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Distribution and chemical coding of neurons in intramural ganglia of the porcine urinary bladder trigone

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Abstract: This study presents the distribution and chemical coding of neurons in the porcine intramural ganglia of the urinary bladder trigone (IG-UBT) demonstrated using combined retrograde tracing and double-labelling immunohistochemistry. Retrograde fluorescent tracer Fast Blue (FB) was injected into the wall of both the left and right side of the bladder trigone during laparotomy performed under pentobarbital anaesthesia. Ten-μm-thick cryostat sections were processed for double-labelling immunofluorescence with antibodies against tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), neuropeptide Y (NPY), somatostatin (SOM), galanin (GAL), vasoactive intestinal polypeptide (VIP), nitric oxide synthase (NOS), calcitonin gene-related peptide (CGRP), substance P (SP), Leu^5-enkephalin (LENK) and choline acetyltransferase (ChAT). IG-UBT neurons formed characteristic clusters (from a few to tens neuronal cells) found under visceral peritoneum or in the outer muscular layer. Immunohistochemistry revealed four main populations of IG-UBT neurons: SOM- (*ca.* 35%), SP- (*ca.* 32%), ChAT- and NPY- immunoreactive (-IR) (*ca.* 23%) as well as non-adrenergic non-cholinergic nerve cells (*ca.* 6%). This study has demonstrated a relatively large population of differently coded IG-UBT neurons, which constitute an important element of the complex neuro-endocrine system involved in the regulation of the porcine urogenital organ function.

Key words: Intramural ganglia - Urinary bladder trigone - Retrograde tracing - Neuropeptides - Neurotransmitter synthesising enzymes - Pig

Introduction

The function of the lower urinary tract to store and periodically release urine is dependent on neural circuits located in the brain, spinal cord and peripheral ganglia. This dependence on the central nervous control distinguishes the lower urinary tract from many other visceral structures (*e.g.* the gastrointestinal tract and cardiovascular system) that maintain a certain level of function even after elimination of the extrinsic neural input. It has long been known that ganglion cells are present within the wall of the bladder in addition to those in the caudal mesenteric ganglion (CaMG) and pelvic ganglia. There is some interspecies variation in the occurrence of these intramural ganglia. They are numerous in the guinea-pig [6], whereas fewer are found in cats, ferrets and rabbits [15, 18].

It has been well recognized so far that the innervation of the urinary bladder is supplied by three sets of peripheral nerves: sacral parasympathetic (pelvic nerves; consisting of mainly preganglionic fibres supplying the intramural ganglia as well as of postganglionic fibres; [5, 6, 17, 22, 34, 42]), thoracolumbar sympathetic (hypogastric nerves; their fibres are mainly postganglionic, and a few preganglionic supply so called "short adrenergic neurons" found within ganglia located very close to pelvic organs; [14, 16]) and sacral sensory (pudendal nerves; [8, 9]). Thus, urinary bladder-projecting neurons (UB-PN) are located in different autonomic and sensory ganglia including intramural ganglia of the urinary bladder trigone (IG-UBT).

Since our knowledge on the distribution and chemical coding of the IG-UBT neurons in the pig is very limited, combined retrograde tracing and double-immunolabelling were used to elucidate the exact localisation and neurochemical features of IG-UBT neurons involved in this neural pathway.

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Materials and methods

The study was performed on 5 juvenile female pigs (10 kg. of body weight) of the Large White Polish breed. All the animals were housed and treated in accordance with the rules approved by the local Ethical Commission (conforming to the "Principles of Laboratory Animal Care", NIH publication No. 86-23, revised 1985).

