IJAE
Vol. 118, n. 2: 223-239, 2013
ITALIAN JOURNAL OF ANATOMY AND EMBRYOLOGY

Research Article: Histology and Cell Biology

Double labelling immunohistochemistry on the sympathetic trunk ganglia neurons projecting to the extrinsic penile smooth musculature of the pig: an experimental study on the retractor penis muscle

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Submitted December 10, 2012; accepted revised April 20, 2013

Summary

Retrograde neuronal tracing and double labelling immunofluorescence methods were used to define the neurochemical content of sympathetic trunk ganglia neurons projecting to the pig retractor penis muscle, which was taken as an experimental model of the male genital smooth musculature.

After the injection of Fast Blue into the bulbo-penile portion of the retractor penis muscle, the eventual co-existence of the catecholaminergic marker tyrosine hydroxylase with calcitonine gene related peptide, leu-enkephalin, neuropeptide Y, neuronal nitric oxide synthase, substance P, vasoactive intestinal polypeptide or vesicular acetylcholine transporter was studied in the ipsilateral S1 sympathetic trunk ganglia, which resulted to contain the greatest number of autonomic retractor penis muscle projecting cells.

The observation of Fast Blue positive neurons under the fluorescent microscope allowed the identification of different subpopulations of catecholaminergic and non-catecholaminergic retractor penis muscle-projecting neurons. The majority of catecholaminergic cells contained tyrosine hydroxylase alone, while the remaining part showed co-localization of tyrosine hydroxylase with all the other tested markers. These last neurons were immunoreactive, in decreasing percentages, for neuropeptide Y, leu-enkephalin, neuronal nitric oxide synthase, substance P, calcitonine gene related peptide, vasoactive intestinal polypeptide and vesicular acetylcholine transporter.

The majority of non-catecholaminergic neurons were immunonegative for all the tested markers. The remaining non-catecholaminergic cells contained, in decreasing percentages, neuropeptide Y, neuronal nitric oxide synthase, leu-enkephalin, vasoactive intestinal polypeptide, vesicular acetylcholine transporter, substance P and calcitonine gene related peptide.

Our findings documented the complexity of the neurochemical interactions that regulate both the motor functions of RPM and the blood flow through the muscle.

Key words

Immunohistochemistry; retrograde tracing; sympathetic trunk ganglia; genital smooth musculature; retractor penis muscle; pig.

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Key to abbreviations:

CGRP: Calcitonine Gene-Related Peptide
CMG: caudal mesenteric ganglion
FB: Fast Blue
LENK: Leu-Enkephaline
nNOS: neuronal Nitric Oxide Synthase
NPY: Neuropeptide Y
RPM: retractor penis muscle
SG: spinal ganglia
SP: Substance P
STG: sympathetic trunk ganglia
TH: Tyrosyne hydroxylase
VAChT: Vesicular Acetylcholine Transporter
VIP: Vasoactive Intestinal Peptide

Introduction

The smooth, paired retractor penis muscle (RPM) is a genital muscle associated to the penis, that originates from the ventral side of the first coccygeal vertebrae (boar, stallion, bull, goat) or from the rectus (ram, rat), and terminates on the urethral side of the body of the penis (Basset, 1961; Palmieri et al., 1983; Dail et al., 1990; Bo Minelli et al., 1993). The muscle is absent in the rabbit (Langley and Anderson, 1895; Craigie, 1948), some primates (Paulet, 1877) and man (Nishi, 1938).

Differently from the other smooth muscles of genital organs, the RPM does not form one of the organ layers and it is in tonic contraction in the resting phase and relaxed in the active phase. For these peculiarity, the RPM of different species has been used as an experimental model to evaluate the neurological responses of the smooth genital musculature when submitted to particular chemical and physical stimuli, and to study the neurotransmitters involved in nervous transmission (Klinge et al., 1970; Eränkö et al., 1976; Cottrell et al., 1978; Gillespie et al., 1981; Bowman and Drummond, 1984; Alaranta et al., 1989; Liu et al., 1991; Sheng et al., 1992; Martin et al., 1993; Sjöstrand et al., 1993; Majewski et al., 1999; La et al., 2001).

