Double labelling immunohistochemical characterization of autonomic sympathetic neurons innervating the sow retractor clitoridis muscle

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Retrograde neuronal tracing and immunohistochemical methods were used to define the neurochemical content of sympathetic neurons projecting to the sow retractor clitoridis muscle (RCM). Differently from the other smooth muscles of genital organs, the RCM is an isolated muscle that is tonically contracted in the rest phase and relaxed in the active phase. This peculiarity makes it an interesting experimental model.
The fluorescent tracer fast blue was injected into the RCM of three 50 kg subjects. After a one-week survival period, the ipsilateral paravertebral ganglion S1, that in a preliminary study showed the greatest number of cells projecting to the muscle, was collected from each animal. The co-existence of tyrosine hydroxylase with choline acetyltransferase, neuronal nitric oxide synthase, calcitonin gene-related peptide, leu-enkephalin, neuropeptide Y, substance P and vasoactive intestinal polypeptide was studied under a fluorescent microscope on cryostat sections. Tyrosine hydroxylase was present in about 58% of the neurons projecting to the muscle and was found to be co-localized with each of the other tested substances. Within fast blue-labelled cells negative to the adrenergic marker, small populations of neurons singularly containing each of the other enzymatic markers or peptides were also observed.
The present study documents the complexity of the neurochemical interactions that regulate the activity of the smooth myocytes of the RCM and their vascular components.

Key words: immunohistochemistry, retrograde tracing, peripheral autonomic neurons, genital smooth muscle, vascular smooth muscle, pig.

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The retractor clitoridis muscle (RCM), absent in primates, dogs and some rodents, is a paired band of smooth muscle that in the sow originates directly from the smooth muscle of the anus and terminates at the base of the clitoris.

Differently from the other smooth muscles of genital organs, it does not form one of their layers, nor is it a component of their fibromuscular stroma. Moreover, the RCM is in tonic contraction in the rest phase and relaxed in the active phase. This peculiarity, and the fact that the muscle is easily identifiable and of simple and versatile preparation, makes it an interesting experimental model which we have been employing for the past several years to define its innervation. Using the Bielschowsky method and gold chloride or silver impregnation techniques (Panu *et al.,* 1995), we demonstrated the presence of vegetative and sensory innervation in the RCM of some ungulates (sow, ewe, goat, mare) in fascial connective tissue. With the use of the retrograde neuronal tracer fast blue (FB) (Panu *et al.,* 2001) we also demonstrated that the peripheral autonomic and sensory neurons innervating the sow RCM are localized bilaterally in the lumbosacral paravertebral ganglia (PaG) and in the caudal mesenteric ganglia (CMG), and ipsilaterally in the sacral dorsal root ganglia (DRG). Moreover, by combining FB and single labelling immunofluorescence methods (Bo Minelli *et al.,* 2002a), we performed a screening that revealed the presence of several biologically active substances within the sow RCM-projecting neurons. In this screening, tyrosine hydroxylase (TH), the rate limiting enzyme of catecholamine synthesis, was shown to be the most abundant substance. By double labelling immunofluorescence methods (Gazza *et al.,* 2005), we tentatively concluded that TH-immunoreactive (-IR) fibres amply supplied both the smooth myocites of the sow RCM and its blood vessels and that, in some cases, they also contained neuropeptide Y (NPY).

On the basis of these data, the present study was

aimed at defining, within the neurons of the S1 paravertebral ganglion (PaG S1), (shown to contain the highest number of sympathetic neurons supplying the muscle (Panu *et al.,* 2001)), the pattern and the percentage of co-existence of TH, with: 1 choline acetyltransferase (ChAT), a marker of cholinergic neurons, 2, neuronal nitric oxide synthase (nNOS), a marker of nitric oxide, and 3, one of the following biologically active peptides, i.e. calcitonin gene related peptide (CGRP), leucine-enkephalin (LENK), neuropeptide Y (NPY), substance P (SP) and vasoactive intestinal peptide (VIP).

This study was carried out in the swine a species which is frequently used as a research model (Kaleczyc *et al.,* 1993, 1997; Majewski *et al.,* 1995, 1999).

Preliminary data of this investigation have been published in abstract form (Bo Minelli *et al.,* 2002b).

