large veins) where they ran in both longitudinal and circular directions (Fig. 1b, c). Body and dome regions had a similar, but less dense, distribution pattern to the fibres in the base of the bladder. Intraepithelial axons were not observed in either of these regions.

CGRP

The subepithelium showed a dense plexus of nerve fibres which had different sizes and orientations (Fig. 2). The muscle layers (longitudinal and circular) of the base showed dense (+ + + +) immunoreactive axons compared to the less dense (+ + +) PACAP-IR fibres in the same area. Otherwise, all other regions of the bladder had similar patterns of innervation to PACAP.

SP

Although SP varicosities had the same overall distribution as CGRP fibres, the fibres were smaller and less dense than in CGRP axons (Fig. 2b, d).

Table 1. Semi-quantitative estimation of PACAP, CGRP, SP and VIP-IR nerve densities in different regions of young (3 months) and old (24 months) rat urinary bladder. –, Absent; +, sparse; + +, sparse to moderate; + + +, moderate; + + + +, dense

	Age	3 Months				24 Months			
	Region	Intraepithelium	Subepithelium	Muscle layer	B.V.	Intraepithelium	Subepithelium	Muscle layer	Blood Vessels
PACAP	Dome	–	+	+	+	–	+	+	+
	Body	–	+ +	+ +	+	–	+ +	+ +	+
	Base	+	+ + + +	+ + +	+ +	+	+ +	+ +	+ +
CGRP	Dome	–	+	+	+	–	+	+	+
	Body	–	+ +	+ +	+	–	+ +	+ +	+
	Base	+	+ + + +	+ + + +	+ +	+	+ + + +	+ + +	+ +
SP	Dome	–	+	+	+	–	–	+	+
	Body	–	+ +	+ +	+	–	+	+ +	+
	Base	+	+ + +	+ + +	+	–	+ + +	+ +	+
VIP	Dome	–	–	–	+	–	–	–	+
	Body	–	–	–	+	–	–	–	+
	Base	–	+	+	+	–	+	+	+

Table 2. Relative density of PACAP, CGRP, SP and VIP-IR nerves in young (3 months) and old (24 months) rat distal ureter. –, Absent; +, sparse; + +, sparse to moderate; + + +, moderate; + + + +, dense

Age/ Site	3 Months				24 Months			
	PACAP	CGRP	SP	VIP	PACAP	CGRP	SP	VIP
Intraepithelium	–	–	–	–	–	–	–	–
Subepithelium	+ + +	+ + + +	+ + +	+	+ +	+ + + +	+ +	+
Muscles								
Inner	+ + +	+ + +	+ +	+	+ +	+ + +	+ +	+
Outer	+ + +	+ + + +	+ + +	+	+ +	+ + + +	+ +	+
Blood Vessels	+ +	+ +	+ +	+	+ +	+ +	+ +	+

