Distribution and immunohistochemical properties of autonomic neurons supplying the ovine hip joint capsule

W. Sienkiewicz[1]*, A. Dudek[1], A. Chroszcz[2], M. Janeczek[2], J. Kaleczyc[1]

[1]Faculty of Veterinary Medicine, University of Warmia and Mazury, Olsztyn, Poland
[2]Faculty of Veterinary Medicine, Wroclaw University of Environmental and Life Sciences, Wroclaw, Poland

*Corresponding author: waldemar.sienkiewicz@uwm.edu.pl

ABSTRACT: Combined retrograde tracing and double labelling immunohistochemistry were applied to study the distribution and chemical coding of autonomic neurons projecting to the ovine hip joint capsule. As revealed by retrograde tracing, fast blue-positive autonomic neurons supplying the lateral side of the hip joint capsule and the medial side of the hip joint capsule were located within the lumbar and sacral of the ipsilateral sympathetic chain ganglia and within the caudal mesenteric ganglion. Immunohistochemistry revealed that nearly all (sympathetic chain ganglia: 96% and caudal mesenteric ganglion: 98.8%) the neurons were adrenergic in nature (positive for dopamine β-hydroxylase). Many retrogradely labelled neurons also displayed immunoreactivity to neuropeptide Y (approximately 34% of fast blue-positive neurons within caudal mesenteric ganglion and sympathetic chain ganglia). Populations of Met-Enk⁺ (20%) and Leu-Enk⁺ (6%) neurons were present only in the sympathetic chain ganglia while within caudal mesenteric ganglion no enkephalinergic-labelled neurons were noted. Only a small population (2.2%) of hip joint capsule-projecting neurons were Gal-IR and they were observed only within the caudal mesenteric ganglion. No cholinergic neurons involved in the innervation of the hip joint capsule were found. However, fast blue-positive nerve cell bodies were surrounded by numerous cholinergic nerve fibres often forming basket-like formations. Single Gal⁺ nerve fibres were found in the intraganglionic connective tissue. Substance P-positive or calcitonin gene-related peptide-positive intraganglionic nerve terminals were very numerous and formed "baskets" surrounding fast blue-positive perikarya within sympathetic chain ganglias and caudal mesenteric ganglion.

Keywords: sheep; hip joint capsule; tracing; autonomic neurons; immunohistochemistry

The autonomic nervous system controls the blood flow to joints as well as vascular permeability, directly or indirectly, in cooperation with leukocytes (Schaible and Straub 2014). It is known that the sympathetic nervous system supports the development of inflammation, but it may also reduce inflammation of chronic duration. In addition to local vascular effects in the joint, the sympathetic nervous system influences numerous immune processes in the joint and in lymphoid organs. Hence, the net effect of the sympathetic nervous system on inflamed tissue results from local sympathetic effects in the joint as well as from sympathetic influences on major systemic immune processes (Janig and Green 2014; Schaible and Straub 2014).

The available literature contains data dealing with the distribution of the autonomic neurons supplying the shoulder joint (Yoshida et al. 1995), elbow joint (Widenfalk et al. 1988), knee joint (Widenfalk and Wiberg 1989; Catre and Salo 1999) and temporo-mandibular joint (Widenfalk and Wiberg 1990; Uddman et al. 1998; Casatti et al. 1999) in the rat and the elbow and knee joint in the monkey (Lee et al. 1991; Wiberg and Widenfalk 1991) and in

the rabbit (Lee et al. 1991). All these studies were performed utilising the retrograde tracing method. In the literature there are two papers describing the distribution and chemical coding of autonomic neurons projecting to the knee joint (Catre and Salo 1999) and temporo-mandibular joint (Uddman et al. 1998) in the rat.

There are also many papers regarding the immunohistochemical properties of the nerve fibres present in the joint capsule. Most of them are concern only with sensory innervation. This topic was studied in the human hip joint capsule (Saxler et al. 2007) and in the knee joint in mice (Buma 1994), cats (Marshall et al. 1994; Heppelmann et al. 1997), dogs (Tamura et al. 1998) and humans (Wojtys et al. 1990; Witonski and Wagrowska-Danielewicz 1999). The presence of sensory nerve fibres was also described in the human temporo-mandibular joint (Yoshida et al. 1999) and rat shoulder joint (Yoshida et al. 1995). Some papers reported the presence of autonomic nerve fibres located within the capsule of the rat knee joint (Ahmed et al. 1994; Ahmed et al. 1995; Ackermann et al. 2001), the facet joint of the lumbar spine (Ahmed et al. 1993) and the temporo-mandibular joint (Uddman et al. 1998).

Our study was carried out on sheep, which are frequently used as an animal model for orthopaedic studies. Disorders of the hip joint and also methods of their treatment are one of the most frequently studied areas in orthopaedics (Muhr et al. 1974; Bergmann et al. 1984; Bergmann et al. 1999; Miozzari et al. 2004; Bialecki et al. 2014). Concerning the role of the autonomic innervation of joints within the inflammation, this very important factor should be taken under consideration during such studies.

