Innervation of the patella

An immunohistochemical study in mice

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In the mouse, arthritis was induced by a single subpatellar intraarticular injection of bacterial collagenase. This procedure induces also patellar malalignment. A rich innervation of thin varicose calcitonin gene-related peptide (CGRP) and substance P (SP) immunoreactive fibers was found in the joint capsule, in the periosteum of the patella, in the synovial tissues at the lateral border of the patella, in the femoral groove, and in the subchondral bone of the patella and femur. Moreover, fibers were found in plica tissues between the quadriceps and patellar tendon, and the femoral groove.

After the collagenase treatment, the general innervation pattern was comparable to that of the controls, but CGRP and SP innervation was no longer detectable with the antibodies in the plica tissues, and was to a lesser extent detectable in the fat pad of the patella, in the lateral borders of the patella and in the proliferated synovial tissues. Signs of degenerated axonal profiles were observed in these locations with a polyclonal antibody to the growth-associated protein GAP-43/B-50. At all the other peripheral locations, such as the muscles, the GAP-43/B-50 distribution was normal.

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For some considerable time it has been well-known that bone, the medullary space in bones and the periosteum are innervated (Kuntz and Richings 1945, Miller and Kashara 1963, Sherman 1963, Milgram and Robinson 1965, Reimann and Christensen 1977). Particularly since the application of immunocytochemistry to hard tissues, it has become evident that different chemically-defined types of fiber are involved in the innervation of the skeleton. Nerve fibers containing vasoactive intestinal polypeptide, substance P (SP), calcitonin gene-related peptide (CGRP), neuropeptide Y and tyrosine hydroxylase have been found in mineralized bone, the medullary space, the periosteum and in peri- and intraarticular soft tissues (Grönblad et al. 1984, 1988, Bjurholm et al. 1988a, b, 1989). Particularly fibers containing CGRP and SP are involved in the propagation of nociceptive impulses to the spinal cord (for refs, see Wojtys et al. 1990).

I studied the innervation of the patella in mice in which pathological conditions were induced chemically with a single intraarticular injection of bacterial collagenase (van der Kraan et al. 1990). After this procedure, arthritis develops in the tibio-femoral joint. Osteophytes are formed on the margins of the patella and in the collateral ligaments, the synovium is proliferated and malalignment of the patella is always observed.

An antibody to a neuron-specific protein (GAP-43 or B-50), located in the inner face of axonal membranes, was used as a general marker for neuronal fibers (Kristjansson et al. 1982, Gorgels et al. 1989, van Lookeren Campagne et al. 1991).

Animals and methods

10 adult male C57BL10 mice, 10 weeks old, were used. The right knee was injected intraarticularly once through the patellar ligament with 6 μL of a filtered (0.2-μm bacteria filter) solution of 2% (W/V) bacterial collagenase (248 μ/mg, Worthington Biochemical Corporation, Freehold, NJ, U.S.A.; van der Kraan et al. 1990). The left control knee was injected with a similar volume of physiological saline. In a separate experiment, the possible effect of the injection on the innervation pattern was tested: 5 mice were injected in the right knee with physiological saline, the left knee remained untreated.

Histology

After 35 days the mice were anesthetized with sodium pentobarbitone (ca 60 mg/kg body weight) and perfused via the left ventricle with 0.9% NaCl (0.5–2 min) and with 4% freshly prepared paraformaldehyde

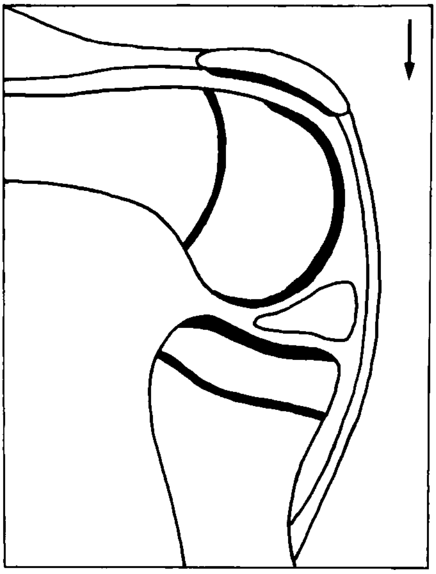


Figure 1. The knee joint of the mouse. The arrow indicates the direction of the sectioning. All joints were sectioned in the frontal plane.