Moreover, the fact that the muscle is easily identifiable and of simple and versatile preparation makes it an interesting experimental model, that we have used for several years to define its innervation. Using gold chloride or silver impregnation techniques we have shown the presence of a sensory innervation and of autonomic, presumibly parasympathetic neurons in the pig, horse, bull, goat and ram RPM and in its fascial connective tissue (Palmieri et al., 1983; Bo Minelli et al., 1993). Using the retrograde neuronal tracer Fast Blue (FB) we have documented the site of the peripheral, autonomic postganglionic neurons and of the spinal, sensitive neurons projecting to the pig RPM. The former are located bilaterally in the lumbosacral (L2-S3) sympathetic trunk ganglia (STG) and in the prevertebral caudal mesenteric ganglia (CMG), the latter, also bilaterally, in the sacral (S1-S3) spinal ganglia (SG) (Panu et al., 2003). The association of the retrograde neuronal tracer FB and single labelling immunofluorescence allowed us to make a preliminary screening on the neurochemi-

cal content of the peripheral STG, CMG and SG neurons related to the pig RPM (Botti et al., 2006a). In this screening, tyrosine hydroxylase (TH), the rate limiting enzyme of catecholamine synthesis, was shown to be the most abundant substance in the autonomic neuron subset.

On the basis of these data, the present study was aimed at defining, within the neurons of the S1 STG, the eventual co-existence of TH with: calcitonine gene related peptide (CGRP), leu-enkephalin (LENK), neuropeptide Y (NPY), neuronal nitric oxide synthase (nNOS), substance P (SP), vasoactive intestinal polypeptide (VIP) or vesicular acetylcholine transporter (VAChT).

This 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 neuro-anatomical studies (Merighi et al., 1990; Timmermans et al., 1993; Kaleczyc et al., 2002; Boratynski and Welento, 1996; Majewski et al., 1999; Panu et al., 2001, 2003; Botti et al., 2006a, 2006b, 2009, 2012).

Preliminary data of this investigation have been published in abstract form (Botti et al., 2002).

Matherials 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. For 24 h prior to surgery, the animals were given no food and were administered preventive antibiotic therapy with intramuscular Ceftiofur (Naxcel 5 mg/kg; Pfizer, Sandwich Kent, U.K.). The animals were sedated by intramuscular injection of azaperone (4-10 mg/10 kg; Stresnil, Janssen Cilag, Cologno Monzese, Italy) and ketamine (Ketavet 100, 150 mg/10 kg;, Intervet Italia, Aprilia, Italy). After recumbency and venous catheterization (*vena auricularis caudalis*), anaesthesia was induced by the administration of propofol (Rapinovet, 2-6 mg/kg; Schering Plough, Segrate, Italy) and maintained with 1.5% to 2% isofluorane in 100% oxygen delivered by an open circuit, via a cuffed Magill orotracheal tube. The post-operative anti-inflammatory effect was achieved by a daily intramuscular injection of tolfedine (Temgesic, 2 mg/kg; Schering Plough) for 5 days post operatively.

The study was carried out on the RPM of 4 castrated 50 Kg-weighting pigs, by means of the retrograde neuronal tracer Fast Blue (FB) technique combined with double labelling immunofluorescence methods. The castration was carried out when the pigs are 2-3 days old, while our experimentation was carried out when they are 3 months old, so the surgical trauma should not have influenced the results.

Under general anaesthesia, the RPM was identified in the uro-genital region through an incision in the skin and tela subcutanea. The bulbo-penile portion (Panu et al., 2003) of the left muscle was inoculated with 50 μl of 2% w/v FB, a fluorescent tracer with cytoplasmatic affinity. The fascial connective tissue of the muscle represented a barrier capable of preventing the dispersion of the tracer in the surrounding tissues.

