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Sensory innervation of the cat knee articular capsule and cruciate ligament visualised using anterogradely transported wheat germ agglutinin–horseradish peroxidase

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ABSTRACT

Wheat germ agglutinin–horseradish peroxidase conjugate (WGA–HRP) was injected into the dorsal root ganglia (L5–S1) of the cat and used as an anterograde tracer substance for intra-axonal labelling of peripheral nerve endings in joint capsule and cranial (anterior) cruciate ligament (CCL). We believed that the high specificity of WGA–HRP for neural tissue along with the high visibility of its reaction product could help resolve controversies concerning the sensory innervation of the cruciate ligaments. Substantial amounts of WGA–HRP were transported in tibial nerve axons to the level of the knee. However, using standard HRP histochemistry we found that the capsular tissue and ligament synovia disintegrated during the incubation reaction. This problem was avoided by air drying the tissue slices on glass slides prior to reaction. Abundant labelling occurred in the posterior capsule with dense filling of axons and terminal endings. Sensory endings displayed features consistent with Ruffini endings and pacinian corpuscles. Sensory endings were located throughout the CCL in its sagittal plane, in the subsynovial layers and between collagen fascicles. In each CCL we observed 5–17 ovoid and elongated endings with dense terminal arborisations. These endings were between 100 and 150 μm long, were encapsulated, and gave rise to 1 or 2 axons. Large (up to 1.5 mm in maximum extent) elongated regions of dense, inhomogeneous labelling were found in the body of several CCLs. These resembled Golgi tendon-like endings, with the exception of their large size. We conclude that anterograde transport of HRP to the knee is a useful technique for labelling mechanoreceptors and axons in knee tissue. However, recently developed immunohistochemical analysis of peripheral tissue using protein gene product 9.5 appears to be the method of choice and should be employed for further study of human and animal cruciate ligament innervation.

Key words: Joint mechanoreceptors.

INTRODUCTION

Human and animal cruciate ligaments of the knee contain encapsulated and corpuscular sensory endings supplied by myelinated axons. Putative sensory endings have been observed using light microscopy (Boyd, 1954; Skoglund, 1956; Sjölander et al. 1989; Haus & Halata, 1990; Marinozzi et al. 1991; Yahia & Newman, 1991), immunohistochemical techniques (Rivard et al. 1993; Aune et al. 1996), and ultrastructural examination (Halata & Haus, 1989). Histological evidence for mechanoreceptors is consistent with electrophysiological recordings of mechanoreceptive afferents from nerves supplying the cranial (anterior) cruciate ligament (CCL) in the cat (Pope et al. 1990; Krauspe et al. 1992; Madey et al. 1993; Khalsa & Grigg, 1996). These signals may supply information used for leg motor control, but we lack conclusive evidence to support such a theory. The sensory role of the anterior cruciate ligament (ACL) in human motor control is of particular interest given the high injury incidence of this structure, and strong suspicion of a sensory role (Skoglund, 1956; Madey et al. 1993).

Although the presence of sensory endings in cruciate ligaments is well established, fundamental

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characteristics of this innervation remain controversial. Light microscopic studies using a variety of embedding and staining techniques, including gold chloride and ultrastructural examination of human ACL and cat CCL, report or imply only a few to several encapsulated sensory endings per ligament (Boyd, 1954; Skoglund, 1956; Freeman & Wyke, 1967; Schultz et al. 1984; Sjölander et al. 1989; Haus & Halata, 1990). Similar findings exist for the fibular collateral ligament (de Avila et al. 1989). In contrast, other studies employing gold chloride staining reported between 19 and 37 receptors per ligament in cat cruciate ligament (Gomez-Barrena et al. 1992) and 'abundant' receptors in canine ligament (Yahia & Newman, 1991). Marinozzi et al. (1991) reported large numbers of receptors in rat cruciate ligament using acetylcholinesterase stains.

