BMC Veterinary Research

RESEARCH Open Access

Immunohistochemical characterization of nerve fibers supplying canine elbow joint capsule

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Abstract

Introduction Only a few studies on the innervation of the dog's elbow joint have been described in veterinary literature. Consequently, there is a lack of information on the distribution and density of sensory nerve fibers within the dog's elbow joint capsule. In the current study, the density of nerve fibers and their functional characteristics within the dog's elbow joint capsule were determined by using immunohistochemical techniques.

Material and Methods The study material consisted of isolated joint capsules of the right and left elbow joints of 10 dogs, was divided into four quadrants: cranial, caudal, lateral and medial. The prepared material was cut with cryostat in to 14-16µm thick sections and next subjected to double immunohistochemical staining. Primary antisera directed against acetylated tubulin (AccTub), substance P (SP), calcitonin gene-related peptide (CGRP), vesicular acetylcholine transporter (VAChT), vasoactive intestinal peptide (VIP), nitric oxide synthase (NOS), galanin (GAL), and dopamine beta-hydroxylase (DβH) were used for the study. The obtained preparations were analyzed using a Zeiss LSM 700 confocal microscope.

Results Immunoreactive fibers for all studied substances were located in all quadrants of the elbow joint capsule.

Conclusion The created preliminary model of the innervation of the dog's elbow joint provides a basis for expanding the scope of research on the innervation of this joint. Additionally, it may contribute to the search for new or improvement of existing surgical techniques within this joint.

Keywords Joint capsule, Nerve fiber, Immunohistochemistry, Elbow joint

Introduction

Osteoarthritis (OA) is one of the most frequently diagnosed musculoskeletal disorders in dogs, associated with chronic pain and gradually leading to deterioration in physical fitness and permanent decline in quality of life. Approximately 37% of lameness cases in dogs are caused by OA [1]. The etiology of the disease is multifactorial and is characterized by progressive damage to the articular cartilage and underlying bone, as well as changes in the periarticular soft tissues. It is diagnosed in over 20% of dogs as young as one year old [2]. With age, the percentage of affected animals increases, reaching 45–80%

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of the population above 8–12 years of age [3]. Therapeutic management of OA in the elbow joint is particularly challenging because the diagnosed changes are often advanced, irreversible, and involve various joint structures, which is due to the joint's complex anatomical structure. Treatment focuses mainly on improving the dog's quality of life by alleviating pain and limiting disease progression. In veterinary medicine, there are many treatment protocols for OA of the elbow joint, but none of them leads to complete symptom resolution or cure of the patient. This is why new, better options for pain management in patients with OA are still being sought. From a point of view of pain relief in the joint, it is important to know how exactly is the joint innervated. The joint capsule is innervated by periosteal nerve fibers, nerve fibers from muscles, and fibers that are branches of the main nerves innervating the joint [4]. According to veterinary anatomical atlases, the joint is innervated by four nerves: the musculocutaneous nerve, the median nerve, the radial nerve, and the ulnar nerve [5]. In the available literature, there are only a few studies describing in detail the articular branches of these nerves to the dog's elbow joint capsule [4, 6–8]. Consequently, information regarding the presence and density of sensory fibers within the dog's elbow joint capsule is lacking. To fill in this gap, we labeled nerve fibers within the dog's elbow joint capsule by using immunohistochemical techniques with primary antisera directed against selected neuropeptides. The findings can be taken as a starting point for a discussion about the possible functional properties of the labeled nerve fibers.

Primary sera against AccTub, SP, CGRP, NOS, GAL, DβH, VIP and VAChT were used in the study. Acetylated tubulin (AccTub) plays a role in intracellular transport and is a building block of nerve cells. The purpose of staining with an antibody directed against acetylated tubulin was to determine the overall density of nerve fibers within the capsule. SP and CGRP are widely considered pain mediators. The use of antibodies directed against SP and CGRP allowed for the imaging of sensory fibers. In our study, we decided to use an antibody directed against (DβH), because the presence of this enzyme was found in most autonomic neurons supplying the hip joint [9]. Galanin is a neuropeptide involved in sensory processes [10]. The presence of GAL was demonstrated in studies of the sheep hip joint capsule, localizing this substance in sensory and autonomic neurons [9, 11]. The presence of VIP has been found in sensory fibers supplying, among others, the knee joint capsule in cats and mice [12, 13], particularly in fibers surrounding synovial blood vessels [11]. VACHT is a marker of the cholinergic system, which plays a significant role in joint function. Studies conducted by Dudek et al. 2017 [14] in sheep confirmed the presence of VAChT+ neurons innervating the hip joint capsule, located in the spinal ganglia. Nitric oxide (NO), a gas, is produced from L-arginine by various isoforms of nitric oxide synthase (NOS). NO is an unconventional neurotransmitter because it is a gas, is not stored in synaptic vesicles, and is synthesized on demand by neurons. It has many physiological functions, including being a neurotransmitter, neuromodulator, and vasodilator [15].

Materials and methods

The study material consisted of isolated capsules of the left and right elbow joints of dogs. They were collected within 1 h after euthanasia. The joint capsules were obtained from 10 dogs. Dogs in the age range (9–13 years), eight males, two females, with a similar body weight in the range of 35–50 kg body weight. The dogs belonged to different breeds - Labrador Retriever, German Shepherd, the remaining dogs were mixed breeds. The animals were euthanized in veterinary clinics for reasons unrelated to elbow joint diseases. According to Article 1, Paragraph 2 of the Act on the Protection of Animals Used for Scientific or Educational Purposes, veterinary services and clinical veterinary studies are not experimental procedures and do not require the approval of the Ethics Committee. The elbow joint capsules used as research material were divided into 4 quadrants: cranial, medial, lateral, and caudal. (Fig. 1).The material for immunohistochemical studies was fixed by immersion in a 4% paraformaldehyde solution in phosphate buffer (PO4, 0.1 M, pH 7.4) at a temperature of 4 °C for 30 min. After this time, the sample was rinsed in phosphate buffer (0.1 M, pH 7.4) and transferred to a 30% buffered sucrose solution (PB) (0.1 M, pH 7.4). The fixed capsules were embedded in medium (OCT, Cell Path) and then frozen at -24 °C. The material was then sectioned using a cryostat microtome (Leica Crycut 1800) into 14–16 µm thick sections and mounted on chromalum-coated slides. The slides were stored at -24 °C. The prepared material on the slides was subjected to double immunohistochemical staining. Primary antisera from mice, rabbits, and rats directed against acetylated tubulin (AccTub), substance P (SP), calcitonin gene-related peptide (CGRP), vesicular acetylcholine transporter (VAChT), vasoactive intestinal peptide (VIP), nitric oxide synthase (NOS), galanin (GAL), and dopamine beta-hydroxylase (DβH) were used in the study (Table 1). The sections were blocked (for 60 min) with 10% goat serum. It contained 1% bovine serum albumin and 0.5% Tween 20 in phosphate-buffered saline (PBS) x NaCl (0.1 M, pH 7.4). After blocking, the slides were rinsed in PBS x NaCl (0.1 M, pH 7.4) 3 times for 15 min each, and then a mixture of primary antibodies diluted in PBS x 2NaCl (0.1 M, pH 7.4) was applied to the sections. Incubation with the primary antibodies lasted 24 h in a humid chamber at room temperature.