After 7 days the animals, under general anaesthesia, were perfused intracardially with heparinized physiological solution followed by fixative solution (4% w/v of paraformaldehyde in 0.1 M phosphate buffer, pH 7.4).

The ipsilateral S1 STG, that contained the greatest number of FB+ neurons (Panu et al., 2003), was removed from each animal, post-fixed by immersion in the same fixative for 2 hours at 4 °C, rinsed with phosphate buffer (pH 7.4) and transferred into a 10% w/v buffered sucrose solution (pH 7.4) for 24 hours. 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.

Serial sections 12 mm thick were stained, using a double immunofluorescence method, to test the possible co-localization of TH with CGRP, LENK, NPY, nNOS, SP, VIP or VAChT (see Table 1). The same combinations of primary antisera were applied to sections separated by at least 96 mm from each other. This strategy eliminated the likelihood of testing the same neuron twice for the same couple of antisera. All the labelling procedure was carried out at room temperature; primary antisera and secondary reagents are listed in Table 1. After air-drying for 30 minutes 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 hour, to reduce non-specific back ground staining. Then they were incubated with the combination of primary antisera

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

Primary antibody	Raised in	Code no.	Dilution	Supplier
Anti TH	Mouse (monoclonal)	T 2928	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Anti CGRP	Rabbit (polyclonal)	C 8198	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Anti LENK	Rabbit (polyclonal)	L 8516	1:5	Sigma, St. Louis, Missouri, U.S.A.
Anti n-NOS	Rabbit (polyclonal)	AB 5380	1:1500	Chemicon International, Inc., Temecula, CA
Anti NPY	Rabbit (polyclonal)	N 9528	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Anti SP	Rabbit (polyclonal)	S 1542	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Anti VAChT	Rabbit (polyclonal)	V 5387	1:500	Sigma, St. Louis, Missouri, U.S.A.
Anti VIP	Rabbit (polyclonal)	V 3508	1:4000	Sigma, St. Louis, Missouri, U.S.A.
Secondary reagents				
Anti rabbit IgG/FITC	Goat	F 0382	1:40	Sigma, St. Louis, Missouri, U.S.A.
Anti mouse IgG/Biotin	Sheep	RPN 1001	1:100	Amersham Pharmacia Biotech Sweden
Streptavidin/Texas Red		RPN 1233	1:100	Amersham Pharmacia Biotech Sweden
Normal serum				
Goat serum		G 9023		Sigma, St. Louis, Missouri, U.S.A.

(overnight), further with a mixture of fluoroscein isothiocyanate (FITC)-conjugated goat antirabbit IgG and biotinylated goat anti-mouse IgG (1 hour), finally with Texas Red-conjugated streptavidin (1 hour) and mounted in buffered glycerin (Bio-Optica, Milan, Italy). Each step of immunolabelling was followed by rinsing the sections with PBS (3x5 min; pH 7.4). Standard tests (preabsorption with the neuropeptides, omission of the primary antisera) were applied to control the specificity of immunofluorescence. The labelled sections were studied and photographed with a Zeiss Axioskop 2 plus fluorescence microscope equipped with epi-illumination and appropriate filter sets for FB (excitation wavelenght 390-420 nm; emission wavelenght 450 nm), FITC (excitation wavelenght 450-490 nm; emission wavelenght 515-565 nm) and Texas Red (excitation wavelenght 530-585 nm; emission wavelenght 615 nm). Relationships between immunohistochemical staining and FB distribution were examined directly by interchanging filters.

In each ganglion, the relative number of FB+ neurons were counted in every third sections and only the perikarya with clearly visible nucleus were counted.

The relative percentages of neurons containing different combination of the markers were calculated on the total number of FB+ cells tested for each couple of primary antisera. Data are expressed as means ± standard error among the 4 animals.

