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Localization of regulatory peptides in the male urogenital apparatus of domestic equidae: a comparative immunohistochemical study in *Equus caballus* and *Equus asinus*

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Summary. An immunohistochemical study was carried out on specimens of testis, excurrent duct including the male accessory glands and urethra in its various tracts in the horse and the donkey, in order to localize nine regulatory peptides. Immunoreactivities were tested by means of Labelled Strept Avidin-Biotin (LSAB) method. The study has shown that Equine male genitalia are supplied by many peptide immunoreactive nerves containing NPY-, VIP-, leu- and met-Enkephalin-, Substance P-, CGRP- and Bombesin/GRP-like peptides, each of them having a characteristic distribution pattern. These neurotransmitters were localized in nerve fibers running in the connective tissue or in contact with the smooth muscle cells, as well as in sub- and intra-epithelial nerve terminals, and in perivascular nerve fibers. In addition, leu- and met-Enkephalin-like-immunoreactive endocrine cells were shown in the bulbourethral gland of the horse. In both species it was evident that an extensive utilization of NPY and VIP exists. A contingent of NPY- and VIP-ir nerve fibers may have an intrinsic origin. The other regulatory peptides tested show a characteristic distribution pattern, limited to some organs and peculiar to each of the two species of Equidae. Differences observed comparing *E. caballus* and *E. asinus* might be related to the species-specific balance of the accessory neurotransmitters which in turn accompany adrenergic innervation. In both species it is noteworthy the complete absence of any regulatory peptide in the testis, with the exception of the perivascular localization of NPY-ir nerve fibers.

Key words: Regulatory peptides, Peptidergic innervation, Immunohistochemistry, Male urogenital apparatus, Domestic equidae

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Introduction

Regulatory peptides may coexist with the classical neurotransmitters in the autonomic nervous system, sometimes being colocalized in the same nervous terminal (Burnstock, 1976; Lundberg and Hökfelt, 1983). They possibly act as neurotransmitters or neuromodulators (for review see Burnstock et al., 1979). These substances may be localized in epithelial cells also, where they exert a hormone or hormone-like local activity (endocrine or paracrine function).

The male and female genital tracts have been proven to contain such hormone-like substances both in components of the peripheral nervous systems and in epithelial cells, principally in laboratory mammals and man (Alm et al., 1978, 1980; Del Fiacco, 1982; Shu-Dong et al., 1982; Gu et al., 1983b; Adrian et al., 1984; Huang et al., 1984; Vaalasti et al., 1986; Amenta et al., 1992; Properzi et al., 1992; Tainio, 1995). Also in the genital tracts, regulatory peptides serve as neurotransmitters and/or neuromodulators (Fahrenkrug et al., 1989).

Studies dealing with presence and localization of regulatory substances are sporadic in domestic mammals (Rodríguez-Martinez, 1990; Vittoria et al., 1990, 1992; Tamaki et al., 1992; Lakomy et al., 1994, 1995). Thus, the aim of this work was to localize a large number of regulatory peptides throughout the male genital apparatus of *Equus caballus* and *Equus asinus*. The comparative study carried out on the male genitalia of these two species of equidae having a very close zoological relationship might show different patterns, as we have already demonstrated in previous ultrastructural works, examining different morphophysiological aspects of the same apparatus (Arrighi et al., 1991, 1994). Moreover, this immunohistochemical study will be the basis for future comparison of the peptidergic innervation pattern in other domestic mammals, such as

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bovine and pig.

Materials and methods

Samples of testis, epididymis, ductus deferens and its ampulla, vesicular gland, prostate, bulbourethral gland and several tracts of pelvic and penile urethra were collected at slaughtering from healthy stallions 1.5-4 years old. Five horses and three donkeys were used. Fragments of the specimens were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS) 0.1M at pH 7.4 at 4 ºC for 24 h. Pieces were then rinsed in PBS and cryoprotected overnight by infiltration with a 20% sucrose solution at 4 ºC. After freezing in isopenthane cooled by liquid nitrogen, cryosectioning was performed on a CRYO-CUT microtome (American Optical Corporation). 12-14 μm-thick sections were collected on polylysinated glass slides (Poly-d-lysine, Sigma, Italy, 0.1% in PBS pH 7.6) (Huang et al., 1983) and air-dried for a couple of hours. Sections were then treated by 0.05% bovine trypsin (Trypsin TPCK cod. 3740, Worthington Biochemical Corporation, USA) in Tris (hydroxymethyl-aminomethane)-HCl buffer 0.05M at pH 8.1 for 15 min at 37 ºC (pH at this temperature 7.8), in order to unmask the antigens and increase the immunostaining of the peptides tested. A post-fixation was then administered by a SUSA liquid modified according to Stevens and Shaw (1982) for 20 min at room temperature. After washing, the immunohistochemical procedure was performed. The primary antisera tested are indicated in Table 1.

Each primary serum was used at various dilutions until the optimal one (see below) was found. Incubations were carried out in humidity chamber at 4 ºC for 18-22 h. Thereafter, a Labelled StreptAvidin Biotin (LSAB) system was used, utilizing biotinylated swine anti-rabbit immunoglobulins (DAKO, code E 353) as secondary serum, followed by StreptABComplex/HRP (DAKO, code K377). Tris-Buffered-Saline, pH 7.6 (TBS: 0.05M Tris/HCl, 0.15M NaCl), was used for dilutions and rinses throughout the whole procedure. Either 3,3'-diaminobenzidine tetrahydrochloride (DAB) or 3-amino-9-ethylcarbazole (AEC) were employed as chromogens. Sections were counterstained with Mayer's hematoxylin and mounted using Entellan or an aqueous mounting media (DAKO Glycergel, code C 563), if AEC was used as chromogen.

Samples of small or large intestine were also collected and sections were used as positive control.

The negative controls included (1) preabsorption of the 1st layer antisera with related and unrelated antigens (Peninsula, U.K.), (2) the use of non-immune rabbit serum in place of a primary antiserum at the same dilution and (3) omission of the 1st layer.

Slides were observed and photographed under an Ortoplan photomicroscope (Leitz, Germany). Evaluations of staining intensities were based on subjective estimates of both authors, after examination of many sections per slide of all the animals tested.

Table 1. Primary antisera tested, all raised in rabbits.

PRIMARY ANTISERA TESTED	SOURCE	CODE
Anti-synthetic neuropeptide Y (NPY)	Amersham, UK	RPN 1702
Anti-porcine vasoactive intestinal peptide (VIP)	Amersham, UK	RPN 1582
Anti-synthetic substance-P	Amersham, UK	RPN 1572
Anti-human calcitonin gene-related peptide (CGRP)	Peninsula, UK	RAS 6009 N
Anti-rat calcitonin gene-related peptide (CGRP)	Peninsula, UK	RIN 6006
Anti-synthetic methionine-enkephalin	Amersham, UK	RPN 1562
Anti-leucine-enkephalin	Peninsula, UK	RAS 8601 N
Anti-synthetic amphibian bombesin/ gastrin releasing peptide (GRP)*	Amersham, UK	RPN 1692
Anti-synthetic somatostatin	Amersham, UK	RPN 1612

*: the anti-bombesin serum used does not cross-react with Subtance P.