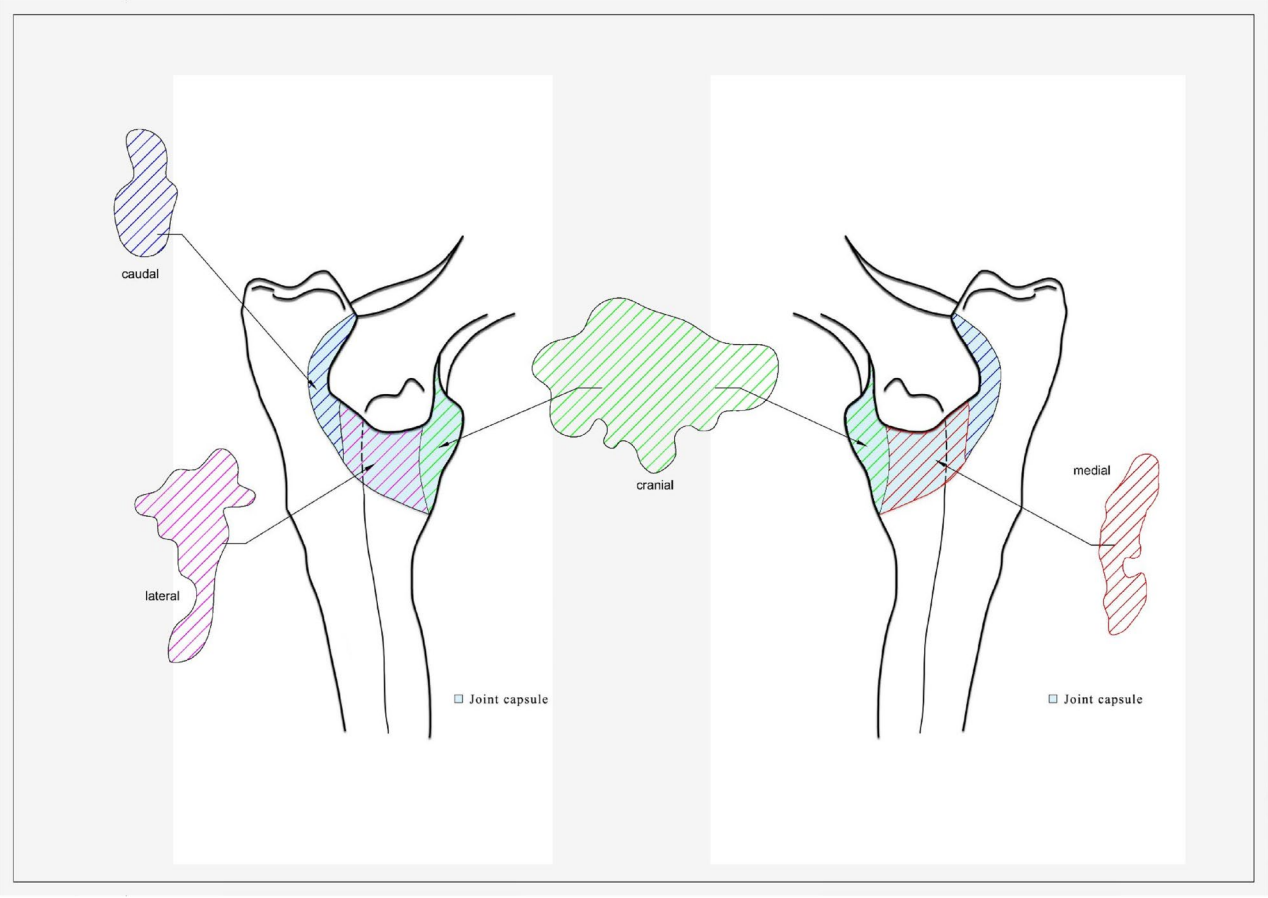

Fig. 1 Illustrative figure showing fragments of the capsule of a canine's elbow joint

Table 1 Antisera used in the study

Antigen	Host	Type	Dilution	Cat. No.	Lot/Batch	Supplier
Primary antisera						
Acetyl-alpha Tubulin	mouse	monoclonal	1:500	#32-2700	#TC240595	Invitrogen
VACHT	rabbit	polyclonal	1:4000	V5387	095K4751	Sigma
CGRP	rabbit	polyclonal	1:2000	11,535	2659 F	Cappel
n-NOS	mouse	monoclonal	1:100	N2280	081K4815	Sigma-Aldrich
VIP	mouse	monoclonal	1:500	MaVIP	91,278	East Acres Biologicals
SP	rat	monoclonal	1:150	8450 – 0505	NC134	ABD Serotec, UK
Gal	rabbit	polyclonal	1:2000	RIN7153	990921-2	Peninsula Lab.
DßH	*rabbit*	monoclonal	1:500	DZ1020-0050	0606031668	Biomol, Exeter, UK
Secondary antisera						
Host		Fluorochrom	Dilution	Code	Lot	Supplier
Goat-anti-rabbit IgG (H + L)		Alexa fluor 488	1:500	A11008	51,385 A	Invitrogen
Goat-anti-mouse IgG (H + L)		Alexa fluor 488	1:500	A11001	632,115	Invitrogen
Goat-anti rabbit IgG (H + L)		Alexa fluor 568	1:500	A11011	623,962	Invitrogen
Goat-anti-mouse IgG (H + L)		Alexa fluor 568	1:500	A11004	49,399 A	Invitrogen
Goat-anti-rat IgG (H + L)		Alexa fluor 555	1:500	A21434	1,670,155	Invitrogen

