Target Specific Organization and Neuron Types of the Dog Pelvic Ganglia: A Retrograde-Tracing and Immunohistochemical Study*

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Summary. The major pelvic ganglion in both the rat and guinea pig has been extensively studied because of its anatomical simplicity. To clarify the target specific neural pathway in the diffusely distributed pelvic ganglia of larger animals, the pelvic plexus of the female dog was investigated by retrograde tracing and immunohistochemistry. The whole mount staining of the pelvic plexus with acetylcholinesterase histochemistry revealed 70-100 ganglia of varying sizes. Neurons retrogradely labeled from the rectum were mainly found in ganglia located in the dorso-caudal part of the plexus. The majority of these were non-catecholaminergic, immunoreactive for either calbindin (Calb) or neuropeptide Y (NPY), and characteristically associated with baskets of enkephalin (ENK)-immunoreactive varicose fibers. Neurons projecting to the utero-vaginal walls were distributed in ganglia located in the ventro-caudal part of the plexus. These mainly consisted of two major neuron groups: catecholaminergic Calb-immunoreactive neurons, and non-catecholaminergic neurons containing nitric oxide synthase (NOS) and/or vasoactive intestinal peptide (VIP), which were preferentially associated with a network of ENK-immunoreactive varicose fibers. Neurons retrogradely labeled from the urinary bladder mainly occurred in ganglia located around the junction between the ureter and the bladder. These consisted of catecholaminergic Calb neurons and non-catecholaminergic neurons containing Calb or NOS. Only a few ENK-immunoreactive fibers were found within the clusters of catecholaminergic neurons. These results indicate that organ specific neurons are located in separate ganglia and have both a distinctive composition of neuron types as well as different innervation by preganglionic fibers.

The pelvic plexus plays an important role in the autonomic regulation of the pelvic viscera, including the lower digestive, urinary and genital organs. While it is generally composed of a network of nerve branches deriving from hypogastric and pelvic nerves and ganglia lying within the network, the anatomical structure of the pelvic plexus varies among species of animals. In man, the macaque, rabbit, cat and dog, the plexus of the pelvis forms a dense network of nerve strands, and a large number of ganglia are widely incorporated throughout the network (LANGLEY and ANDERSON, 1896; WOŹNIAK and SKOWROŃSKA, 1967). In contrast with these larger species, the pelvic plexus of smaller species such as the rat or guinea pig is relatively simple. It has one distinct large major ganglion and some small accessory ganglia (LANGWORTHY, 1965; COSTA and FURNESS, 1973; PURINTON et al., 1973). Because of this anatomical simplicity, the major pelvic ganglion of the rat and guinea pig has been used as a model for the investigation of ganglionic neurons regulating the pelvic viscera. In the pelvic ganglia of these animals have been found cholinergic and noradrenergic nerve cells containing a variety of different peptides (DAIL et al., 1983; DAIL and DZIURZYNSKI, 1985; MATTIASSON et al., 1985; PAPKA et al., 1985; MORRIS and GIBBINS, 1987; DHAMI and MITCHELL, 1991; KEAST, 1991, 1995; KEAST and CHIAM, 1994; KEAST et al., 1995). Immunohistochemical and retrograde-tracing studies of the major pelvic ganglion have shown that neurons projecting to the distal colon, urinary bladder, and reproductive organs have varied distributions within the ganglion and show some similarities and differences

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in the chemical coding of their cell bodies and nerve terminals associated with these neurons (KEAST and DE GROAT, 1989; DHAMI and MITCHELL, 1994; DOMOTO and TSUMORI, 1994). However, there have yet to be any studies on the diffusely distributed pelvic ganglia of larger animals, particularly analyses of the viscerotopic organization of the pelvic ganglia and of the histochemical characteristics of neurons projecting to each pelvic viscera of the dog.

The present study aimed to: 1) provide a detailed description of the arrangement of the pelvic ganglia in the dog; 2) ascertain the topographic relationship between the pelvic ganglion neurons and specific target viscera such as the rectum, urinary bladder, uterus and vagina; and 3) determine the chemical coding of ganglionic neurons innervating the specific pelvic viscera and of the nerve fibers associated with the ganglion neurons. Thus, histochemical staining for acetylcholinesterase (AChE) and nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and immunohistochemistry for tyrosine hydroxylase (TH), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), enkephalin (ENK), nitric oxide synthase (NOS) and calbindin (Calb) combined with retrograde axonal tracing techniques were performed. Calb-immunoreactivity was used as a marker for a subpopulation of the postganglionic neurons, since a previous study demonstrated that a special population of neurons in the inferior mesenteric ganglion of the dog contains Calb (LI and MASUKO, 1997).