The surgery was performed under pentobarbital (Vetbutal, Biowet, Poland; 20 μl/kg b.w., i.v.) anaesthesia. Thirty minutes prior

Table 1. Antisera used in this study

Antigen	Host	Code	Dilution	Supplier
Primary Antisera				
DBH	rabbit	DZ 1020	1:500	Affiniti, UK
CGRP	rabbit	RPN 1842	1:1600	Amersham, UK
TH	mouse	1017381	1:40	Boehringer Mannheim, GER
ChAT	rabbit	AB 143	1:800	Chemicon, UK
GAL	rabbit	4600-5004	1:1600	Biogenesis, UK
NOS	rabbit	B 220-1	1:2000	Euro Diagnostica
SOM	rat	8330-0009	1:30	Biogenesis, UK
SP	rat	8450-0505	1:250	Biogenesis, UK
VIP	rabbit	20077	1:200	Incstar, MO, USA
NPY	rabbit	NA 1233	1:400	Affiniti, UK
NPY	rat	NZ 1115	1:200	Affiniti, UK
LENK	rabbit	EA 1149	1:600	Affiniti, UK
Secondary Reagents				
FITC-conjugated goat anti-rabbit IgG			1:400	Jackson Immunores. Lab., USA
FITC-flourolink goat anti-mouse IgG			1:400	Jackson Immunores. Lab., USA
FITC-flourolink goat anti-rat IgG			1:400	Jackson Immunores. Lab., USA
Biotinylated goat anti-rabbit IgG			1:400	Dako, DK
Biotinylated goat anti-rat IgG			1:400	Amersham, UK
Biotinylated goat anti-mouse IgG			1:400	Amersham, UK
Cy3-conjugated streptavidin			1:4000	Dianova, Hamburg, GER

to the main anaesthetic was given, the pigs were pretreated with propionylpromazine (Combelen, Bayer, Germany; 0.4 mg/kg b.w., i.m.). During a mid-line laparotomy the dorsal wall of the urinary bladder was gently exposed and the fluorescent retrograde neuronal tracer Fast Blue (FB; Dr K. Illing KG & Co, Gro-Umstadt, Germany) was injected into the left and right side of the bladder trigone, in a total volume of 50 µl of 5% FB solution. Each side was injected 25 times (1 µl of the dye solution per 1 injection, under the serosa, by a Hamilton syringe equipped with a 26G needle) along the whole extension of the urinary bladder trigone keeping a similar distance between the places of the injections. To avoid leakage, the needle was left in place for one minute. The wall of the injected organ was then rinsed with physiological saline and gently wiped with gauze.

After a survival period of three weeks, the animals were reanaesthetised and transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). The urinary bladder trigones were cut out and the pieces postfixed by immersion in the same fixative for 2 h. Then, they were washed with phosphate buffer (pH 7.4), and finally transferred to and stored in 18% buffered (pH 7.4) sucrose solution until further processing.