The slides were then rinsed again three times for 15 min in PBS x NaCl (0.1 M, pH 7.4). After rinsing the samples, a mixture of secondary antibodies (Table 1) was applied to the sections and incubated for 60 min in a humid chamber at room temperature. After incubation with the secondary antibodies, the preparations were rinsed three times for 15 min in PBS x NaCl (0.1 M, pH 7.4). The sections were then covered with a mixture of glycerol and PB (0.1 M, pH 7.4), mixed in a 1:1 ratio, and covered with coverslips. The prepared slides could then be evaluated

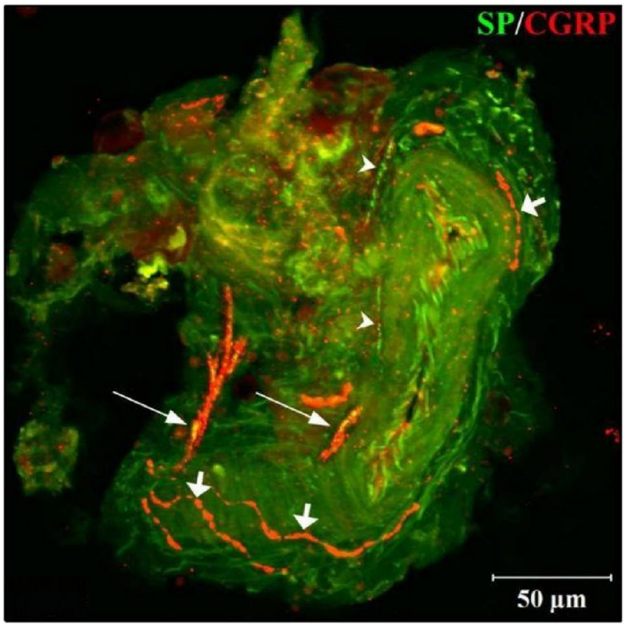

Fig. 2 Confocal laser scanning microscope images showing the distribution of CGRP [CGRP+; red; Alexa 555 visualization (A555)] and SP [SP+ , green; Alexa 488 visualization (A488)] nerve fibres in the elbow joint capsule; green and red channels were digitally superimposed, so nerve fibres that stained for both markers are yellow to orange. Moderate numbers of nerve fibres exhibited immunoreactivity to CGRP only (short arrow), and moderate number of nerve fibres stained to SP only (arrowhead). Nerve fibers immunopositive for SP/CGRP are marked with a long arrow

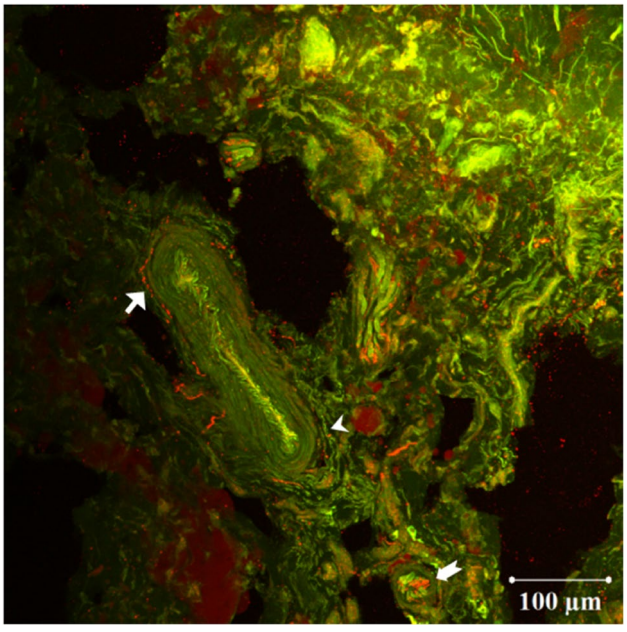

Fig. 3 Section from the fibrous membrane of the canine elbow joint stained with antibodies against SP (arrowhead) and CGRP (short arrow). In the photo we can see a bundle of nerve fibers (double tailed)

under a confocal microscope (Zeiss LSM 700). Evaluation of the preparations was performed with the microscope equipped microscope lens 20x magnification, 0.8 aperture. Samples were scored by a single experienced investigator using a semi-quantitative analysis of the obtained data, rigorously applying the following fiber count scoring: 0 = no fibers, 1 = a few (single in the preparation) 2 = some, 3 = numerous, 4 = very numerous.

The goal of our work was primarily clinical. The goal was to determine whether sensory fibers were evenly distributed within each capsule fragment, or whether differences existed. The semiquantitative system (using the scale described above) is sufficient effectively capture these crucial joint innervation thresholds.

Photographic documentation was performed using the aforementioned microscope, with the ZEN 2009 software. Images from double staining were obtained by overlaying color channels.

Results

Immunoreactive fibers for SP, CGRP, and SP/CGRP were evenly distributed in all quadrants of the elbow joint capsule (Figs. 2 and 3). There were few fibers immunoreactive for SP (SP-1), some fibers immunoreactive for CGRP (CGRP-2), and few (SP/CGRP-1) or some (SP/CGRP-2) immunoreactive for SP/CGRP. SP and CGRP positive fibers were distributed in the fibrous layer of the joint capsule and in the synovial layer, with a slight predominance of their density in the former layer (Fig. 4). In the synovial layer, a substantially higher density of double-positive SP/CGRP fibers was observed, with many fibers observed (SP/CGRP-3), in contrast to only few fibers in the fibrous layer of the joint capsule (SP/CGRP-1). A higher density of SP and CGRP positive fibers was observed around blood vessels, where some or many fibers were noted.