MATERIALS AND METHODS

Animals

Female adult dogs (6–10 kg) were used. The animal experiments were approved by the Ethics Committee for Use of Experimental Animals at Saga Medical School. All experimental operations and perfusion fixation of animals were performed under deep anesthesia with an intraperitoneal injection of sodium pentobarbital (Nembutal, 25 mg/kg).

Tracer experiment

To investigate neurons projecting to the pelvic viscera, three animals were anesthetized as described above, and 0.5% Cholera toxin subunit B (CTB, List Biological Lab., Campbell, CA, USA), a retrograde tracer of the axonal transport, was injected into the walls of the rectal ampulla. In two other animals, CTB was injected into the walls of the bladder neck, and 0.5% Fast Blue (FB, Sigma, St. Louis, MO, USA), a fluorescent retrograde tracer, was simultaneously injected into the walls of the uterine cervix and the

proximal vagina (utero-vaginal walls). Conversely, one animal was injected with FB into the urinary bladder and CTB into the utero-vaginal walls. Multiple (three to four) bi-lateral injections with a total tracer volume of 20–30 μ l were made into each organ using a 30-gauge needle.

Histochemistry

One week after the application of tracer, experimental animals and control animals ($n=4$) were deeply anesthetized as described above and perfused through the ascending aorta, first with 1 liter of phosphate-buffered saline (PBS), and then with 4–6 liters of a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The pelvic plexus with the rectum, urinary bladder, and utero-vagina were removed and immersed in the same fixative for an additional 4–16 h at 4°C.

For fluorescence immunohistochemistry, the fixed tissues were soaked in 30% sucrose in PBS for 10–12 h at 4°C, and cryotome sections (20 μ m) were made. Free-floating sections were preincubated with PBS containing 1% bovine serum albumin, 1% normal swine serum and 0.1% Triton X-100, and were then incubated at 4°C for 14–16 h with one of several mixtures of various combinations of goat anti-CTB serum (List Biological Lab., Campbell, CA, USA; dilution 1:5000), monoclonal antibodies to TH (HATANAKA and ARIMATU, 1984; dilution 1:2), or monoclonal antibodies to Leu-ENK (Sera-Lab Limited, Sussex, UK; dilution 1:500) together with one of the following polyclonal antisera raised in rabbits: anti-Calb (YAMAKUNI et al., 1984; dilution 1:2000), anti-NPY (Peninsula Laboratories., Belmont, CA, USA; RAS 7172 already diluted), anti-TH (Eugene Tech International, Allendale, CA, USA; dilution 1:4000), anti-VIP (INCSTAR, Stillwater, MN, USA; dilution 1:4000) or anti-NOS (Euro-Diagnostica, Arnhem, Netherlands; dilution 1:4000). After incubation with the primary antiserum, sections were washed with PBS and exposed to a mixture of fluorescein isothiocyanate (FITC)-conjugated swine anti-goat IgG (Tago, Biosource, Camarillo, CA, USA; dilution 1:100) and tetramethylrhodamine (TMR)-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark; dilution 1:100) or a mixture of FITC-conjugated swine anti-mouse IgG (Nordic Immunological Labo., Tiburg, Holland; dilution 1:100) and TMR-conjugated swine anti-rabbit IgG for 2 h at room temperature. Sections were then rinsed in PBS, mounted on glass slides, covered with Perma Fluor Aqueous Mountant (Lipshaw Immunon, Pittsburgh, PA, USA), and viewed with a Nikon fluorescence microscope fitted with V-1A, G-1B and B21 filter

Fig. 1 A. Whole-mount preparation of the right pelvic plexus of the female dog stained for acetylcholinesterase, showing nerve trunks and ganglia, lateral view. *Hn* hypogastric nerve, *Pn* pelvic nerve. Scale bar=5 mm. **B.** Schematic diagram showing the arrangement of the plexus and pelvic viscera based on Figure 1A. Ganglia numbered 1-10 were mainly examined in this study.

blocks that allowed selective viewing of the FB, TMR, and FITC fluorescence, respectively.

The specificity of the immunoreaction was tested by replacing a specific antiserum with a non-immunized serum, or by preabsorption of the antiserum with an excess amount of antigens (1-100 mg/diluted antisera).

Histochemical staining for NADPH-d, a histochemical marker for NOS, was performed as described by VINCENT et al. (1983). Some free floating sections were incubated in 0.1 M Tris buffer (pH 7.6) containing 1.0 mg/ml β -NADPH (Sigma), 0.2 mg/ml nitro blue tetrazolium (Sigma), 2.7 mg/ml L-malic acid (Sigma) and 0.1% Triton-X 100 at 37°C. After

incubation for 1 h, the sections were rinsed several times in PBS and processed for fluorescence immunohistochemistry as described above.