Materials and Methods

All procedures were approved by the local Ethics Committee for Animal Experimentation and by the Italian Ministry of Health. Precautions aimed at avoiding unnecessary suffering were taken at all stages of the experiment.

The study was carried out on the RCM of three 50 kg sows combining the retrograde neuronal tracer FB and double labelling immunofluorescence techniques.

The central part of the left RCM of each animal, under general anaesthesia, was inoculated with 50 µl of 2% w/v FB, a fluorescent tracer with cytoplasmatic affinity, by the use of a Hamilton syringe.

After a 7-day survival time, the animals, under general anaesthesia, were intracardially perfused, first with heparinized physiological solution and afterwards with fixative solution (4% w/v paraformaldehyde in 0.1 M phosphate buffer (PBS), pH 7.4). Before collecting the samples, macroscopic and microscopic examination of the RCM and adjacent tissues revealed that the distribution of FB was confined to the muscle and no evidence of the tracer in the surrounding tissues was found. This confirmed that the fascial connective tissue of the muscle represents a barrier capable of preventing the dispersion of the tracer in the surrounding tissues.

The ipsilateral PaG S1 was removed from each animal, post-fixed by immersion in the same fixative for 2 hr at 4°C, rinsed with PBS,pH 7.4 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 ganglion was placed flat in the cryostat mould and serially cut along its longest axis, in order to obtain a large number of cells per section.

The 12 µm thick sections were stained by a double labelling immunofluorescence method to test the occurrence and co-localization of TH with ChAT, nNOS, CGRP, LENK, NPY, SP and VIP.

The same combinations of primary antisera were applied to sections at least 96 µm away from each other to eliminate the likelihood of testing the same neuron twice for the same antisera. After air-drying at room temperature (rt) for 30 min, the sections were incubated with a solution containing 0.25% Triton X-100, 1% bovine serum albumin and 10% normal goat serum in PBS for 1 hr (rt), to reduce non-specific background staining. They were then incubated with the combination of primary antisera (overnight, rt), further incubated with a mixture of fluorescein isothiocyanate (FITC)-conjugated goat antirabbit IgG and biotinylated-goat anti-mouse IgG (1 hr; rt) and, finally, incubated with Texas Red-conjugated streptavidin (primary antisera and secondary reagents are listed in Table 1) and mounted in buffered glycerin (Bio-Optica). Each step of immunolabelling was followed by rinsing the sections with PBS (3×5 min; pH 7.4). In control experiments, no immunoreactivity was detected in sections incubated in the absence of primary antisera, replaced by PBS, and in sections incubated with the rabbit antisera which had been adsorbed with excess (100 µg/mL) of the respective antigens, when these were available [rat CGRP (Sigma), porcine NPY (Sigma), synthetic VIP (Sigma)].

The labelled sections were studied and photographed with a Zeiss Axioskop 2 plus fluorescence microscope equipped with epi-illumination and appropriate filter for FB (excitation wavelength 390-420 nm; emission wavelength 450 nm), FITC (excitation wavelength 450-490 nm; emission wavelength 515-565 nm) and Texas Red (excitation wavelength 530-585 nm; emission wavelength 615 nm). Relationships between FB distribu-

tion and immunohistochemical staining were examined directly by interchanging filters. The observations were made by a single operator.

In each ganglion, the relative percentages of RCM-projecting neurons containing different combinations of the markers were calculated on the total number of FB-labelled (FB^+) cells tested for each couple of primary antisera. Data are expressed as means ± S.E.M.

Results

Each combination of primary antisera was tested on a mean number of 179.05±12.93 FB+ neurons of the PaG S1 from each animal. 57.91±10.03% of the FB^+ neurons showed TH-IR. The immunofluorescence varied from strong to moderate and was uniformly distributed through the perikarya, sometimes also being evident along the neuronal processes.

Double labelling immunofluorescence showed that TH was co-localized in different proportions with each one of the other tested substances (Table 2).

NPY showed the highest percentage (34.14±6.83%, n=167±30.75) of co-localization with TH (Figure 1, A1-A3). The neuronal cell bodies immunoreactive (IR) for NPY showed a granular labelling. These cell bodies were randomly distributed throughout the ganglion, sometimes forming small cell groups, but in general they were seen as single perikarya. Many of them had IR processes.