A semi-quantitative analysis of the obtained data was conducted by applying the following scoring of the number of fibers was used: 0 = no fibers, 1 = a few (single in the preparation) 2 = some, 3 = numerous, 4 = very numerous. Among 216 specimens, there were 31 cases where at least one of the evaluations for SP, CGRP, or SP/CGRP was missing. Table 2 presents the frequencies and percentages of different combinations of scores (in the order: SP, CGRP, SP/CGRP; for instance, "122" means SP = 1, CGRP = 2, SP/CGRP = 2) for 185 specimens with all three evaluations. The result presented in the table indicate that, in 155 (83.79%) of the cases, there was no score 3 ("numerous") for any of the three evaluations. In other words, in 83.79% of the cases, the highest score for any of the evaluations was equal to at most 2 ("some"). In 29 (15.67%) of the cases, there was a score of 3 ("numerous") for one of the evaluations, and in only one case (0.54%) there was one score of 4 ("very numerous"), noted for SP.

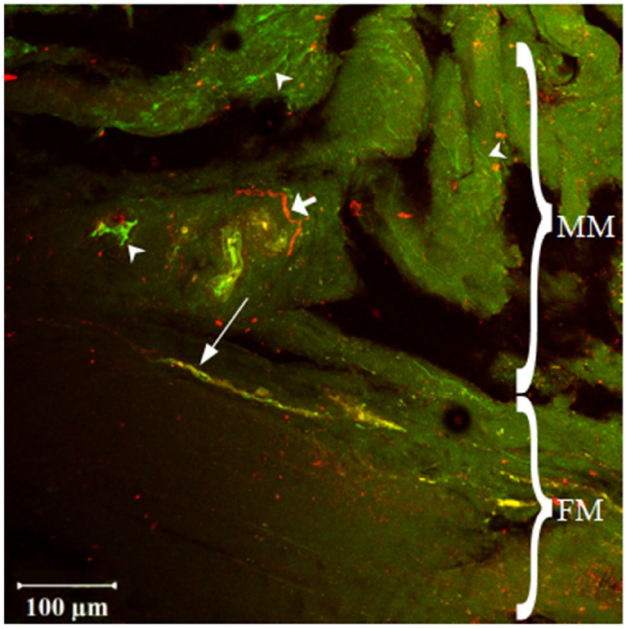

Fig. 4 Confocal laser scanning microscope images showing the distribution of CGRP [CGRP+; red; Alexa 555 visualization (A555)] and SP[SP+ , green; Alexa 488 visualization (A488)] nerve fibres in sections from mucose membrane (MM) and fibrous membrane(FM). SP-immunopositive nerve fibres are stained green (arrowhead), and CGRP-immunopositive fibers are stained red (short arrow). SP/CGRP double fibers are stained orange (long arrow)

Table 2 Frequency and percentage of specimens with different results ("Combination") of assessment of SP, CGRP, and SP/CGRP (in that order). The following scoring of the number of fibers was used: 0 = no fibers, 1 = a few (single in the preparation), 2 = some, 3 = numerous 4 = very numerous. For instance: 212 means "some fibers for SP, a few for CGRP, some for SP and CGRP"

Combination	Frequency	Percent
020	1	0.54
111	28	15.14
112	10	5.41
121	54	29.19
122	34	18.38
211	4	2.16
212	6	3.24
221	2	1.08
222	16	8.65
123	1	0.54
131	4	2.16
132	11	5.95
213	2	1.08
232	9	4.86
322	2	1.08
421	1	0.54
Total	185	100.00

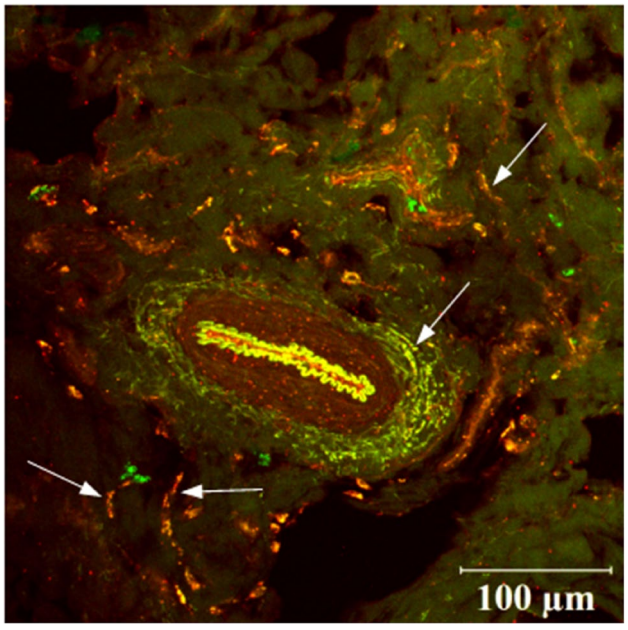

Fig. 5 Double-stained fibers immunopositive for AcTub/DBH [AcTub+ , green; Alexa 488 visualization (A488)][DBH+; red; Alexa 555 visualization (A555)]around a blood vessel (long arrow -we can observe fibers colored in various shades from yellow to orange)

The distribution of the various combinations of scores was similar for all four quadrants of the joint, as illustrated in Table S1 in Supplementary Materials (note that no formal statistical test to confirm similarity of distributions was applied due to the sparsity of the table).

During microscopic examinations, the presence of some immunoreactive fibers for acetylated tubulin AcTub (AcTub-2) was found, with a similar density in all quadrants of the joint capsule. There were many (Acc Tub-3) or a lot (Acc Tub-4) fibers immunoreactive to AcTub around blood vessels (Fig. 5). A few AccTuB/DBH (AccTuB/DBH +) fibers were present in all quadrants of the capsule (Fig. 6).

Among 216 specimens, there were 53 cases where at least one of the evaluations (AccTuB or AccTuB/DBH) was missing. Table 3 presents the frequencies and percentages of different combinations of scores (in the order: AccTuB, AccTuB/DBH; for instance, "21" means AccTuBP = 2, AccTuB/DBH = 1) for 163 specimens with both evaluations. It indicates that the most prevalent combination (96 specimens, 58.90%) was "21", i.e., the case when for AccTuB a score of 2 ("some") was noted, while for AccTuB/DBH a score of 1 ("a few") was given. Overall, in 147 (90.18%) of the cases, the highest score observed for any of the two evaluations was 2 ("some"). It is worth noting that, for AccTuB/DBH, the scores of 3 and 4 were not noted.