and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4, at 20 °C, for 15 minutes. The knee joints were excised and further fixed for 48 h in the same fixative. All the joints were decalcified in 10% EDTA in 0.1 M phosphate buffer (adjusted to pH 7.4) at 4 °C. The EDTA solution was changed twice a week. After complete decalcification (2 weeks), the joints of 4 mice were embedded in polymethyl-methacrylate (PMMA), sectioned in the frontal plane (see Figure 1), after which alternating 7 µm sections were stained with hematoxilin-eosin, Safranin O and PAS/alcian blue. The joints of 6 mice were transferred to 30% sucrose, deep-frozen and sectioned (50 µm) with a freeze microtome. Sections were collected in Tris-buffered saline (TBS; 0.05 M Tris, 0.9% NaCl, pH 7.4) for free-floating immunohistochemical processing.

Immunohistochemistry

Immunostaining was performed with polyclonal rabbit antibodies to CGRP (1:6000; Peninsula, RAS 6009–N), SP (1:6000; UCB, i675/002) and to GAP–43/B–50 (a gift of Dr. Oestreicher, 1:8000; see Oestreicher and Gispen 1986, Gorgels et al. 1987, 1989 for specificity tests of this antibody). The sections were stained with (1) the antiserum, overnight, (2) peroxidase-conjugated swine immunoglobulins to rabbit immunoglobulins (1:50, 90 min, Dakopatts) and (3) with 0.04% 3,3-diaminobenzidine-4HCl (Merck), 0.1% nickel ammonium sulfate and 0.003% H_2O_2 in 0.05 M Tris-buffer (pH 7.6) for 10 min. All the antibody dilutions were made in TBS, to which 0.5% bovine-serum albumin, 0.5% Triton–X–100 and 1% normal goat serum were added. Before, between and after each incubation step, the sections were washed with three 20-min changes of TBS. All the incubations were performed at room temperature during continuous gentle agitation. After the staining procedure, the sections were mounted on gelatin-coated slides, air-dried, dehydrated and cover-slipped with Entellan.