Results

Slight differences were found in the immunoreactivities to the different regulatory peptides in the two equine species. Tables 2 and 3 summarize the results respectively in *Equus caballus* and *Equus asinus*.

Since cross-reactivity with unknown peptides containing the antigenic sequences cannot be excluded, the immunoreactive material is more properly referred to as «peptide-like» immunoreactivity (ir).

NPY-like immunoreactivity

NPY-like immunoreactivity was present all over the urogenital apparatus both in *Equus caballus* and *Equus asinus*. It was constantly localized in thin, varicose perivascular nerve fibers, which contacted the muscular layer of small arteries and arterioles in all the examined organs (Fig. 1a,b). This perivascular localization was the only one present in the *testis* and in the *urethra* of the horse, as well as in the *testis*, *urethra* and *epididymis* of the donkey.

In the horse *epididymis* long varicose NPY-ir nerve fibers were seen running in the peritubular connective tissue (Fig. 2). They were also seen contacting the muscular layer of the duct. Sometimes single NPY-ir fibers were identifiable in small nerve bundles. In the *ductus deferens* NPY-ir nerve fibers distributed to the muscular sheet and lamina propria of the mucosa. In the *ampulla ductus deferentis* too the distribution of NPY-ir nerve fibers was in the muscular layer, as well as in the axis of the glands. In the epididymis, ductus deferens and ampulla ductus deferentis of the donkey the distribution and localization of NPY-immunoreactivity were quite similar, except that it was less abundant than in the horse. In the *prostate* gland of the horse and donkey intensely immunoreactive nerve fibers were frequent. Fiber bundles (Fig. 3a) surrounded the

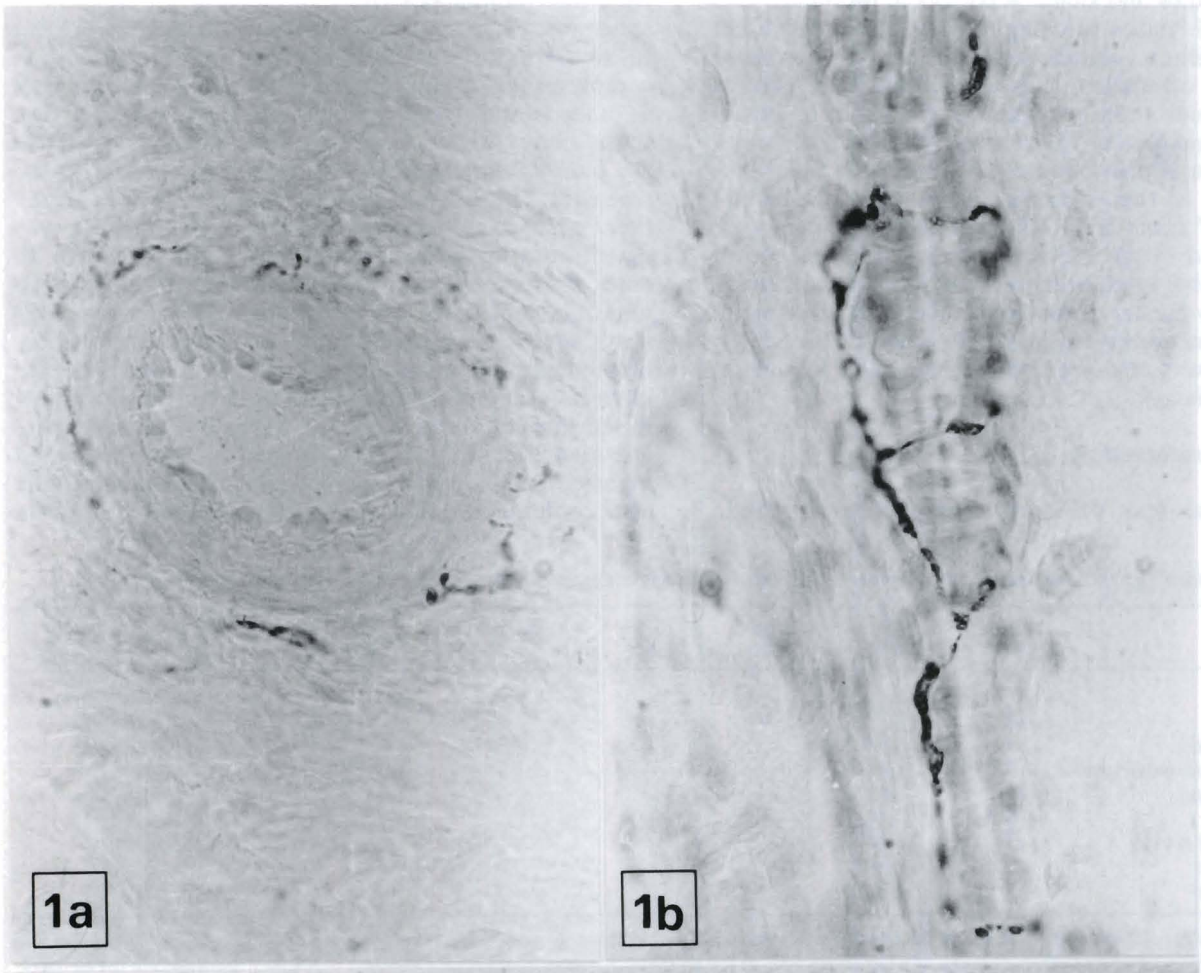


Fig. 1. NPY-like-immuno-reactive perivascular nerve fibers. **a.** Transverse section of a small artery in the ductus deferens of *Equus caballus*. x 510. **b.** An NPY-ir nerve fiber surrounds an arteriole in the epididymis of *Equus caballus*. Note its varicose aspect. x 800