The distribution of the various combinations of scores was similar for all four quadrants of the joint, as illustrated in Table S2 (note that no formal statistical test to

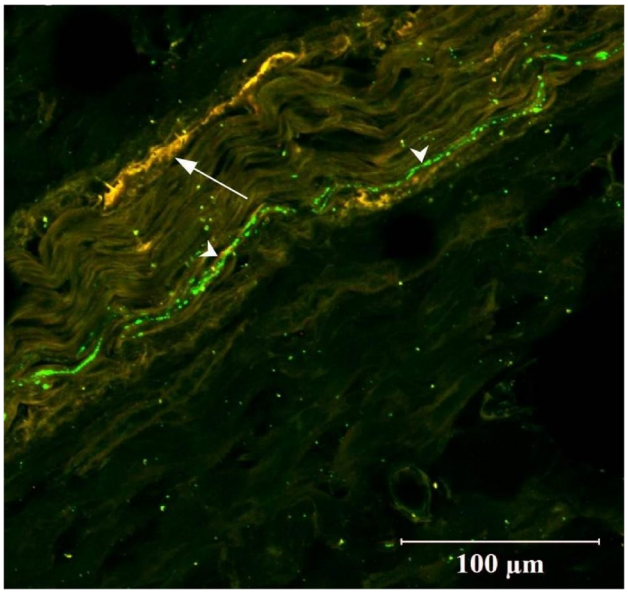

Fig. 6 Confocal laser scanning microscope images showing the distribution of DBH[DBH+; red; Alexa 555 visualization (A555)] and AcTub [AcTub+, green; Alexa 488 visualization (A488)] nerve fibres in the fibrous membrane of the joint capsule, fiber immunopositive for AcTub and DBH (long arrow),fiber immunopositive for AcTub (arrowhead)

Table 3 Frequency and percentage of specimens with different results ("Combination") of assessment of AccTuB and accTuB/DBH (in that order). The following scoring of the number of fibers was used: 0 = no fibers, 1 = a few (single in the preparation), 2 = some, 3 = many, 4 = a lot. For instance: 21 means "some fibers for AccTuB, a few for AccTuB/DBH"

Combination	Frequency	Percent
00	3	1.84
10	4	2.45
11	43	26.38
21	96	58.90
22	1	0.61
31	11	6.75
32	1	0.61
41	3	1.84
42	1	0.61
Total	163	100.00

confirm similarity of distributions was applied due to the sparsity of the table).

Few nerve fibers immunoreactive for nitric oxide synthase (NOS) (NOS-1) were observed in all quadrants of the joint capsule. Their density increased around blood vessels, where some (NOS-2) or many (NOS-3) were observed. There were a few nerve fibers positive for galanin (GAL-1), and they were evenly distributed in all quadrants of the joint capsule (Fig. 7). The observed density of GAL-positive fibers was higher in the synovial layer of the capsule (GAL-2) than in the fibrous layer (GAL-1) (Fig. 8). A few double immunoreactive fibers for

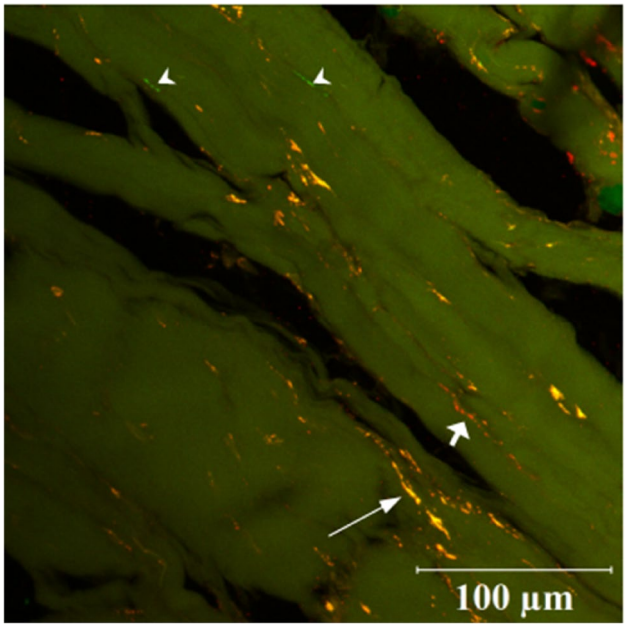

Fig. 7 Confocal laser scanning microscope images showing the distribution of NOS [NOS+;green; Alexa 488 visualization (A488)] and GAL [GAL+, red; Alexa 555 visualization (A555)] nerve fibres in sections from fibrous membrane. NOS-immunopositive nerve fibres are stained green (arrowhead), and GAL-immunopositive fibers are stained red (short arrow). NOS/GAL positive fibers are stained orange (long arrow)

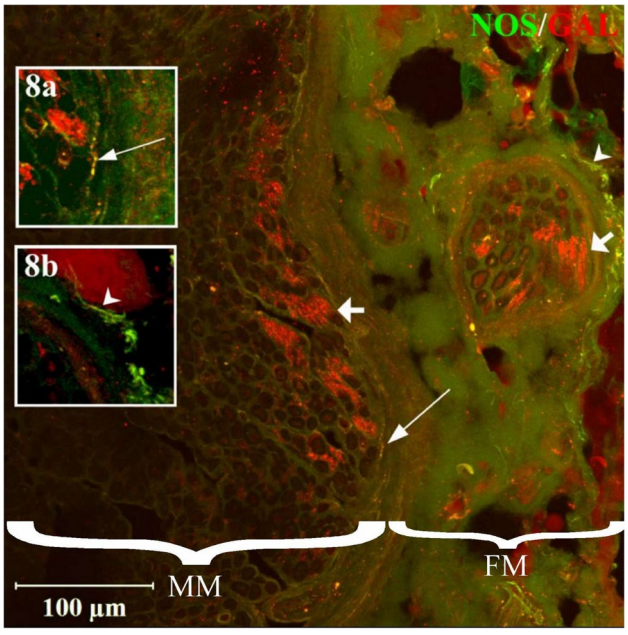

Fig. 8 Confocal laser scanning microscope images showing the distribution of GAL[GAL+; red; Alexa 555 visualization (A555)] and NOS [NOS+ , green; Alexa 488 visualization (A488)] nerve fibres in the elbow joint capsule;GAL nerve fibers immunopositive in the synovial (MM) and fibrous membrane (FM) of the joint capsule (red, short arrow). **a** Double-stained fibers for NOS and GAL at the interface of the membranes (orange, long arrow). **b** NOS fibers positive in the fibrous membrane of the capsule (green, arrowhead)

NOS/GAL were observed, with a slight predominance in the lateral quadrant of the joint capsule.