The location of receptors also remains controversial. In several studies receptors in the human anterior cruciate ligament were reported to cluster either at the tibial insertion (Zimny et al. 1986) or the femoral insertion (Schultz et al. 1984). In the cat receptors have been reported both at the tibial and at the femoral insertions (Freeman & Wyke, 1967; Sjölander et al. 1989), and throughout the ligament (Halata & Haus, 1989; Haus & Halata, 1990).

Finally, 3 types of corpuscular or encapsulated endings have been described, corresponding roughly to pacinian corpuscles (lamellar capsule), Ruffini-like endings (small multiple globular or ovoid corpuscles), and Golgi-like (large, fusiform corpuscle). In cat CCL some investigators reported only Golgi tendon organ-like endings (Skoglund, 1956; Freeman & Wyke, 1967; Koch et al. 1995) while others reported all 3 endings (Sjölander et al. 1989). Recent work in human ACL identified only Ruffini-like and pacinian corpuscles (Halata & Haus, 1989; Haus & Halata, 1990).

Conflicting results among studies of ligament receptors may be attributed to limitations of staining techniques (cf. Bancroft & Stevens, 1990), poor fixation and staining, and mistaken identification of nonneural structures such as blood vessels (cf. de Avila et al. 1989; Halata & Haus, 1989; Haus & Halata, 1990; Koch et al. 1995). The abundance of nonneural argyrophilic structures in joint tissue requires conservative criteria to identify neural elements stained with impregnated silver or gold salts, and the need to follow structures in serial sections (de Avila et al. 1989; Koch et al. 1995).

Anterograde transport of wheat germ agglutinin–horseradish peroxidase (WGA–HRP) after injection into extracellular spaces in sensory ganglia has been employed successfully to visualise cutaneous sensory nerve endings in the rat hind paw (Robertson & Aldskogius, 1982; Aldskogius et al. 1986) and in rat facial skin, cornea, and dental tissues (Marfurt & Turner, 1983). In these studies HRP was transported in large amounts both by large and by small axons to axonal terminals and provided high specificity for neural tissue, high visibility, and ease of interpretation.

The purpose of the present study was to determine whether WGA–HRP could be used as an anterograde tracer substance for intra-axonal labelling of peripheral nerve endings in cat joint capsule and ligaments. Immunohistochemical analysis of peripheral sensory innervation, for example using antibodies for protein gene product 9.5, came into widespread use since the present work was completed and, in our opinion, should be employed for further study of cruciate ligament innervation (e.g., Rivard et al. 1993; Aune et al. 1996).

METHODS

Five adult cats were used in these experiments, employing a protocol approved by our Institutional Review Board. Animals were initially anaesthetised with ketamine (22 mg/kg intramuscularly, with 10 mg acepromazine and 1 g xylazine), then given an initial injection of 30 mg sodium pentobarbital followed by continuous infusion at 10 mg/h through an internal jugular catheter. Animals were ventilated at a rate of 20 ($I/E = 0.3$) and rectal temperature maintained at 37 °C. Vital signs and depth of anaesthesia were monitored every 2 h.

The pelvis was rigidly fixed with the animal in the prone position and a laminectomy was performed to expose the L5–S1 dorsal root ganglia bilaterally. 5–10 μl of 2 % WGA–HRP (Sigma) were injected into each dorsal root swelling over a period of about 15 s with a 50 μl Hamilton syringe. In 2 cats normal saline was injected into the ganglia on one side as a control to assess the presence of artefactual labelling. Incisions were closed and the animals maintained under general anaesthesia for 30–36 h.

Animals were euthanised with sodium pentobarbital i.v. and immediately perfused to fix the tissues. The aorta was cannulated just distal to the arch and the animal was perfused with 700 ml normal saline with 3 ml of 3 % H_2O_2 followed by 2 l of cool 3 % paraformaldehyde in a phosphate buffer over 45 min, and then 6 l cold, graded phosphate-buffered sucrose solutions until a concentration of 30 % was achieved.