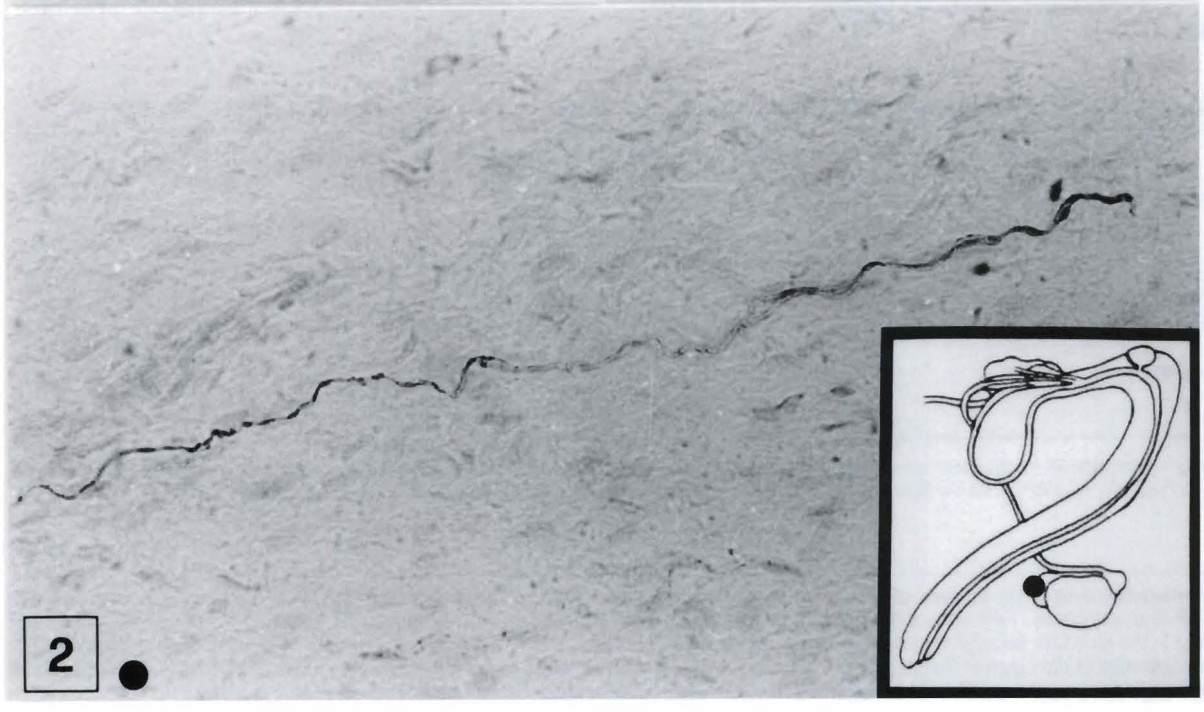


Fig. 2. A long NPY-like-immuno-reactive nerve fiber can be seen in the peritubular connective tissue of horse epididymis. x 400. Schematic drawing at the corner of this and the following figures indicates the site/s of sampling. All of them have been re-drawn from Nickel et al. (1975) and are assumed to be valid both for *Equus caballus* and *Equus asinus*.

glandular lobules and single nerve fibers insinuated into the interstitial spaces reaching the glandular epithelium (Fig. 3b,c). Single, subtle nerve fibers often contacted the muscular component (Fig. 3d,e). Intramural ganglia were also found in the prostate, in which cell bodies as well as intraganglionic fibers were NPY-ir (Fig. 3e). A similar pattern of distribution of NPY-ir nerve fibers was also present in the *vesicular gland* (Fig. 4a,b) and *bulbourethral gland* (Fig. 4c). NPY-ir nerve fibers were also localized in the *pelvic urethra*. The presence of immunoreactive structures in these latter localizations was generally greater in the horse than in the donkey. In the donkey, nevertheless, a subtle network of NPY-ir nerve fibers was also seen among the small lobules of the urethral glands.

VIP-like-immunoreactivity

There was less VIP-like immunoreactivity than NPY-like-immunoreactivity throughout the urogenital apparatus. Perivascular VIP-ir nerve fibers were present in small arteries and arterioles in the glandular complexes and in the pelvic urethra in the horse, as well as in the various tracts of urethra in the donkey (Fig. 5a). In the horse, VIP-ir nerve fibers were present mostly in the *bulbourethral gland*, and to a lesser extent in the *prostate*, the *vesicular gland* and *ampulla ductus deferentis*. In all these localizations this peptide was distributed in the interstitial spaces in contact with smooth muscle cells, sometimes approaching the glandular epithelia. In the *ductus deferens* the VIP-immunoreactivity was limited to sparse nerve fibers distributed in the muscular coat. In the donkey anti-VIP immunoreactivity was localized in nerve fibers running in the interstitial spaces of the prostate (Fig. 5b). Rare VIP-ir nerve fibers were also seen in other examined organs. In the horse and in the donkey intramural ganglia also contained a slight VIP-immunoreactivity both in

Table 2. Immunolocalization of regulatory peptides in the male urogenital organs of *Equus caballus*. Optimal dilutions of antisera are indicated.

	NPY 1:1200	VIP 1:1000	SUB-P 1:600	CGRP 1:5000	(met)-ENK 1:800	(leu)-ENK 1:2000	BOMB/GRP 1:600	SOM 1:1000
Testis	+[c]	-	-	-	-	-	-	-
Epididymis	++[b,c]	-	+[b]	++[b]	-	-	-	-
Ductus deferens	++[b,c]	±[b,c]	-	-	+[b]	+[b]	+[b]	-
Ampulla ductus deferentis	++[b,c]	±[b,c]	-	-	+[b]	+[b]	-	-
Vesicular gland	++[b,c]	±[b]	-	±[b]	-	-	-	-
Prostate	++[b,c,d]	±[b,c]	±[b]	±[b]	-	-	-	-
Bulbourethral gland	++[b,c]	+[b,c]	-	-	+[b,c,a]	+[b,a]	±[c]	-
Pelvic urethra	+[b,c]	±[b,c,d]	-	-	-	-	-	-
Penile urethra	+[c]	±[b]	-	-	-	-	-	-

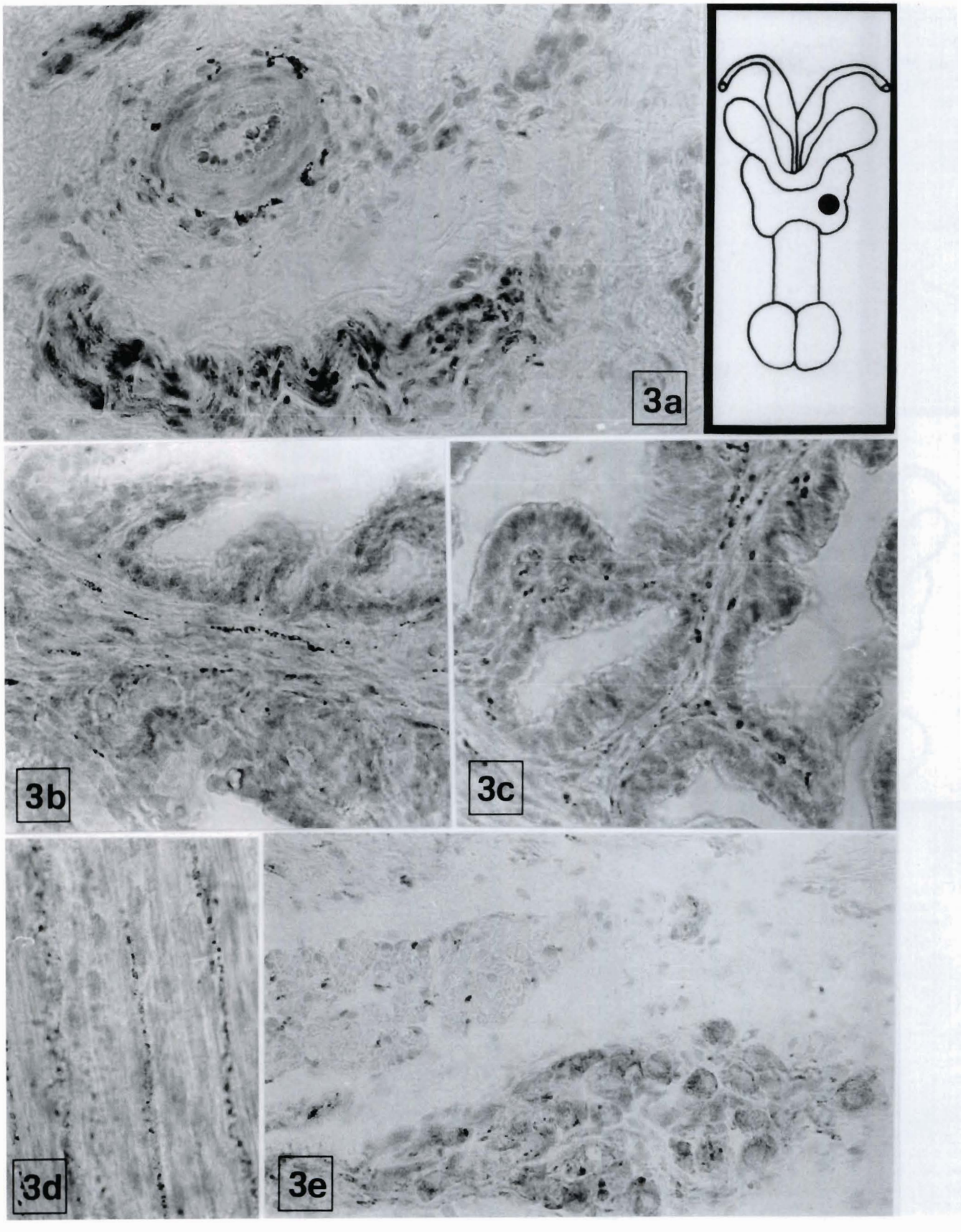
Intensity of staining: -, unreactive; ±, reaction not always detected in all the samples, or unevenly present in a section; +, detectable reaction; ++, intense reaction; +++, strong reaction; [a]: endocrine/paracrine cells in the epithelia; [b]: nerve fibers and/or bundles of fibers; [c]: perivascular nerve fibers; [d]: intramural ganglia.

