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Uterus-innervating neurones of paracervical ganglion in the pig: immunohistochemical characteristics

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Immunohistochemical characteristics of neurones innervating the porcine uterus located in paracervical ganglia were studied with a combination of retrograde fluorescent tracing and immunofluorescence. Retrograde fluorescent tracer Fast Blue (FB) was injected into the uterine horn and uterine cervix. The presence of biologically active substances, tyrosine hydroxylase (TH), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), galanin (GAL), Met-enkephalin-Arg-Gly-Leu (MEAGL) and calcitonin gene-related peptide (CGRP) was studied in FB-positive neurones localised in paracervical ganglia. FB-positive neurones containing TH, NPY, VIP and MEAGL were numerous, while those containing CGRP were scarce. The results pointed to some species-related differences in immunohistochemical coding of neurones of paracervical ganglion responsible for uterus innervation.

key words: neuropeptides, tyrosine hydroxylase, uterus, paracervical ganglion, pig, innervation

INTRODUCTION

The female pelvic plexus contains numerous nerve ganglia located in the parametrium at the utero-vaginal junction. This collection of ganglia is termed the paracervical ganglion or Frankenhauser's ganglion. Adrenergic and non-adrenergic neurones located in the paracervical ganglion innervate the reproductive organs, urinary bladder, ureter and associated blood vessels [3–5, 13, 15, 19]. Sympathetic nerve cells of paracervical ganglion are characterised as "short" adrenergic neurones to distinguish them from sympathetic nerve cells located in pre- and paravertebral ganglia [18, 19]. Physiological studies suggest that non-adrenergic neurones located in the ganglia of the female pelvic plexus are responsible for a variety of cholinergic actions, including vasodilatation of the uterine arteries [3, 4]. There is also direct morphological evidence that neurones of the paracervical ganglion, which contain choline acetyltransferase (an acetylcholine-synthesising enzyme), send their axons to the uterus of the rat [23].

Immunohistochemical studies of the paracevical ganglion of the rat, cat and guinea pig revealed the existence of several biologically active substances in its neurones. The substances include vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY) [1, 7, 12, 24], galanin [21], enkephalins [10] and others.

Fluorescent tracers, such as Fast Blue (FB), are particularly suitable for combining tracing technique with immunohistochemical detection of biologically active substances. They allow determination of the

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content of the biologically active substances (so-called immunohistochemical coding) in specific neurones responsible for innervation of a particular tissue or organ. As tracing techniques revealed that the paracervical ganglion is partially responsible for the autonomic innervation of the uterus in the rat [11], guinea pig [2] and pig [26], we decided to study the immunohistochemical characteristics of the uterus-innervating neurones of the paracervical ganglion in the pig. We decided to study the presence of neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), galanin (GAL), Met-enkephalin-Arg-Glu-Leu (MEAGL), calcitonin gene-related neuropeptide (CGRP) and tyrosine hydroxylase (TH) in the pelvic neurones innervating the uterus.

MATERIAL AND METHODS

6 gilts of the White Large Polish breed (c. 10 kg of body weight, age approx. 10 weeks) were purchased from a commercial fattening farm. Gilts were divided into two experimental groups. Laparotomy was performed under pentobarbital anaesthesia (Vetbutal, Biowet — Poland, 30 mg/kg of body weight, i.v.) and a total of 10 μl of 5% Fast Blue (FB) suspension (Dr Illing, Germany) was injected into the wall of the appropriate part of the uterus. 5 portions of 2 μl each of FB suspension were injected using a Hamilton microsyringe equipped with a 26 G needle. Three animals were injected into the dorsal wall of the uterine cervix, and three animals were injected into the middle part of the uterine horn. Injections into the wall of the uterine horn were performed on the side opposite to the *ligamentum latum uteri.* To avoid leakage the needle of the syringe was left in place for one minute. The wall of the uterus was then rinsed with physiological saline and gently wiped with gauze. After 3 weeks animals were deeply anaesthetised (as for abdominal surgery) and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The animals were then dissected and the uterine cervici with paracervical ganglia (PG) were collected. Tissues were post-fixed for 2 hours in the same fixative, transferred to 18% sucrose in 0.1 M phosphate buffer (pH 7.4) and stored at 4°C.