As clearly seen from the cited literature, the available data are incomplete and concern only human or laboratory animals. There is no comprehensive study dealing with the distribution and immunohistochemical features of the autonomic innervation of the hip joint capsule in any species, including sheep.

MATERIAL AND METHODS

The study was carried out on ten sexually mature sheep, weighing around 30–40 kg each. The animals were housed and treated in accordance with the rules approved by the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Sciences and Higher Education). The animals were divided into two groups (*n* = 5), LAT and MED. The anaesthesia was achieved by an intravenous injection of a ketamine (10 mg/kg body weight; Bioketan®), diazepam (0.02 mg/kg body weight) and fentanyl (Fentanyl®) mixture. During the surgery, 250 ml of 0.9% sodium chlorate in infusion was administered and the saturation and pulse were controlled using a pulse oximeter (Nonin®). The sheep in the LAT group then received a 20-µl injection of the fluorescent retrograde tracer fast blue (FB) (5% suspension of FB in distilled water) into the lateral aspect of the right hip joint capsule (HJC), while the animals of the MED group received an injection in the medial aspect of the HJC. The tracer was administered in ten injections of 2 µl of FB each. After a survival period of five weeks, the animals were deeply anesthetised (following the same procedure as described above) and then, they were transcardially perfused with 0.5 l of preperfusion solution containing 0.9% sodium chloride (Chemia, Gliwice, Poland), 2.5% polyvinylpyrrolidone (Sigma, Deisenhofen, Germany), 0.5% procaine hydrochloride (Polfa, Warsaw, Poland), and 20 000 IU of heparin (Heparinum; Polfa; added extempore), followed by 8–10 l of 4% ice-cold buffered paraformaldehyde (pH 7.4). No tracer contamination with FB was found in the neighbouring tissues or in the muscles surrounding the sites of tracer injections. Sympathetic chain ganglia (SChG) and caudal mesenteric ganglia (CaMG) tissue blocks were collected and then post-fixed by immersion in the same fixative for 30 min, rinsed with phosphate buffer (pH 7.4) and transferred to and stored in 18% buffered sucrose solution (pH 7.4) until further processing.

The tissue blocks were cut into 14-µm-thick cryostat sections, mounted on glass slides and viewed under a fluorescent microscope equipped with a filter block for FB. Fast blue-positive (FB$^+$) neurons were counted in every third section to avoid double analysis. The selected sections comprising FB$^+$ neurons were processed for the double immunofluorescence method using primary antisera against dopamine-β-hydroxylase (DβH), vesicular acetylcholine transporter (VACHT), (SP), neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), met-enkephalin (Met-Enk), leu-

Table 1. Antisera used in the study

	Antigen	Host	Type	Dilution	Cat. No.	Supplier
Primary antisera	DβH	mouse	monoclonal	1 : 500	MAB308	Millipore
	NPY	rabbit	polyclonal	1 : 400	NA1233	Biomom, Exeter, UK
	Gal	rabbit	polyclonal	1 : 2000	RIN 7153	Peninsula Lab. INC. Belmont, USA
	SP	rat	monoclonal	1 : 150	8450-0505	ABD Serotec, Oxfordshire, UK
	CGRP	rabbit	polyclonal	1 : 2000	11535	Cappel, Aurora, USA
	VACHT	rabbit	polyclonal	1 : 5000	V5387	Sigma-Aldrich, Saint Louis, USA
	VIP	mouse	monoclonal	1 : 500	MaVIP	East Acres Biologicals, Southbridge, USA
	Met-5-ENK	rabbit	polyclonal	1 : 500	RPN 1562	Amersham, Buckinghamshire, UK
	Leu-5-ENK	rabbit	polyclonal	1 : 500	RPN 1552	Amersham, Buckinghamshire, UK
	Host	fluorochrome		dilution	code	supplier
Secondary antisera	Goat-anti-rabbit IgG (H+L)	Alexa Flour 488		1 : 500	A11008	Invitrogen, Paisley, UK
	Goat-anti-mouse IgG (H+L)	Alexa Flour 488		1 : 500	A11001	Invitrogen, Paisley, UK
	Goat-anti-rabbit IgG (H+L)	Alexa Flour 568		1 : 500	A11011	Invitrogen, Paisley, UK
	Goat-anti-mouse IgG (H+L)	Alexa Flour 568		1 : 500	A11004	Invitrogen, Paisley, UK
	Goat-anti-rat IgG (H+L)	Alexa Flour 488		1 : 500	A11006	Invitrogen, Paisley, UK

CGRP = calcitonin gene-related peptide, DβH = dopamine-β-hydroxylase, Gal = galanin, Leu-5-ENK = leu-5-enkephalin, Met-5-ENK = met-enkephalin, NPY = neuropeptide Y, SP = substance P, VACHT = vesicular acetylcholine transporter, VIP = vasoactive intestinal polypeptide

5-enkephalin (Leu-Enk), galanin (Gal), calcitonin gene-related peptide (CGRP) and substance P (SP) (Table 1) according to a method described previously (Dudek et al. 2011).