TH and nNOS were co-localized in a lower proportion (6.90±3.18%; n=185.33±40.34). A similar percentage of co-localization was found for TH and SP (6.76±5.11%; n=178±41.02), but was observed only in two of the three subjects. The immunofluorescent labelling of both nNOS-IR (Figure 1, B1-B3) and SP-IR (Figure 1, C1-C3) cells was uniformely distributed throughout the perikarya.

The percentage of co-localization found for TH and LENK was 4.49±2.86% (n=210.67±54.77, Figure 1, D1-D3). Similarly, the proportion of co-localization found for TH and ChAT was 4.28±2.16% (n=179±39.25; Figure 1, E1-E3), but this co-existence was observed only in two sub-

Table 1. Antisera and dilutions used in the experiments.

Primary antisera	*Raised in*	*Code no.*	*Supplier*	*Dilution*
Anti-tyrosine hydroxylase (TH)	mouse (monoclonal)	T 2928	Sigma, St. Louis, Missouri, U.S.A.	1:4000
Anti-choline acetyltransferase (ChAT)	rabbit (polyclonal)	AB 5042	Chemicon International, Inc., Temecula, CA	1:3000
Anti-neuronal nitric oxide synthase (n-NOS)	rabbit (polyclonal)	AB 5380	Chemicon International, Inc., Temecula, CA	1:1500
Anti-calcitonin gene related peptide (CGRP)	rabbit (polyclonal)	C 8198	Sigma, St. Louis, Missouri, U.S.A.	1:4000
Anti-leu-enkephalin (LENK)	rabbit (polyclonal)	L 8516	Sigma, St. Louis, Missouri, U.S.A.	1:5
Anti-neuropeptide Y (NPY)	rabbit (polyclonal)	N 9528	Sigma, St. Louis, Missouri, U.S.A.	1:4000
Anti-substance P (SP)	rabbit (polyclonal)	S 1542	Sigma, St. Louis, Missouri, U.S.A.	1:4000
Anti-vasoactive intestinal polypeptide (VIP)	rabbit (polyclonal)	V 3508	Sigma, St. Louis, Missouri, U.S.A.	1:4000
Secondary antisera				
Anti rabbit IgG/FITC	goat	F 0382	Sigma, St. Louis, Missouri, U.S.A.	1:40
Anti mouse IgG/biotin	sheep	RPN 1001	Amersham Pharmacia Biotech, U.K.	1:100
Streptavidin/Texas red		RPN 1233	Amersham Pharmacia Biotech, U.K.	1:100

Table 2. Number (No.) of RPM-projecting (FB+) cells tested for each pair of primary antisera and relative percentages (RP) of FB+ neurons showing different combination of positivity to each pair of tested primary antisera. Data are expressed as means ± S.E.M.

Tested combination of primary antisera	*TH^+/Ab^+ (RP)*	*TH^+/Ab^- (RP)*	*TH^-/Ab^+ (RP)*	*TH^-/Ab^- (RP)*	FB^+ tested cells (No.)
TH-CGRP	3.10±1.74%	63.64±7.33%	0.34±0.18%	32.93±8.99%	173.33±38.85
TH-LENK	4.49±2.86%	55.69±6.11%	1.86±1.45%	37.96±5.57%	210.67±54.77
TH-nNOS	6.90±3.18%	49.92±11.36%	2.53±1.34%	40.65±11.95%	185.33±40.34
TH-NPY	34.14±6.83%	28.28±2.92%	11.10±1.71%	26.49±10.76%	167±30.75
TH-SP	6.76±5.11%	54.33±5.37%	0.00±0.00%	38.91±9.53%	178±41.02
TH-ChAT	4.28±2.16%	55.49±5.85%	0.55±0.37%	39.68±7.92%	179±39.25
TH-VIP	2.48±2.48%	49.54±10.66%	0.00±0.00%	47.98±13.14%	160±24.56