Results

In the studied ganglion (S1 STG sx) there were 1228.08±449.01 RPM projecting neurons. Serial sections 12 mm thick allowed to test 470.11±12.78 neurons for the different couples of antisera. Among the FB+ neurons tested for different combinations of markers, two subpopulations of either catecholaminergic (TH+) (59.19%±11.31) or non-catecholaminergic (TH-) (40.81%±11.31) cells were identified. The highest proportion of co-localization was found for TH and NPY (22.18%±1.62%, n=466.5±143.47) (Fig. 1 a, b, c). TH and LENK resulted co-localized in appreciable but lower percentage (13.10%±7.94%, n=424±169.67) (Fig. 1 d, e, f). Minor percentages of co-localization were found for TH and nNOS (4.32%±1.50%, n=523.5±182.41) (Fig. 1 g, h, i), TH and SP (3.41%±1.54%, n=491.75±164.24) (Fig. 1 l, m, n), TH and CGRP (2.80%±2.26%, n=488.25±207.91) (Fig. 1 o, p, q), TH and VIP (2.61%±1.40%, n=456.5±259.53) (Fig. 1 r, s, t) and TH and VAChT (2.42%±1.46%, n=440.25±169.91) (Fig. 1 u, v, z).

Among FB+ paravertebral, non-catecholaminergic neurons an appreciable proportion was NPY+ (8.87%±3.74.2%), while small numbers were nNOS+ (4.53%±3.55%), LENK+ (1.54%±0.95%) and VIP+ (1.16%±0.69%). Neurons immunoreacrtive for VAChT (0.76±0.76%), SP (0.37%±0.37) and CGRP (0.08±0.08%) were found only in one subject. (see Table 2).

Discussion

The combined use of the retrograde neuronal tracer FB and double labelling immunofluorescence allowed us to define the neurochemical characteristics of autonomic STG neurons projecting to the pig RPM. In particular, among the FB+ neurons two subpopulations were identified, catecholaminergic (TH+) and non-catecholaminergic (TH-) cells.