Both knees were harvested and stored in a phos-

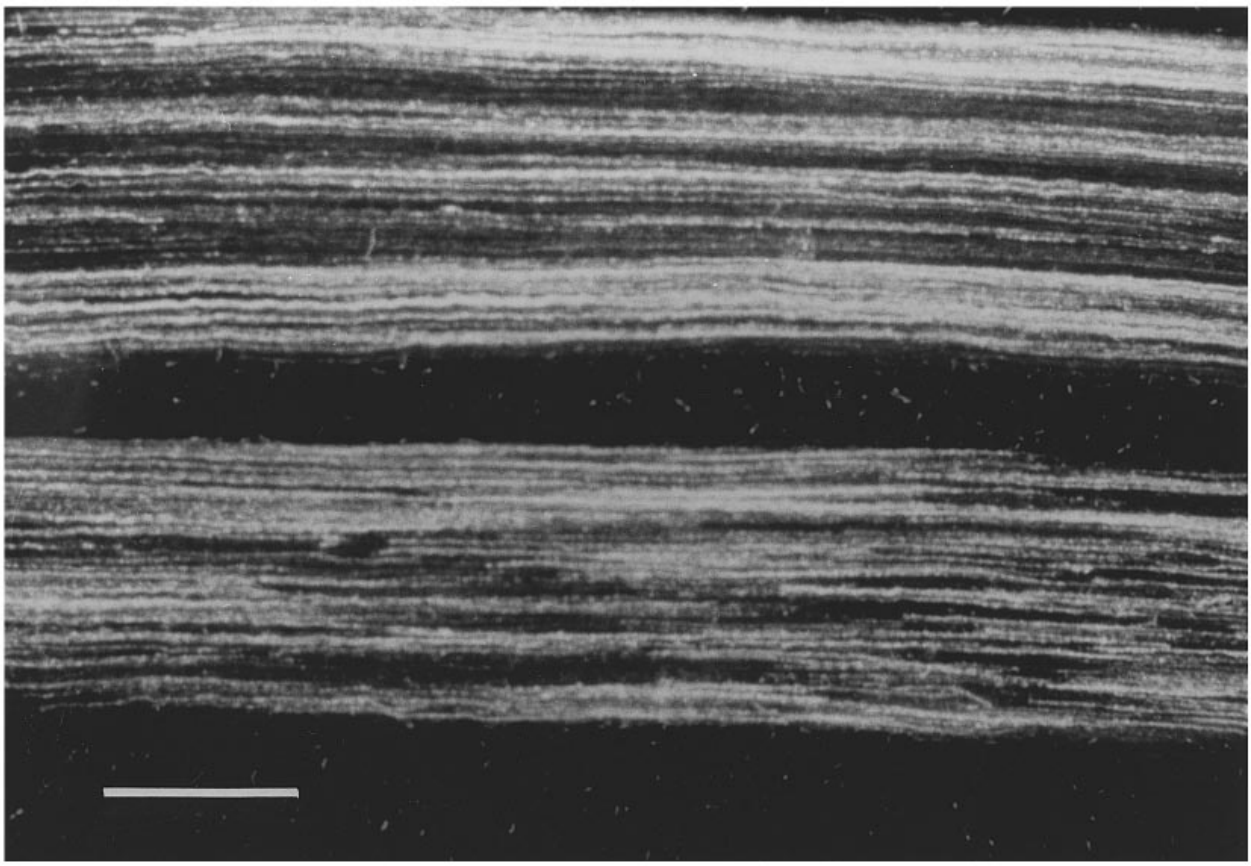

Fig. 1. Tibial nerve 4 cm distal to the knee. Note densely filled (white) axons. Filled axons presumably belong to primary sensory neurons. Darkfield micrograph. Bar, 250 μm.

phate buffer containing 30 % sucrose. The dorsal root ganglia, tibial nerve, cranial cruciate ligament and posterior joint capsule were removed and processed to determine the presence of HRP. Ligaments were removed from their bony insertions under an operating microscope, using a sharp scalpel to ensure as complete a removal as possible. Tissues were frozen-sectioned serially along the longitudinal axis of the ligaments at 20–50 μm thickness. We also attempted in pilot studies to decalcify bone by exposure to 7% ethylene-diamine tetra-acetate (EDTA) at 4 °C for 24 or 48 h.