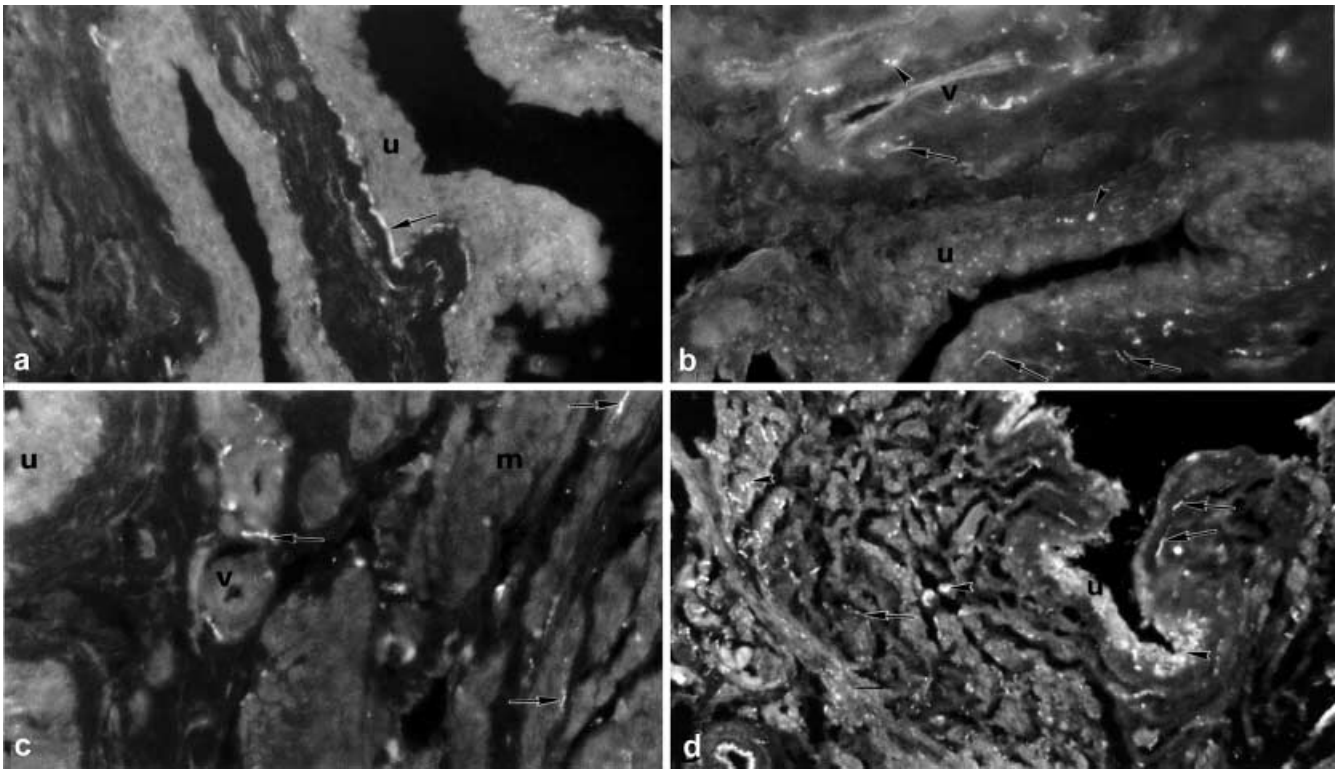

Fig. 1. Pituitary adenylate cyclase activating polypeptide-immunoreactivity (PACAP-IR) in the base of the urinary bladders of young (**a**, **c**) and aged (**b**, **d**) rats. Note the PACAP nerve fibres (*arrows*) in the subepithelial region (**a**, **b**, **d**), muscle tissues (**b**, **c**) and around blood vessels (**b**, **c**). Age-pigment (*arrowheads*) was observed in aged rat urinary bladders (**b**, **d**). *u*: urothelium; *m*: muscles; *v*: vessels. Magnification: **a**, ×180; **b**, ×180; **c**, ×279; **d**, ×128

VIP

The bladder base, in both age groups, showed few axons in the subepithelium and muscle tissues. With the exception of a few perivascular VIP axons, no VIP-IR nerve structures could be detected in the body or in the dome of young and aged rat bladders.

Distal ureter

Epithelium

No immunoreactivity was observed in the intraepithelial regions. In contrast, the subepithelium was densely innervated by CGRP axons (Fig. 3c) and most of the varicosities ran longitudinally. SP and PACAP showed moderately dense levels of immunoreactive fibres (Figs. 3a, e), while VIP had very few varicosities (Fig. 3g).

Muscle layers

The muscular layers showed different degrees of immunostaining. While, the outer longitudinal layer had relatively more CGRP and SP varicosities than the inner group of muscle, PACAP showed relatively moderate immunoreactive axons in both layers.

Vascular tissues

All sections showed few to moderate varicosities. These ran mainly in the muscle tissues (Fig. 3a, c, d). They were single axons with small varicosities, and some perivascular immunoreactive nerve fibres were also been observed.

Effect of age

Urinary bladder

The average weight ($\pm$SD) of young rat urinary bladder was 108 ± 15 mg, while that of aged rat bladder was 222 ± 25.5 mg. With the exception of PACAP innervation of the aged bladder base, the three bladder regions (base, body and dome) did not show a large difference with age in their densities of peptidergic innervation. However, the PACAP innervation density showed a marked decrease in the bladder base subepithelium and, to a lesser extent, in the muscle layers of aged animals (Fig. 1). In contrast to the immunohistochemical findings, PACAP radioimmunoassay showed that the mean concentration of PACAP-38 (pmol/g wet weight) in the urinary bladder of young rats (2.68 ± 0.5) was not significantly different ($P > 0.05$) from the mean concentration (2.8 ± 0.3) of PACAP-38 in aged rat urinary bladders.