To confirm the macroscopic structure of the pelvic plexus and the arrangement of the ganglia, a whole-mount preparation of the pelvic plexus was stained with AchE histochemistry (KARNOVSKY and ROOT, 1964). After removal of the associated adipose tissue, the peritoneal folds containing the pelvic plexus were incubated with a medium consisting of 1.7 mM acetylthiocholine iodide, 5 mM sodium citrate, 3 mM CuSO₄, 0.5 mM potassium ferricyanide, 0.1% Triton X-100, and 65 mM sodium hydrogen maleate buffer, pH 6.0, for 1 h at room temperature. The tissues were then rinsed in distilled water, dehydrated in ethanol, and cleared in xylene.

Cell counting

For quantitative analyses, serial sections (20 μ m) were cut along the plane that included the largest sectional area of the ganglion and incubated with the mixtures of various combinations of goat anti-CTB serum together with rabbit antisera to Calb, TH, NPY, VIP, and NOS, as described above. Each incubation group contained at least two or more sections for each ganglion. For each section, retrogradely labeled neurons with FB and/or CTB as well as cell bodies immunoreactive to the neuronal marker were counted. The total number of neuronal cell bodies was also counted in each of five or more sections that included a larger sectional area of each ganglion and had been used for the counting described above. Thus, the total numbers of retrogradely labeled neurons, the percentages of retrogradely labeled cells as well as immunoreactive neurons in each ganglion and of the immunoreactive marker appearing in retrogradely labeled neurons, and the average number of neuronal perikarya per sectional plane could be calculated for each ganglion.

RESULTS

General arrangement of pelvic ganglia

The dog pelvic plexus is formed by a joining of the hypogastric nerves and the visceral branches of the sacral nerves (pelvic nerves) within the peritoneal folds reflected from the sides of the bladder to the lateral walls of the pelvis (lateral ligaments of bladder). The plexus extends caudally along the sides of the rectum, and the urethra and the vagina in the female (WOŹNIAK and SKOWROŃSKA, 1967). After staining of a whole-mount preparation of the plexus

with AchE histochemistry (Fig. 1A), it was apparent that the dog pelvic plexus was composed of 70–100 ganglia of varying sizes and a complex network of nerve bundles associated with the pelvic viscera. Although the absolute number and position of these ganglia varied between animals and even between sides of the same animal, it was usually possible to distinguish 10 to 20 relatively large ganglia under a dissection microscope. Among these ganglia, ten representative ganglia (PG1–PG10, corresponding to the ganglia numbered 1–10 in Fig. 1B) that could be identified at a similar position of the plexus in each animal were primarily examined in this study.

Distribution of retrogradely labeled neurons

Although the number of retrogradely labeled neurons in the pelvic ganglia varied among the organs injected with a tracer as well as between animals, a consistent distribution pattern of the labeled neurons from each organ was apparent. There was no difference in the distribution pattern between CTB and FB as the tracer (Table 1).

After injection of the tracer into the walls of the rectum, 1353 labeled neurons on average (range 965–1802; $n=3$ animals) were found in the pelvic ganglia taken from a uni-lateral side. Most of these labeled neurons (average 92.1%, range 78.9%–99.8%; $n=3$) were found within the PG8–10, with a peak in the PG9. These ganglia were located along the side of the rectum in the dorsal part of the pelvic plexus (Fig. 1B). Only a few, if any, labeled neurons were found in the PG1–7 (Table 1).

After injection of the tracer into the walls of the uterine cervix and vagina, 707 labeled neurons on average (range 517–983, $n=3$) were found. These were preferentially distributed in the PG4–6 (average 85.6%, range 64.3%–98.2%) located along the nerve bundles that traveled in the ventro-caudal part of the plexus to connect the lateral margin of the cervico-vaginal walls (Fig. 1B, Table 1).

Tracer injected into the urinary bladder revealed 1056 labeled neurons on average (range 727–1273, $n=3$) throughout the pelvic plexus, but many of these (average 76.3%, range 71.4%–84.1%) were found in the PG1–3. These ganglia were located around the junction between the ureter and urinary bladder or more proximally along the nerve bundles running toward the bladder in the ventro-rostral part of the plexus (Fig. 1B). Some labeled cells were also found in the PG4–10 (Table 1).