In pilot studies, we found that the joint capsule tissue and delicate synovial membrane and subsynovial tissue of the ligaments virtually disintegrated during subsequent incubation with tetramethyl benzidine (3,3′,5,5′-tetramethylbenzidine; TMB Sigma) and reaction with H_2O_2 if the sections were floated in the reactants according to standard procedures (Mesulam, 1982). We attempted to fix perfused tissue further by placing it in a bath of 2% paraformaldehyde for varying durations before transferring the tissue of the phosphate buffer prior to sectioning. These attempts severely decreased the amounts of observable reaction product and were abandoned (see Mesulam, 1982). Ligament and capsular tissues were preserved better when we placed the sections on slides and allowed them to air dry for 1/2 to 1 h before incubation with TMB. The slides containing the tissue then were washed in double distilled water and incubated with 5% TMB dissolved in sodium acetate buffer at 4 °C for 20 min. A reaction product was created by adding 1.0 ml of a 0.3 % H_2O_2 solution per 80 ml of incubation mixture. After 20 min specimens were washed with cold sodium acetate buffer, run through graded alcohols, cleared with xylene, and mounted on slides with synthetic mounting medium.

Sections were viewed with dark field, bright field, and polarised light microscopy. Reaction product appeared as dense clusters of fine granules that were easy to distinguish from scant background artefact, which consisted of much larger, and more sparsely distributed crystals. Inclusion criteria as neuronal elements required the identification of concentrated accumulations of reaction product on serial sections. We did not observe any labelled red blood cells; in unperfused tissue red blood cells were frequently