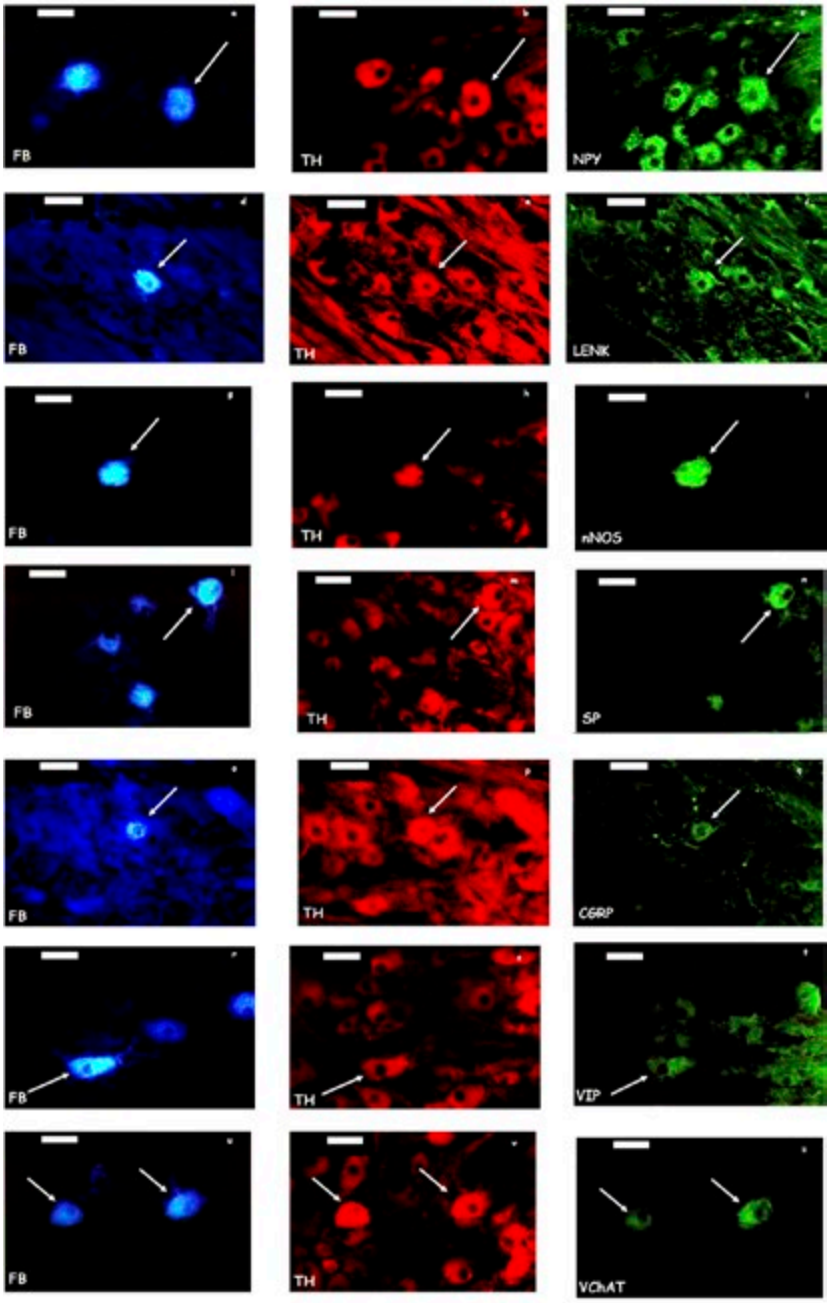

Figure 1 – Photomicrographs showing the labelled neurons of the left, ipsilateral, S1 sympathetic trunk ganglion projecting to the pig retractor penis muscle. The left column represents Fast Blue staining, the central column represents thyrosine hydroxylase immunolabelling and the right column represents the labelling for different neurotramsnitters, as follows: NPY (c), LENK (f), nNOS (i), SP (n), CGRP (q), VIP (t), VAChT (z). Scale bars = 50 μm.

Table 2 – Percentages of immunoreactivity (mean ± standard error among 4 animals) for the combinations of antibodies tested on Fast Blue positive (FB+) neurons in the sympathetic trunk ganglion S1. The meaning of the abbreviations used for the neurotransmitter molecules is given in the key to abbreviations.

Neurotrasmitter and enzyme antigens (AG) tagged together with TH	TH+/AG+	TH+/AG-	TH-/AG+	TH-/AG-	FB+ cells
CGRP	2.80 ± 2.26%	56.39 ± 10.26%	0.08 ± 0.08%	40.73 ± 11.36%	488.25 ± 207.91
LENK	13.10 ± 7.94%	40.74 ± 3.27%	1.54 ± 0.95%	44.61 ± 8.15%	424 ± 169.67
nNOS	4.32 ± 1.50%	44.90 ± 6.71%	4.53 ± 3.55%	46.26 ± 8.11%	523.5 ± 182.41
NPY	22.18 ± 1.62%	32.34 ± 8.90%	8.87 ± 3.74%	36.60 ± 6.51%	466.5 ± 143.47
SP	3.41 ± 1.54%	54.19 ± 8.34%	0.37 ± 0.37%	42.03 ± 9.59%	491.75 ± 164.24
VAChT	2.42 ± 1.46%	49.63 ± 7.69%	0.76 ± 0.76%	47.19 ± 8.56%	440.25 ± 169.91
VIP	2.61 ± 1.40%	48.88 ± 6.99%	1.16 ± 0.69%	47.35 ± 7.90%	456.5 ± 259.53