Horizontal 12 μm frozen sections of the uterine cervix were mounted on chrome alum-gelatine-coated slides, dried, and stored desiccated at –70° C. Sections were processed for immunofluorescence. Slides were hydrated in phosphate buffered saline, pH 7,4 (PBS) and incubated with appropriate primary antibodies. Stainings were done with the following primary antibodies: NPY, VIP, GAL, MEAGL, CGRP and TH. Antibodies used are listed in Table 1. Incubations were carried out overnight (16–18 h) at room temperature (RT). Slides were rinsed in PBS and incubated with secondary antibody: rhodamine-conjugated swine anti-rabbit IgG (R156, DAKO, Denmark, 1:50) for slides incubated with NPY, VIP, GAL, MEAGL and CGRP or fluoresceine-conjugated goat anti-mouse IgG (1211–3291, Cappel, USA, 1:200) for slides incubated with TH antibody. Incubations were carried out for 1 h at RT. Slides were rinsed in PBS, covered with 1:1 mixture of glycerol and PBS, coverslipped and examined under Leitz Aristoplan epi-fluorescence microscope for the presence of FB--containing (FB+) with filter set D. Fluorescence of rhodamine- and fluoresceine-labelled structures was observed using filter sets N2.1 and I3, respectively.

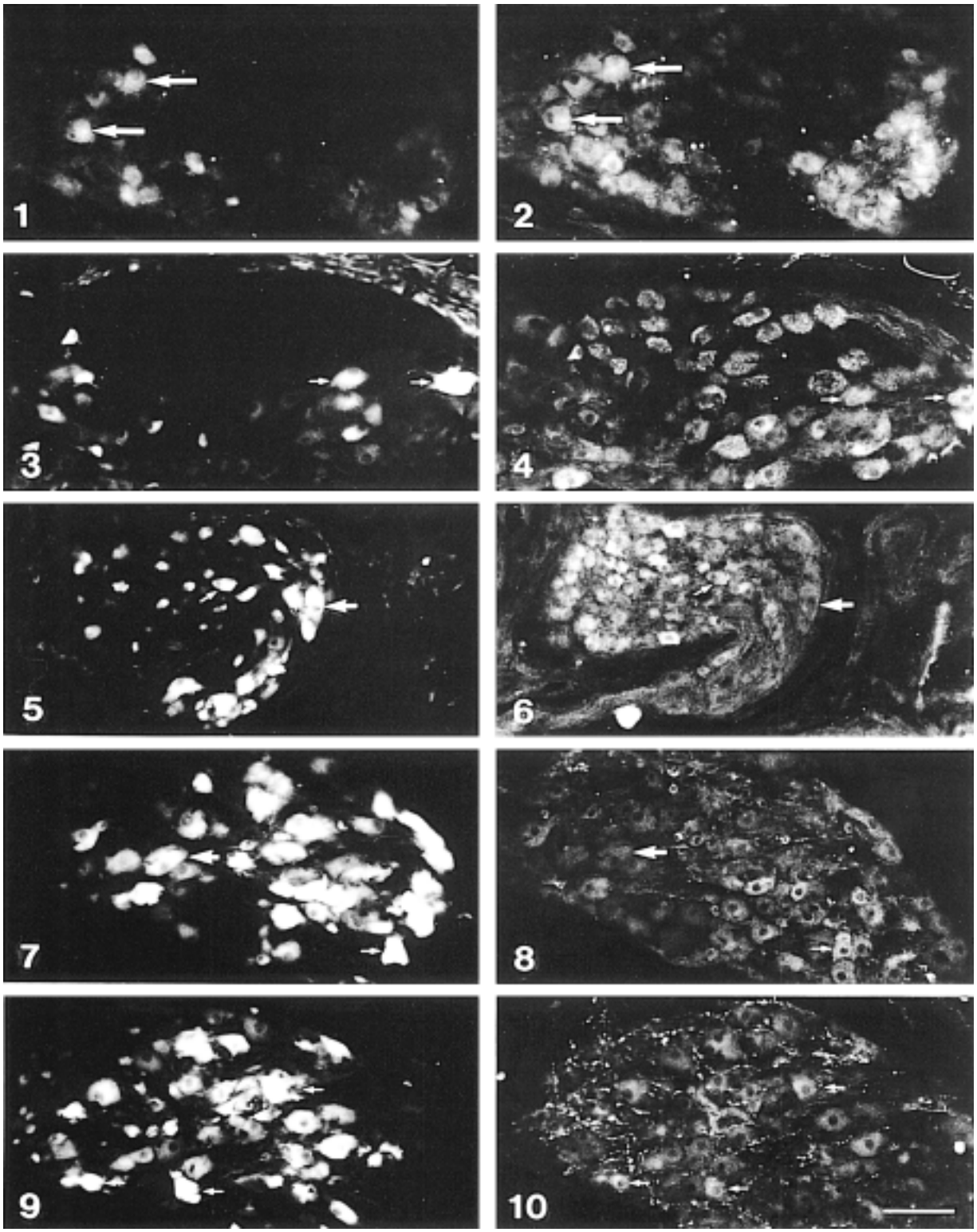
In the experiments, the principles of laboratory animal care followed the National Institute of Health Guide (NIH publications No. 80–23, Rev. 1978) and national law on animal experimentation.

