IJAE
Vol. 117, n. 3: 175-189, 2012
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

Research Article: Histology and Cell Biology

Immunohistochemical characteristics of the nerve fibres of sow retractor clitoridis muscle

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Submitted April 27, 2012; accepted May 23, 2012

Summary

The occurrence of several biologically active neuropeptides (calcitonine gene-related peptide, leu-enkephaline, neuropeptide Y, substance P, and vasoactive intestinal peptide) or nitric oxide-synthesizing enzymes (neuronal nitric oxide synthase), tyrosine hydroxylase, vesicular acetylcholine transporter, and their co-localization with tyrosine hydroxylase were investigated by immunohistochemistry in the retractor clitoridis muscle of slaughtered sows. Single immunolabelling revealed that tyrosine hydroxylase and neuropeptide Y immunoreactive nerve fibres were the most numerous, followed by the neuronal nitric oxide synthase and calcitonine gene-related peptide immunoreactive ones, the vasoactive intestinal peptide, substance P and leu-enkephaline immunoreactive nerve fibres were few and vesicular acetylcholine transporter immunoreactivity were observed only in single fibres. Double immunolabelling revealed the only co-localization of tyrosyne hydroxylase with neuropeptide Y. The most reliable labelling of nerve fibres of the retractor clitoridis muscle was observed around blood vessels, followed by non-vascular smooth muscles. The present data indicate that the sow retractor clitoridis muscle receives nerve fibres that exhibit different chemical codes and, likely, differences in their chemical coding depend on the target-structure.

Key words

Immunohistochemistry; retrograde tracing; nerve fibres; genital smooth musculature; retractor penis muscle; sow.

Key to abbreviations

CGRP: Calcitonine Gene-Related Peptide
LENK: Leu-Enkephaline
nNOS: neuronal Nitric Oxide Synthase
NPY: Neuropeptide Y
RCM: retractor clitoridis muscle
SP: Substance P
TH: Tyrosyne hydroxylase
VAChT: Vesicular Acetylcholine Transporter
VIP: Vasoactive Intestinal Peptide

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Introduction

The innervation of female genital organs has been extensively studied in different species. In particular, the use of degenerative methods and retrograde neuronal tracing techniques has allowed to characterize the innervation of the genital tract of sheep (Flieger, 1977), sow (Welento *et al.*, 1984; 1987a; Boratynski *et al.*, 1988; Flieger *et al.*, 1988a,b; Wasowicz *et al.*, 1998), cow (Welento *et al.*, 1987b), rat (Inyama *et al.*, 1986; Berkley *et al.*, 1988; Nance *et al.*, 1988), guinea-pig (Alm and Lundberg, 1988, Papka *et al.*, 1991) and cat (Kawatani *et al.*, 1990, 1994; Kawatani and de Groat, 1991).

In spite of these numerous observations, there are scarce informations about the innervation of the genital smooth musculature, even if our previous studies have documented the site (Panu *et al.*, 2001) and the neurochemical content (Bo Minelli *et al.*, 2002) of the neurons projecting to the smooth retractor clitoridis muscle (RCM) of the sow. We used the RCM, as experimental model for the smooth genital musculature, because, under specific hormonal stimuli, it reacts in a similar way as the uterine smooth musculature (Basset, 1961). The RCM, absent in primates, dogs and some rodents, is a band of smooth muscle, related to the lateral wall of the vagina, that estends from the coccygeal vertebrae or from the anus to the clitoris (Basset, 1961; Panu *et al.*, 1995).

The aim of the present research was to define the immunohistochemical characteristics of the nerve fibres, that are present in the RCM and to establish a basis for further anatomical investigations dealing with the nerve control of the intrinsic and vascular musculature of genital muscles.

Our study was carried out on the pig, which is frequently used as a model in biomedical researches (Dodds, 1982; Swindle *et al.*, 1992; Crissinger *et al.*, 1994), including neuroanatomical studies (Boratynski *et al.*, 1988; Flieger *et al.*, 1988a,b; Merighi *et al.*, 1990; Timmermans *et al.*, 1993; Kaleczyc *et al.*, 1995, 1999, 2002; Boratynski and Welento, 1996; Kaleczyc, 1998; Majewski *et al.*, 1999; Panu *et al.*, 2001, 2003; Botti *et al.*, 2006, 2009).