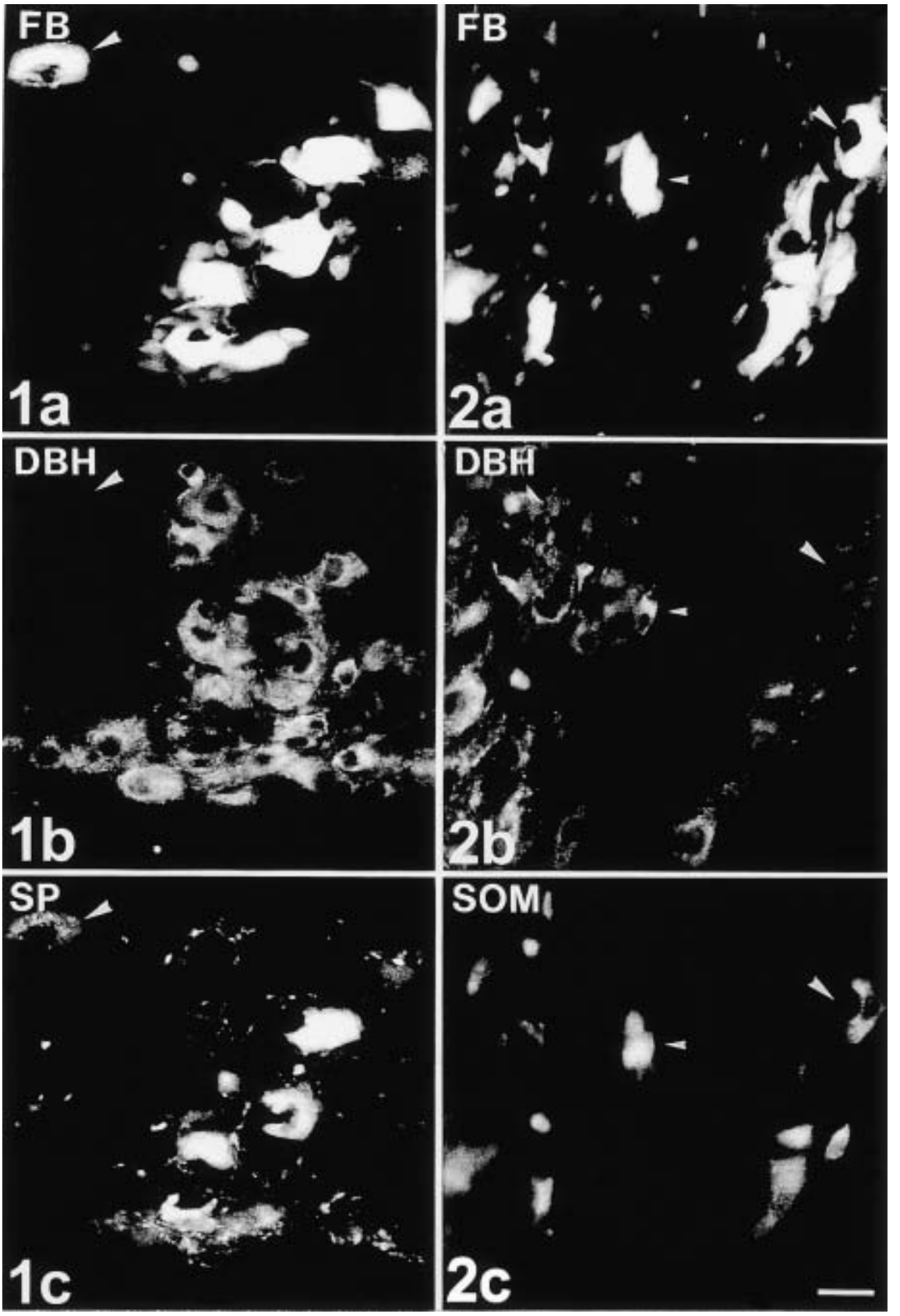
Transversal sections of the trigone of the urinary bladder were cut into 10 µm-thick serial cryostat sections. FB-labelled cell counts were done prior to immunohistochemistry. To determine the relative number of the IG-UBT UB-PN, the neurons were counted in every fifth section of the ganglia in all the animals. Only neurons with a clearly visible nucleus were considered. All the sections containing retrogradely labelled nerve cells were processed for double-labelling immunofluorescence with antibodies listed in Table 1 and labelling techniques were applied as described previously [39].

Standard tests (preabsorption for the neuropeptide antisera, omission and replacement by non-immune sera of all the primary antisera used) were applied to control the specificity of immunofluorescence. The labelled sections were examined and photographed with a Zeiss Axiophot fluorescence microscope equipped with epi-illumination and an appropriate filter set for FITC, Texas Red and FB, and with confocal microscope (Bio-Rad Microradiance MR 2). Relationships between immunohistochemical staining and FB distribution were examined directly by interchanging filters.

Results

Up to 80% of IG-UBT, found under visceral peritoneum or in the outer muscular layer of the trigone were formed by clusters of neuronal cells (from a few to tens of perikarya). The remaining ganglia were distributed on the opposite site of the wall of the urinary bladder. They

Fig. 1a-c. Intramural ganglia of the urinary bladder trigone (IG-UBT). Some FB-positive neurons (**a**) co-exhibit DBH- (**b**; TXR visualisation, applies to all the figures) and SP-immunoreactivity (**c**; FITC visualisation, applies to all the figures; arrowhead shows SP-positive neuron only). Scale bar = 25 µm. **Fig. 2a-c.** IG-UBT. A loose cluster of FB-positive neurons (**a**), one of them (small arrowhead) is DBH-(**b;** small arrowhead) and SOM-positive (**c,** small arrowhead; large arrowhead shows a neuron which was SOM-positive only). Scale bar = 25 µm.