TH - The abundance of FB+ neurons immunoreactive only for TH was a costant finding, as other researchers have already documented in the STG ganglia of the pig (Hill and Helde, 1989; Lakomy et al., 1994a, Zalecki, 2012), the guinea pig (Heym et al., 1990), the rat (Carrillo et al., 1991; Vanhatalo et al., 1996; Kepper and Keast, 1997) and the cat (Lindh et al., 1989a). Perhaps the most unexpected finding of our study was the relatively small percentage of immunoreactivity for TH. This result could be due to the fact that the animals used in our experiments were castrated. However the use of intact animals of the same age would lead to the same results. In fact it is known that the immunohistochemical properties of sympathetic neurons can change with age (Maslyukov et al., 2007) and that the autonomic neurons innervating the genital organs are influenced by steroid hormones (Keast, 1999; 2000; Sienkiewicz, 2010). Colenbrander et al. (1978) studied the changes in serum testosterone concentration in male pigs during development, and found it to be highest in the 2nd and 3rd week after birth and decline thereafter. Serum testosterone concentration then remained relatively low until the 18th week of age, when it rose significantly. Therefore, the low level of serum testosterone in three-month old boars are similar to the one of castrated pigs of the same age and would lead to the same percentages of catecholaminergic neurons that we found in our study.

The catecholaminergic neurons, that we found, are involved in excitatory functions, in fact it is commonly accepted that they influence the contraction of the smooth muscular coat of the organs (Owman and Stejernquist, 1988; Andersson, 2000; Andersson et al., 2000) and the blood flow through the organs (Kaleczyc et al., 1993; Majewski et al., 1995; Czaja et al., 1996; Andersson and Stief, 1997). However, the contraction of the smooth muscles might not be the only possible function for the labelled catecholaminergic neurons observed. In fact, in accordance with what has been reported by Zalecki (2012) about the innervation of the porcine pylorus,

the sympathetic fibers have an excitatory function mediated by beta-adrenoreceptors located on postganglionic cholinergic fibers, the activation of which result in acetylcholine release, but they may also have an inhibitory function mediated mainly by alpha-adrenoreceptors located on the excitatory cholinergic nerves and by beta-adrenoreceptors present in the muscles.

TH and/or NPY - Among the catecholaminergic RPM projecting paravertebral neurons, the higher proportion of co-localization was found for TH and NPY (22.18%±1.62%). Similar results had already been found in the pig STG (Lindh et al., 1989a; Hill and Elde, 1989; Kaleczyc et al., 1993; Lakomy et al., 1994a; Zalecki, 2012). The neurons immunoreactive for TH and NPY, that we found, could be the predominant source of the TH+/NPY+ fibres found by Majewski et al. (1999) surrounding penile blood vessels and in lower proportion supplying intrinsic smooth musculature of the RPM, according to Dail et al., 1989; Diederichs et al., 1990; Carrillo et al., 1991. These neurons could have a vasoconstrictor function, according to earlier research conducted on other species (Heym et al., 1990; Trudrung et al., 1994; McLachlan, 1995; Majewski, 1999; Gibbins and Morris, 2000).

Some FB+ STG neurons immunoreactive for NPY innervating the porcine RPM were non-catecholaminergic (8.87%±3.74%). Our finding is in agreement with other studies reporting NPY as a transmitter not only of the catecholaminergic but also of the non-adrenergic non-cholinergic (NANC) component of the penile innervation (Schmalbruch and Wagner, 1989; Carrillo et al., 1991; Crowe et al., 1991), that is thought to have a role in the control of local blood flow and in the motor function of the genital tract.

TH and/or LENK - TH and LENK were co-localized in discrete percentages within the RPM projecting STG neurons (13.1%±7.94%). TH+/LENK+ neurons had been already found in porcine STG and prevertebral ganglia (Lakomy et al., 1994b; Kaleczyc et al., 1993; 1995). We think that these RPM-projecting paravertebral neurons might be the source of the TH+/LENK+ fibres found by Majewski et al. (1999) in the RPM.