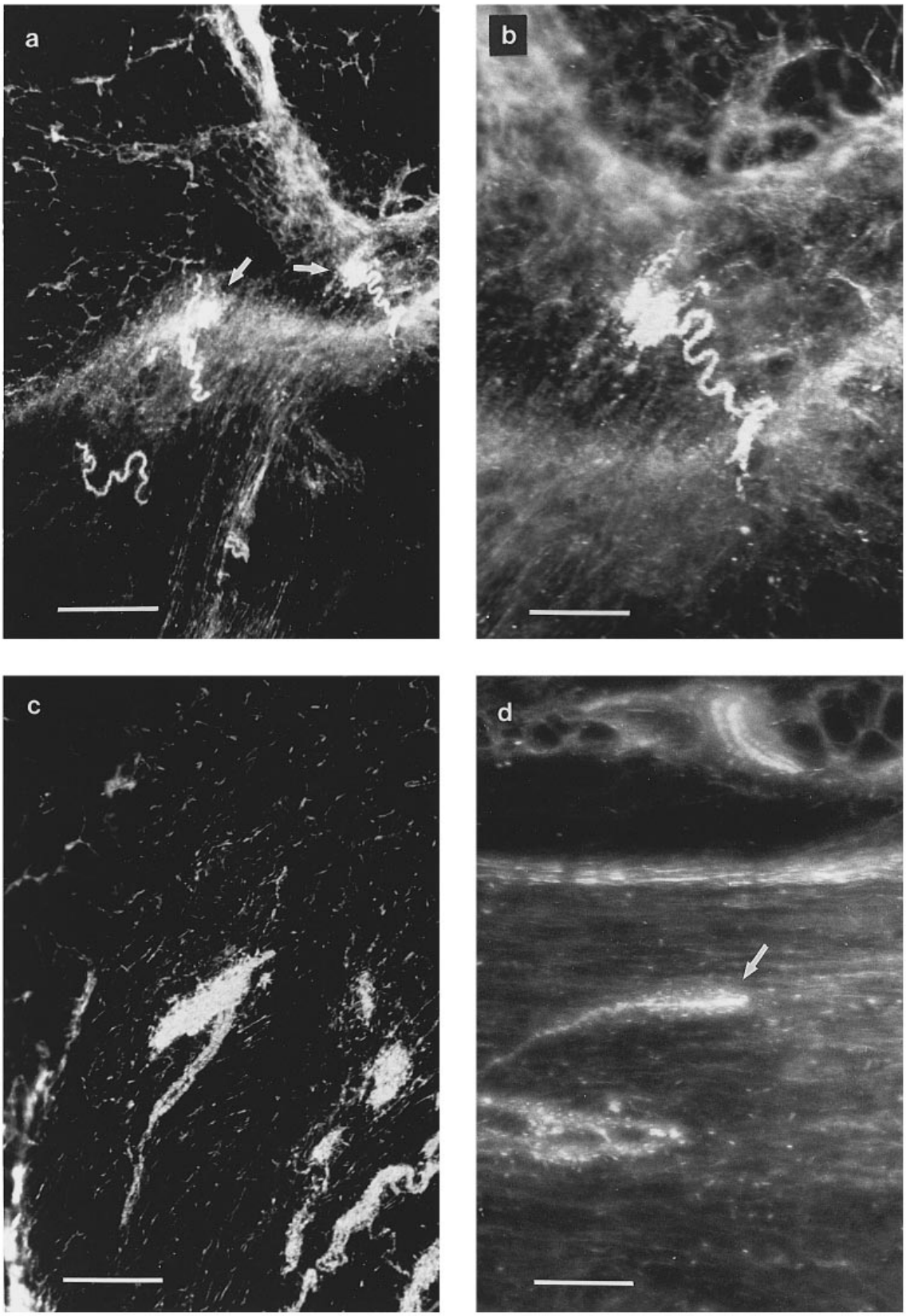

Fig. 2. Posterior articular capsule of the knee joint. Darkfield micrographs. (*a*) Two Ruffini-like endings with axons and a segment of axon located at the junction between the stratum fibrosum (lower) and subsynovial-areolar tissue (upper). (*b*) Higher magnification of the right-most ending in *a*. Note the broadly labelled region, which is presumed to indicate multiple arborisations forming the terminal endings. (*c*) Ruffini-like ending in the stratum fibrosum showing a 'spray' shape. Two axons enter the ending. (*d*) Pacini-like ending (arrow). Note that a single axon supplies the receptor, which shows labelling consistent with a single central axon. Reaction crystals surrounding the central axon may reflect migration of HRP. Bars: *a*, 250 μm; *b–d*, 100 μm.

labelled and identified easily based on their biconcave shape.

Counterstaining with neutral red substantially decreased reaction production and was abandoned (see also Mesulam, 1982; Robertson & Aldskogius, 1982).

RESULTS

All 5 animals transported substantial amounts of WGA–HRP in tibial nerve axons to the level of the knee, as evidenced by dense filling of presumptive primary sensory axons with reaction product (Fig. 1). Injected DRGs showed dense labelling of virtually all cell bodies and axons on serial sections. The tibial nerve showed groupings of completely filled axons, and axons devoid of reaction product in a topographic relationship that would be expected in a mixed nerve. This was true for all tibial nerve samples (up to 4 cm distal to the knee) taken from animals with dorsal root ganglia injected with WGA–HRP. All control tissue (saline injected side—DRG, tibial nerve, articular capsule, ligament) showed no labelling except a sparse, even distribution of crystals consistent with background artefact from nonspecific precipitation of reaction product.

Posterior joint capsule of the knee

The posterior articular capsule contained abundant nerve bundles and single axons coursing through serial sections that were filled densely and uniformly with reaction product (Fig. 2). Neural elements were found only within fibrous connective tissue, never within adipose tissue. Axons often followed tortuous courses.