FB
1a
FB
2a
DBH
1b
DBH
2b
SP
1c
SOM
2c

also consisted of up to tens neuronal cells. Numbers of counted Fast Blue-positive (FB^+) neurons in all the ganglia studied were pooled and then presented as mean ±SEM. Of 74 IG-UBT studied in the present study, 63 were found inside and 11 outside of the trigone. In all the ganglia under study, altogether 6173 of FB^+ neurons were found, *i .e.*, 1235 ± 106.3 per animal.

Of all the neurons studied, approximately 52% belonged to the subset of noradrenergic cells (they contained colocalized TH and DBH), approximately 23% were cholinergic (as may be judged from the existence of ChAT in their somata) and approximately 6% has been considered NANC, on the basis of the comparison of consecutive sections.

Further immunolabelings revealed that, taking into considerations the presence of studied neuropeptides, FB^+ neuronal cells may be divided into four main subpopulations: SOM-immunoreactive (IR) (35.7 ± 1.8% of all FB^+ perikarya; *e.g.*, Figs. 2, 9), SP-IR (31.8 ± 2.1%; Figs. 1, 7), cholinergic and peptidergic simultaneously - ChAT- and NPY-IR (23.3 ± 2.6%; Fig. 4), and, as was compared on the serial sections, non-adrenergic, non-cholinergic (NANC) nerve cells (5.7 ± 1.3%; Fig. 5), that lacked all of studied neuropeptides.

When the existence of catecholamine-synthetizing enzymes and particular peptides was taken into consideration, it has been found that two different subpopulations of FB/SOM-positive neurons occurred: TH/DBH-negative (20.5 ± 2.1%) and TH/DBH-positive (15.5 ± 2.1%; Fig. 2).

Many of FB/SP-positive neurons were TH/DBH-positive (23.1 ± 3.3%), while less numerous population was SP-IR, but TH/DBH-negative (8.1 ± 3.3%; Fig. 1).

Neurons that contained immunoreactivity to NPY were simultaneously noradrenergic (TH/DBH-positive; 7.4 ± 1.2%; Fig. 3) or cholinergic (CHAT-positive; 16.5 ± 0.5%; Fig. 4).

Among NANC nerve cells, the most numerous population were NOS-positive neurons (30.7 ± 2.2% of all NANC perikarya; Fig. 5). VIP-, GAL- and/or CGRP-IR, FB^+ neurons were not found (Fig. 6).

The IG-UBT received a moderate ChAT-, GAL-, TH/DBH-, SP- and/or CGRP-IR nerve supply (Figs. 7, 8). These nerve terminals formed dense meshwork, that was evenly distributed within the investigated ganglia. It should be mentioned that nerve fibres immunoreactive to SP and/or CGRP formed basket-like structures, surrounding TH/DBH-IR neurons, whereas nerve terminals exhibiting other neuropeptides were less numerous. The TH/DBH-IR nerve terminals surrounded very often SOM- or SP-positive nerve cells. Only very few nerve terminals were found to be immunoreactive to NOS, LENK, SOM and NPY (Figs. 7, 8, 9).

No VIP-positive terminals were found.

Discussion

It is well known that the innervation of the urinary bladder originates not only from the CaMG and pelvic plexus ganglia, but also from intramural ganglia distributed within the wall of the urinary bladder. These intramural ganglia consist of both noradrenergic as well as cholinergic neurons. It is known that between mammals there are some differences in the distribution of the UBG. They are numerous in the guinea-pig [17] and less numerous in the cat [15, 20] ferret, rabbit [17] and horse [40]. No or very few UBG have been found in the mouse [19] and rat [18].