Among 216 specimens, there were 53 cases where at least one of the evaluations (NOS, GAL, or NOS/GAL) was missing. Table 4 presents the frequencies and percentages of different combinations of scores (in the order: NOS, GAL, NOS/GAL; for instance, "120" means NOS = 1, GAL = 2, NOS/GAL = 0) for 163 specimens with all three evaluations. It indicates that the most prevalent combination (102 specimens, 62.58%) was "111", i.e., the case when for all evaluations it was concluded that there were only "a few" fibers. Overall, in 158 (96.83%) of the cases, the highest score observed for any of the three evaluations was 2 ("some"). In 5 (3.07%) of the cases, there was one score of 3 ("numerous"), noted for NOS.

The distribution of the various combinations of scores was similar for all four quadrants of the joint, as illustrated in Table S3 (note that no formal statistical test to confirm similarity of distributions was applied due to the sparsity of the table).

Immunoreactive fibers for VIP and VAChT, VIP/VAChT were present in all quadrants of the joint capsule (Figs. 9 and 10). There were few (VIP-1) or some (VIP-2) fibers immunoreactive to VIP. Some VAChT fibers (VAChT-2) and some double VIP/VAChT fibers (VIP/VAChT-2) were observed. A slight predominance of fiber density was found in the cranial and medial quadrants of the joint capsule. Their density also increased around blood vessels, with many or a lot fibers present (Fig. 11). A slight predominance of VIP-positive fiber density was observed in the synovial layer of the capsule, with many fibers (VIP-3), compared to the fibrous layer of the joint capsule, where only some VIP-positive fibers were observed (VIP-2).

Among 216 specimens, there were 59 cases where at least one of the evaluations (VACHT, VIP, or VACHT/VIP) was missing. Table 5 presents the frequencies and percentages of different combinations of scores (in the order: VACHT, VIP, VACHT/VIP; for instance, "231" means VACHT = 2, VIP = 3, VACHT/VIP = 1) for 167 specimens with all three evaluations. It indicates that in 119 (71.16%) of the cases, there was no score 3 ("numerous") in the combination. In other words, in 71.16% of the cases, the highest score for any of the three evaluations was at most 2 ("some"). On the other hand, in 15 (8.98%) of the cases, there was at least on score of 4 ("very numerous") for one of the evaluations, with 6 (3.59%) cases where "numerous" fibers were noted for all three evaluations.

The distribution of the various combinations of scores was similar for all four quadrants of the joint, as illustrated in Table S4 (note that no formal statistical test to confirm similarity of distributions was applied due to the sparsity of the table).

Table 4 Frequency and percentage of specimens with different results ("Combination") of assessment of NOS, GAL and NOS/GAL (in that order). The following scoring of the number of fibers was used: 0 = no fibers, 1 = a few (single in the preparation), 2 = some, 3 = numerous, 4 = very numerous. For instance: 120 means "a few fibers for NOS, some for GAL, and none for NOS/GAL"

Combination	Frequency	Percent
110	9	5.52
111	102	62.58
112	1	0.61
120	1	0.61
121	7	4.29
200	1	0.61
210	1	0.61
211	20	12.27
221	10	6.13
222	6	3.68
310	2	1.23
311	3	1.84
Total	163	100.00

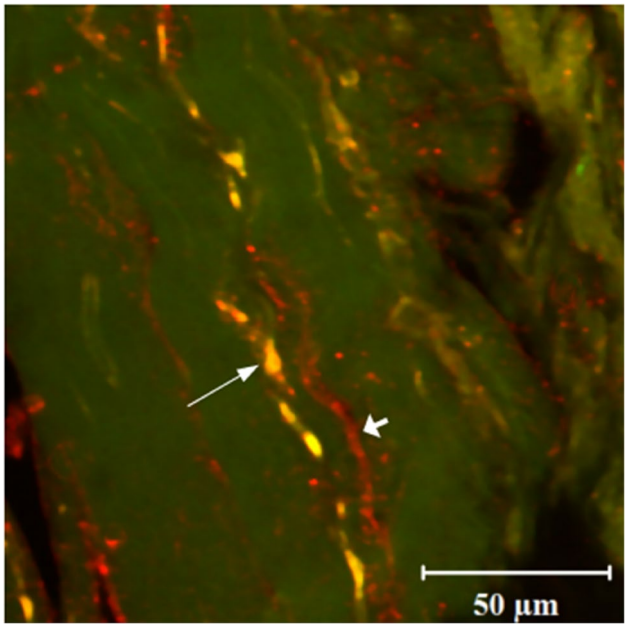

Fig. 9 Confocal laser scanning microscope images showing the distribution of VIP [VIP+; green; Alexa 488 visualization (A488)] and VACHT [VACHT+, red; Alexa 555 visualization (A555)] nerve fibres in sections from the elbow joint capsule; green and red channels were digitally superimposed, so nerve fibres that stained for both markers are yellow to orange (VIP/VACHT; long arrow) or. against VACHT only (red; short arrow)

Discussion

Immunohistochemical staining using neuron-specific markers allows visualization of their morphology, distribution, and innervation density. This technique requires the appropriate selection of a primary antibody specific to the desired antigen marker (in our case, a neuropeptide in nerve fibers) and the appropriate selection of a