These results indicate that neurons projecting to individual organs have preferential distributions within the pelvic plexus. Thus, we here refer to PG1–

Table 1. Occurrence of retrogradely labeled neurons in the pelvic ganglia after the injection of tracers into the rectum, utero-vagina, and urinary bladder

Animal	Injection site (Tracer)	Percentage of total number of neurons labeled in pelvic ganglia										Total number of neurons labeled
		PG1	PG2	GP3	PG4	GP5	GP6	GP7	PG8	GP9	PG10	
Rectum												
A	(CTB)	–	0	0	–	0	0	0.2	13.3	70.5	16.0	965
B	(CTB)	0	0	0	0.2	0.7	–	1.6	15.1	57.7	24.6	1292
C	(CTB)	2.3	0.7	2.6	–	5.6	4.0	5.9	23.6	55.3	–	1802
Utero-vagina												
D	(CTB)	0	0	3.2	50.2	8.0	6.1	3.0	13.7	15.7	–	622
E	(FB)	1.5	0	1.2	–	75.0	19.2	1.2	0	1.9	0	517
F	(FB)	0	1.8	–	39.0	41.4	17.8	–	0	0	–	983
Urinary bladder												
D	(FB)	37.0	14.7	19.7	7.7	1.2	0.6	10.0	8.4	0.7	–	727
E	(CTB)	36.2	27.7	9.4	–	2.2	4.8	2.0	9.8	5.5	2.4	1273
F	(CTB)	65.7	18.4	–	5.4	2.5	3.3	–	3.8	0.9	–	1169

PG1-10 correspond to ganglia numbered 1-10 in Figure 1B. –: ganglion not detected.

Table 2. Distribution of immunoreactivity in the pelvic ganglia and retrogradely labeled neurons

Pelvic ganglia		Total number of neurons per sectional plane	Number of immunoreactive neurons per sectional plane (Percentage of total number of neurons)				
			Calb	TH	NPY	VIP	NOS
Rectal PG 8, 9, 10 (n=11 ganglia)		371±49	214±28 (57.2±2.3)	1±1 (0.3±0.1)	91±14 (26.0±1.4)	4±1 (1.2±0.2)	15±2 (4.2±0.6)
Utero-vaginal PG 4, 5, 6 (n=12 ganglia)		175±16	39±5 (23.8±1.8)	43±4 (26.0±2.1)	5±1 (2.7±1.0)	25±3 (14.4±0.5)	118±16 (63.7±3.8)
Vesical PG 1, 2, 3 (n=14 ganglia)		143±13	38±4 (25.7±1.2)	15±2 (9.6±0.5)	2±0.4 (1.1±0.3)	3±0.3 (2.7±0.2)	25±3 (16.9±0.9)
PG 7 (n=4 ganglia)		87±20	32±7 (36.6±6.9)	1±0.2 (0.5±0.5)	8±5 (8.4±8.8)	3±1 (4.1±0.6)	6±2 (8.0±2.4)
Pelvic ganglia	Injection site	Total number of neurons labeled per sectional plane (Percentage)	Number of immunoreactive neurons labeled per sectional plane (Percentage of total number of neurons labeled)				
			Calb	TH	NPY	VIP	NOS
Rectal (n=8 ganglia)	Rectum	80±22 (28.6±4.2)	47±15 (60.2±3.3)	0 (0)	22±7 (27.8±1.7)	0 (0)	0 (0)
Utero-vaginal (n=8 ganglia)	Utero- vagina	30±7 (17.5±4.2)	6±1 (18.7±2.7)	7±2 (23.3±2.8)	1±0.3 (3.9±1.2)	5±2 (14.6±2.8)	11±3 (40.6±7.0)
Vesical (n=8 ganglia)	Urinary bladder	41±4 (26.5±4.0)	9±2 (20.6±1.4)	8±3 (19.9±3.0)	0.4±0.3 (1.0±0.3)	1±0.3 (1.6±0.9)	0.4±0.3 (1.0±1.1)

The number of neurons was counted in sections including a larger sectional area of each ganglion (4-8 ganglia from 3-5 animals). All values are presented as the mean±S. E. M.

Fig. 2. Retrogradely labeled neurons (A, C, E, G, I) showing immunoreactivity for Calb (B), TH (D), NPY (F), VIP (H) and NOS (J) in the rectal pelvic ganglia, after injecting CTB into the walls of the rectum. Double staining of the ganglion neurons showing separate distributions of Calb- and TH-immunoreactive cells (K, L) and the co-localization of VIP and NADPH-d in some neurons (M, N). Arrowheads indicate identical neurons in each section. Scale bar = 50 μ m

3 as vesical, PG4-6 as utero-vaginal, and PG8-10 as rectal pelvic ganglia, respectively. In contrast with these target specific ganglia, PG7, which was located more centrally in the plexus (Fig 1B), showed no specific relation to any pelvic organs. A small popula-

tion of neurons in PG7 projected to each pelvic organ examined (Table 1).