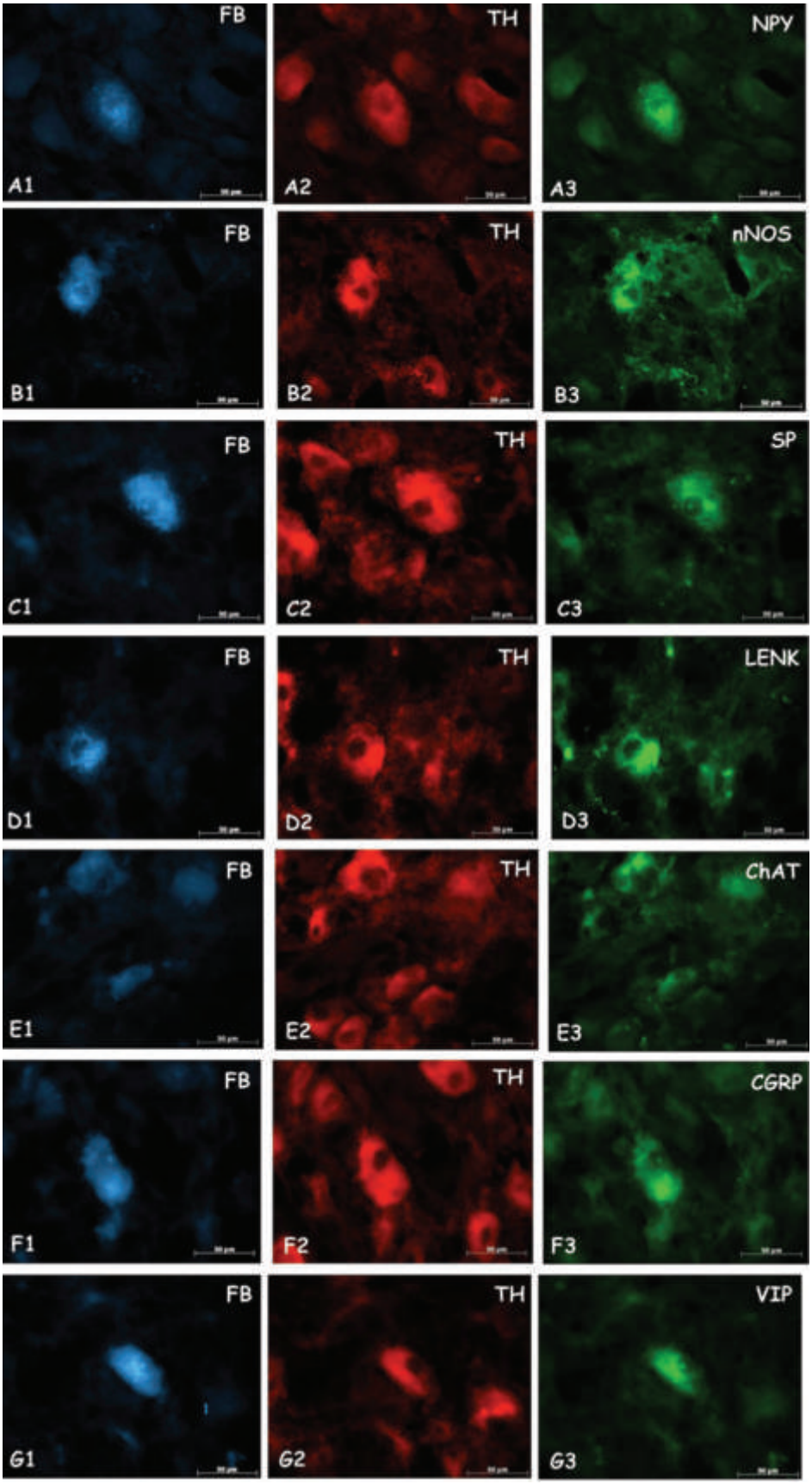

Figure 1. Fluorescence micrographs of PaG S1 FB⁺ cells showing positivity to the different couples of tested antisera Respectively: A= a FB⁺ cell (A1) showing also positivity to TH (A2) and NPY (A3); B= a FB⁺ cell (B1) showing also positivity to TH (B2) and nNOS (B3); C= a FB⁺ cell (C1) showing also positivity to TH (C2) and SP (C3); D= a FB⁺ cell (D1) showing also positivity to TH (D2) and LENK (D3); E= three FB⁺ cells (E1) showing also positivity to TH (E2) and ChAT (E3); F= a FB⁺ cell (F1) showing also positivity to TH (F2) and CGRP (F3); G= a FB⁺ cell (G1) showing also positivity to TH (G2) and VIP (G3). Scale bar = 50 μm. Magnification, 40X

jects, as was that of the TH and CGRP co-localization (3.10±1.74%, n=173.33±38.85; Figure 1, F1-F3); while the co-existence of TH and VIP was detected in only one subject (2.48±2.48%, n=160±24.56; Figure 1, G1-G3).