Table 3. Immunolocalization of regulatory peptides in the male urogenital organs of *Equus asinus*. Optimal dilutions of antisera are indicated.

	NPY 1:1200	VIP 1:1000	SUB-P 1:600	CGRP 1:5000	(met)-ENK 1:800	(leu)-ENK 1:2000	BOMB/GRP 1:600	SOM 1:1000
Testis	±[c]	-	-	-	-	-	-	
Epididymis	+[c]	-	+[b]	++[b]	+[b]	-	-	
Ductus deferens	+[b,c]	±[b]	-	-	+[b]	+[b]	±[c]	
Ampulla ductus deferentis	+[b,c]	±[b]	-	±[b]	+[b]	+[b]	±[c]	
Vesicular gland	+[b,c]	±[b]	+[b]	-	+[b]	±[b]	±[b]	
Prostate	++[b,c,d]	+[b,d]	+[b,c]	+[b]	±[b]	±[b]	-	
Bulbourethral gland	+[b,c]	±[b]	-	-	-	-	-	
Pelvic urethra	±[b,c]	±[b,c]	-	+[e]	-	-	-	
Penile urethra	±[c]	±[c]	-	±[e]	-	-	-	

Intensity of staining: -, unreactive; ±, reaction not always detected in all the samples, or unevenly present in a section; +, detectable reaction; ++, intense reaction; +++, strong reaction; [b]: nerve fibers and/or bundles of fibers; [c]: perivascular nerve fibers; [d]: intramural ganglia; e: intraepithelial nerve fibers.

Fig. 3. NPY-like-immunoreactivity in the prostate gland. **a.** An immunoreactive nerve fiber bundle is running in the connective tissue surrounding the glandular lobules, in *Equus caballus*. Perivascular ir-nerve fibers can also be seen surrounding a small artery. x 410. **b** and **c.** Immunoreactive nerve fibers are distributed in the glandular septa in *Equus asinus* (b) and *Equus caballus* (c). x 470. **d,** Immunoreactive nerve fibers are seen in close contact with the smooth muscle cells of the organ in *Equus caballus*. x 510. **e.** Immunoreactive cell bodies and subtle nerve fibers can be seen in an intramural ganglion. Immunoreactive nerve fibers are also seen contacting the transversally-sectioned muscular coat. *Equus caballus*. x 320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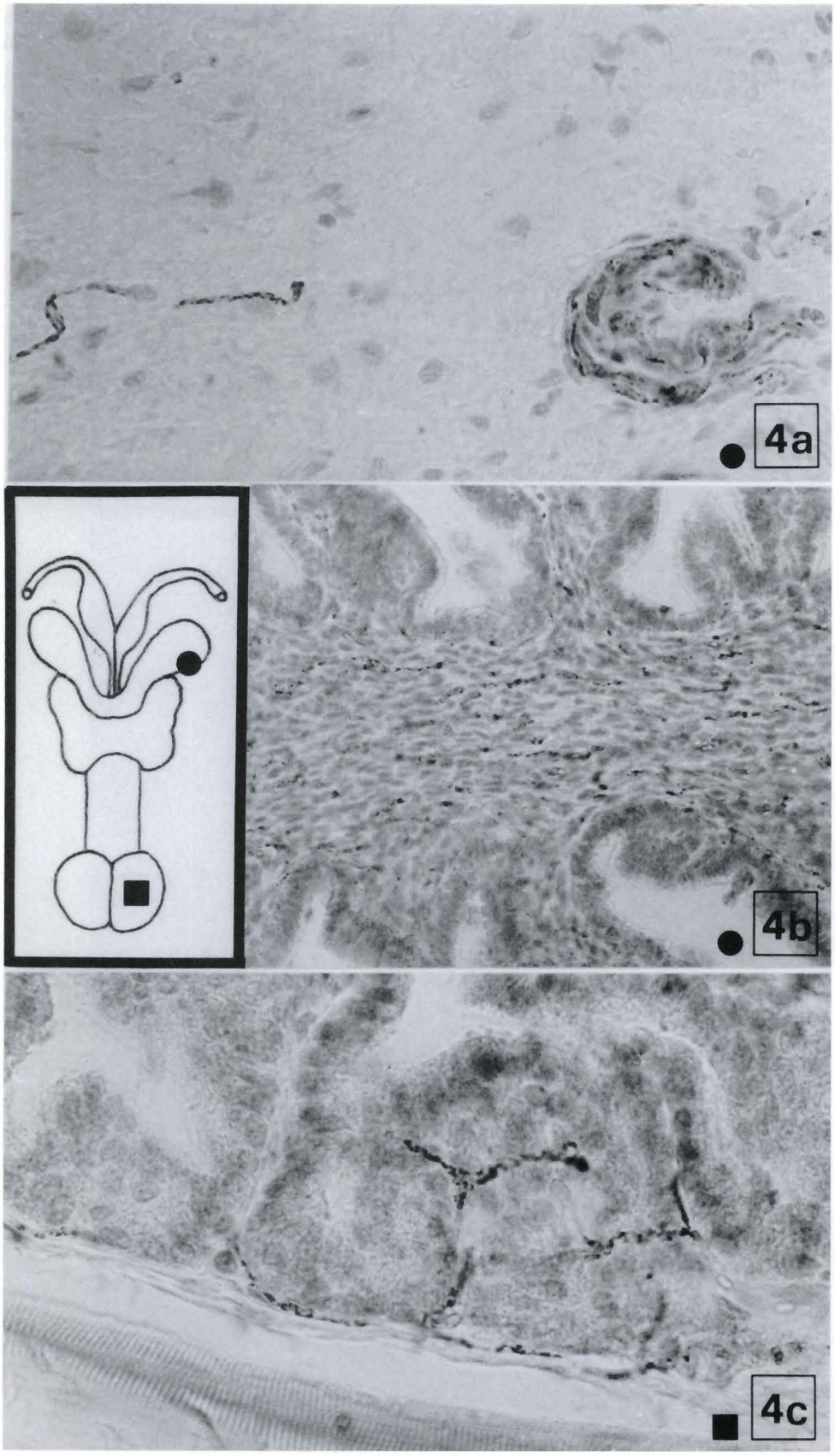