RESULTS

Retrograde tracing

SChG. FB-positive (FB$^+$) neurons were found within the ipsilateral lumbar (L) and sacral (S) sympathetic chain ganglia (SChG) from L2 to S2 in animals of the LAT and MED groups. These neurons were round- or oval-shaped with a longitudinal axis of approximately 35 μm and a short axis of approximately 25 μm. The neurons were evenly distributed throughout the ganglia. The average number of FB$^+$ neurons was 312 ± 99 and 954 ± 54 in sheep from the LAT and MED groups, respectively. The largest average number of FB$^+$ neurons in animals of the LAT group was found within the L4 SChG (126 ± 28.3), while the S2 ganglion contained the smallest number of nerve cells (3.8 ± 0.8). In L2, L3, L5, L6
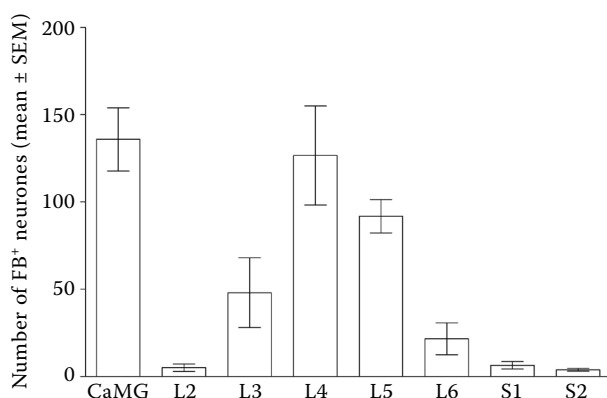

Figure 1. Number of fast blue-positive (FB$^+$) neurons within the caudal mesenteric ganglion (CaMG) and particular sympathetic chain ganglia in sheep injected with FB on the lateral side of the hip joint capsule
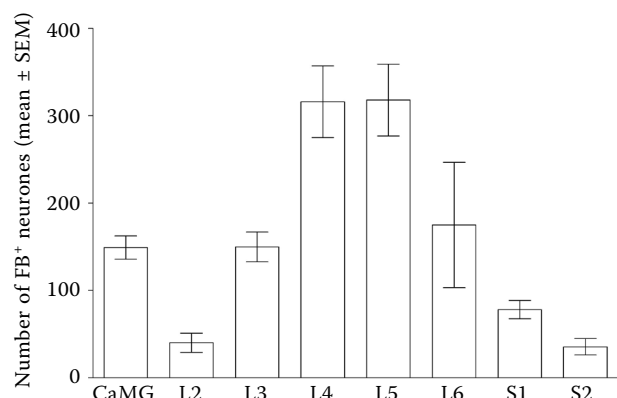

Figure 2. Number of fast blue-positive (FB$^+$) neurons within the caudal mesenteric ganglion (CaMG) and particular sympathetic chain ganglia in sheep injected with FB on the medial side of the hip joint capsule

and S1 ganglia, the number of retrograde-labelled neurons amounted to 5 ± 2.1, 48 ± 20, 91.8 ± 9.6, 21.6 ± 9 and 6.4 ± 2.1, respectively (Figure 1). In sheep of the MED group, the largest number of FB$^+$ nerve cells was found within L5 (318 ± 41.1), while the lowest number of cells was found within the S2 ganglion (35.4 ± 9.4). In L2, L3, L4, L6 and S1 ganglia, the number of retrogradely labelled neurons amounted to 40 ± 10.9, 149 ± 17.1, 316 ± 41, 174 ± 71.7 and 78 ± 10.5, respectively (Figure 2).

CaMG. Within CaMG, the numbers of labelled cells amounted to 135.8 ± 18.1 and 149 ± 13.4 in animals of the LAT and MED groups, respectively (Figures 1 and 2). These cells were round- or oval-shaped with a longitudinal axis of approximately 35 μm and a short axis of approximately 25 μm. The neurons were evenly distributed throughout the ganglia.

Immunohistochemistry

SChG. Immunohistochemistry revealed that nearly all the neurons (96 ± 1.8%) were adrenergic in nature (dopamine-β-hydroxylase (DβH$^+$)-positive) (Figures 3 and 4). Retrogradely labelled neurons also exhibited immunoreactivity to neuropeptide Y (NPY; 34 ± 5.4%, Figures 3 and 5) and the enkephalins met-enkephalin (Met-Enk; 20 ±