Among FB^+ paravertebral non-catecholaminergic neurons, a discrete proportion was NPY-IR (11.10±1.71%), while smaller percentages of cells were nNOS-IR (2.53±1.34%) or LENK-IR (1.86±1.45%). Very few neurons exhibited immunoreactivity for ChAT (0.55±0.37%) or CGRP (0.34±0.18%). No non-catecholaminergic RCM-projecting neuron, in the studied ganglia, showed immunoreactivity for VIP or for SP alone.

Discussion

TH-immunoreactivity

On the basis of the presence of the catecholamine-synthetizing enzyme (TH), the majority of the autonomic paravertebral RCM-projecting neurons is catecholaminergic in character. These neurons are probably the major source of the high number of TH-IR fibers that we have previously found in the RCM (Gazza *et al.,* 2005).

However, among the FB^+ tested cells, there was a conspicuous population of neurons that did not express positivity to TH and that therefore, should be considered either cholinergic or non-catecholaminergic/non cholinergic in character.

Noradrenaline is excitatory in nature and causes contraction of smooth muscle cells (Owman and Stjernquist, 1988; Keast, 1999; Andersson, 2000). As noradrenergic fibers are usually found in the muscular coat or around blood vessels of genital organs (Kaleczyc *et al.,* 1993; Czaja *et al.,* 1996; Andersson and Stief, 1997), and of the RCM in particular (Gazza *et al.,* 2005), it may be concluded that the TH-IR RCM-projecting neurons exert the same influence both on the smooth muscle cells of the muscle and its blood vessels.

NPY-immunoreactivity

NPY was the substance that showed the highest proportion of co-localization with TH. Our previous observations (Gazza *et al.,* 2005) of high numbers of TH/NPY-IR fibers in the sow RCM are in accordance with the present finding. Moreover, high levels of NPY in noradrenergic neurons have already been found by Lundberg *et al.* (1983) and by Klimczuk (2004) in the male pig sympathetic paravertebral neurons.

In the present study, NPY-immunoreactivity was also found in a significant number of TH-negative (TH-) neurons innervating the porcine RCM. This is in accordance with the proportion of NPY-containing cells (5-10% of the gangliar neurons) found by Lakomy *et al.* (1994) in the thoraco-lumbar paravertebral ganglia of female piglets.

Our finding is not only in agreement with the large number of NPY-IR nerve fibres observed in the RCM (Gazza *et al.,* 2005), but also, more generally, in different porcine female genital organs such as the uterus, oviduct and ovary (Häppölä *et al.,* 1991).

Since NPY is a potent vasoconstrictor of many mammalian arteries (McLachlan and Llewellyn-Smith, 1986; Lindh *et al.,* 1989; Lundberg *et al.,* 1990), these NPY-positive neurons, whether containing TH or not, could play a role in blood flow regulation (Gibbins, 1991, Lundberg *et al.,* 1983). Moreover, in the female, NPY also regulates the contractile activity of non-vascular smooth muscle cells of the uterus and oviduct, as well as the secretory function of the ovary (Markiewicz *et al.,* 2003). These regulatory functions might be performed by inhibiting the release of acetylcholine (ACh) from cholinergic nerve endings by a presynaptic mechanism (Stjernquist *et al.,* 1987; Lundberg *et al.,* 1990), or by enhancing the contractile effects of noradrenaline directly affecting postsynaptic receptors (Ekblad *et al.,* 1984).

nNOS-immunoreactivity

In the present study, nNOS showed a moderate degree of co-localization with TH within the porcine paravertebral RCM-projecting neurons. This type of immunoreactivity has not been observed either in the nerve fibres supplying the sow RCM (Gazza *et al.,* 2005) or in the fibers and neurons innervating the porcine female reproductive organs (Majewski *et al.,* 1995). Differently, the co-localization of nNOS with TH had earlier been observed in some paravertebral neurons projecting to the bulbospongiosus muscle of the male pig (Gazza *et al.,* 2003) and, but only exceptionally, in a very small number of paravertebral neurons of the cat (Anderson *et al.,* 1995), guinea-pig (Fischer *et al.,* 1996), and man (Klimaschewski *et al.,* 1996). Finally NOS has been shown to be co-localized with dopamine-beta-hydroxylase (DβH)

in the dopaminergic neurons of the bovine cranial cervical ganglion (Sheng *et al.,* 1993).