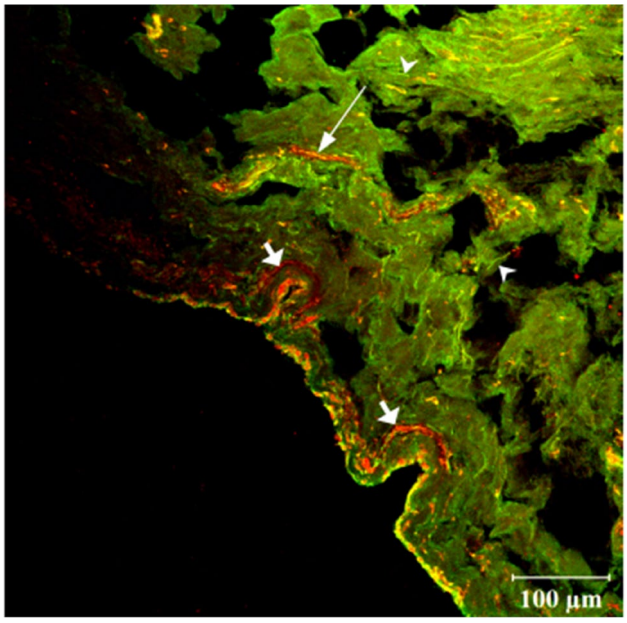

Fig. 10 Confocal laser scanning microscope images showing the distribution of VIP [VIP+; green; Alexa 488 visualization (A488)] and VACHT [VACHT+, red; Alexa 555 visualization (A555)] nerve fibres in sections from the elbow joint capsule; Fibers immunopositive for VIP stained green (arrowhead). Fibers immunopositive for VACHT stained red (short arrow). Double-stained fibers containing VIP/VACHT can be observed in orange (long arrow)

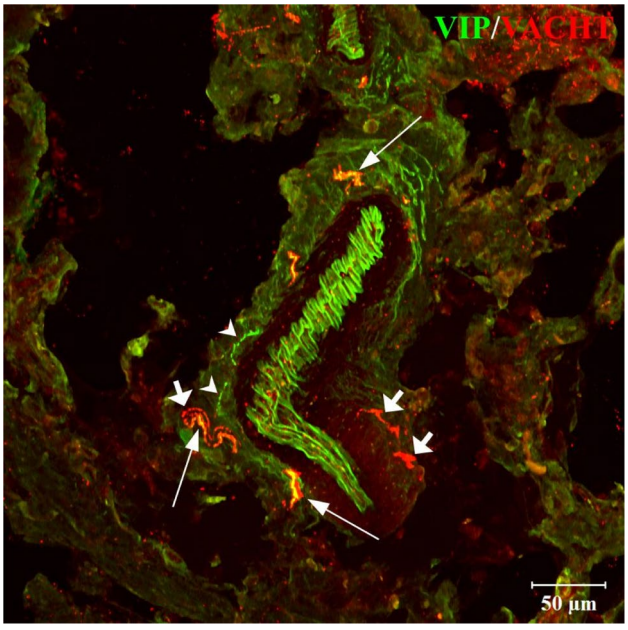

Fig. 11 Confocal laser scanning microscope images showing the distribution of VIP [VIP+; green;Alexa 488 visualization (A488)] and VACHT [VACHT+, red;Alexa 555 visualization (A555)] nerve fibres in sections from the elbow joint capsule; Nerve fibers around a blood vessel - fibrous layer of the joint capsule. Fibers immunopositive for VACHT stained red (short arrow), for VIP (arrowhead)). Fibers doubly stained, containing VIP/VACHT - orange (long arrow)

Table 5 Frequency and percentage of specimens with different results ("Combination") of assessment of VACHT, VIP and VACHT/VIP (in that order). The following scoring of the number of fibers was used: 0 = no fibers, 1 = a few (single in the preparation), 2 = some, 3 = many, 4 = a lot. For instance: 231 means "some fibers for VACHT, many for VIP, and a few for VACHT/VIP"

Combination	Frequency	Percent
000	1	0.60
010	2	1.20
111	32	19.16
112	1	0.60
121	8	4.79
122	2	1.20
211	4	2.40
220	1	0.60
221	9	5.39
222	59	35.33
223	1	0.60
231	2	1.20
232	5	2.99
233	4	2.40
322	3	1.80
323	1	0.60
333	17	10.18
334	3	1.80
343	1	0.60
432		1.20
433	2	1.20
443	1	0.60
444	6	3.59
Total	167	100.00

secondary antibody, which will reveal the antigen-antibody complex under the microscope. The use of these primary antibodies allows determining the functional properties of nerve fibers. In our study, primary antisera directed against acetylated tubulin (AccTub), substance P(SP), calcitonin gene-related peptide (CGRP), nitric oxide synthase (NOS), galanin (GAL), dopamine beta-hydroxylase (DβH), vasoactive intestinal peptide (VIP), and vesicular acetylcholine transporter (VAChT) were used.

We observed an even distribution of immunopositive fibers for all the examined substances in all evaluated fragments of the dogs' elbow joint capsules. The highest density in all quadrants of the joint capsules was observed for immunopositive fibers for AccTub, with a slightly lower, but still substantial, presence of fibers immunopositive for SP, CGRP, and SP/CGRP. The presence of nerve fibers for all the examined substances importantly increased around the blood vessels of the joint capsule. Immunopositive fibers for all the substances were present in both the synovial and fibrous layers of the joint capsule. The presence of galaninergic, vipergic, and cholinergic nerve fibers in the synovial layer of the joint

capsules suggests that they may influence synovial fluid production.

AccTub is present in the cell bodies and axons of neurons in both the central and peripheral nervous systems [16]. In our study, the staining showed the presence of many, evenly distributed nerve fibers in all quadrants of the capsule.

SP and CGRP are involved in the transmission of sensory stimuli through peripheral nerves [17]. It is believed that SP also transmits pressure and temperature [18]. SP and CGRP immunopositive fibers play an important role in joint innervation. Fortier and Nixon in 1997 [19] detected sensory fibers immunopositive for SP in areas of remodeled bone in the course of osteoarthritis in horses, suggesting that these fibers play a role in transmitting pain information and enhancing inflammation in the course of this disease. In our study, SP and CGRP immunopositive fibers were the most frequently occurring nerve fibers within the joint capsule. Additionally, the SP and CGRP fibers were evenly distributed in all quadrants of the joint capsule, suggesting an even distribution of sensory fibers.