Figure 4. Fast blue-positive (FB$^+$) neurons innervating the lateral side of the hip joint capsule, located in the fourth lumbar sympathetic chain ganglia, immunoreactive for dopamine-β-hydroxylase (DßH) and surrounded by no cholinergic vesicular acetylcholine transporter (VACHT$^+$) nerve fibres
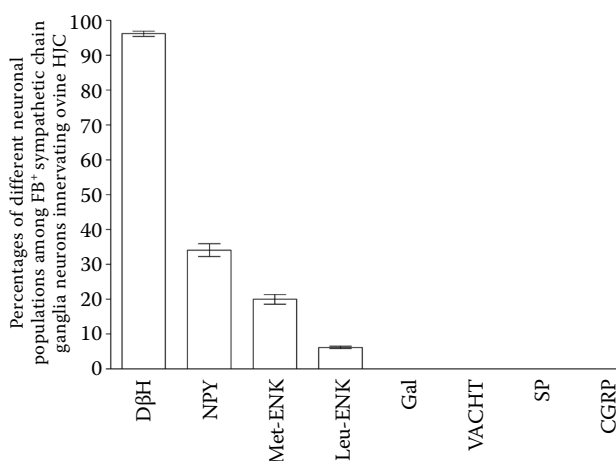

Figure 3. Chemical coding of fast blue-positive neurons supplying the hip joint capsule (HJC) located within the sympathetic chain ganglia

CGRP = calcitonin gene-related peptide, DβH = dopamine-β-hydroxylase, Gal = galanin, Leu-ENK = leu-5-enkephalin, Met-ENK = met-enkephalin, NPY = neuropeptide Y, SP = substance P, VACHT = vesicular acetylcholine transporter
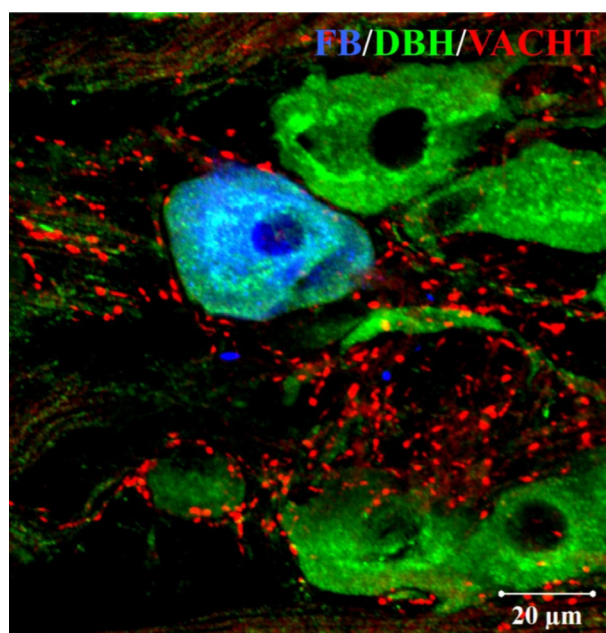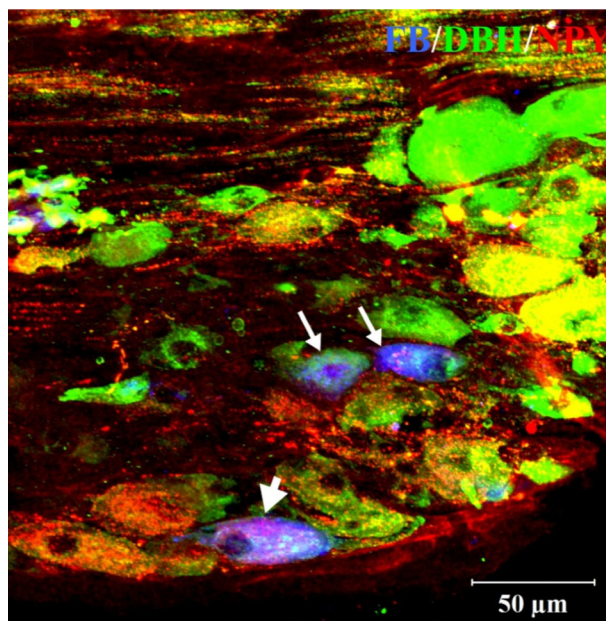

Figure 5. Fast blue-positive (FB$^+$) neurons innervating the medial side of the **hip joint capsule**, located in the fifth sympathetic chain lumbar ganglion and immunoreactive for **dopamine-β-hydroxylase** (DßH) (long arrows) or DßH/neuropeptide Y (NPY) (short arrow). Note the presence of numerous NPY$^+$ nerve fibres

3.9%) and leu-5-enkephalin (Leu-Enk; 6.2 ± 0.8%, Figures 3 and 6). The analysis of double-stained tissue sections revealed that all NPY-, Leu-Enk- and Met-Enk-immunoreactive FB+ perikarya were simultaneously DβH-positive. No cholinergic neurons involved in the innervation of the HJC were found. However, FB+ nerve cell bodies were surrounded by numerous cholinergic nerve fibres often forming basket-like formations (Figure 4). The labelled perikarya were galanin-negative (Gal⁻) but single Gal+ nerve fibres were found in the intraganglionic connective tissue (Figures 3 and 7). Calcitonin gene-related peptide-positive (Figure 8) or substance P-positive (Figure 9) intraganglionic nerve terminals were very numerous. These fibres formed "basket-like structures" surrounding FB+ perikarya located in SChG-s.