Materials and methods

The study was carried out on the RCM of 4 slaughtered sows. The muscles were immediately fixed for 4 hours at 4°C with a solution of 4% w/v, ice-cold, phosphate buffered paraformaldehyde (pH 7.4). Then the samples were rinsed with phosphate buffer (pH 7.4), divided into small pieces and transferred into a 10% w/v buffered sucrose solution (pH 7.4) for 24 h. Afterwards, they were transferred into a 30% w/v buffered sucrose solution (pH 7.4), where they were stored (at 4 °C) for at least three days or until further processing.

Each piece of the muscle was placed flat in the cryostat mould and cut in 12 µm thick serial sections.

The sections were stained, using a single and double labelling immunofluorescence method, in order to test for the presence and co-existence of tyrosyne hydroxylase (TH), as a marker for the presence of noradrenaline (NA), with vesicular acetylcholine transporter (VAChT), as a marker for cholinergic neurons, neuronal nitric oxide synthase (nNOS), the enzyme synthesizing nitric oxide, and the following biologically active peptides: calcitonine gene-related peptide (CGRP), leu-enkephaline (LENK), neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal peptide (VIP).

Table 1 – Primary antisera, secondary reagents and dilutions used.

Primary antibody	raised in	Code no.	Dilution	Supplier
Anti TH	mouse (monoclonal)	T 2928	1:8000	Sigma, St. Louis, Missouri, U.S.A.
Anti VAChT	rabbit (polyclonal)	V 5387	1:1000	Sigma, St. Louis, Missouri, U.S.A.
Anti n-NOS	rabbit (polyclonal)	AB 5380	1:3000	Chemicon International, Inc., Temecula, CA
Anti CGRP	rabbit (polyclonal)	C 8198	1:8000	Sigma, St. Louis, Missouri, U.S.A.
Anti LENK	rabbit (polyclonal)	L 8516	1:10	Sigma, St. Louis, Missouri, U.S.A.
Anti NPY	rabbit (polyclonal)	N 9528	1:8000	Sigma, St. Louis, Missouri, U.S.A.
Anti SP	rabbit (polyclonal)	S 1542	1:8000	Sigma, St. Louis, Missouri, U.S.A.
Anti VIP	rabbit (polyclonal)	V 3508	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Secondary antibody				
Anti rabbit IgG/FITC	Goat	F 0382	1:80	Sigma, St. Louis, Missouri, U.S.A.
Anti rabbit IgG/Texas Red	Goat	TI 1000	10 µg/ml	Vector Laboratories, Burlingame CA, U.S.A.
Anti mouse IgG/Texas Red	Horse	TI 2000	10 µg/ml	Vector Laboratories, Burlingame CA, U.S.A.

The same primary antiserum or couple of antisera were applied to sections separated by at least 96 µm from each other, to eliminate the likelihood of testing the same fibre twice for the same antisera.

In case of single immunolabelling, sections were air-dried at room temperature (rt) for 30 min., rinsed (3X5 min.) with phosphate buffered saline (PBS; pH 7.4) and incubated with a blocking mixture containing 0.25% Triton X-100, 1% bovine serum albumine and 10% normal goat serum in PBS for 1 h (rt), to reduce non-specific background staining. The sections were then incubated overnight in a humid chamber with one of the aforesaid primary antisera, then they were incubated (1h; rt) with fluoroscein isothiocyanate (FITC)-conjugated goat antirabbit IgG, Texas Red-conjugated goat antirabbit IgG, or Texas Red-conjugated horse antimouse IgG, according to the primary antiserum used (primary antisera and secondary reagents are listed in Table 1) and mounted in buffered glycerin (Bio-Optica).

In case of double labelling, after incubation in the blocking medium (see above), sections were incubated (overnight; rt) in a humid chamber with a mixture of two primary antisera (TH with each of the aforesaid antisera). Then the sections were incubated with a mixture of FITC-goat antirabbit and Texas Red horse antimouse secondary antibodies (1h; rt), and mounted as described above. Each step of single- or double- labelling was followed by rinsing the sections with PBS (3x5 min).

The single- or double-labelled sections were studied and photographed with Zeiss axiophot fluorescence microscope equipped with epi-illumination and appropriate filter sets for FITC and Texas Red.

Controls. Standard tests (preabsorption for the neuropeptide antisera, omission and replacement of all the primary antisera used by PBS) were applied to control the specificity of immunofluorescence.