RESULTS

After tracing to the uterine cervix, approx. 80% of FB-positive neurones in pelvic ganglia were TH-positive (Figs. 1, 2), versus 50% of total number of neurones of paracervical ganglion displaying immunoreactivity to TH. These neurones were distributed both in small neuronal clusters where only single FB-positive neurones were visible and in big clusters where up to 30% of neurones were FB-positive. FB-positive neurones located in small clusters were usually TH-negative, while in big clusters virtually all FB-positive neurones were TH-positive. 65% of FB+ neurones contained NPY (Figs. 3, 4), versus

Table 1. List of primary antibodies

Substance	Type	Dilution	Code	Manufacturer
TH	Mouse, monoclonal	1:50	1017381	Boehringer Mannheim
NPY	Rabbit, polyclonal	1:500	RPN1702	Amersham
VIP	Rabbit, polyclonal	1:500	8827009	Incstar
GAL	Rabbit, polyclonal	1:500	RAS7153N	Peninsula
MEAGL	Rabbit, polyclonal	1:2000	R-0171	N. Yanaihara, Japan
CGRP	Rabbit, polyclonal	1:500	RPN1842	Amersham



Figures 1–10. Horizontal pairs of photographs show the same section in which FB (left) or rhodamine/fluoresceine (right) was visualised; FB-positive neurones (Fig. 1) show immunofluorescence to TH (Fig. 2; arrows); FB-positive neurones (Fig. 3) show immunofluorescence to NPY (Fig. 4; arrows); FB-positive neurones (Fig. 5) show immunoreactivity to VIP (Fig. 6; small arrow). Big arrow points FB-positive neurone which is VIP-negative; FB-positive neurones (Fig. 7) show immunoreactivity to MEAGL (Fig. 8; small arrow). Big arrow points FB-positive neurone which is MEAGL-negative; FB-positive neurones (Fig. 9) show immunoreactivity to CGRP (Fig. 10; arrows). Bar 100 μm in Figs. 1– 4, 7, 8–10 and 150 μm in Figs. 5, 6.

45% of total number of neurones in paracervical ganglia. The distribution of NPY-positive neurones innervating the uterus was more even. They occurred equally often in small and big neuronal clusters. Immunoreactivity to NPY was seen either as smooth, diffuse immunostaining, or as brightly fluorescent floccules surrounding the nucleus, located in cytoplasm. 35% of FB-positive neurones (versus 40% of total population) contained VIP (Figs. 5, 6). Those neurones displayed rather even, diffuse immunostaining of the cytoplasm. 55% of FB-positive neurones (versus 45% of total population) contained immunoreactivity to MEAGL (Figs. 7, 8) which was visible as delicate sand-like granules located in cytoplasm. 10% of FB-positive neurones were CGRP-positive (Figs. 9, 10), and the percentage of CGRP-positive neurones in total population of neurones in paracervical ganglia was also close to 10%. Immunoreactivity to CGRP was seen in these neurones both as diffuse immunostaining and as delicate granules in the cytoplasm. No FB-positive neurones contained simultaneously GAL+.

After tracing to the uterine horn, virtually all FB-positive neurones were TH-positive. They were present only in big clusters of neurones. 43% of FB-positive neurones were NPY-positive, 39% were VIP-positive, 44% were MEAGL-positive. No CGRP- and GAL positive/FB-positive neurones were detected in paracervical ganglia after tracing to the uterine horn (data not shown).