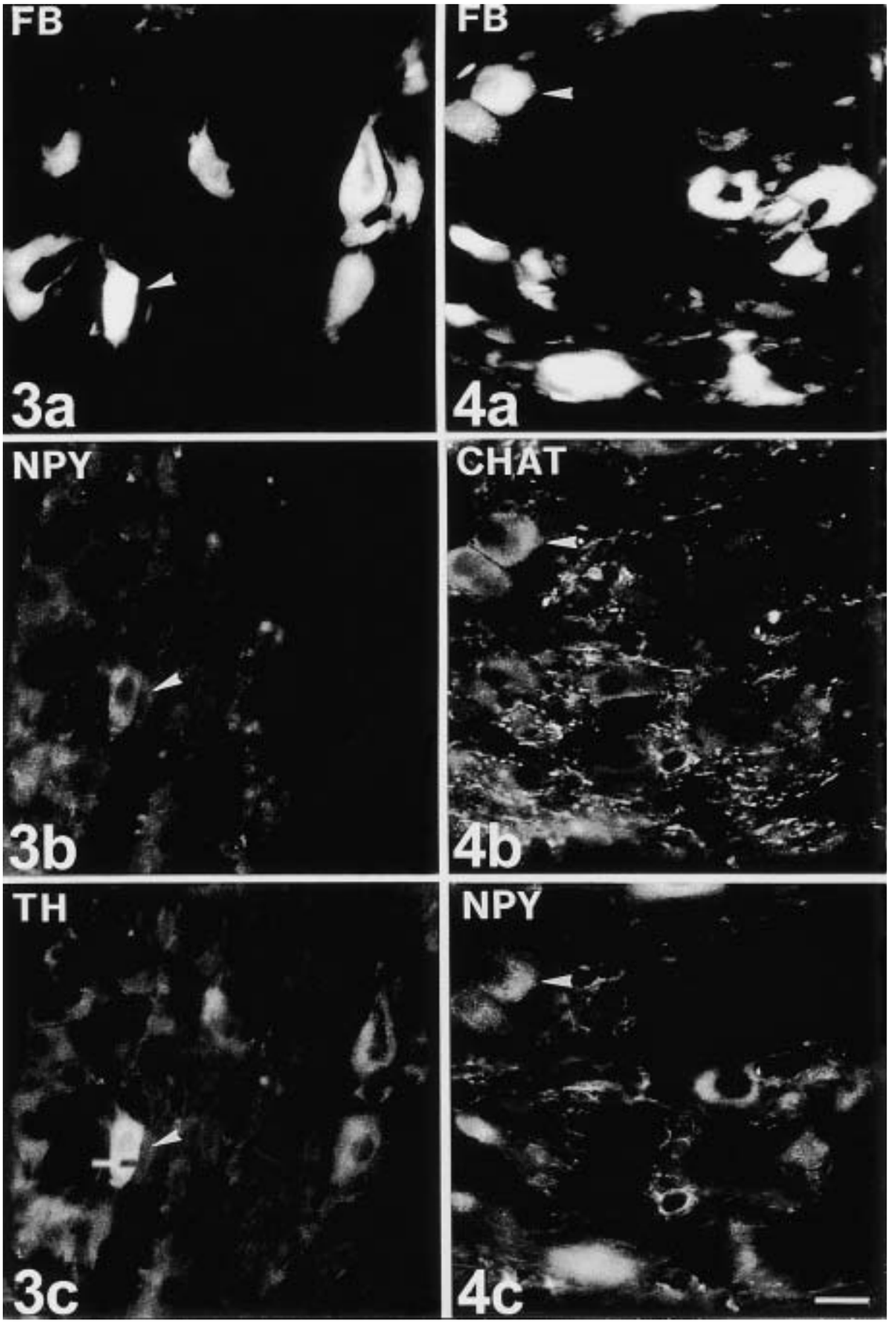
This study has demonstrated numerous ganglia mainly arranged in the wall of the urinary bladder trigone. However, some neurons have been found located within the ventral wall of the urinary bladder. This observation has been confirmed by earlier investigation of the pig urinary bladder [5] revealing neurons in the intramural ganglia, however they were less numerous as compared to those found in this study. The differences in the number of neurons resulted probably from the fact that animals of different ages were used in these experiments.