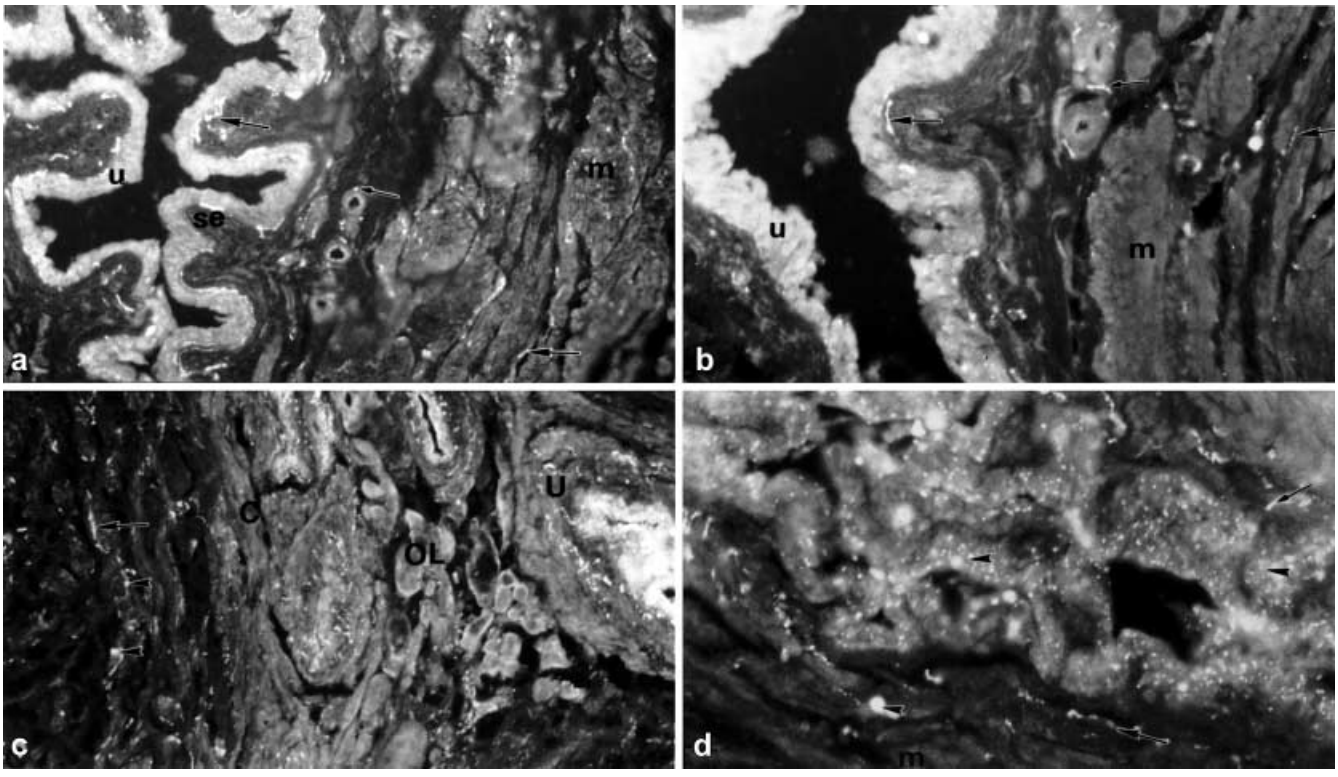

Fig. 2. Young (3 month-old) (**a**, **b**) and aged (24 month-old) (**c**, **d**) rat urinary bladder bases immunostained with CGRP (**a**, **c**) and SP (**b**, **d**). Immunoreactive fibres (*arrows*) were observed in the subepithelial region (**a**, **b**, **d**), muscle tissues (**a–c**) and surrounding blood vessels (**a**, **b**). Lipofuscin (age-pigment) was seen mainly inside the epithelium (**d**) and in the muscle layers (**c**). *u*: urothelium; *se*: subepithelium; *m*: muscles; *C*: circular; *OL*: outer longitudinal; *U*: ureter. Magnification: **a**, ×120, **b**, ×272, **c**, ×100, **d**, ×203

Distal ureter

The subepithelium and the muscular tissues of aged ureters contained less axons immunorective to PACAP and SP compared to the young rat ureters (Fig. 3a, b, e, f), but no apparent changes in CGRP-IR and VIP-IR nerve densities were observed in the aged animals.