DISCUSSION

The pelvic ganglia of the female did not attract much attention in the past and our knowledge of the immunohistochemical characteristics of neurones located there is limited. Papka et al. [20] described TH-, NPY-and VIP-positive neurones in the rat paracervical ganglion, while neurones immunoreactive to enkaphalins and CGRP were absent. Similarily, Morris and Gibbins [17] did not detect any immunoreactivity to enkephalins and CGRP in the paracervical ganglion of the guinea pig. Papka and McNeill [22] reported absence of neurones immunoreactive to CGRP and GAL in the rat paracervical ganglion. On the other hand, Häppölä et al. [10] detected in the porcine paracervical ganglion a distinct population of neurones containing MEAGL, which suggests species-related differences in immunohistochemical characteristics of neurones located in the ganglia of the female pelvic plexus.

Even less information is available on the immunohistochemical characteristics of neurones responsible for the innervation of the uterus which are located in the pelvic plexus. However, Alm and Lundberg [2] revealed that in the guinea pig all neurones innervating the uterus were adrenergic, which is similar to the situation found in the pig, where the majority of uterus-innervating neurones displayed immunoreactivity to TH. The results suggest that the paracervical ganglia preferentially supply the uterus with adrenergic nerve fibres. This fact is consistent with the finding that FB-positive neurones located in pelvic ganglia are more often NPY-positive (65%) than the total population of paracervical neurones (45%) and NPY is known to co-exist with noradrenaline in sympathetic neurones [25].

VIP is regarded as a co-transmitter present in cholinergic neurones [14]. VIP-positive uterus-projecting neurones found in the porcine paracervical ganglion may be responsible for the cholinergic, vasorelaxatory action of the neurones of the paracervical ganglion [4]. What must be, however, elucidated is the content of TH in the population of uterus-projecting neurones of the paracervical ganglion. Our finding that the majority of FB-positive neurones is TH-positive suggests that at least a significant fraction of VIP-positive neurones of the paracervical ganglion innervating the uterus must be TH-positive (although not necessarily sympathetic).

MEAGL is a member of the enkephalin family, which is thought to play a vasomotor role in peripheral tissues. MEAGL-positive nerve fibres were detected in the porcine uterus around blood vessels and in muscular membrane [9, 10]. The relatively high number of MEAGL-positive neurones projecting to the uterus found in pelvic ganglia suggests that this source contributes significantly to the innervation of porcine uterus with MEAGL-positive nerve fibres.

CGRP is a peptide being a co-transmitter in afferent fibres [6], but it exerts also a potent vasodilatory effect [16]. Finding CGRP-positive neurones responsible for the innervation of the uterus located in pelvic ganglia suggests that the peptide may mediate the vasomotor influence of paracervical ganglion neurones on the uterus. It suggests also significant species-related differences in the system of CGRP-positive nerve fibres supplying the uterus, as no CGRP-positive neurones were found in the paracervical ganglia of the rat and guinea pig [17, 20]. Because CGRP was found also in uterus-projecting neurones in porcine spinal ganglia (Wasowicz, unpublished) it is clear that two distinct populations of CGRP-positive nerve fibres exist in the porcine uterus [8].

The data on the presence of GAL in the neurones innervating the porcine uterus are consistent with

those obtained by other authors. Papka and Traurig [21] did not find the paracervical ganglion to be an origin of GAL-positive nerve supply of the uterus. In the porcine paracervical ganglia no GAL-positive, uterus-projecting neurones were detected. This means that this ganglia cannot be a source of GAL-positive uterine nerve fibres. It corresponds with an extremely low number of GAL-positive nerve fibres found in the porcine uterus (Wasowicz, unpublished). While Papka & Traurig [21] found spinal ganglia to be responsible for supply of the rat uterus with GAL-positive nerve fibres, we did not find GAL in porcine spinal ganglia (Wasowicz, unpublished).

Our findings contribute to the knowledge of the biologically active substances in the autonomic ganglia of animals and may deliver clues about the significance of autonomic ganglia in the physiological processes in internal organs, in this case the female reproductive system. Our findings point also to some species-related differences which must be taken into consideration when adopting results of studies on the reproductive system of laboratory animals to diagnostics and therapy in medicine.

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