Fig. 4. NPY-like-immunoreactivity in horse glandular complexes. **a.** In the vesicular gland immunoreactive nerve fibers can be seen in the connective tissue, running both single and in bundles. x 420. **b.** Varicose nerve fibers are present in the mucosal lamina propria of the vesicular gland. x 360. **c.** Subepithelial nerve fibers can be seen in the bulbourethral gland. x 570

neurons and in subtle nerve fibers.

Substance P-like-immunoreactivity

In the horse, Substance P-like-immunoreactivity was found in the *epididymis* in long, varicose nerve fibers running in the interstitial spaces among the sections of the duct (Fig. 6a). Single Substance P-ir-fibers were present in nerve bundles in the connective tissue at the periphery of the organ (Fig. 6a). Anti-Substance P-immunoreactivity appeared to decrease toward the distal parts of the epididymis. This general pattern was the same in the epididymis of the donkey. Among the glandular complexes, the *prostate* only showed the presence of sparse nerve fibers immunoreactive to Substance P antiserum, running in the axis of the glandular folds (Fig. 6b), as well as in the interstitium contacting the smooth muscle cells (Fig. 6c).

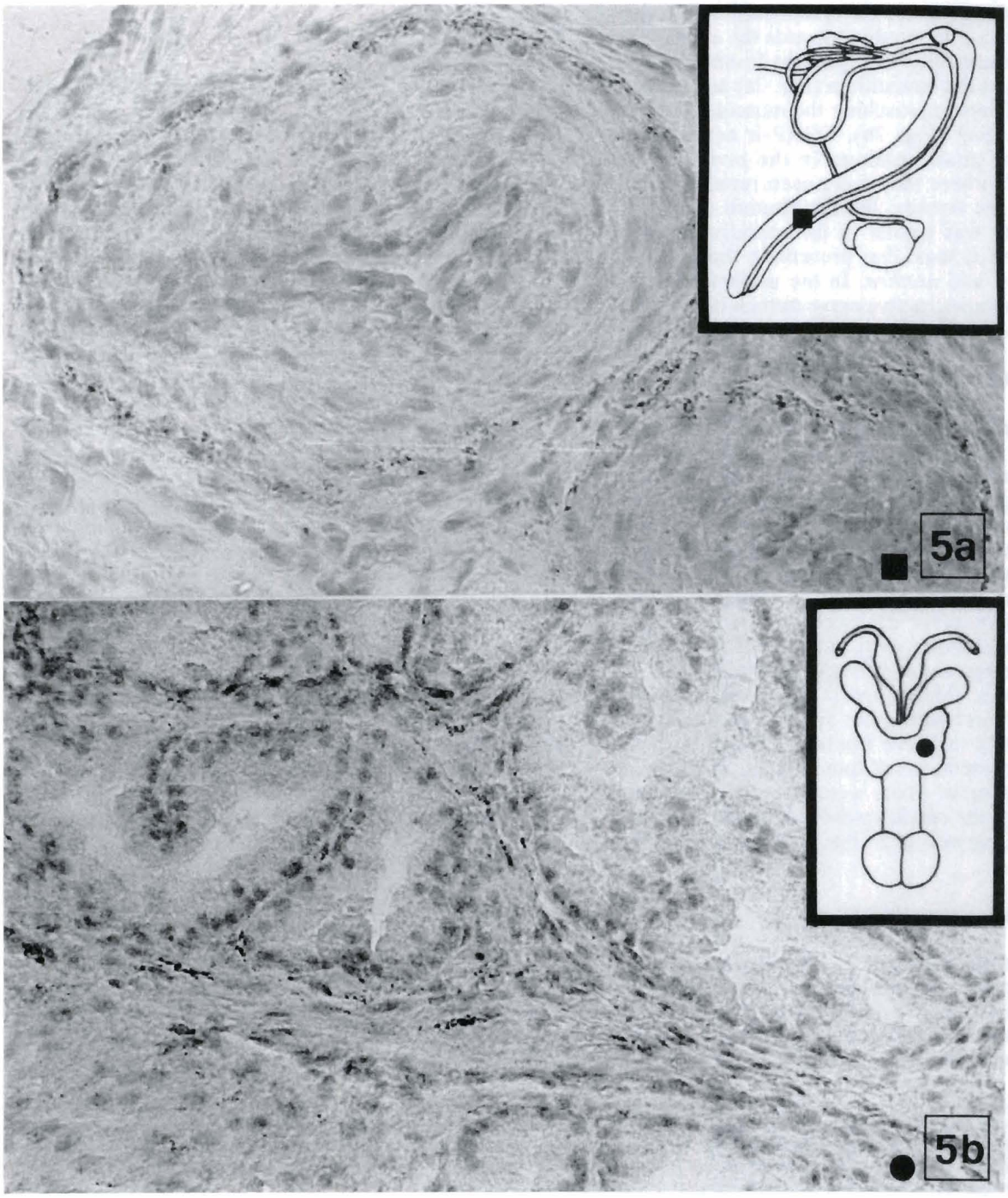


Fig. 5. VIP-like immunoreactivity in *Equus asinus*. **a.** Perivascular nerve fibers can be seen in small arteries of the penile urethra. x 510. **b.** Immuno-reactive nerve fibers run to the glandular epithelium in the prostate. x 640

Immunoreactive nerve fibers were also seen in contact with the muscle layer of small arteries (Fig. 6d), as well as in perivascular localizations. Limited to the donkey, Substance P-ir nerve fibers were also detected in the vesicular gland, with a pattern of distribution similar to that seen in the prostate.

CGRP-like-immunoreactivity

In the horse, CGRP-like-immunoreactivity was detectable in nerve fibers of the *epididymis*, where they appeared to decrease in number towards the cauda. The CGRP-immunoreactivity was present in small nerve bundles running in the interstitium (Fig. 7a) and in long, varicose nerve fibers approaching the muscular layer of the epididymal duct (Fig. 7b). CGRP-ir nerve fibers were present in small numbers in the *prostate* and *vesicular gland*, where they were seen running in the interstitium. In the donkey, the distribution of CGRP-immunoreactivity was similar in the *epididymis* and in the *prostate*, but it was also present in the *ampulla ductus deferentis* and *urethra*. In the urethra sub- and intraepithelial immunoreactive nerve terminals were also seen (Fig. 7c,d).