Fig. 3. Retrogradely labeled neurons (A, C, E, G, I) showing immunoreactivity for Calb (B), TH (D), NPY (F), VIP (H) and NOS (J) in the utero-vaginal pelvic ganglia, after injecting FB into the utero-vaginal walls. Double staining of the ganglion neurons showing the co-localization of Calb and TH (K, L) and the co-localization of VIP and NADPH-d (M, N) in some neurons. Arrowheads indicate identical neurons in each section. Scale bar = 50 μ m

Immunohistochemistry of ganglion neurons

Immunostaining of ganglia associated with the pelvic viscera for neuropeptides and other neuronal markers revealed a target organ specific pattern of immunoreactivity in the ganglion neurons.

The rectal pelvic ganglia (PG8-10) were relatively large in size. Of these ganglion cells (Table 2, Fig. 2), Calb-immunoreactive neurons were the most prominent, followed by NPY-immunoreactive and NOS-immunoreactive neurons. Only a small number of VIP-immunoreactive neurons and sporadic TH-immuno-

Fig. 4. Retrogradely labeled neurons (A, C, E, G, I) showing immunoreactivity for Calb (B), TH (D), NPY (F), VIP (H) and NOS (J) in the vesical pelvic ganglia, after injecting FB into the walls of the urinary bladder. Double staining of the ganglion neurons showing the co-localization of Calb and TH (K, L) and the co-localization of VIP and NADPH-d (M, N) in some neurons. Arrowheads indicate identical neurons in each section. Scale bar = 50 μ m

reactive cells were present in these ganglia. On average, 28.6% (range 11.4–40.5%) of neurons in these ganglia were retrogradely labeled from the rectal walls. Of these labeled neurons, 60.2% were immunoreactive to Calb and 27.8% were NPY-immunoreactive. No immunoreactivity for NOS, VIP, or TH was found in these labeled cells.

The utero-vaginal pelvic ganglia (PG4–6) were medium in size. In these ganglia (Table 2, Fig. 3), there were two major populations of ganglion cells. The first of these was neurons immunoreactive to TH and Calb. The double immunohistochemical staining showed that most (95.4%) Calb neurons in these ganglia were TH-immunoreactive. The other

Fig. 5. Double staining of the pelvic ganglia showing the relationship between ENK- or VIP-immunoreactive nerve fibers and certain immunoreactive neuronal perikarya. **A-F.** In the rectal pelvic ganglia, dense baskets of ENK-immunoreactive varicose fibers surround most of the Calb (**A, B**) and some NPY (**C, D**) neurons, but not NOS (**E, F**) neurons. **G-L.** In the utero-vaginal pelvic ganglia, dense ENK-immunoreactive varicose fibers are distributed around NOS (**G, H**) neurons, but not TH (**I, J**) neurons, while VIP-immunoreactive fibers are distributed throughout the ganglia, including around TH neurons (**K, L**). **M-R.** In the vesical pelvic ganglia, ENK-immunoreactive varicose fibers are found around NOS (**M, N**) and TH (**O, P**) neurons, while VIP-immunoreactive fibers are distributed throughout the ganglia, including around TH neurons (**Q, R**). Arrowheads indicate identical neurons in each section. Scale bar=50 μ m

major population consisted of NOS-immunoreactive and partly VIP-immunoreactive neurons. The double histochemical staining revealed that VIP and NADPH-d were colocalized in 54.3% of VIP-immunoreactive and 10.9% of NADPH-d positive neurons. Each major neuron group formed clusters and showed a distinctly separate distribution. A small population of NPY-immunoreactive cells was present, and 69% of these were immunoreactive to TH. Of the neurons in these ganglia, 17.5% on average (range 7.1–43.7%) were retrogradely labeled from the utero-vaginal walls. These labeled cells also showed immunoreactivity for TH, Calb, NOS, VIP and NPY, with a similar propor-

tion found in the total population of these ganglia.