Results

The sow RCM was supplied by nerve fibres with different chemical coding and that were observed around the smooth muscle cells, around the blood vessels and/or in the interstitial connective tissue of the muscle (Table 2).

Table 2 - Distribution of immunoreactivity within tissue.

	Smooth muscle cells	Blood vessels	Interstitial connective tissue
TH	+++	+++	+++
NPY	+++	+++	
nNOS	+	++	
CGRP			++
VIP			+
SP			+
LENK			+
VAChT		+/-	+/-
TH/NPY	+++	+++	

TH: Tyrosyne hydroxylase; NPY: Neuropeptide Y; nNOS: neuronal Nitric Oxide Synthase; CGRP: Calcitonine Gene-Related Peptide; VIP: Vasoactive Intestinal Peptide; SP: Substance P; LENK: Leu-Enkephaline; VAChT: Vesicular Acetylcholine Transporter.

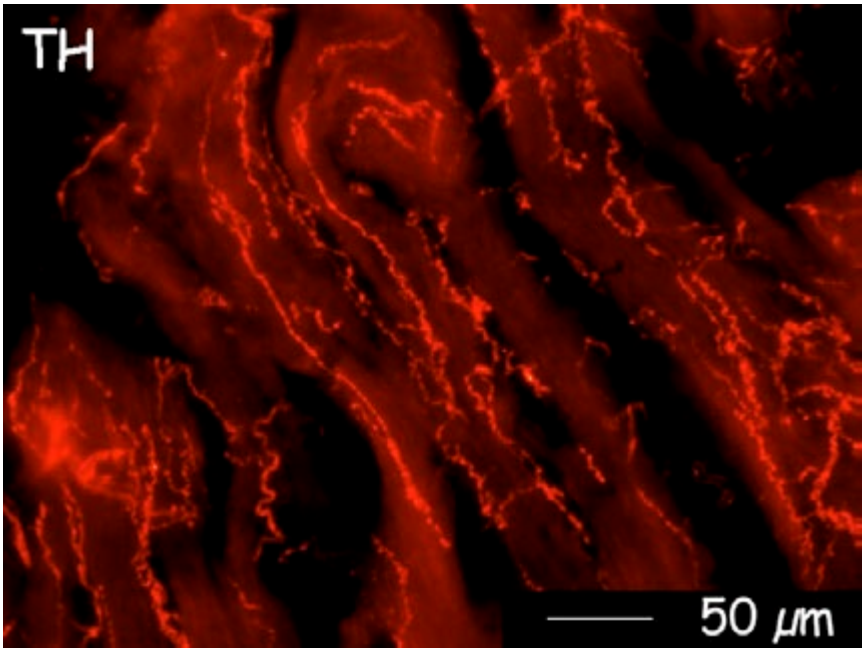

Fig. 1 – TH+ fibres in association with the intrinsic smooth muscle cells of the RCM. X 40, bar = 50 µm.

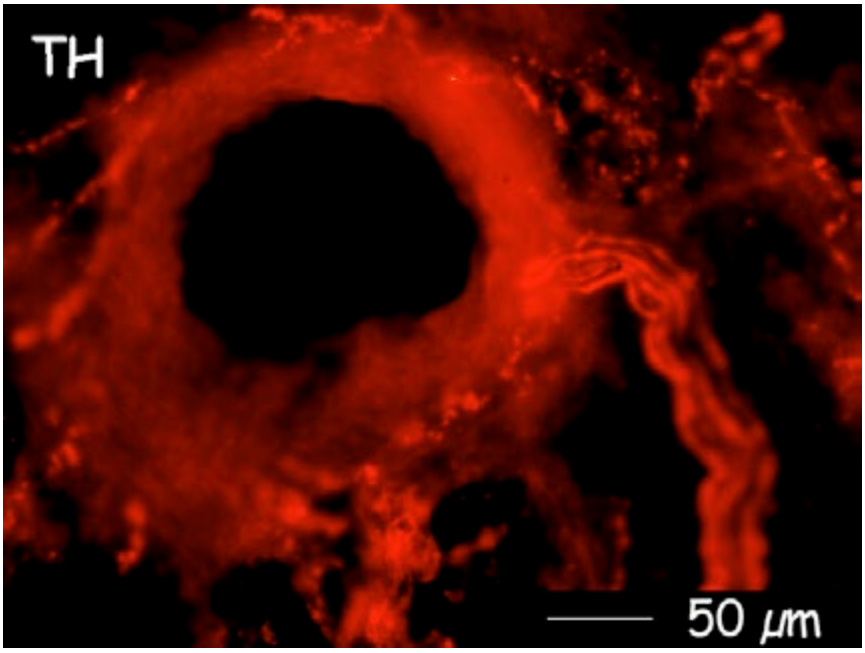

Fig. 2 – TH+ fibre in association with the muscular coat of RCM blood vessels. X 40, bar = 50 µm.