Enkephalin-like-immunoreactivity

[Met-]- and [Leu]-Enkephalin-like-immuno-reactivities had a similar pattern of distribution in the horse urogenital apparatus. The respective antisera marked extremely subtle and tortuous nerve fibers in the muscular coat of the *ductus deferens* and its ampulla (Fig. 8a), insinuating sometimes into the subepithelial connective tissue. Immunoreactive nerve fibers were also found in the *bulbourethral gland* (Fig. 8b), where scarce immunoreactive endocrine cells were also detected with both antisera (Fig. 8c), as well as thin Met-enkephalin-immunoreactive perivascular fibers.

In the donkeys, [Met-]- and [Leu]-Enkephalin-like-immunoreactivities were quite similar and localized almost exclusively in nerve fibers in contact with the smooth muscle components of some organs. Enkephalin-immuno-reactive nerve fibers were present in the outer muscular layer of the *cauda epididymis*, *ductus deferens* and its *ampulla*, as well as in the vesicular gland (Fig. 8d).

Bombesin/GRP-like immunoreactivity

The immunolocalization of Bombesin/GRP-like peptide was sporadic in both species. In the horse the immunoreactivity for this peptide was found only in nerve fibers of the muscular coat of the *ductus deferens* and in rare perivascular nerve fibers in arterioles in the *bulbourethral gland*. In the donkey, Bombesin/GRP-immunoreactivity was sometimes found in perivascular nerve fibers of the *ductus deferens* and its *ampulla*, as well as in sparse interstitial nerve fibers in the vesicular gland.

An immunoreactivity to Somatostatin peptide was never detected in any nervous or epithelial component, in neither equine species, despite its presence in positive controls.

Discussion

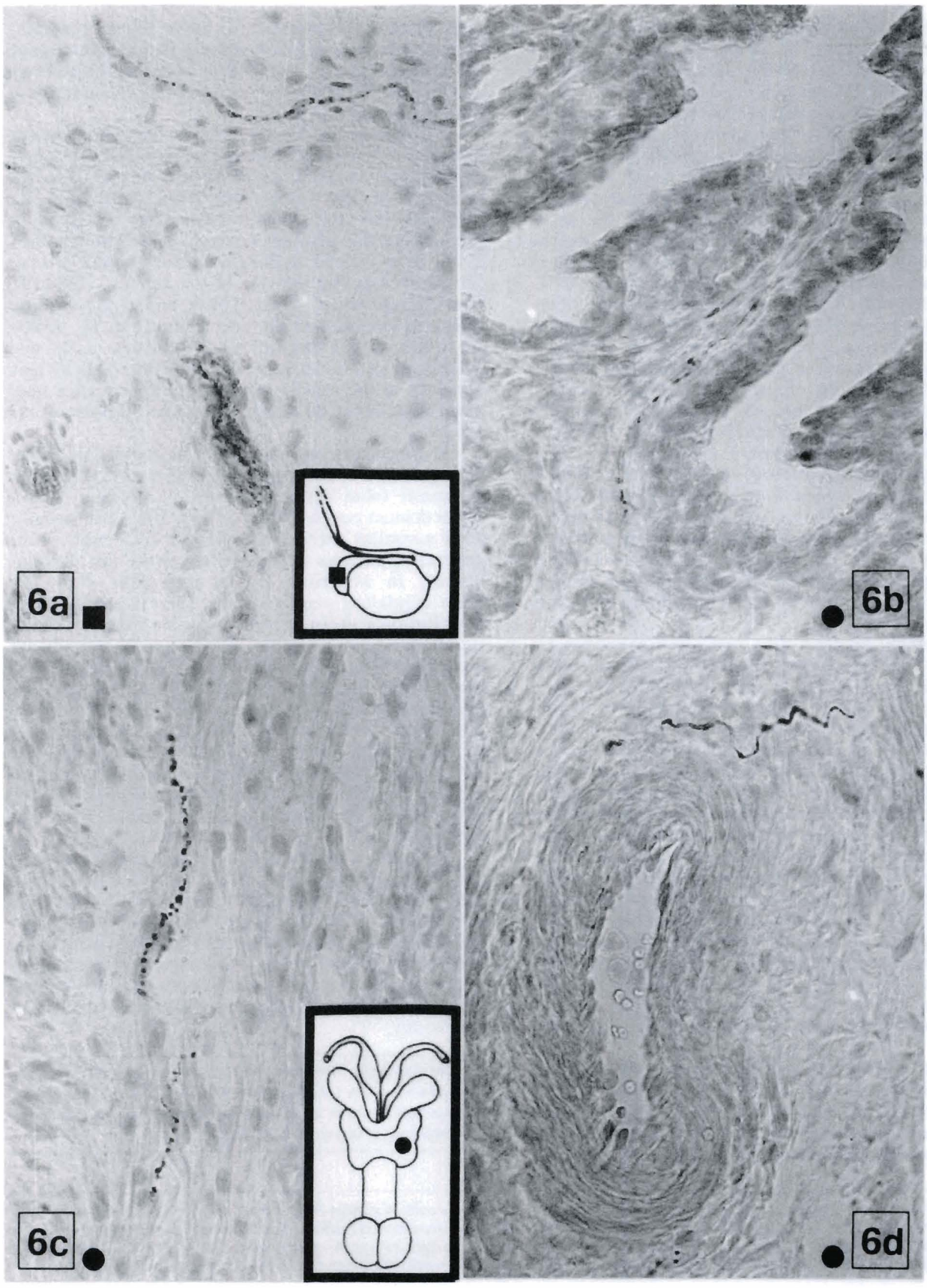
In this study it has been shown that Equine male genitalia are supplied by a rich network of peptidergic nerve fibers. The immunoreactive nerve fibers often show a characteristic varicose appearance, which is consistent with a possible release of regulatory peptides all along the axons («boutons en passant») in addition to their terminal endings. A relatively small number of nerve fibers show a smooth appearance.

Among the different urogenital organs, the glandular complexes, above all the prostate, were particularly supplied by peptide-containing nerves. In both species it was evident that an extensive utilization of neuropeptide Y exists. This was particularly apparent in *Equus caballus*, less evident in *Equus asinus*.

NPY-ir nerves have been reported to exist in male genitalia of man (Adrian et al., 1984; Tainio, 1995), guinea-pig and rat (Lamano-Carvalho et al., 1986), being the major peptide-containing neuronal component. As it is known that innervation of the male genital apparatus is almost completely adrenergic (Sundler et al., 1986; Kujat et al., 1993), it is conceivable that NPY in these organs, as in many other body regions might be colocalized in monoamine-storing nerve terminals. NPY is a vasoconstrictor peptide, so it might be implicated in the regulation of smooth muscle activity and local blood flow. In the glandular complexes this peptide might be implicated in the control of epithelial functions also. Finally, the presence of NPY-ir neurons and nerve fibers in intramural ganglia is consistent with the hypothesis of a possible intrinsic origin of NPY-ir neural structures.