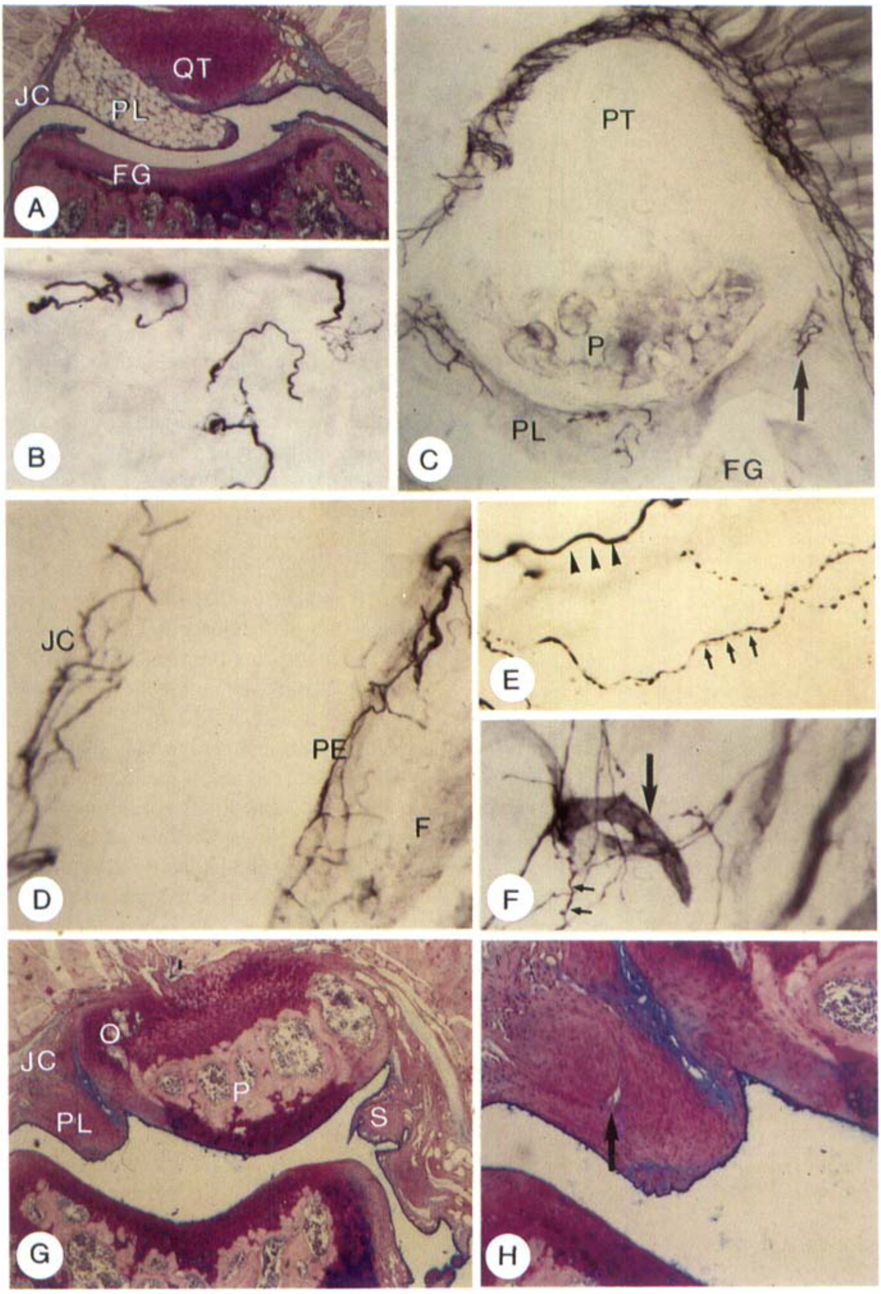
The method specificity of the antisera was determined using the conventional method of substituting normal rabbit preimmune serum for the primary antiserum and omitting the primary antiserum step. The specificity of the CGRP antisera was further tested by the preadsorption of 5 mL of the diluted antiserum with 0.1 mg CGRP (6009, Peninsula), 5 mL of the diluted SP antiserum was preadsorbed with 0.1 mg SP (7451, Peninsula). As a reference for the effect of EDTA on the immunostaining, the brains were dissected and cut into 2 halves in the mid-sagittal plane. After 48 h of fixation, the left half was transferred to 0.1 M phosphate-buffered (pH 7.4) 30% sucrose, allowed to sink and rapidly deep-frozen in liquid nitrogen, and stored at –80 °C. The right halves of the brains were transferred to 10% EDTA in the buffer, at 4 °C. After 1, 3, and 6 weeks, the right halves of the brains were treated in the same way as the left halves. Free-floating tissue sections of comparable levels of both halves of the brains were sequentially incubated in the same vials with the antibodies. The EDTA sections were marked with a small cut for recognition.

Results

No differences were observed between the brains treated with EDTA and the controls. After 6 weeks in the decalcification solution, the staining pattern and intensity were comparable with the controls. In the joints, labeled fibers could be easily recognized, the immuno-background staining was low or absent, and all controls were completely negative. Most of the fibers were free-running through the connective tissues. The CGRP and SP immunoreactivity were located in the same areas, but generally more CGRP fibers were found as compared to SP fibers. Therefore, the following description of the innervation pattern applies to both the CGRP and SP fibers.

A rich innervation of CGRP and SP fibers was found directly outside the joint capsule (Figures 2 and 3), in the transition area of the joint capsule with the

Figure 2. PAS/Alcian blue-stained (A, G, H) and immuno-stained sections (B–F) with antibodies to CGRP (B–D), SP (E) and GAP-43/B-50 (F) of the patellar joint of control (A–E) and arthritic (F–H) mice.



A–C. Note the innervated synovial plica tissues (PL) between the quadriceps tendon (QT) or patella (P) and the femoral groove (FG), the intense innervation of the outside of the joint capsule (JC) and the innervation (arrow) just under the attachment site of the joint capsule (JC) on the patella (P). A) ×25. B) Enlargement of plica tissue shown in C. ×175. C) ×50.