CaMG. Immunocytochemistry revealed that 98.8 ± 0.3% (Figures 10 and 11) of all labelled perikarya observed within the CaMG were adrenergic in nature (DBH+). Over one third of them (33.6 ± 3.5%, Figures 10 and 12) were simultaneously NPY+ while only 2.2 ± 0.4% contained galanin simultaneously (Figures 10 and 13). No cholinergic (VACHT+) perikarya nor perikarya containing
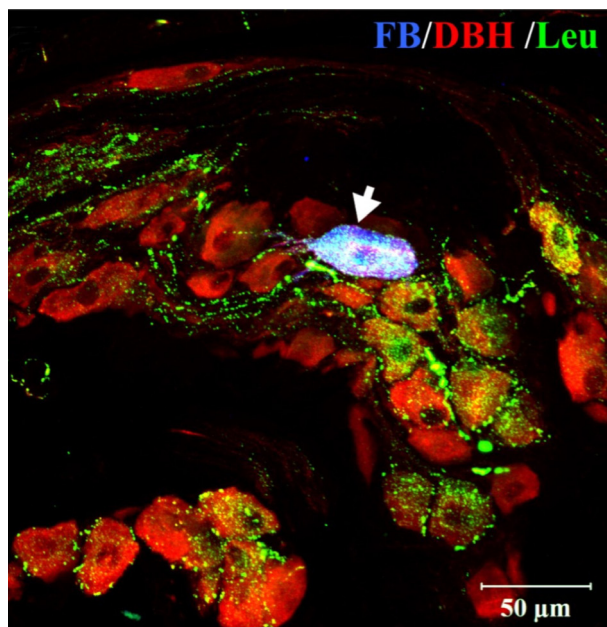

Figure 7. Fast blue-positive (FB+) neuron innervating the lateral side of the hip joint capsule, located in the sixth sympathetic chain lumbar ganglion and immunoreactive for dopamine-β-hydroxylase (DßH) but immunonegative for galanin (Gal) (long arrow). The short arrow indicates a neuron that was immunonegative for both studied substances
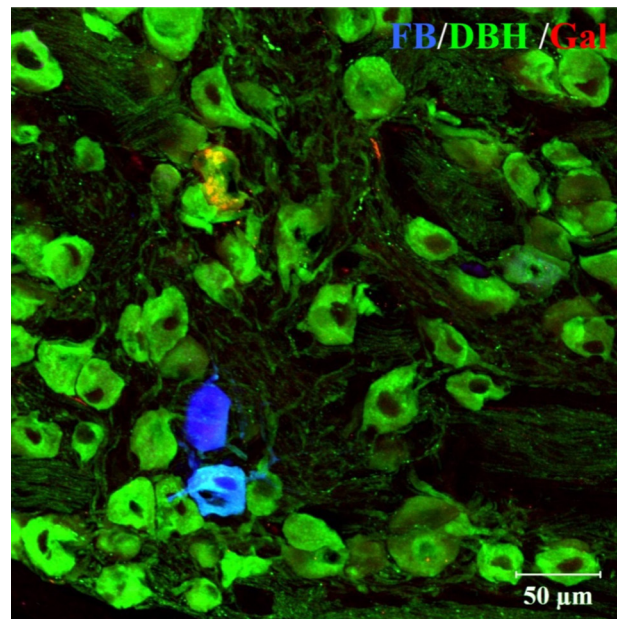

Figure 6. Fast blue-positive (FB+) neuron innervating the medial side of the hip joint capsule, located in the sixth sympathetic chain lumbar ganglion and immunoreactive for dopamine-β-hydroxylase (DßH) and leu-5-enkephalin (Leu) (short arrow). The immunoreactive neuron is richly supplied by Leu nerve fibres
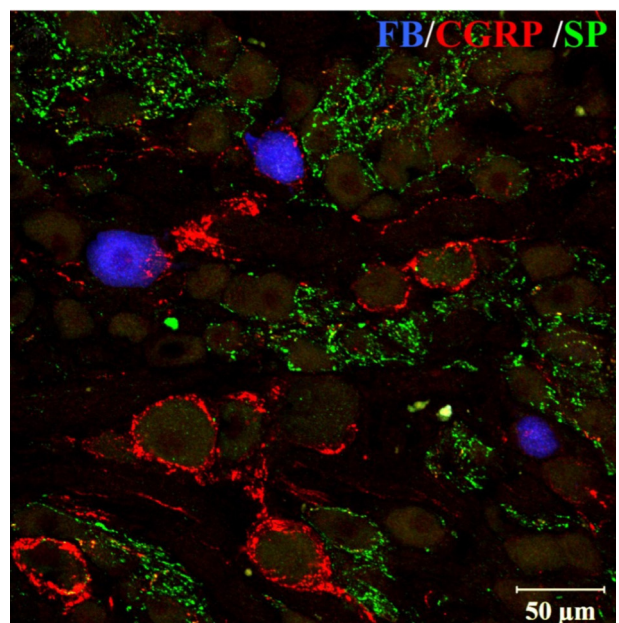