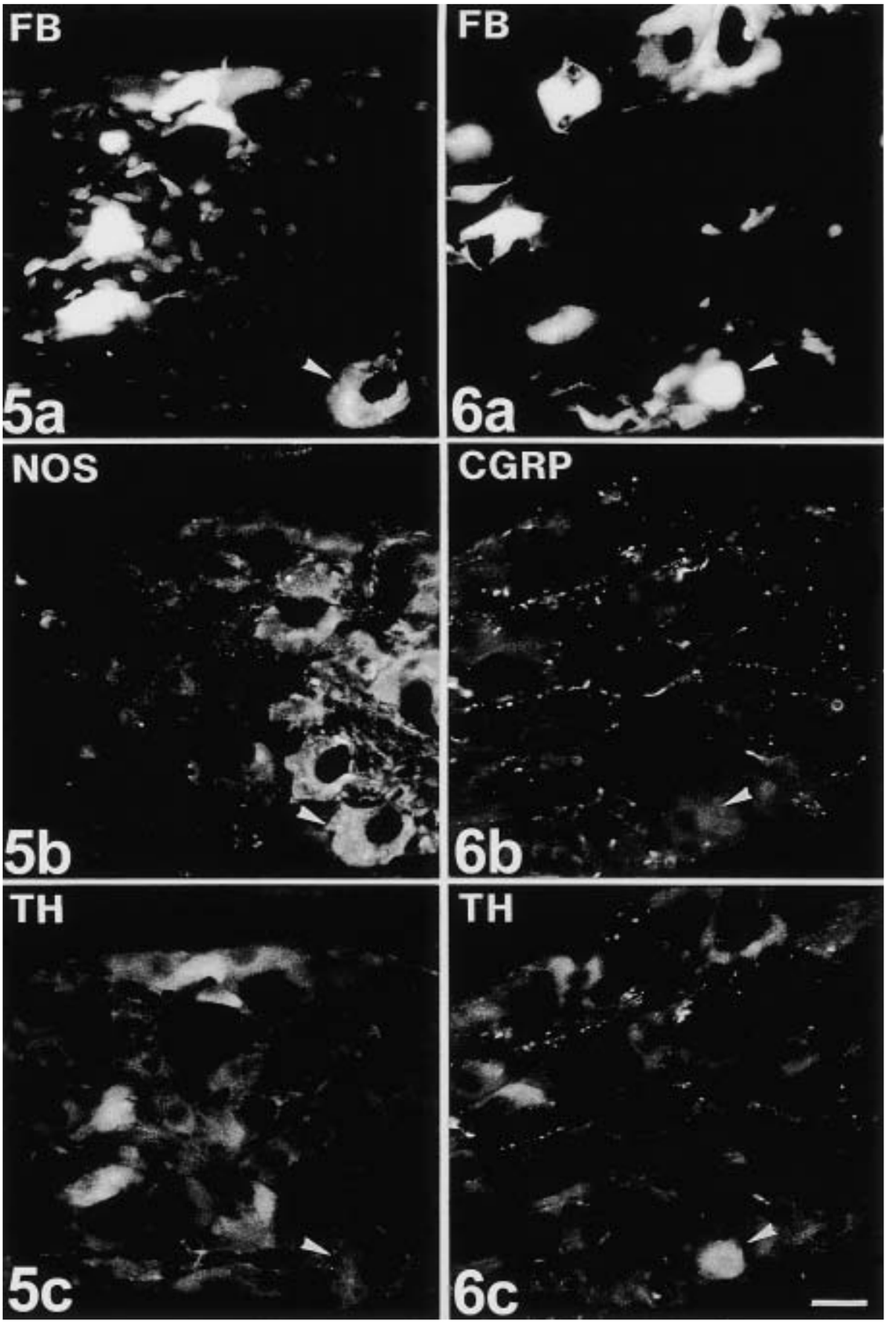
Immunohistochemical investigations performed in the present study have revealed that the most numerous population of UBG neurons are those immunoreactive to SOM. These results correspond well with data obtained in other mammals and dealing with immunohistochemical characteristics of IG neurons in the UBT [3, 5, 6, 25, 27, 28]. Since the relaxing effect of SOM in the detrusor muscle is very low [4, 26] it seems possible that neurons containing SOM could act in the local intraganglionic transmission or have a modulatory or inhibitory effect on the action of other transmitters (*e.g.* ATP [26]).