In one type of ending the parent axon often arborised into 2 or 3 axons prior to entering expanded regions of dense reaction product (Fig. 2*c*). We presume that these expanded regions of dense labelling reflect multiple terminal axonal arborisations. These endings were roughly ovoid (Fig. 2*a*, *b*) or 'spray-shaped' (Fig. 2*c*), with a maximum extent between 50 and 150 μm. No capsule was visible under dark or brightfield microscopy. The border of each corpuscle was rough and TMB crystals appeared to contact collagen bundles when visualised under polarised light. These sensory endings typically were found within the stratum fibrosum and sometimes near its junction with subsynovial tissue (Fig. 2*a*, *b*). A second type of ending was small and elongated (50×100 μm), and appeared to have a densely stained central core fed by a single axon (Fig. 2*d*). The dense core was sometimes surrounded by a light, diffuse cloud of reaction product. Endings were not segregated by type, as both types of endings were found in the same regions of the capsule.

The diameters of axons in these 2 types of endings were from 7 to 10 μm, based on measurement of the reaction product extent. These measures were taken at 100 to 200 μm before the axon entered the presumed region of the sensory ending.

Cranial cruciate ligament

The cranial cruciate ligament exhibited accumulations of reaction product consistent with sensory endings with dense terminal arborisations. Most endings were found in the body of the ligament (Fig. 3*b*, *c*). On occasion endings were found in subsynovial tissue (Fig. 3*a*). Two distinct types of sensory endings were observed. The first ending was ovoid or elongated in shape with a maximal extent of about 100 μm. It had a smooth border (Fig. 3*a–c*) that appeared to represent a capsule when viewed at high magnification (not shown). Occasionally one pole of the ending appeared bifurcate (Fig. 3*b*). The long axes of endings found within the body of the ligament were always parallel to the collagen fascicles. These endings consistently exhibited homogeneous accumulations of reaction product from border to border with no discernible internal structure. Between 5 and 17 of these small endings were observed in each of the 5 cranial cruciate ligaments on examination of serial sections. Sensory endings were found along the entire length of the ligament, from the femoral to tibial insertions. Three endings in total were found in subsynovial tissue. We also observed several ovoid endings in the subsynovial tissue between the CCL and PCL.

The second type of ending was much larger, usually with a length of approximately 1000–1500 μm. The long axis was parallel to the long axis of the ligament. These endings had a smooth border and, when viewed at high magnification, appeared surrounded by a capsule. Reaction product was accumulated in discrete regions (Fig. 3*d*) in contrast to the smaller ovoid endings. These regions seemed to swirl around one another within the confines of the capsule. From 1 to 3 endings of this type were found in the body of each of 4 ligaments.

The distribution of neural elements within the CCL differed from the posterior capsule. Several sections of CCL showed 1 or 2 single axons travelling within the substance of the ligament or in the subsynovial connective tissue. These were always traced to a receptor. We did not observe bundles of multiple