Figure 8. Micrograph showing a section stained with antibodies against substance P (SP) and calcitonin gene-related peptide (CGRP). Fast blue-positive (FB+) neurons innervating the medial side of the hip joint capsule are located in the fifth sympathetic chain lumbar ganglion and are richly supplied by CGRP+ nerve fibres
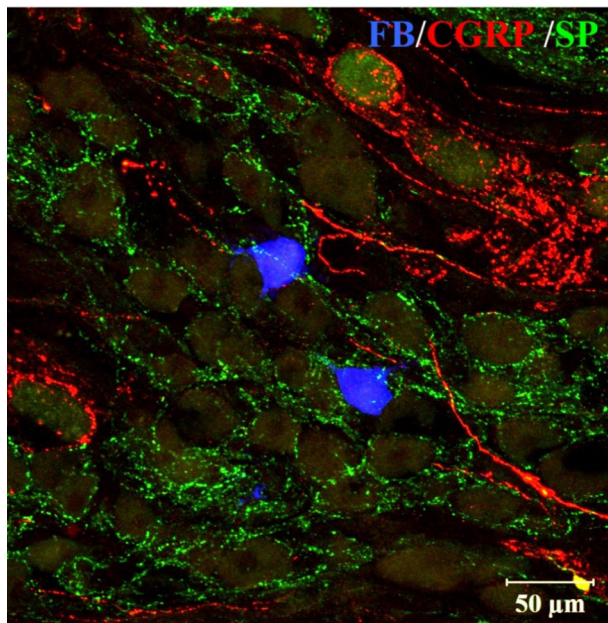
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Figure 9. Micrograph showing a section stained with antibodies against substance P (SP) and calcitonin gene-related peptide (CGRP). Fast blue-positive (FB+) neurons innervating the medial side of the hip joint capsule are located in the sixth sympathetic chain lumbar ganglion and are richly supplied by SP+ nerve fibres

SP, CGRP, enkephalin- or VIP-immunoreactivity were found. Dense networks of VACHT+ nerve fibres were noted in the vicinity of FB+ nerve cells
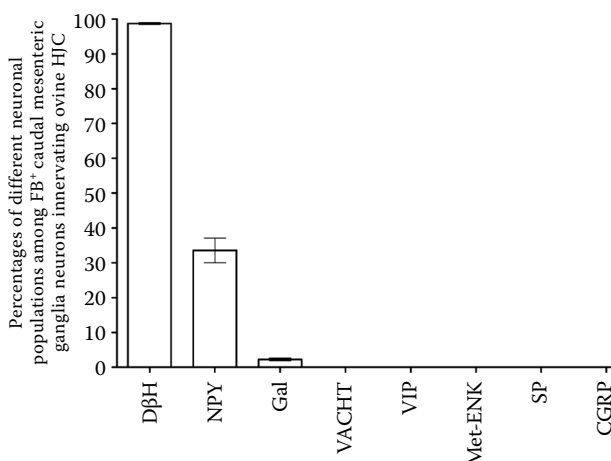

Figure 10. Chemical coding of fast blue-positive neurons supplying the hip joint capsule (HJC) located within the CaMG

CGRP = calcitonin gene-related peptide, DβH = dopamine-β-hydroxylase, Gal = galanin, Met-ENK = met-enkephalin, NPY = neuropeptide Y, SP = substance P, VACHT = vesicular acetylcholine transporter, VIP = vasoactive intestinal polypeptide
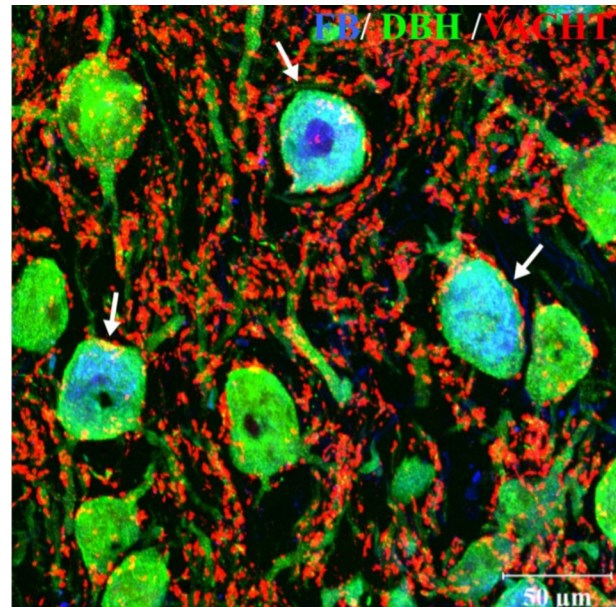

Figure 11. Fast blue-positive (FB+) neurons innervating the lateral side of the hip joint capsule are located in the caudal mesenteric ganglion, are immunoreactive for dopamine-β-hydroxylase (DßH) (long arrows) and are surrounded by high numbers of no cholinergic vesicular acetylcholine transporter (VACHT+) nerve fibres

(Figure 11). Populations of SP+, CGRP+ (Figure 14), Gal+ and enkephaligernic nerve fibres were also observed.