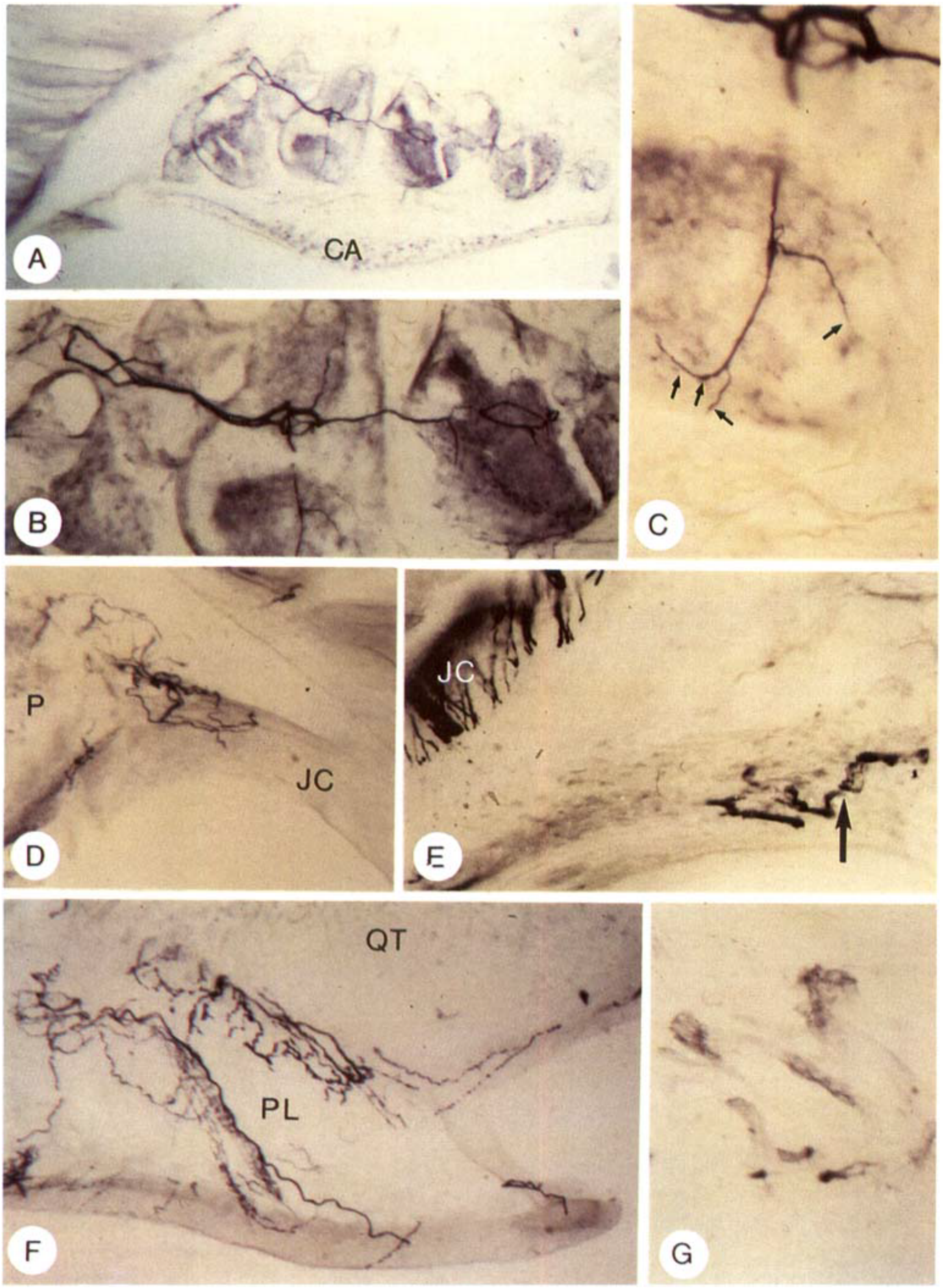
D. Innervation of the joint capsule (JC) and the periosteum (PE) of the femur (F). ×80.

E, F. High magnification of fibers in the fat pad of the patella. E) Note 2 types of fiber, viz., thick fibers (arrowheads) and thin varicose fibers (small arrows). F) Effect of collagenase treatment on fibers in the fat pad. Note very characteristic structures (large arrow) and normal fibers(small arrows). D–F ×225.