The vesical pelvic ganglia (PG1–3) usually showed a small round configuration. In these ganglion neurons (Table 2, Fig. 4), Calb, TH and NOS were predominant. TH-immunoreactive neurons in these ganglia had a much smaller cell body and tended to be clustered near the periphery of the section. About 34.1% of them were also immunoreactive to Calb, and virtually all Calb-immunoreactive cells within the cluster of smaller neurons were TH-immunoreactive. However, many (86.4%) of the Calb neurons in these ganglia were TH negative and were preferentially distributed in the central part of the gan-

glion. A few NPY-immunoreactive neurons were present, with some of them also being TH-immunoreactive. A small cluster of VIP-immunoreactive neurons was occasionally found in the peripheral part of the section, and 60% of them were NADPH-d positive. Of the neurons in these ganglia, 26.5% on average (range 13.2–46.4%) were retrogradely labeled from the urinary bladder. In these labeled neurons a substantial number of neurons immunoreactive for TH as well as Calb and a few neurons immunoreactive for NPY, NOS and VIP were mainly found in the peripheral areas of the ganglion. Most of the remaining labeled neurons were distributed in the central part of the ganglion and showed no immunoreactivity for any markers examined.

The pelvic ganglia located in the central part of the plexus (PG7) were irregular in size and shape. In the PG7, Calb-immunoreactive neurons were predominant, and some NPY-, NOS- and VIP-immunoreactive neurons and a few TH-immunoreactive neurons were present (Table 2). A small population of neurons in the PG7 was retrogradely labeled from each of the pelvic organs, and the labeled cells showed mainly Calb immunoreactivity.

Immunohistochemistry of varicose nerve fibers

Varicose nerve fibers immunoreactive to ENK or VIP were found in all ganglia examined (Fig. 5). In the rectal ganglia, ENK-immunoreactive varicose fibers were richly distributed among the Calb-immunoreactive as well as NPY-immunoreactive neurons, and occasionally formed a dense basket of varicosities surrounding these immunoreactive neurons. Around the NOS- or VIP-immunoreactive cell bodies, ENK-immunoreactive fibers were sparse. Although VIP-immunoreactive fibers were prominent in the fiber bundles passing through these ganglia, few VIP varicose fibers were distributed among ganglion neurons.

In the utero-vaginal pelvic ganglia, ENK-immunoreactive varicose fibers were distributed within the clusters of NOS and VIP neurons but not within those of TH/Calb neurons. Although these ENK fibers showed a dense network, they rarely formed the basket of varicosities surrounding NOS and VIP neurons. VIP-immunoreactive varicose fibers were richly distributed in these ganglia, and were found around TH/Calb neurons as well as NOS and VIP neurons.

In the vesical pelvic ganglia, a small number of ENK-immunoreactive fibers were found in the peripheral region of these ganglia where NOS and/or VIP neurons as well as TH positive neurons were

clustered, but not around Calb neurons located in the central part of the ganglion. VIP immunoreactive varicose fibers were distributed throughout the ganglia without particular regional differences.

DISCUSSION

The present study has for the first time demonstrated a target organ specific topographical distribution and neurochemical feature of the ganglion neurons in the pelvic plexus of the female dog.

Distribution of the ganglion neuron

The whole mount staining of the plexus with AChE histochemistry has provided detailed anatomical structures of the dog pelvic plexus and ganglia that have not been described by macroscopic anatomy (LANGLEY and ANDERSON, 1896; WOŹNIAK and SKOWROŃSKA, 1967). Nearly one hundred ganglia showed a regular arrangement rather than diffuse distribution in the plexus. This arrangement enabled us to distinguish representative samples among these ganglia. The present retrograde-tracing experiments have revealed that ganglionic neurons projecting to each pelvic organ have a preferential localization in the plexus; i. e., neurons which project to the rectum were mainly present in ganglia located at the dorso-caudal part of the plexus, those which innervate the utero-vaginal walls were distributed in ganglia of the ventro-caudal part of the plexus, and many neurons supplying the urinary bladder were located in ganglia of the ventro-rostral part of the plexus. In the major pelvic ganglion of the male rat, a characteristic pattern of distribution for each pelvic organ-specific neuronal population has been demonstrated: most of the neurons which project to the colon are located in the dorsal part of the major pelvic ganglion near the entrance of the pelvic nerve, whereas neurons innervating the bladder are relatively evenly distributed throughout the ganglion (DAIL et al., 1983; KEAST et al., 1989). This was also the case for the neurons within the dog pelvic plexus innervating the rectum, and interestingly held true for those supplying the bladder, some of which tended to distribute across a wide range of the plexus. Although the morphology of the dog pelvic plexus differs from that of the rat in which ganglionic neurons are converged into one major ganglion and a few small accessory ganglia (LANGWORTHY, 1965; PURINTON et al., 1973), it appears that there are some similarities in the topographical organization of neurons within the pelvic plexus with respect to their target organs. It has also

been mentioned that ganglia close to a target organ contain a high percentage of neurons innervating that organ, whereas ganglia located more centrally in the pelvic plexus contain a more heterogeneous population of cells in the cat (DE GROAT and BOOTH, 1993).