Figure 12. Fast blue-positive (FB+) neurons innervating the medial side of the hip joint capsule are located in the caudal mesenteric ganglion and are immunoreactive for dopamine-β-hydroxylase (DßH) (long arrow) or DßH/neuropeptide Y (NPY) (short arrows)
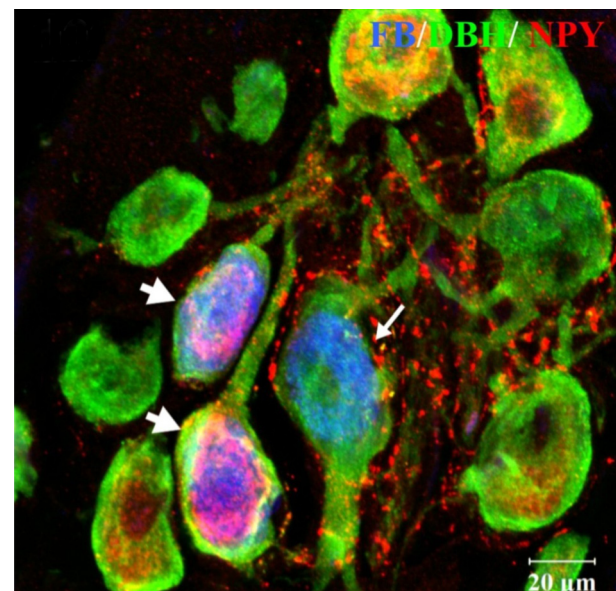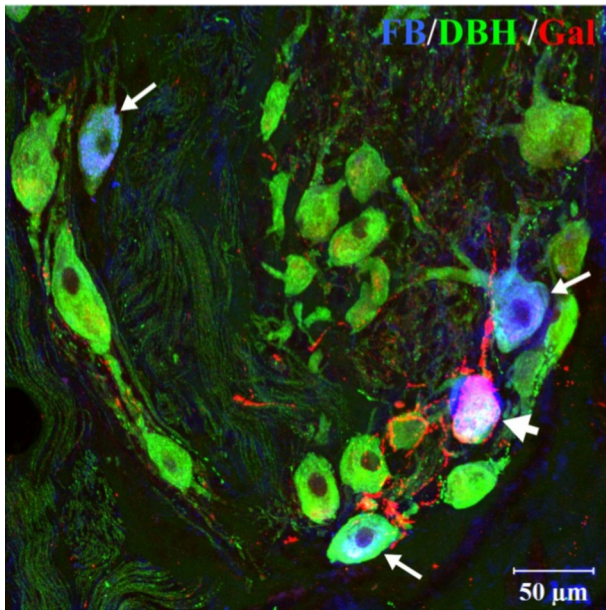
Figure 13. Fast blue-positive (FB⁺) neurons innervating the medial side of the hip joint capsule are located in the caudal mesenteric ganglion and are immunoreactive for dopamine-β-hydroxylase (DßH) (long arrows) or DßH/galanin (Gal) (short arrow)
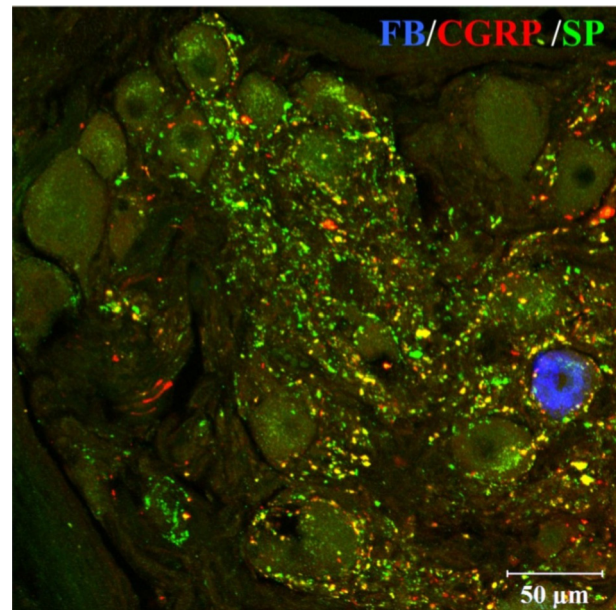

Figure 14. Micrograph showing a section stained with antibodies against substance P (SP) and calcitonin gene-related peptide (CGRP). An fast blue-positive (FB⁺) neuron innervating the medial side of the hip joint capsule is located in the caudal mesenteric ganglion and is richly supplied by SP/CGRP⁺ nerve fibres