We have detected nNOS-immunoreactivity also in a small number of TH neurons projecting to the RCM. This immunoreactivity has already been demonstrated in a small number of paravertebral neurons supplying the gilt genital organs (Majewski *et al.,* 1995), and in non-noradrenergic cells (Anderson *et al.,* 1995; Fischer *et al.,* 1996), where nNOS is often co-localized with VIP or NPY.

In the porcine female genital organs, Majewski et al. (1995) found high numbers of nNOS-IR fibers only in the ovary and moderate numbers of nitrergic fibers in the oviduct, uterus and vagina. These results indicated that NO is particularly involved in the regulation of blood flow through the porcine reproductive tract. It may be postulated that NO also exerts its well known relaxing activity on the smooth muscle cells of the RCM, in addition to causing vasodilatation of its blood vessels. On the other hand, in its co-existence with TH, might play a role of modulator. In fact, it seems seem to lead to an increase in TH activity in postganglionic neurons via both cyclic GMP-dependent and -independent mechanisms (Klimaschewski *et al.,* 1996).

SP-immunoreactivity

We found a moderate degree of co-localization of SP with TH in porcine paravertebral RCM-projecting neurons. This is an unexpected finding because no co-localization of SP with TH was observed in the nerve fibres supplying the sow RCM (Gazza *et al.,* 2005) and, more generally, SP has never been found co-localized with noradrenergic markers in neurons of the female pig sympathetic paravertebral ganglia (Majewski *et al.,* 1995; Wasowicz, 2003). Nevertheless, immunoreactivity to both TH and SP has been observed in a small percentage of paravertebral neurons projecting to the pig bulbospongiosus muscle (Gazza *et al.,* 2003), in moderate numbers of neurons of the porcine urinary bladder trigone intramural ganglia (Pidsudko, 2004) and in the majority of the principal neurons of the rat superior cervical ganglion (Bohn *et al.,* 1984).

In the present study, no positivity for SP has been found in non-catecholaminergic neurons. Although SP has always been considered to be absent in neurons of pig paravertebral ganglia (Häppölä *et al.,* 1993; Lakomy *et al.,* 1994), it has been demonstrated to be present in a very small number of paravertebral neurons projecting to the pig retractor penis muscle (Botti *et al.,* 2006). Among other species, SP has been noticed only in the stellate ganglion of water buffalo (Nasu *et al.,* 2000) and cattle (Nasu *et al.,* 2003).

However, since some studies support the supposition that the failure to find SP may be due to the methods used, which might not be sensitive enough to detect minute amounts of intracytoplasmic SP (Lindh *et al.,* 1989, Häppölä *et al.,* 1993), we would conclude that our data need further investigations. If confirmed, the putative physiological significance of the co-existence of TH and SP in genital organs might be that they play a role in several visceral reflexes and neuroendocrine responses (Traurig *et al.,* 1991; Majewski *et al.,* 1995).

LENK-immunoreactivity

We found immunoreactivity for LENK, an enkephalin belonging to the opioid peptides family, in a fair number of catecholaminergic RCM-projecting cells of the PaG S1. The coexistence of LENK or other enkephalins with TH in pig paravertebral neurons has been observed only in some neurons projecting to the bulbospongiosus muscle (Gazza *et al.,* 2003), but opioid peptides have been detected in noradrenergic nerve terminals supplying both the muscle layer and some arteries of the porcine oviduct (Czaja *et al.,* 1996).