G. Effect of collagenase treatment on patellar joint. Note osteophyte (O) at the attachment site of the joint capsule (JC) on the patella (P), dislocation of the patella (P) and fibrotic plica tissue (PL) of synovial (S) origin. ×25.

H. Higher magnification of same area. Note tear in synovial plica tissue (arrow). ×70.

Figure 3. Sections immuno-stained with antibodies to CGRP (A–D, F) and to GAP–43/B–50 (E, G) of control (A–D, F) and osteoarthritic (E, G) mice.



A–C. Innervation of the patella by a small bundle of CGRP fibers. Note small branches of the fiber (arrows) in the direction of the subchondral bone. A) ×35. B) and C) enlargements of A. ×150 and ×400, respectively.

D. Innervation at the attachment site of the joint capsule (JC) on the patella (P). ×45.

E. Almost same location in contralateral site. Note degenerated fibers (arrow). ×125.

F. Innervation of plica tissue ×150.

G. Same location, but after induction of arthrosis. Note absence of normal fibers and the presence of many degenerative structures. ×160.

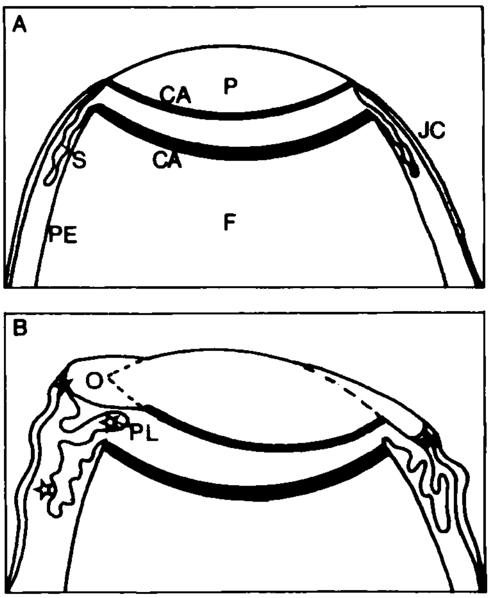


Figure 4. Representative tissue sections of the control patellar joint. CA cartilage, F femur, JC joint capsule, P patella, PE periosteum of femur, S synovium. B Diagrammatic depiction of an arthritic joint, in which the most important changes after induction of arthritis are depicted. Note formation of large synovial plica tissue (PL) in the space between the patella and the femoral groove and the formation of osteophytes (O) at the margins of the patella at the attachment sites of the joint capsule. Asterisks indicate areas in the tissue sections where CGRP and SP innervation were lost, and where GAP–43/B–50 immunopositive degenerative structures were found; at the margins of the patella, the synovial plica tissue between the patella and femoral groove, in the patellar fat pad and in the synovial tissues lateral to the femur.

patella, in the periosteum of the patella, in the anterior surface of the patellar tendon overlying the patella, and in the femoral groove beneath the quadriceps tendon. Many immuno-positive fibers were also found on the anteriolateral periosteum of the femur, partly in association with blood vessels. Particularly the synovial tissues at the margins of the patella were heavily innervated with the fibers. Plica tissues of the lateral and medial synovium, were found at the upper and lower poles of the patella where the tendons insert. Plicas consisted of fat cells separated by connective tissue septae and were covered with a thin synovial lining. No plicas were found in the joint space at the central region of the patella. A rich innervation of CGRP and SP fibers was found in all plica tissue. Most of the fibers were located in the fibrous septae interconnecting the fat cells or in the synovial lining. CGRP and SP fibers were also found in the marrow cavities of the femur and patella. Thick fibers branched, sending very small varicose fibers that were directed into the subchondral bone of the articular cartilage.

The effects of the collagenase injection have been described by van der Kraan et al. (1990). Clinically the mice functioned well. After 5 weeks, it was noticeable that the right (injected) knee was unstable, and the patella was dislocated. The articular cartilage of the patella appeared to be normal, but inspection with polarized light showed some fibrillation of the collagen fibers. The synovial tissues were proliferated (Figure 2). Both the medial and lateral borders of the patella showed osteophytes, and the synovial space was enlarged. Synovial plica tissues were directed into the joint cavity between the femur and patella. The plicas were rather fibrotic, and in some of them, particularly between the patellar tendon and the femoral groove, necrotic zones and tears were present.