A little proportion of FB+ cells immunoreactive for LENK were non-catecholaminergic (1.54%±0.95%). Opioid peptides have been found in the pig both in neurons of the cervical (Happola et al., 1993) and thoraco-lumbar sympathetic trunk (Lakomy et al., 1994b) and in prevertebral ganglia (Lakomy et al., 1993; 1994b; Kaleczyc, 1998).

In catecholaminergic and non-catecholaminergic neurons, LENK probably performs an inhibitory role on the release of noradrenaline and causes significant relaxation, as can be desumed from pharmacological studies (Kastin et al., 1978; Konishi et al., 1979; 1981; Stjernquist et al., 1983; 1987; Katayama and Nishi, 1984; De Groat, 1987; Iravani and Zarr, 1994).

TH and/or nNOS - TH and nNOS were co-localized in a small percentage of FB+ neurons (4.32%±1.5%). The same co-localization was found only in the sow paracervical ganglion (Czaja et al., 2001), in the cat STG ganglia (Anderson et al., 1995), in the bovine cranial cervical ganglion (Sheng et al., 1993) and in the human and guinea pig pelvic ganglion (Jen et al., 1996; Elfvin et al., 1997). Moreover, the co-localization of nNOS with TH had earlier been observed in some STG neurons projecting to the bulbospongiosus muscle (Gazza et al., 2003) of the male pig and to the retractor clitoridis muscle of the sow (Ragionieri et al., 2008). When co-existing with TH it might play a modulator role. In fact, it seems to lead to an increased TH activity in postgangli-

onic neurons via both cyclic GMP-dependent and independent mechanisms (Klimaschewski et al., 1996).

nNOS immunoreactivity was also found in a moderate number of non-catecholaminergic RPM-projecting neurons (4.53%±3.55%).

Our results may explain the origin of part of the nNOS+ terminals within the boar penis (Majewski et al., 1999), that are numerous around blood vessels and scarce around non-vascular smooth muscles. Such distribution may implicate the involvement of NO in the relaxation of penile blood vessels rather than in the control of RPM functions. In fact, NO is known both as a powerful vasodilator and a relaxant substance for non-vascular smooth muscle (Moncada et al., 1991; Andersson and Wagner, 1995; Mizusawa et al., 2001).

TH and/or SP or CGRP - A small proportion of catecholaminergic RPM projecting neurons contained SP (3.41%±1.54%) and CGRP (2.8%±2.26%). These findings are quite unusual, because also if SP and CGRP have been found in the STG neurons projecting to the pig bulbospongiosus muscle (Gazza et al., 2003), the sow retractor clitoridis muscle (Ragionieri et al., 2008) and the human pelvic ganglion (Jen et al., 1996), they are commonly considered markers of the afferent pathways (Maggi, 1995; Czaja, 2000). Indeed, Majewski et al. (1999) classified as sensory the infrequent CGRP+ and SP+ fibres in the porcine RPM, principally supplying blood vessels.

The presence of these two substances in non-catecholaminergic neurons was an incostant finding, occurring only in one subject. On the other hand, the presence of CGRP+ neurons has already been described inside STG of the male pig (Hill and Elde, 1989; Happola et al., 1993; Lakomy et al., 1994a) and in the prevertebral caudal mesenteric ganglia of the female pig (Majewski and Heym, 1992).

TH and/or VIP - A little percentage of catecholaminergic RPM projecting neurons showed VIP immunoreactivity (2.61%±1.4%). VIP has been found co-localized with markers of the presence of noradrenaline in some neurons of the porcine sympathetic chain ganglia (Hill and Helde, 1989) and of the human pelvic ganglia (Jen et al., 1996). In a previous study we had already found VIP in a small number of FB+/TH+ paravertebral neurons projecting to the sow retractor clitoridis muscle (Ragionieri et al., 2008). However this is an unusual co-existence for a peptide commonly thought to be a marker of cholinergic or NANC sympathetic nerve fibres (Majewski et al., 1999; Hedlund et al., 1999; 2000; Andersson, 2000; Andersson et al., 2000; Mizusawa et al., 2001).