Neuron types

The present immunohistochemical studies have demonstrated immunoreactivity for TH, Calb, NPY, VIP and NOS, but not for ENK in the neuronal perikarya of the dog pelvic plexus. On the basis of the expression of these neuronal markers and the specific pattern of coexistence, the following subpopulations of neurons in the plexus were defined: catecholaminergic neurons containing either TH alone (TH/-); both TH and Calb (TH/Calb) or both TH and NPY (TH/NPY); and non-catecholaminergic and possibly cholinergic neurons possessing Calb (-/Calb), NPY (-/NPY), VIP (-/VIP) or NOS (-/NOS). Histochemical studies for NADPH-d, a histochemical marker for NOS (DAWSON et al., 1991; HOPE et al., 1991; WARD et al., 1992), combined with immunohistochemistry, have revealed that NO-synthesizing neurons were further divided into two subpopulations, showing NOS alone and containing both NOS and VIP (-/NOS/VIP).

These peptides and markers for neurotransmitters found in the present study coincide with those that have been demonstrated in the pelvic neurons in the rat and guinea pig (MORRIS and GIBBINS, 1987; KEAST and DE GROAT, 1989; KEAST, 1991, 1995; DHAMI and MITCHELL, 1994; DOMOTO and TSUMORI, 1994), indicating that similar populations of neurons are involved in the pelvic plexus of these species. However, there are striking differences in the distributional patterns of these neuron types between the present observations and previous studies of the rat or guinea pig. In the rat pelvic plexus, where most pelvic neurons converge into a single large ganglion, all neuron types have been found throughout the major pelvic ganglion, though TH/NPY neurons and VIP neurons have showed respective tendencies to aggregate near the hypogastric nerve entrance and close to and within the cavernous nerve (KEAST and DE GROAT, 1989; KEAST, 1995). On the other hand, separate ganglia relating to each pelvic organ of the dog have a distinctive organization of neuron types specific for their target organs, although the ganglia located near the junction of the hypogastric and pelvic nerves (PG7) exhibiting only a tenuous relation to each pelvic organ show mixed features in their organization of neuron types.

Rectal pelvic ganglia

The most prominent feature of neurons projecting to the rectum is that the majority of them are non-catecholaminergic -/Calb (60%) or -/NPY (28%) neurons, and both are characteristically associated with baskets of ENK-immunoreactive varicose fibers. Therefore, the rectal pelvic ganglia, in which most of the neurons supplying the rectum are located, consist mainly of -/Calb and -/NPY neurons. Although sporadic or small numbers of TH, VIP and NOS neurons are present in these ganglia, no projection of these neurons to the rectum has ever been found. It has been reported that in the rat (KEAST and DE GROAT, 1989) and guinea pig (DHAMI and MITCHELL, 1994) pelvic ganglia non-catecholaminergic NPY neurons associated with the basket of ENK fibers project to the colon or rectum. This therefore appears to be a common feature of the pelvic innervation of the lower intestine in the rat, guinea pig, and dog.

In addition to the -/NPY neurons, the present study showed the involvement of the -/Calb neurons in the pelvic projection to the rectum. Calbindin is a cytosolic calcium-binding protein which occurs in distinctive subsets of neurons and has been used as a valuable neuroanatomical marker (FURNESS et al., 1988; KURAMOTO and FURNESS, 1989; CELIO, 1990; BAIMBRIDGE et al., 1992; GRKOVIC and ANDERSON, 1997). Our previous study in the dog (LI and MASUKO, 1997) has demonstrated that Calb-immunoreactivity occurs in a subpopulation of sympathetic postganglionic neurons and that the TH/Calb neurons in the inferior mesenteric ganglion, which receive the viscerofugal fibers from the distal colon, project to the longitudinal muscles and the enteric plexus of the lower intestine. Previous observations on the dog distal colon have also revealed that -/NPY varicose fibers are distributed in the longitudinal muscle layer as well as in the myenteric plexus, and that -/Calb fibers exist in the myenteric plexus (LI and MASUKO, 1997). Thus, the longitudinal muscle and the myenteric plexus appear to be candidates for targets of the projection from pelvic ganglia.