No obvious differences were observed in the CGRP and SP innervation of the periosteum, the joint capsule and the medullary cavity. However, the innervation of the plica tissues between the quadriceps and patellar tendon and the femoral groove was no longer detectable. The sections were also stained with a marker for neuronal elements, the antibody to the growth-associated phosphoprotein GAP–43/B–50. In the control knees, the general staining pattern found with the GAP–43/B–50 antibody was comparable to the CGRP and SP distributions, including the innervation of the plicas, but the innervation pattern was more intense. In the arthritic knee, however, staining was observed in the areas where the CGRP and SP immunoreactivity had been lost (Figure 4). Immunopositive, fairly irregular plate-like structures were observed. The stained structures as a whole did not show any clear morphology of nerve fibers. In the general vicinity of the stained structures, neuronal fibers with a normal morphology were present. These probably degenerative structures were found in the plica tissues, in the proliferated synovial tissues around the patella, in the patellar fat pad, and in the lateral and medial soft tissues which bordered the patella.

Discussion

To avoid damage to the innervation pattern I preferred to produce changes in the joint chemically rather than by trauma. The breakdown of collagen Type I in the collateral ligaments, cruciate ligaments, and menisci and the resulting instability of the joint are probably responsible for the induction of the lesions (van der Kraan et al. 1990). The advantage of this procedure is that it is rapid, the initial damage to the joint is minimal and the results are reproducible.

Previously it was found with light and electron microscopical procedures that SP and CGRP are colocalized (Gulbenkian et al. 1986, Venesio et al. 1987, Bjurholm et al. 1988a, Holzer 1988). Although I made no double-labeling experiments, the similarity in the appearance of the SP- and CGRP-immunoreactive fibers around the patella of the mice, strongly suggests that here also at least a large part of the fibers contains both SP and CGRP. Although other fibers may also propagate sensory information to the spinal cord (Heppelmann and Schaible 1990), particularly SP and CGRP fibers are involved in the transduction of nociceptive impulses to the spinal cord (Hope et al. 1990, Schaible et al. 1990, Wojtys et al. 1990, Hanesch et al. 1991). The presence of CGRP and SP in the periarticular and intraarticular soft tissues around the patella suggests that sensory information can be obtained from all these structures.

A loss of CGRP and SP fibers was particularly striking in the plica tissues between the patellar tendon and the femoral groove, in the soft tissues lateral to the patella, and in the synovial folds. One explanation for the lack of innervation may be lowered SP and CGRP levels in the fibers due to increased release of the peptides into the tissues, decreased transport, and/or lower levels of synthesis of the peptides in the cell bodies (Grönblad et al. 1988, Hukkanen et al. 1991). If peripheral levels of SP and CGRP are low after increased release, the immunocytochemical staining procedure may be too weak to demonstrate the peptides. However, this explanation does not seem plausible, since the GAP–43/B–50 antibody did not reveal normal fibers, which could be expected as the fibers were depleted. Instead, very characteristic structures were stained. Although the remnants of nerve fibers could be recognized, the structures as a whole did not have the morphology of nerve fibers. Although the exact nature of these structures cannot be judged from the light microscopical picture alone, I speculate that the structures are related to nerve trauma or damage. Tetzlaff et al. (1989) found similar structures after an experiment in which the sciatic and facial nerves of the rat were crushed. They speculated that GAP–43/B–50 might be secreted from regenerating axons and might play a role in axon-Schwann cell interactions during axonal maturation.

Joint instability induced by the collagenase can be expected if the protection against excessive movements by cruciates, collateral ligaments and joint capsule is lost. It may induce chronic overload of particular joint structures and/or large deformations of the soft tissues at the attachment sites of the joint capsule on the patella. This may be an important common factor in both the deleterious effects on the tisssues and the nervous innervation. Degenerative neuronal structures were only found at locations where also other tissue damage was found, e.g., necrotic zones in the plica tissue, ruptures, fibrillation of fat tissue.

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