It has also been shown that many neurons in the UBG contain SP and it should be stressed that most of them are noradrenergic in nature (TH/DBH-positive). Because such co-existence was observed only after axotomy and deafferentation [23] or in the short-time cell culture of the sympathetic neurons [21, 23, 29, 30, 31], the present results should be carefully checked in further investigations to eliminate the possibility of an artificial induction of the co-existence of these peptides in neurons studied.

As it has been found in other mammals [35], the present study has also revealed that porcine IG UBT contain a large population of cholinergic neurons

Fig. 3a-c. Scattered FB-positive neurons (**a**) from the IG-UBT; one of them is NPY- (**b**) and TH-positive (**c ;** arrowhead). Scale bar = 25 μm. **Fig. 4a-c.** IG-UBT; many FB-positive neurons (**a**) contain NPY-immunoreactivity (**c;** arrowhead), whereas only small numbers of them are simultaneously CHAT-positive (**b**; arrowhead). Scale bar = 25 μm.

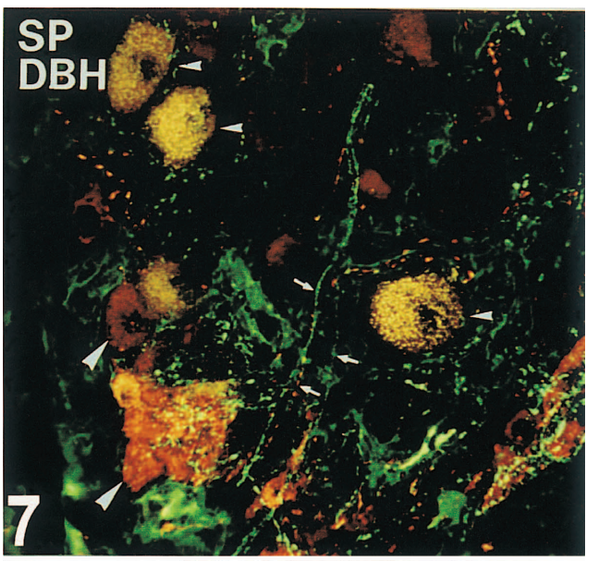

FB
3a
FB
4a
NPY
3b
CHAT
4b
TH
3c
NPY
4c


FB
FB
5a
6a
NOS
CGRP
5b
6b
TH
TH
5c
6c

(CHAT-IR) and that some of them are simultaneously NPY-positive. Although Dixon *et al.* [10, 11, 12, 13] observed that up to 50% of IG-UBT ganglia in human colocalized TH/DBH and VAChT, markers of noradrenergic and cholinergic traits, respectively, this was not the case in the pig. It may be attributed most probably to interspecies differences or to the different stage of the development of this part of peripheral nerve system, that is known to be able to make so-called "transmitter switch" in the presence of particular trophic molecules - *vide* the plasticity of the sweat gland innervation [33].

The present investigation has shown a small population of NANC neurons in the ganglia studied. A prominent proportion of these nerve cells contains immunoreactivity to NOS. This observation agrees with the results of earlier studies performed on the pig urinary bladder [1, 2, 36, 37].

The neurons in the porcine UBG are moderately supplied with CHAT-, GAL-, TH/DBH-, SP-, or CGRP-IR nerve fibres and poorly supplied with NOS-, LENK-, NPY- or SOM-IR nerve terminals. Similar intraganglionic distribution pattern of nerve fibres in the IG-UBT has been described in the pig [5] as well as in other mammals [24, 41, 43] but so far it is not known which ganglia contribute to the nerve supply and the source of the observed nerve terminals remains to be discovered. Correlating the present observations and our own pub-