Discussion

The present study describes the distribution of PACAP and three other sensory neuropeptides (CGRP, SP, VIP) in the young and aged rat urinary bladder and distal ureter by means of immunohistochemistry. A number of peptides have been localised in primary afferent neurones projecting from the pelvic viscera to the spinal cord, among them CGRP, SP and enkephalin [5]. In addition, the distribution pattern of nerve axons in the young bladder and ureter, demonstrated in this study, is similar to that previously reported in young rats (SP and CGRP [23, 27, 31, 32]); (PACAP, CGRP and VIP antibodies [8, 9]). However, the present study shows immunoreactive nerve axons in different regions of the urinary bladder and ureter and in young adult and aged rats, which have not previously been reported. For example, peptidergic immunoreactivity (IR) is frequently demonstrated beneath the epithelium and in muscle tissues of the bladder base but there have been no reports describing their distributions in the dome and the body regions. Moreover, most of the previously published results used only young rat tissues (approximately 2 month-old) rather than adult and aged animals as we used here. In this study, using a tyramide signal amplification method to intensify the antigen antibody binding sites, we extend the study on PACAP in the urinary bladder [8, 9], which has not been localised clearly in previous studies [17, 23]. These previous attempts were not successful in demonstrating PACAP-IR in the ureter, while in the urinary bladder some single varicose nerve terminals were described in relation to smooth muscle bundles and blood vessels.

The possible sites of action of peptidergic immunoreactive nerve axons in micturition reflex include (1) the urothelium and smooth muscle bundles in the urinary bladder, (2) the pelvic ganglia, (3) the dorsal root ganglia and (4) the spinal cord. PACAP is expressed not only in the central nervous system, but also in other regions of the peripheral nervous system and peripheral organs [2]. PACAP has been found in rat dorsal root ganglia [6, 34] where it showed a marked up-regulation after nerve injury. Double labelling revealed that PACAP-containing sensory neurons constitute a subpopulation of those containing CGRP and SP [23]. Although the exact physiological role of PACAP in nerves is still uncertain, the presence of PACAP in sensory neurons as well as in