Apart from its well-known intervention in the penile erection (Yeh et al., 1994), VIP is believed to be involved in muscle relaxation, blood flow and secretion, possibly antagonising the effects of NPY in the reproductive apparatuses. In our study, VIP-ir nerve fibers were present mostly in the glandular complexes, where this peptide might show a direct effect on epithelial secretory cells together with an augmented local blood flow, which might result in enhanced secretion. The demonstration of an immunoreactivity in intramural ganglia in *Equidae* might denote a possible

Fig. 6. Substance P-like immunoreactivity. **a.** A long, varicose nerve fiber and an immunoreactive fiber bundle are shown running in the interstitium of the horse epididymis. x 350. **b.** Thin single immunoreactive nerve fibers are present in the interlobular connective tissue, in the prostate of the donkey. x 570. **c.** Varicose aspect of an immunoreactive nerve fiber contacting smooth muscle cells in the prostate of *Equus asinus*. x 550. **d.** A subtle immunoreactive nerve fiber contacts the wall of a small artery in horse prostate. x 540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intrinsic origin of VIP-ir nerves distributing to the genitalia (Adm et al., 1977, 1980). We cannot exclude a coexistence of VIP- and NPY-ir structures (Schmalbruch and Wagner, 1989), both with intrinsic origin.

The immunolocalizations of Substance P and CGRP were limited in equine male genitalia. Substance P-ir nerve fibers were seen contacting smooth muscle fibers in epididymal duct and prostate in the horse, as well as in donkey vesicular gland. These localizations possibly denote an implication in motor functions. We never found Substance P-ir nerve fibers penetrating the epithelia in such a way as to sustain a sensory significance (Gu et al., 1983a,b; Lamano-Carvalho et al., 1986). Differently, CGRP-ir intraepithelial nerve fibers were seen in the donkey pelvic and penile urethra. In this localization, they may be regarded as sensory fibers and might have a function in pain transmission (Iwanaga et al., 1985). Further, these fibers might monitor characteristics of the lumenal content as it has been hypothesized for intraepithelial CGRP-ir sensory endings in the digestive apparatus (Rodrigo et al., 1994). It is also possible that a relation exist between CGRP-ir nerve fibers and serotonin-ir cells, which are abundant in the urethral epithelium (Arrighi and Domeneghini, 1993), as it has been hypothesized for the dog urethra by Tamaki et al. (1992). A possible dual function (afferent and efferent) of the same CGRP-secreting nerve terminal has also been hypothesized (Ishida-Yamamoto and Tohyama, 1989).

The opioid peptides Leu- and Met-Enkephalin are known to be localized both to endocrine cells and to the autonomic innervation. Their function in the male genital organs could be linked to the control of smooth muscle, and this seems particularly likely in those localizations seen in Equidae, the canalicular organs, where the progression of seminal fluid is also promoted by muscular contractions. It has been hypothesized that adrenergic transmission is facilitated by Substance P and inhibited by enkephalins in the rat vas deferens, in such a way modulating the process of ejaculation (Sastry et al., 1991). We observed the presence of Enkephalin-ir endocrine cells in the horse bulbourethral glands. It is likely that, as in intestinal localizations, the same peptide/s may be released by nerve terminals or endocrine cells, which may, in turn, exert a truly endocrine or paracrine action. A paracrine function has been hypothesized for enkephalins in the seminiferous epithelium (Spiteri-Grech and Nieschlag, 1993).

In the equine male genital apparatus the immunolocalization of Bombesin/GRP was sporadic. This peptide was only found in nerve fibers of the muscular coat of the ductus deferens and in rare perivascular nerve fibers, where it could induce motor effects on the smooth musculature. The presence of nerve fibers containing immunoreactive Gastrin Releasing Peptide (Bombesin) has been previously recorded in both the vas deferens and seminal vesicle of mouse, rabbit and guinea-pig (Stjernquist et al., 1983).

It is noteworthy that we never found a Somatostatin-immunoreactivity either in endocrine cells or in nerve components, neither in horse nor donkey. On the contrary, in the same laboratory conditions we previously detected sporadic somatostatin-ir cells in the bovine and pig prostate (Arrighi and Domeneghini, 1993). These were described also in the human prostate (Di Sant'Agnese and De Mesy Jensen, 1984; Di Sant'Agnese and Cockett, 1994), and in the urethro-prostatic complex of the ram (Vittoria et al., 1990). Absence of somatostatin-ir endocrine cells was underlined also in the horse female urethra, despite their presence in the female urethral epithelium of a large number of other domestic mammals (Vittoria et al., 1992).

In conclusion, the peptidergic innervation of equine male genitalia shows a gender-specific pattern, possibly different from other mammalian species. Nerve fibers contain mainly NPY and VIP. These two peptides have a similar extensive distribution throughout the genital apparatus and possibly share an intrinsic origin, in addition to the extrinsic one. The other regulatory peptides tested show a characteristic distribution pattern, limited to some organs. All the regulatory peptides most likely support the various male genital organs via adrenergic (and cholinergic) nerve fibers. In addition, the presence of few Leu- and Met-Enkephalin-ir endocrine cells in the bulbourethral glands of the horse is shown. Absence of any immunoreactivity to anti-somatostatin serum is a noteworthy feature of the male genital apparatus in both the examined equidae. This feature may be related to the presence of inhibitory peptides other than somatostatin.

Differences observed when comparing *E. caballus* and *E. asinus* mainly concern peptides other than NPY and VIP. They might be related to a species-specific different balance of the accessory neuro-transmitters which in turn accompany adrenergic innervation. In both species the complete absence of any regulatory peptide in the testis, with the exception of the perivascular localization of NPY-ir nerve fibers is noteworthy.