Single labelling. The majority of nerve fibres of the RCM was TH- and NPY- immunoreactive (IR). A minor number of fibres was IR for nNOS and CGRP and then for VIP, SP, LENK and VAChT. In particular the cholinergic fibres were scarce in number.

The fibres IR to TH were observed both as isolated fibres and forming thick nerve bundles. The single fibres were observed in association with the intrinsic smooth muscle cells of the RCM (Fig. 1) or with the muscular coat of its blood vessels (Fig. 2), whereas the TH-IR nerve bundles were observed in the interstitial connective tissue.

NPY-IR fibres travelled parallel to the large axis of the intrinsic smooth muscle fibres (Fig. 3) or were associated with blood vessels.

Morphologically, the TH-IR fibres were more voluminous and varicose in respect to NPY-IR fibres (compare Fig. 1 with Fig. 3).

Numerous fibres IR to nNOS were observed in relation with the blood vessels (Fig. 4), while those innervating non-vascular smooth muscles were moderate in number.

The fibres IR to CGRP and, in decreasing order, to VIP, SP and LENK were found in the interstitial connective tissue of the muscle (Fig. 5). All these fibres had a winding course and varicosities, with the exception of the LENK-IR fibres.

The very low number of fibres IR to VAChT were observed in the interstitial connective tissue and in the muscular coat of the vessels of the RCM (Fig. 6). These fibres had a sinuous course and showed varicosities.

Double labelling. The double immunostaining showed only the co-localization of TH with NPY and these fibres were always observed in relation to the smooth muscle

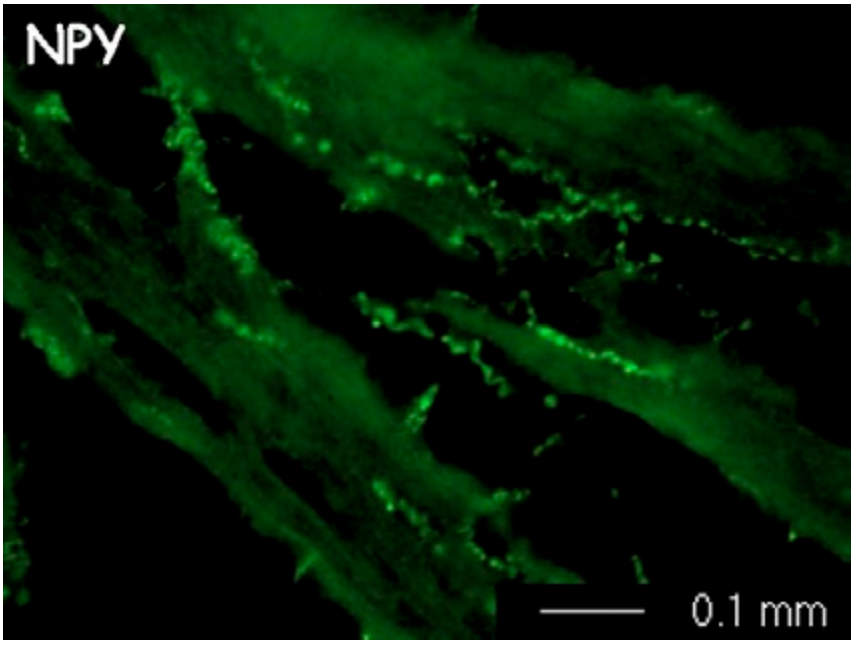

Fig. 3 – NPY+ fibres in association with the intrinsic smooth muscle cells of the RCM. X 40, bar = 50 µm.