We also found LENK immunoreactivity in a small number of FB^{+}/TH^{-} neurons of the PaG S1. In the pig, the presence of opioid peptides has been noticed in neurons of the cervical (Häppölä *et al.,* 1993), thoraco-lumbar (Lakomy *et al.,* 1994) and sacral (Gazza *et al.,* 2003; Botti *et al.,* 2006) paravertebral ganglia, independently of the presence of TH. Moreover, LENK-immunoreactivity has been found in nerve processes in the interstitial connective tissue of the sow RCM (Gazza *et al.,* 2005) and, in general, opioid peptides have been seen in fibres innervating the pig female genital organs (Czaja *et al.* 1996).

The LENK-containing neurons might have a regulatory role in neurotransmission in the RCM and its vessels. In fact, the coexistence of enkephalins with noradrenaline in sympathetic ganglia (Konishi *et al.,* 1981) and in fibres innervating genital organs (De Potter *et al.,* 1987) suggests that these peptides may presynaptically modulate sympathet-

ic inputs at the neuro-effector junction (Czaja *et al.,* 1996). The same coexistence, observed in several noradrenergic perivascular nerve fibers (Owman and Stjernquist, 1988), suggests that enkephalins might also have a vasodilator effect due to their inhibitory action on autonomic neurotransmission (Konishi *et al.,* 1981; Kaleczyc *et al.,* 1997; Kaleczyc, 1998).

CGRP-immunoreactivity

The present study proved the co-localization of CGRP with TH in a small number of RCM-projecting cells. Identically coding neurons have been already observed among the ones projecting to the pig bulbospongiosus muscle (Gazza *et al.,* 2003) and in human paravertebral ganglia (Baffi *et al.,* 1992). However, like SP, CGRP is a tachykinin commonly considered to be a marker of afferent pathways (Majewski *et al.,* 1995; Kaleczyc *et al.,* 1997; Czaja, 2000); therefore, its presence within paravertebral neurons is an unforeseen finding and its co-localization with TH even more so.

The detection of only two $TH^-/CGRP^+$ neurons is another unexpected finding because CGRP-immunoreactivity has already been described in pig (Häppölä *et al.,* 1993; Lakomy *et al.,* 1994; Gazza *et al.,* 2003), cat (Lindh *et al.,* 1989) and horse (Nasu *et al.,* 2003) paravertebral ganglia.

However, our data of a very small number of $CGRP^+$ neurons are in accordance with the infrequent findings of CGRP-IR fibers in the interstitial connective tissue of the sow RCM (Gazza *et al.,* 2005) and in the homologous muscle of the male, the retractor penis muscle, where they principally supply arterial blood vessels (Majewski *et al.,* 1999).

As there is some evidence that a minor proportion of CGRP-containing nerve fibres supplying human genital organs are noradrenergic autonomic structures (Jen *et al.,* 1997), in accordance with Häppölä *et al.* (1993), we propose that they might play a functional role in the modulation of neurotransmission in the target organs of the porcine sympathetic ganglia.

VIP-immunoreactivity

We observed positivity for VIP only in a small number of FB^+/TH^+ paravertebral neurons of one subject, while no non-noradrenergic neurons showed immunoreactivity for VIP. This datum is in agreement with the small number of VIP-IR fibres found in the interstitial connective tissue of the sow RCM (Gazza *et al.,* 2005).

Until now, the co-localization of TH and VIP has been described only in sacral paravertebral neurons projecting to the pig bulbospongiosus muscle (Gazza *et al.,* 2003). However, VIP has already been found in association with DβH in some vasodilatory neurons of the porcine thoracic sympathetic chain ganglia (Hill and Elde, 1989; Majewski, 1999). The coexistence of TH and VIP is unusual for a peptide commonly thought to be a marker of the cholinergic pathway or a classical neurotransmitter of the inhibitory-non adrenergic/non cholinergic (NANC) subdivision of the autonomic nervous system (Itoh *et al.,* 1995).

In general, VIP-immunoreactivity has been found, with distinct cranio-caudal differences, in some neurons of the pig cervical (Häppölä *et al.,* 1993), thoraco-lumbar (Hill and Elde; 1989; Lakomy *et al.,* 1994) and sacral paravertebral ganglia (Botti *et al.,* 2006).