DβH is an essential neurotransmitter-synthesizing enzyme that catalyzes the formation of norepinephrine (NE) from dopamine. NE acts as a neurotransmitter in both the central and peripheral nervous systems. DβH can therefore be assessed as a marker of norepinephrine function in various disorders and diseases. For instance, in humans, a deficiency of this enzyme is associated, among other things, with circulatory disorders, joint hyperflexia, and sluggish deep-tendon reflexes [20]. In our study, DβH immunopositive fibers were present in all parts of the dogs' elbow joint capsules, but their density substantially increased around blood vessels. This may suggest that these neurons are the source of adrenergic innervation primarily for the blood vessels supplying the joint. They may influence the regulation of blood flow within the joint, while simultaneously delivering substances that may affect inflammation modulation within the joint and tissue healing processes. Due to the important role this enzyme plays in blood vessels, its deficiency leads to circulatory disorders, which in turn may also cause disturbances in synovial fluid flow.

GAL is a neuropeptide involved in sensory processes. In the autonomic nervous system, axonal injury leads to an increase in GAL's concentration - it is believed to play a role in enhancing neuron survival and nerve regeneration after injuries and alleviating neurogenic inflammation [21]. GAL is known as a neuropeptide with inhibitory effects on the central nervous system. When administered intrathecally, it causes antinociception and enhances the antinociceptive effects of morphine. It also affects peripheral sensory endings. In our study, GAL immunopositive nerve fibers were present in all quadrants of the dogs' elbow joint capsules, with fiber density increasing around blood vessels and in the synovial layer of the joint capsule. This may suggest the involvement of these fibers not only in sensory and neuroprotective processes, but also in the regulation of blood flow and synovial fluid production in the joint.

In mammals, nitric oxide (NO) is used for many intercellular and intracellular signaling functions, regulation of blood pressure and vascular tone, neuronal signal transmission, cytotoxicity against pathogens and tumors, control of cell growth and differentiation [22] The synthesis of nitric oxide in the body is carried out by NOS, which exists in three main isoforms: NOS-1 (nNOS – neuronal), NOS-2 (iNOS – inducible), and NOS-3 (eNOS – endothelial). Immunohistochemical studies have shown that all three NOS isoforms are present in inflamed synovial membrane. It has been shown that rodents in animal models of arthritis exhibit high levels of NOS and cyclooxygenase, which reflect high levels of NO in serum and urine, developing in connection with disease symptoms. Treatment of these animals with NOS inhibitors or NO scavengers inhibits NO production and effectively eliminates arthritis [23]. NOS immunopositive nerve fibers were observed in our study in all quadrants of the dogs' elbow joint capsules, which is undoubtedly related to the function of this enzyme.

VIP is known as a potent vasodilator. Studies have shown that both a decrease and an excessive increase of this substance in the joint can cause inflammation and degeneration [24]. In a study on rats [25], intra-articular administration of VIP showed that the animals exhibited pain symptoms, even though they were healthy. VIP has been found in sensory fibers supplying, among others, the knee joint capsule of cats and mice [12, 13], particularly in fibers around blood vessels of the synovial membrane [11]. In our study, the density of VIP immunopositive nerve fibers substantially increased around blood vessels within the joint capsule. Additionally, a slight predominance of these fibers was observed in the synovial layer of the capsule, which may suggest the involvement of these fibers in regulating blood flow within the joint and in synovial fluid production.

Acetylcholine is a neurotransmitter synthesized in cholinergic neurons. It is present, among others, in neuromuscular junctions, stimulating skeletal muscles. VAChT is a neurotransmitter responsible for transporting acetylcholine to synaptic vesicles. VAChT expression gradually increases as it approaches the nerve ending (making this molecule a good marker for parasympathetic nerve fibers) [26]. Studies conducted on sheep confirmed the presence of VAChT+ neurons innervating the hip joint capsule located in spinal ganglia [14]. In our study, the presence of VAChT immunopositive nerve fibers was observed in the dogs' elbow joint capsules. With the recent advances

in diagnostic methods, providing a complete and detailed description of the innervation of the dog's elbow joint is an important and interesting research challenge. Especially since there are studies on denervation of the elbow joint in dogs [7, 27], which may be one form of treatment for chronic pain in dogs with diagnosed degenerative disease of the elbow joints. Portela et al. note that for the success of the method, it is extremely important to know the most accurate model of the innervation of the canine elbow joint. In their research, they considered our earlier work related to the macroscopic, characteristic supply of articular branches of the nerves innervating the canine elbow joint. The preliminary model of the innervation of the canine elbow joint obtained by our team - determining the place of departure of the intra-articular branches of the main nerves innervating the elbow joint (ulnar nerve, radial nerve, median nerve and musculocutaneous nerve) into the elbow joint capsule, as well as, as it turned out, the even distribution of sensory nerve fibers within the elbow joint capsule - allows us to initially develop a technique for denervation of the elbow joint, which may therefore contribute to improving the local treatment of chronic elbow joint pain in dogs in the future. Denervation as a treatment for elbow joint pain might be especially indicated for young, large-breed dogs with severe signs of elbow dysplasia, when early surgical intervention has not eliminated chronic pain, the discomfort persists, and pharmacological therapy has become insufficient. We hope that the preliminary model of the innervation of the dog's elbow joint obtained by our team may contribute to the improvement of local treatment for chronic elbow joint pain in dogs in the future.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12917-026-05363-5.

Supplementary Material 1.

Acknowledgements
Additional thanks to Anna Przyborowska and Agata Jacewicz.

Authors' contributions
Jowita JacewiczRoles Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draftWaldemar SienkiewiczConceptualization, Formal analysis, Project administration, Supervision, Validation, Tomasz BurzykowskiFormal analysis, Software, Supervision, Beata DegórskaConceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, All authors reviewed the manuscript.

Funding
The study was financed from the funds of the Department of Small Animal Diseases and Clinic ,Warsaw University of Life Sciences and Department of Animal Anatomy, Faculty of Veterinary Medicine, UWM in Olsztyn.

Data availability
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
According to Article 1, Paragraph 2 of the Act on the Protection of Animals Used for Scientific or Educational Purposes, veterinary services and clinical veterinary studies are not experimental procedures and do not require the approval of the Ethics Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 2 June 2025 / Accepted: 5 February 2026
Published online: 06 March 2026

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