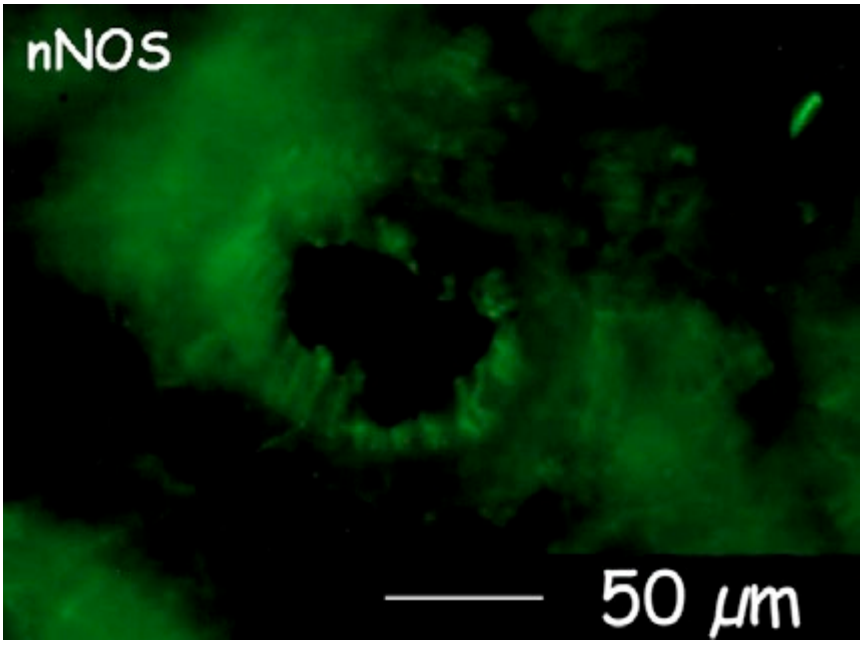

Fig. 4 – nNOS+ fibres in association with the intrinsic smooth muscle cells of the RCM. X 40, bar = 50 µm.

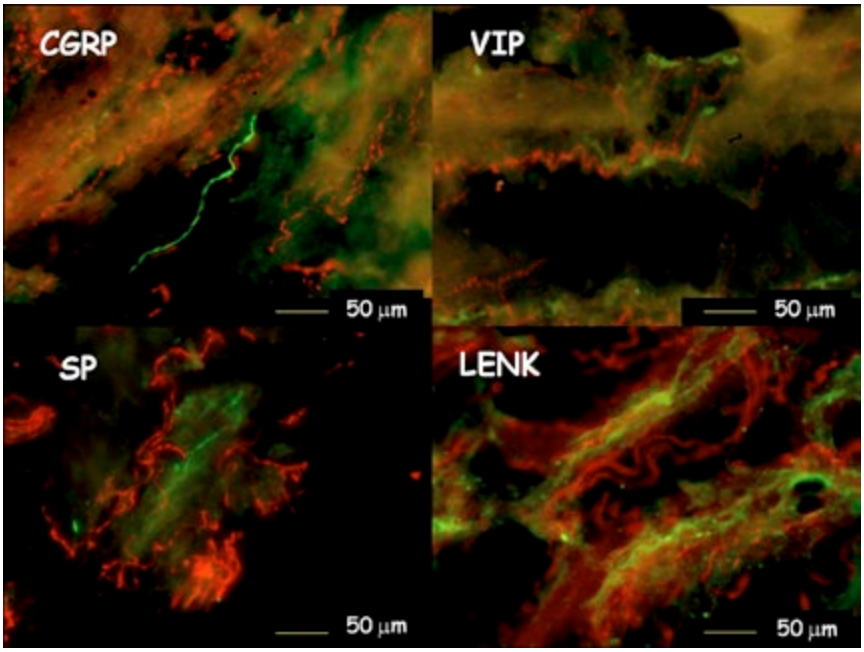

Fig. 5 – CGRP+ (a), VIP+ (b), SP+ (c), LENK+ (d) fibres in association with the interstitial connective tissue of the RCM. The positive fibres are green. X 40, bar = 50 µm.