Utero-vaginal pelvic ganglia

The most significant feature of the utero-vaginal pelvic ganglia is that they mainly consist of discrete clusters of two major neuron groups: non-catecholaminergic neurons containing NOS and/or VIP and catecholaminergic Calb neurons. Although smaller numbers of TH/NPY and -/NPY neurons are present in these ganglia, both non-catecholaminergic

NOS and/or VIP neurons — which are preferentially associated with a dense network of ENK-immunoreactive varicose fibers — and TH/Calb neurons principally project to the utero-vaginal walls. It has been found that all types of varicose nerve fibers immunoreactive for neuronal markers demonstrated in the utero-vaginal pelvic ganglia are distributed in the smooth muscle layers of the utero-vaginal walls, and these varicose nerve fibers, except for Calb-immunoreactive fibers, are present around the vascular walls of the internal genital organs of the dog (our unpublished data).

The projection of non-catecholaminergic neurons, particularly those containing VIP, from the pelvic ganglia to the internal genital organs is a characteristic seen in other species such as the rat, guinea pig, and cat (DAIL et al., 1983, 1986; GU et al., 1984; KAWATANI et al., 1986; KEAST and DE GROAT, 1989; KEAST, 1992; DHAMI and MITCHELL, 1994). Recent evidence has shown that nitric oxide (NO) may act as a non-adrenergic and non-cholinergic inhibitory transmitter and occasionally coexist with VIP in the digestive as well as genital organs (IGNARRO et al., 1990; SANDERS and WARD, 1992; COSTA et al., 1992; FURNESS et al., 1992; DOMOTO and TSUMORI, 1994).

In addition to our previous study on the inferior mesenteric ganglion (LI and MASUKO, 1997), the present study has demonstrated that Calb also exists in a subpopulation of catecholaminergic neurons in the utero-vaginal pelvic ganglia. From these results and our unpublished observation that TH/Calb neurons are sparse in the paravertebral sympathetic ganglia, it appears that TH/Calb neurons are exclusively involved in regulation of the visceral non-vascular smooth muscles.

Vesical pelvic ganglia

In contrast with the rectal or utero-vaginal pelvic ganglia, the vesical pelvic ganglia have a rather complex organization of neuron types. They are comprised of mainly non-catecholaminergic neuron types: -/Calb, -/NOS, and non-immunoreactive for any markers examined (-/-). These ganglia also contain some clusters of VIP (-/VIP and -/VIP/NOS) and TH (TH/-, TH/Calb and TH/NPY) neuron groups situated at their peripheral regions. It is also a characteristic of the vesical ganglia that only a few ENK-immunoreactive fibers are distributed within the clusters of VIP and TH neuron groups. From these ganglia, many neurons of -/- type and an almost equal proportion of TH- and Calb-immunoreactive neurons (possibly TH/Calb type) project to the urinary bladder. Our unpublished observations

have revealed that TH/Calb varicose fibers are distributed in the smooth muscle layers of the bladder walls, but not around intramural ganglionic neurons, while the intramural ganglionic neurons receive TH-, NPY-, VIP-, NOS- and ENK-immunoreactive fibers, and most of the intramural neuronal perikarya are non-immunoreactive for these markers. It is interesting that neurons of -/- type constitute a major population in both the vesical pelvic ganglia and the intramural ganglia. Although we now have no valid explanation for targets or fiber connections of these -/- neurons, they deserve further investigation.

Nerve fibers in the pelvic ganglia

The present study demonstrated the spatial relationship between ENK-immunoreactive varicose nerve fibers and ganglionic neurons of a particular type, and showed that the relationship differs among ganglia projecting to different organs. Since no ENK-immunoreactive cell bodies were found in the dog pelvic ganglia, these ENK fibers are considered to be of an extrinsic, most possibly preganglionic, origin. This would indicate that pelvic neurons projecting to different viscera receive different preganglionic innervation. In contrast with the ENK fibers, varicose nerve fibers immunoreactive for VIP but not NOS showed a relatively uniform distribution throughout every ganglion without target specificity. It is unlikely that these VIP-immunoreactive fibers come from spinal preganglionic neurons or primary afferent neurons in the dorsal root ganglia since no VIP-immunoreactivity has been demonstrated in the preganglionic neurons or in the primary afferent neurons of the dog (unpublished data). Although a possibility of viscerofugal projection can not be completely excluded (LUCKENSMAYER and KEAST, 1996), it is probable that these VIP fibers originate from non-catecholaminergic VIP neurons located within the pelvic ganglia, suggesting a property of the interneuron.

Further studies are required to clarify the target organ specific sacral autonomic pathways involving the pelvic plexus. For this purpose the dog pelvic plexus may be useful, since the present study has shown that each organ specific ganglion makes a separate ganglion group in the vicinity of the organ and has a distinctive composition of neuron types as well as preganglionic fibers specific for the target organ.

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