DISCUSSION

The present work provides detailed information on the distribution and chemical coding of autonomic neurons supplying the hip joint capsule in sheep. This is the first such report in the literature. There are two papers dealing with the localisation and immunohistochemical properties of the autonomic neurons innervating the knee joint (Catre and Salo 1999) and temporo-mandibular joint (Uddman et al. 1998) in the rat. Both of them provide limited data on the chemical coding of the studied neurons. Catre and Salo (1999) found 187 ± 57 retrograde-labelled cells within the ipsilateral lumbar SChG and most of them were localised within L3 and L4 spinal cord segments. Immunostaining only allows description of three neuronal populations: tyrosine hydroxylase (TH)-, vasoactive intestinal peptide (VIP)- and somatostatin (SOM)-positive neurons (Catre and Salo 1999). Temporo-mandibular joint-projecting autonomic neurons were found in the ipsilateral superior cervical ganglion (1741 ± 821) and stellate ganglion (87 ± 100) (Uddman et al. 1998). Information concerning the immunohistochemical properties of this neuron is imprecise and the data describe only the presence of "many" labelled cell bodies in the superior cervical ganglion and stellate ganglion exhibiting NPY-immunoreactivity. It has also been reported that the labelled perikarya innervating the temporo-mandibular joint stained positive for the presence of NOS, but unfortunately no detailed information on the presence of NOS in the autonomic cells and the size of the population were given (Uddman et al. 1998).

Immunohistochemical characterisation of the autonomic nerve fibres located within the joint capsule was described for the rat knee joint capsule (Ahmed et al. 1994; Ahmed et al. 1995; Ackermann et al. 2001), the facet joint of the lumbar spine (Ahmed et al. 1993) and the temporo-mandibular joint (Uddman et al. 1998). Sympathetic neurons supplying joints are similar in their immunohistochemical properties to those controlling the activity of blood vessels within the muscles (Janig 2006); thus, we can assume that they are responsible for controlling the vascular tone in articular blood vessels and this way can influence blood flow (Schaible and Straub 2014).

In recent studies, almost all (96% within the SChG and 98.8% within the CaMG) the HJC-

projecting autonomic neurons were adrenergic in nature (DβH⁺). Results obtained in the CaMG are in agreement with our previous findings in which the general properties of CaMG neurons were described (Sienkiewicz et al. 2015). Our observations are not in congruence with data obtained in the rat (Catre and Salo 1999). The autonomic neurons innervating the rat knee joint were distributed within the SChG (mainly in L3 and L4) and the population of adrenergic neurons (TH⁺) amounted only to 33%. This incongruence can be explained by the fact that these data concern the perikarya innervating the entire patellofemoral joint (Catre and Salo 1999), whereas in our study only neurons supplying the joint capsule were described. Another explanation could be that the used primary antibodies differ (DBH in our study and TH in the study of Catre and Salo (1999)) or that the inconsistencies are because of interspecies differences.

All the NPY⁺ neurons in the present study were simultaneously adrenergic. Many of the rat temporo-mandibular joint-projecting sympathetic neurons were also found to be NPY-immunoreactive (Uddman et al. 1998). NPY is known to intensify/prolong the vasoconstrictive activity of adrenergic neurons (Lundberg et al. 1989a; Lundberg et al. 1989b; Sienkiewicz et al. 1995), so we can assume that it plays a similar role in neurons innervating the HJC in sheep.

The populations of enkephalinergic neurons (20% Met-Enk and 6% Leu-Enk-immunoreactive neurons, respectively) innervating HJC in the sheep were found only within the SChG. No enkephalin-immunoreactive neurons were observed within the CaMG. Simultaneously, enkephalinergic nerve fibres in the close vicinity of FB⁺ neurons were observed within the CaMG.

The enkephalins are known to act as vasodilators. Leu-Enk exerts agonistic action at opioid receptors and inhibits the secretion of noradrenalin by the synapses, which results in inhibition of blood vessel contraction (Ruth et al. 1984; Yuan et al. 1994).

Only a small population (2.2%) of galaninergic neurons innervating the HJC in the sheep was located in the CaMG. However, the presence of single Gal⁺ nerve fibres in the vicinity of labelled neurons was noted within the SChG. A similar-sized population (2%) of porcine trapezius-projecting autonomic neurons was previously reported (Dudek et al. 2014). It is known that galanin has vasoconstrictive properties (Preston et al. 1995).

In the present paper, we have described for the first time the distribution and chemical coding of the autonomic neurons innervating the hip joint capsule in sheep and have provided the most comprehensive data concerning their immunohistochemical characteristics.

Immunostaining analysis shows that in general the neurons innervating the HJC in sheep are similar with respect to their immunohistochemical properties to those described in previous reports, and based on the data described in the literature, we can assume that they are responsible for the control of the blood flow within the HJC: adrenergic or DBH/NPY⁺ and galaninergic neurons are responsible for vasoconstriction, while enkephalinergic ones are responsible for vasodilatation.

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