VIP-ergic neurons play a role in blood flow regulation, because, like NO, VIP acts as vasodilator on genital organ blood vessels (Polak *et al.,* 1981; Morris *et al.,* 1985; Mayewski *et al.,* 1995), and displays a physiological antagonism with NPY in the control of blood flow (Morris *et al.,* 1985).

ChAT-immunoreactivity

The co-localization of cholinergic and catecholaminergic markers, detected in a small number of RCM projecting neurons has been observed only in paravertebral neurons projecting to the pig bulbospongiosus muscle (Gazza *et al.,* 2003). On the other hand, ChAT and DβH have been found co-localized in the foetal pig superior cervical ganglion (Wang *et al.,* 1995). It is known that, during foetal development, some neurons transiently express the noradrenergic phenotype but they subsequently lose their noradrenergic characteristics, becoming cholinergic cells (Schäfer *et al.,* 1997; Keast, 1999; Masliukov and Timmermans, 2004). If so, we may suggest that, in the gilt sympathetic neurons observed herein, the development of the cholinergic marker might not have been paralleled by a complete disappearance of the various catecholamine markers.

A very scarce number of non-catecholaminergic neurons projecting to the RCM were ChAT-IR. This finding is in accordance with the fact that only single cholinergic fibers have been observed in the

interstitial connective tissue and in the muscular coat of the vessels of the pig RCM (Gazza *et al.,* 2005) and of the male homologous retractor penis muscle (Majewski *et al.,* 1999).

Only small groups of cholinergic neurons have also been observed among the paravertebral neurons projecting to the RPM in the male pig (Botti *et al.,* 2006) and in the sympathetic chain ganglia of other species such as the cat (Lindh *et al.,* 1989), rat (Schäfer *et al.,* 1997) and man (Masliukov and Timmermans, 2004). This is in accordance with the fact that the cholinergic paravertebral neurons target principally eccrine sweat glands or hindlimb blood vessels (Majewski, 1999). The small number of cholinergic structures found in the above-mentioned studies might also be due to the limited availability and sensitivity of a suitable marker for cholinergic neurons in the peripheral nervous system (Kaleczyc, 1998; Klimczuk *et al.,* 2005), or to the fact that single cholinergic nerve cell bodies and small acetylcholinesterase-positive ganglia are localized within the muscle itself, rather than in other autonomic ganglia, as has been shown in species such as the goat (Sjöstrand *et al.,* 1993) or bull (Alaranta *et al.,* 1989). Finally, in many species, including the pig, ChAT- and VAChT (vesicular acetylcholine transporter)-immunoreactivity are present in a number of endothelial cells (Haberberger *et al.,* 2000). Therefore, there would be a second source of ACh that could take part in some regulatory mechanisms.

The principal function of cholinergic innervation in the urogenital system might be the presynaptic inhibition of the adrenergic excitatory neurotransmission (Klinge and Sjöstrand, 1977; Sjöstrand *et al.,* 1993), and this could not require a high number of cholinergic neurons and nerve fibers, that, at least as far as the control of the blood flow is concerned, could be helped by the endothelial cells releasing ACh.

Conclusions

The RCM is essentially an isolated bundle of smooth musculature that, differently from other smooth muscles associated with the genital organs, is not a layer of a tubular organ or a part of the fibromuscular stroma of a gland. For this reason, we can be sure that the neurons labelled by the retrograde tracer are the ones exclusively directed to the smooth myocytes of the RCM or of its vessels and to no other tissue component (e.g. glands) of a genital organ. The simplicity of the preparation and the reliability and reproducibility of the obtained data make the RCM a useful model for the study of the innervation of the smooth muscles associated with genital organs.

The present findings suggest that the neurons of the PaG S1, that mainly contributes to the sympathetic innervation of the sow RCM, is involved, first of all, in the maintaining of the muscle tonus of both non-vascular and vascular smooth myocytes. A minor proportion of gangliar paravertebral cells may participate to the modulation (regulation) of neurotransmission or they may act as inhibitory neurons on the activity of non-vascular smooth myocytes, while neurons with only a vasodilator function seem to be very scarce.

Finally, our findings seem to confirm the presence of a previously nearly unknown small population of CGRP- and SP-IR catecholaminergic sympathetic neurons, and indicate the existence of a small proportion of tachykinergic/non-catecholaminergic sympathetic neurons.

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