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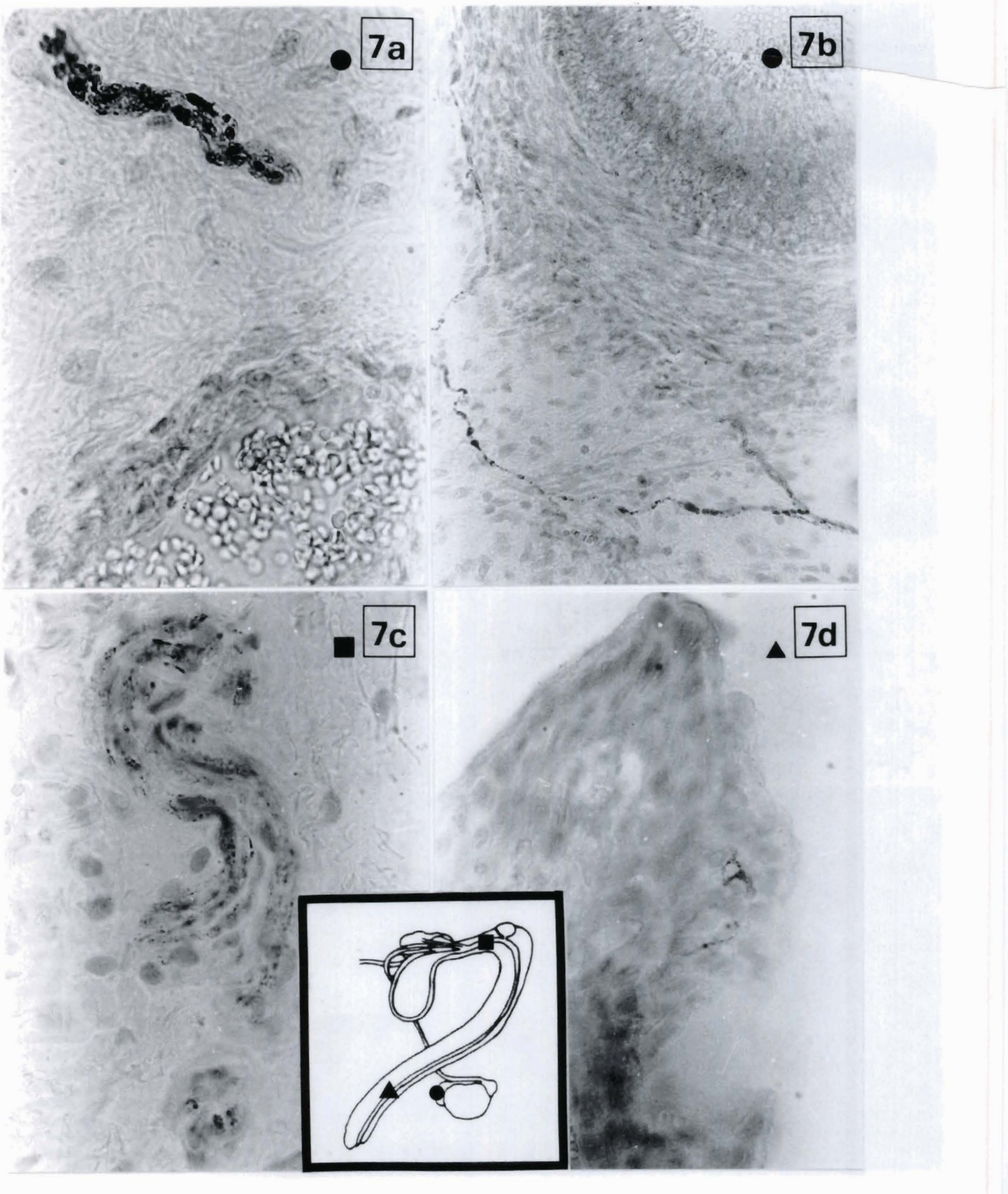
Fig. 7. CGRP-like immunoreactivity. **a.** A small, intensely immunoreactive nerve fiber bundle is present in the intertubular connective tissue of the horse epididymis, in the vicinity of a small vein. x 810. **b.** A long, varicose immunoreactive nerve fiber contacts the muscular coat of the epididymal duct in the horse by two branches. x 320. **c.** Immunoreactive nerve fibers are seen in a nerve bundle running in the mucosal lamina propria of the donkey pelvic urethra. x 810. **d.** Subtle and tortuous CGRP-ir terminals can be seen in the epithelium that lines the mucosa of the donkey penile urethra. x 810


7a
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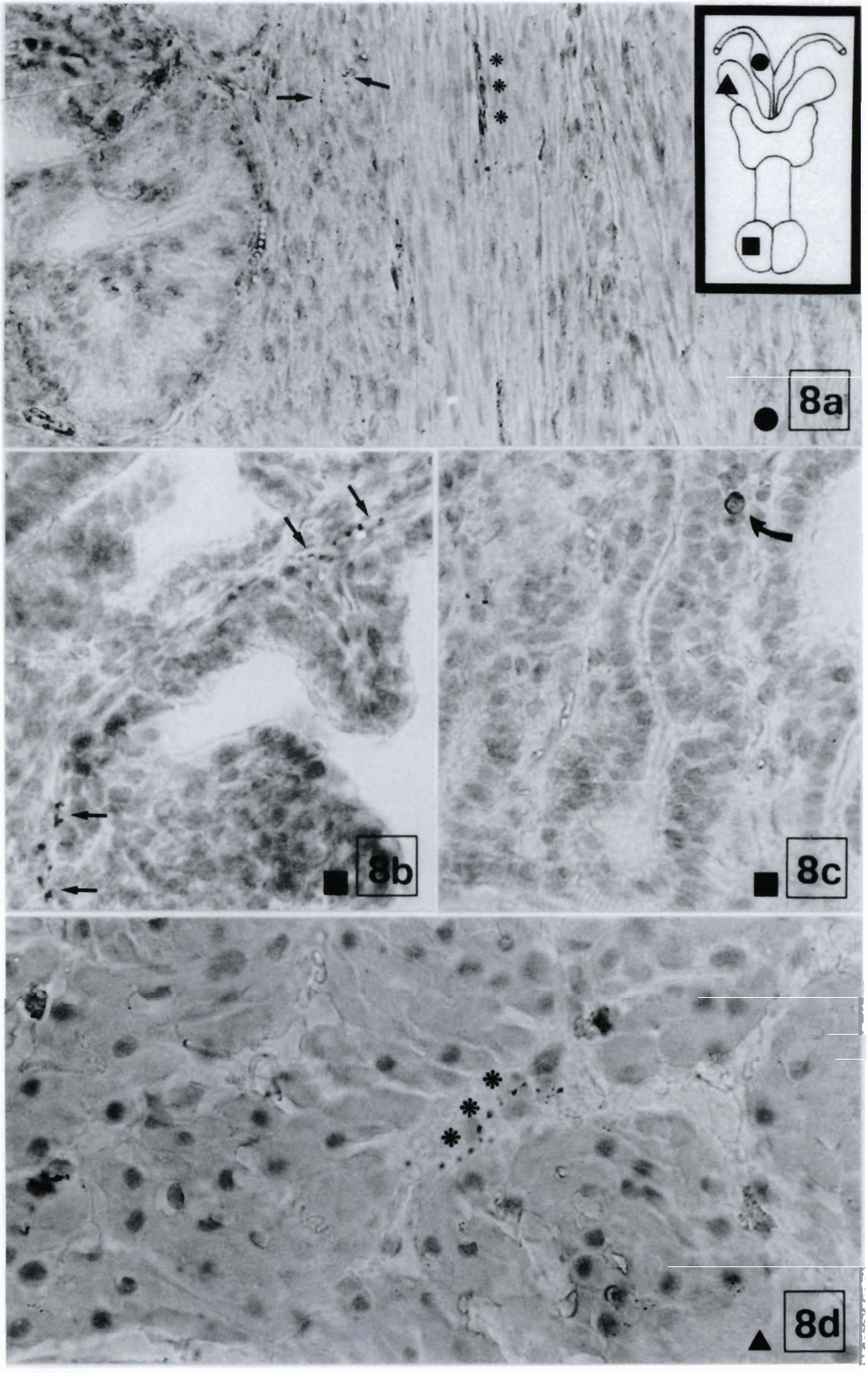

Fig. 8. Enkephalin-like-immunoreactivity. **a.** Sparse, extremely subtle met-Enkephalin-ir nerve fibers are running in the muscular coat of the ampulla ductus deferentis in *Equus caballus*. They are longitudinally (asterisks) or transversally (arrows) sectioned in relation to the arrangement of the muscle cells. **b.** Subtle met-Enkephalin-ir nerve fibers can be seen in the narrow interstitial spaces supporting the horse bulbourethral gland epithelium. **c.** One met-Enkephalin-ir endocrine cell (arrow) can be seen in the horse bulbourethral gland. **d.** A met-Enkephalin-ir nerve fiber with varicose appearance (asterisks) is running in the muscular layer of the vesicular gland in *Equus asinus*. x 570

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