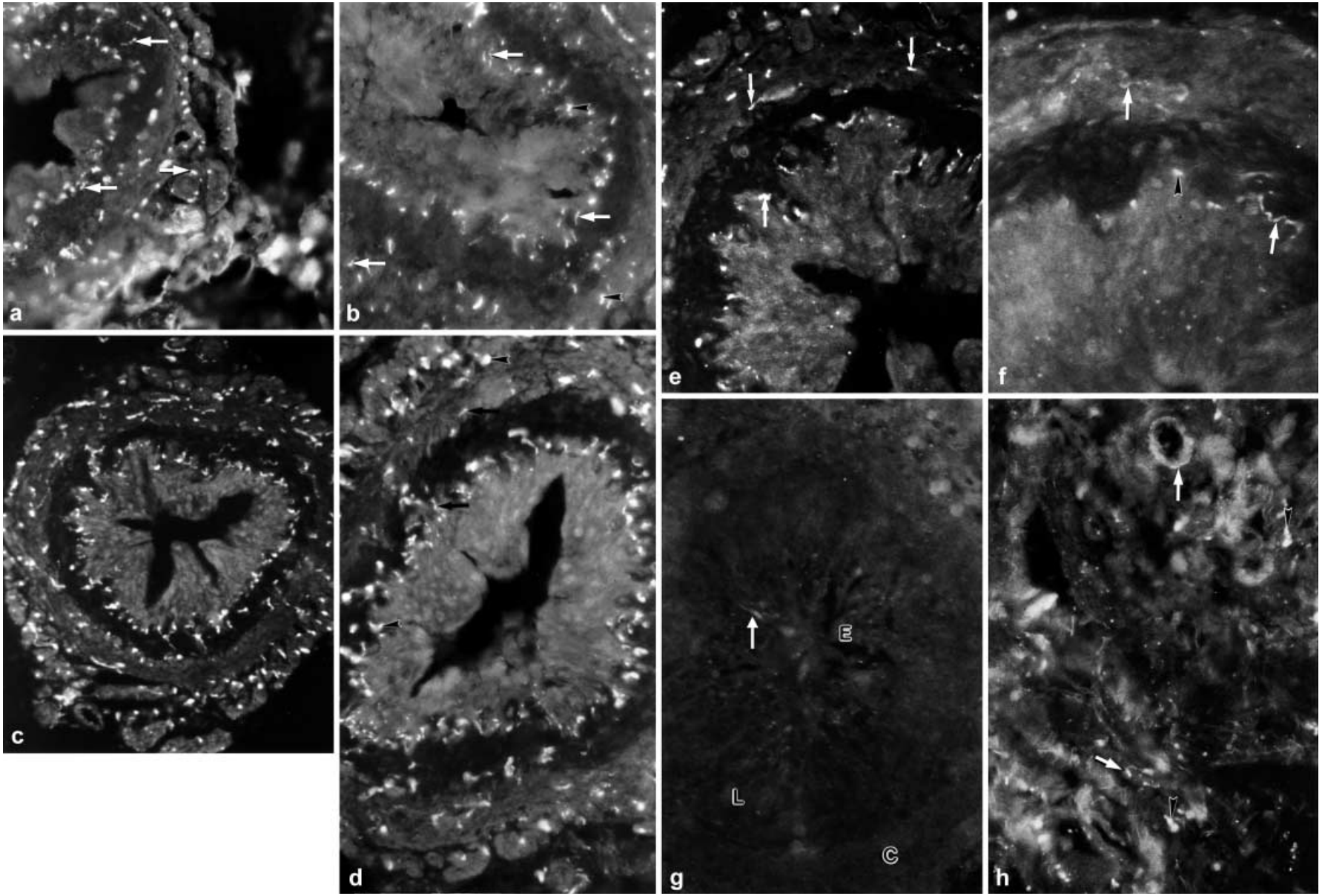

Fig. 3. Young (**a**, **c**, **e**, **g**) and aged (**b**, **d**, **f**, **h**) rat ureters immunostained with PACAP (**a**, **b**), CGRP (**c**, **d**), SP (**e**, **f**) and VIP (**g**, **h**). Note the immunoreactive axons (*arrows*) in the subepithelium (**a–h**) and muscular layers (**a–f**) and (**h**). Age-pigment (*arrowheads*) was also noticed in all sections of aged rat ureters. *E*: epithelium; *L*: inner longitudinal; *C*: outer circular muscle layers. Magnification: **a** ×108, **b** ×160, **c** ×112, **d** ×200, **e** ×240, **f** ×340, **g** ×180, **h** ×276

sympathetic and parasympathetic neurons strongly suggests that PACAP functions as a neurotransmitter or neuromodulator [2]. In rat models of nociceptive neurotransmission, intrathecal administration of PACAP depressed a C fibre-evoked flexion reflex [33]. Thus, PACAP may play a physiological role as a transmitter or modulator in sensory C fibres. In addition, its coexistence with CGRP and SP in sensory afferent fibres and its depletion following capsaicin treatment [8, 9, 23, 24, 33] suggest a sensory function of this peptide in the urinary tract. Moreover, Ishizuka et al. [17] showed that PACAP given intrathecally or intra-arterially (but not intravenously) to conscious rats facilitated spontaneous bladder contractions and produced a decrease in the bladder capacity. The lack of effects of PACAP on isolated preparations of rat bladder detrusor muscle suggested the possibility that PACAP stimulated the micturition pathways both at the spinal cord and ganglionic levels. In addition, Lissbrant et al. [19] have shown, using laser doppler flowmetry on anaesthetised adult rats, that PACAP has strong vasodilatory effects in the testis and moderate effects in the epididymis. It is therefore likely that the PACAP innervation of the vesical and ureteric vasculature may also be vasodilatory.

Effects of age

Few studies have investigated the morphological aspects of these neuropeptides in the rat lower urinary tract and the responses that they produce at the peripheral tissues in ageing. In capsaicin-sensitive primary afferents, which contain several peptides derived from large polypeptide molecules, a decrease in peptide contents has been reported [13]. In addition, a decrease in peptides with age has also been reported in other organs such as the gastrointestinal tract or the airways. SP in the jejunum only and VIP in all compartments of the small intestine are lowered in senescent rats [11]. However, the present study shows that PACAP immunoreactivity decreased considerably with age in the base and to a lesser degree in the muscle layers of the male rat urinary bladder. Moreover, the distal part of the ureter reveals relatively less PACAP- and SP-immunoreactive fibres in the subepithelium and muscular tissues of aged rat tissues. Thus, the subepithelial and muscular declines of PACAP-IR with age, which were not revealed by radioimmunoassay of whole bladder, raise the possibility that PACAP may be involved in the perturbation of the sensation or the transmission of sensory information from the aged bladder as has been reported in another electrophysiological study [16].

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