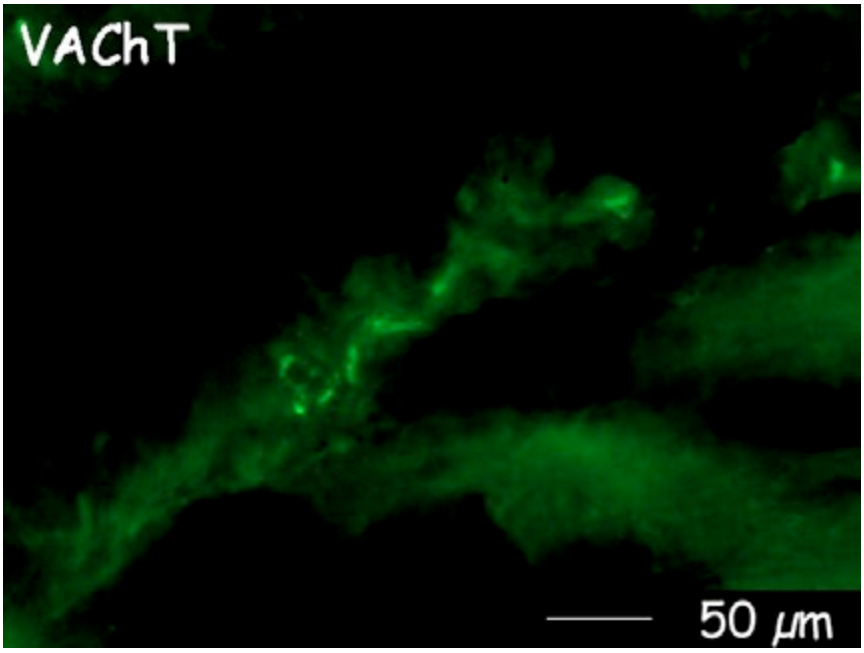

Fig. 6 – VAChT+ fibre in association with the interstitial connective tissue of the RCM. X 40, bar = 50 µm.

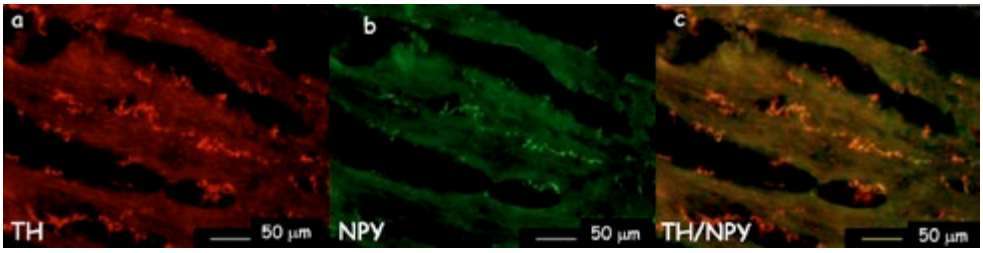

Fig. 7 – TH+/NPY+ fibres in association with the intrinsic myocells of the RCM. a: TH immunoreactivity; b: NPY immunoreactivity; c: TH+/NPY+ immunoreactivities. X 40, bar = 50 µm.

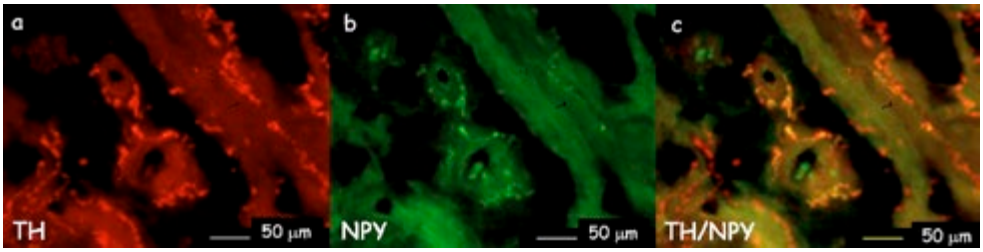

Fig. 8 – TH+/NPY+ fibres in association with the blood vessels of the RCM. a: TH immunoreactivity; b: NPY immunoreactivity; c: TH+/NPY+ immunoreactivity. X 40, bar = 50 µm.

cells of the RCM (Fig. 7) and blood vessels (Fig. 8). In both cases the double labelled fibres were voluminous and showed varicosities.

Discussion

Distribution and co-localization of particular neurotransmitters

Single labelling revealed a reliable axonal immunostaining for TH and NPY, followed by immunostaining for nNOS, while immunoreaction for CGRP, VIP, SP and LENK was restricted to less numerous subpopulations of mostly interstitial connective nerves. VAChT-containing nerve fibres were observed only sporadically.