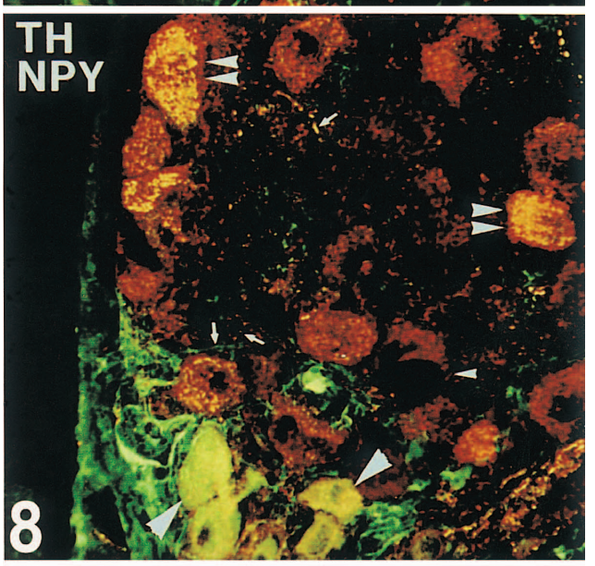


Fig. 5a-c. IG-UBT, a small group of FB-positive neurons (**a**) but only one of them exhibits immunoreactivity to NOS (**b**) and is TH-negative (**c;** arrowhead). Scale bar = 25 μm. **Fig. 6a-c.** IG-UBT; many FB-positive neurons (**a**) contain TH-immunoreactivity (**c;** arrowhead), but are CGRP-negative (**b**; arrowhead). Scale bar = 25 μm.

Fig. 7. IG-UBT, many neurons and nerve terminals immunoreactive for SP (FITC visualisation) and DBH (TXR visualisation). Large arrowheads show DBH-positive neurons and small arrowheads show neurons simultaneously immunostained for SP and DBH. Arrows show a dense meshwork of SP-positive nerve fibres and some DBH-IR nerve terminals. The images were digitally superimposed (double-labelled nerve cells and terminals are yellow). Scale bar = 25 μm. **Fig. 8.** IG-UBT, many neurons and nerve terminals immunoreactive for TH (FITC visualisation) and NPY (TXR visualisation). Large arrowheads show TH-positive neurons and a small arrowhead shows neurons immunostained only for NPY. Double large arrowheads indicate neurons simultaneously immunostained for TH and NPY. Virtually all NPY-positive neurons contain also TH-immunoreactivity but a small number of them are only TH-positive. Arrows show a dense meshwork of TH-positive nerve fibres and some nerve terminals containing only DBH-immunoreactivity. Single nerve fibres contain both substances. The images were digitally superimposed (double-labelled nerve cells and terminals are yellow). Scale bar = 25 μm. **Fig. 9.** IG-UBT; many neurons and nerve terminals immunoreactive for DBH (FITC visualisation) and SOM (TXR visualisation). Large arrowheads show DBH-positive neurons and a small double arrowhead shows neurons immunostained only for SOM. Large double arrowheads show neurons simultaneously immunostained for DBH and SOM. Arrows show a dense meshwork of DBH-positive nerve fibres. The images were digitally superimposed (double-labelled nerve cells and terminals are yellow). Scale bar = 25 μm.

lished data it can be assumed that the nerve fibres containing such peptides as SP, CGRP and GAL are collaterals of sensory neurons, the nerve terminals containing immunoreactivity to CHAT-, NOS-, and LENK probably represent preganglionic axons [7, 32], whereas at least some TH/DBH-, NPY- and/or SOM-IR nerve fibres are processes of CaMG neurons [38]. However, this hypothesis should be verified using anterograde tracing method (*e.g.* anterograde tracer DiI injected directly into the CaMG).

In the present study, there were no VIP-IR nerve fibres found around FB^+ neurons. This is contradictory to data of Crowe and Burnstock [5], who described this population as the second biggest one in these ganglia. This could be explained either by the use of different antisera, animals of different age, or the procedure used.

In summary, the porcine IG-UBG have been found to contain many neurons projecting to the urinary bladder trigone. This study has revealed a relatively large population of differently coded IG-UBT neurons, which constitute an important element of the complex neuroendocrine system involved in the regulation of the porcine urogenital organ function.

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