VIP was also found in a very few percentage of non-catecholaminergic neurons (1.16%±0.69%). This marker has been already described in the pig cervical (Happola et al., 1993) and thoracic (Hill and Helde, 1989; Lakomy et al., 1994a) STG and in the pig prevertebral ganglia (Lakomy et al., 1993; 1996). As many authors affirm that the numerous VIP+ intrapenile fibres originate from local pelvic ganglia, being therefore parasympathetic (Dail et al., 1983; Keast and De Groat, 1989; Domoto and Tsumori, 1994), we think that the few VIP+ RPM projecting neurons, that we found, cannot represent the only source of the large number of VIP+ (predominantly NANC) fibres found by Majewski et al. (1999) in the boar penis and RPM.

TH and/or VAChT - A rather small proportion of RPM projecting neurons were immunoreactive for TH and VAChT (2.42%±1.46%). The co-existence of adrenergic and cholinergic markers is quite unusual. It is possible that this finding is rooted in embryology, in fact it is known that there is a developmental switch from catecholaminergic to cholinergic cells (Landis and Keefe, 1983; Landis, 1983; Schotzinger and

Landis, 1988; Schafer et al., 1997; Dixon et al., 1999). In particular it has been proved that catecholaminergic and cholinergic markers are co-localized in the fetal pig superior cervical ganglion (Wang et al., 1995) and in the STG neurons projecting both to the pig bulbospongiosus (Gazza et al., 2003) and to the sow retractor clitoridis (Ragionieri et al., 2008) muscles. So we think that this switch might be incomplete in the animals that we used, because they were 3 or 4 months old and the development of the cholinergic marker might not yet have been paralleled by complete disappearance of the cathecolamine marker.

The presence of non-catecholaminergic VAChT+ neurons was an incostant finding, observed only in one subject. This datum is in accordance with the moderate number of cholinergic terminals found by Majewski et al. (1999) in the porcine RPM and with the small number of cholinergic neurons found in the caudal mesenteric ganglia of the pig (Pidsudko et al., 2001), guinea pig (Sann et al., 1995; Elfvin et al., 1997), rat and ferret (Sann et al., 1995) and in the STG of the cat (Lindh et al., 1989a, b), rat (Morales et al., 1995; Schafer et al., 1997; 1998) and man (Jarvi et al., 1989). However it is generally believed that the cholinergic pathway operate through neurons of the pelvic plexus (Dail and Hamill, 1989; Sjostrand et al., 1998; Andersson, 2000; Andersson et al., 2000).

In conclusion, the present study documented the complexity of the neurochemical interactions that regulate both the motor functions of RPM and the blood flow through the muscle. Immunohistochemistry has revealed two subpopulations of catecholaminergic and non-catecholaminergic cells. Both the catecholaminergic and non-catecholaminergic neurons often express NPY. An appreciable proportion of the noradrenrgic neurons has shown also immunoreactivity to LENK and nNOS, whereas only a small proportion of them are SP+, CGRP+, VIP+ or VAChT+. Additionally, a very small proportion of the non-catecholaminergic RPM-projecting cells are cholinergic. We conclude that the pig STG are a prominent source of catecholaminergic innervation and that co-transmission represent a mechanism commonly employed by the sympathetic autonomic neurons to modulate the release of noradrenaline and achieve different and precise control of both vascular and non-vascular smooth musculature. Moreover, as non-catecholaminergic fibres are commonly thought to be parasympathetic and originating in pelvic ganglia in close proximity to the target organs, our finding of a few non-catecholaminergic NPY+, VIP+, nNOS+, CGRP+ or VAChT+ neurons in the pig STG raises the possibility that cells stimulating vasodilation or inhibiting smooth musculature contraction may not only be attributed to the parasympathetic innervation.

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