TH-IR nerve fibres were one of the most numerous subsets of the autonomic nerve fibres found within the sow RCM. These fibres were prevalently found around the blood vessels, but single noradrenergic varicosities were also found around the intrinsic miocytes. Our finding is in accordance with Majewsky *et al.* (1999), who found noradrenergic fibres not only around all arteries and veins, but also in the interstitial tissue of the boar penis and retractor penis muscle, the male analogous of the RCM. Noradrenaline is a typical sympathetic neurotransmitter with excitatory activity. In fact it causes the contraction of smooth musculature (Owman and Stjernquist, 1988; Andersson, 2000; Andersson *et al.*, 2000) and in particular the contraction of blood vessel musculature (Majewski *et al.*, 1999).

The present study has also revealed that the sow RCM receive an extensive NPY-IR innervation distributed to the intrinsic and vascular smooth musculature. Our result is supported by what has been found in man (Wespes *et al.*, 1988; Kirkeby *et al.*, 1991; Hauser-Kronberger *et al.*, 1994), monkey (Schmalbruch and Wagner, 1989), rat (Lamano Carvalho *et al.*, 1986; Carrillo *et al.*, 1991), guinea-pig (Lamano Carvalho *et al.*, 1986) and pig (Majewsky *et al.*, 1999) penis and retractor penis muscle. NPY is a neuropeptide that show excitatory activity, in fact it enhances the contractile effects of noradrenaline (Ekblad *et al.*, 1984; Lundberg, 1991) and is a potent vasoconstrictor (Lundberg and Hokfelt, 1986; McLachlan and Llewellyn-Smith, 1986; Morris and Gibbins, 1987; Lundberg *et al.*, 1990; Majewski *et al.*, 1995).

Our finding of numerous nNOS-IR fibres around blood vessels and less number around non-vascular smooth muscles is in agreement with that has been found by Majewsky *et al.* (1999) in the porcine retractor penis muscle. nNOS is the enzyme producing nitric oxide (NO), a molecule having relaxing activity, in particular involved in the autoregulation of local blood flow through direct effects on vascular smooth muscle or through inhibition of perivascular sympathetic nerve activity (Melikian *et al.*, 2009). The nNOS distribution pattern, that we found, may implicate its involvement in the relaxation of the RCM blood vessels rather than the control of the same muscle functions.

Also CGRP-IR was found within moderate numbers of fibres that were distributed in the interstitial connective tissue of the sow muscle. This peptide has been found in the male genital organs, prevalently in the penis with interspecies differences in its existence and distribution. In fact, it has been found in the rat cutis glandis (Lamano Carvalho *et al.*, 1986), in the rat and dog urethral epithelium (Lamano Carvalho *et al.*, 1986; Iwanaga *et al.*, 1985), and in the pig cutis glandis and urethral epithelium (Majewski *et al.*, 1999). CGRP is commonly considered a marker of the afferent pathways (McNeill *et al.*, 1992; Maggi, 1995; Majewski *et al.*, 1995; Kaleczyc, 1997; Czaja, 2000). The distribution pattern of CGRP-IR nerve fibres in the interstitial connective tissue may implicate its involvement as contraction evoking agent in the sow RCM methabolism.

We also found VIP, SP and LENK, but within scarce nerve fibres innervating the porcine RCM. All these substances were distributed prevalently in the interstitial connective tissue of the muscle. The presence of VIP-IR nerve fibres has been found in the porcine retractor penis muscle (Majewski *et al.*, 1999), that is heavily innervated by this peptide. This peptide principally acts, like nitric oxide, as vasodilator on blood vessels (Lynch *et al.*, 1980; Clark *et al.*, 1981; Polak *et al.*, 1981; Polak and Bloom, 1984; Morris *et al.*, 1985; Majewski *et al.*, 1995), but it also plays a relaxatory role in the muscular wall of the uro-genital tract (Lynch *et al.*, 1980; Walles *et al.*, 1980; Helm *et al.*, 1981; Polak *et al.*, 1981; Stjernquist and Owman, 1984; Palle *et al.*, 1989; Owman and Stjernquist, 1988; Yoshiyama and de Groat, 2008). Its distribution pattern in the interstitial connective tissue of the RCM may suggest its involvement in both functions.

Also the presence of SP-IR fibres has been documented in the boar penis (Majewski *et al.*, 1999), even if it has usually been observed around blood vessels. However, we can not exclude this destination for SP-IR nerve fibres that we found. SP is considered a marker of the afferent pathways (Maggi, 1995; Majewski *et al.*, 1995; Kaleczyc, 1997; Czaja, 2000), like CGRP, and is an excitatory neuropeptide (Majewski *et al.*, 1995).

We also reported, in our study, the occurrence of LENK, an opioid pentapeptide that has been observed throughout male internal genital organs (Owman and Stjernquist, 1988) and in the retractor penis muscle (Majewski *et al.*, 1999), the male homologous of the RCM. The enkephalines seem to have a vasodilator effect due to their inhibitory nature on autonomic neurotransmission (Konishi *et al.*, 1979; 1981; Katayama and Nishi, 1984; Kaleczyc *et al.*, 1997; Kaleczyc, 1998).

We have also observed single nerve fibres immunoreactive to VAChT, that were in the interstitial connective tissue and around blood vessels. Also results of previous studies have suggested a rather scarce cholinergic innervation of the pig retractor penis muscle (Majewsky *et al.*, 1999). So the paucity of cholinergic terminals in these two homologous smooth muscles, associated to the porcine female and male genital organs, may be explained by two hypotheses. First, the porcine RCM and retractor penis muscle receive axons from paravertebral and prevertebral neurons (Panu *et al.*, 2001; 2003), and the paravertebral and prevertebral cholinergic nerve cells (Bo Minelli *et al.*, 2002; Botti *et al.*, 2003) do not contribute to the innervation of the muscles. Second, according to Majewski *et al.* (1999), the intramuscular cholinergic terminals may contain too low amounts of VAChT to be detected by immunofluorescence, thus leading to false negative results.

Finally the double labelling allowed to document only the co-localization of TH and NPY within the sow RCM nerve fibres, that were observed in relation to both the muscle cells of the RCM and of its blood vessels. This co-localization has been already detected by Majewski *et al.* (1999) in relation with the vessels (arteries and veins) and the intrinsic smooth muscle of the boar penis. The co-localized sympathetic axons, that we found, could determinate the contraction of intrinsic and extrinsic smooth musculature of the sow RCM.

Putative origin of particular subsets of RCM nerve fibres

Our previous study (Panu *et al.*, 2001) has documented that the sow RCM is innervated by fibres originating from neurons located in the sacral (S1-S4) spinal ganglia, in the lumbo-sacral (L5-L6 or L6-L7 and S1-S3) sympathetic trunk ganglia and in the prevertebral caudal mesenteric ganglia. Moreover an our previous study (Bo Minelli *et al.*, 2002) has documented the neurochemical content of the above mentioned neurons, that showed IR for all the substances studied, even if with differences dealing from the category of ganglia.

On the basis of these researches we could hypothesize that the vast majority, if not all, of the numerous TH- and NPY-IR nerve fibres originate from the neurons located in the sympathetic trunk and prevertebral ganglia, where these two substances are mainly present.

Also the nNOS-IR fibres could prevalently originate from autonomic neurons projecting to the sow RCM. However, since McNeill *et al.* (1992) have suggested that some of penile sensory nerves may serve as an additional source of NO and Bo Minelli *et al.* (2002) have found a discrete percentage of sensory neurons, projecting to the RCM, IR to nNOS, we could hypothesize that the nNOS-IR fibres originate also from spinal ganglia.

The CGRP-IR nerve fibres in the sow RCM could be afferent and originate only from the neurons of spinal ganglia, because, in our previous study (Bo Minelli *et al.*,

2002), this peptide has been found in a very little percentage of sympathetic trunk ganglia and has not been detected in caudal mesenteric ganglia.

The VIP-, SP- and LENK-IR nerves could originate from sensory and autonomic neurons that project to the sow RCM, because they have been observed in spinal, sympathetic trunk and prevertebral ganglia (Bo Minelli *et al*., 2002).

Finally VAChT immunoreactive fibres could originate only from the autonomic ganglia, because this substance has not been documented in the sensory neurons innervating the RCM (Bo Minelli *et al*., 2002).

In conclusions, the present results document that the sow RCM intrinsic and vascular smooth muscles receive a heterogeneous innervation in relation to the respective functional specializations. In addition, our study confirms the finding that we have documented in sensory and autonomic neurons projecting to the sow RCM (Bo Minelli *et al*., 2002). In fact in the nerve fibres of the muscle we have found the same neuroactive substances of the neurons. Nevertheless, further studies are necessary to elucidate the exact physiological relevance of these substances.

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