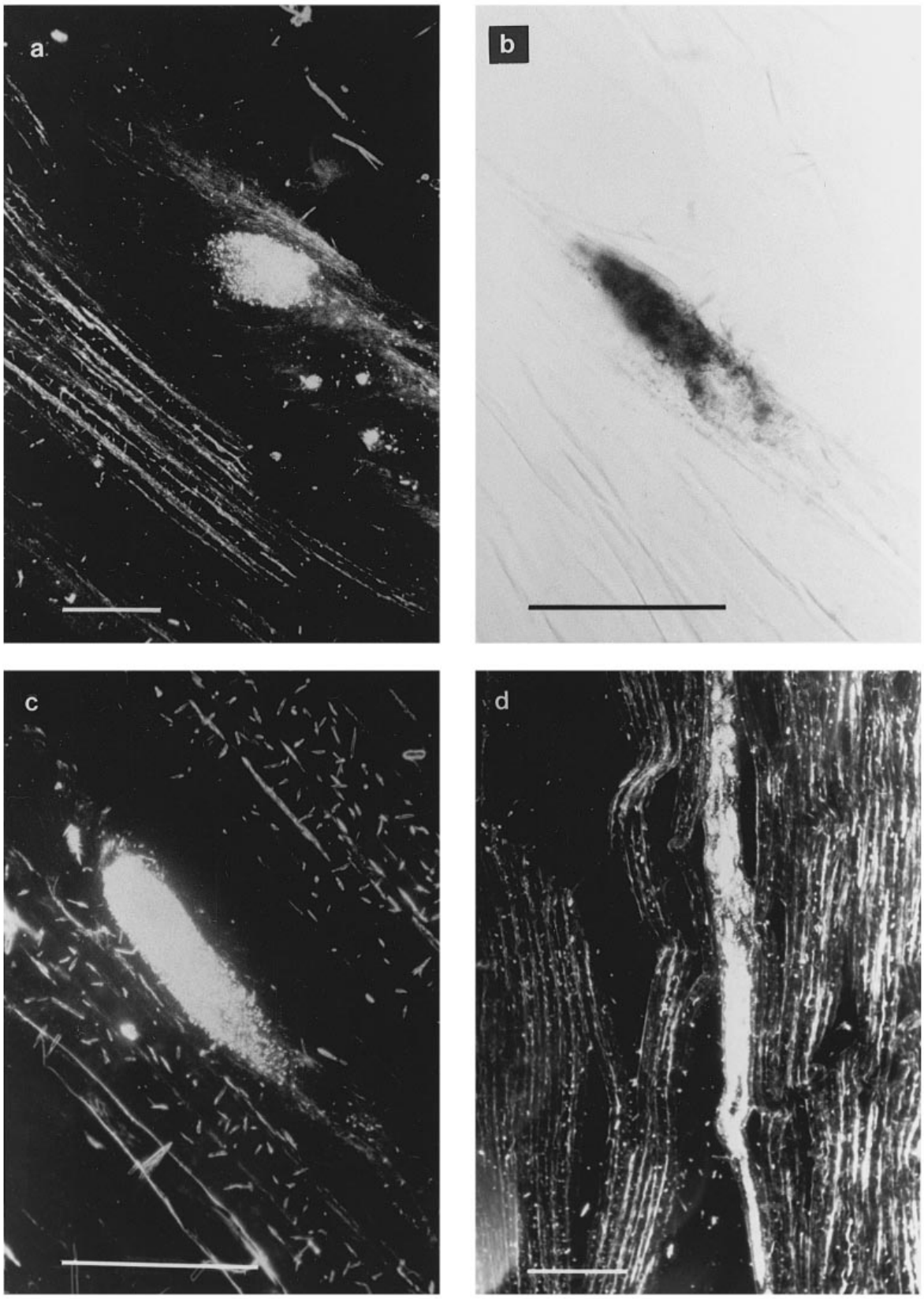

Fig. 3. Cranial cruciate ligament. (*a*) Oval-shaped sensory ending located in subsynovial tissue. The smooth border is consistent with a perineurial capsule. Darkfield micrograph. (*b*) Sensory ending running parallel with, and between collagen fascicles. Note labelling consistent with 1 or possibly 2 axons at one pole and reaction crystals apparently external to the ending that may reflect HRP leakage or migration. Bright field micrograph. (*c*) Sensory ending in parallel with collagen fascicles. Smooth borders to the labelled regions may indicate a perineurial capsule. Darkfield micrograph. (*d*) Portion of a large elongated region of labelling located among collagen bundles. Fine reaction crystals are contained in multiple compartments. Darkfield micrograph. Bars: *a–c*, 100 μm; *d*, 200 μm.

axons in the ligament. Endings in CCL were found widely separated from other endings, and the axons of one ending did not branch to other endings.

DISCUSSION

This study was undertaken to examine sensory innervation of articular tissue, particularly the cruciate ligaments, using a method that is sensitive and specific for axons and their terminal endings. Although we were successful in the use of HRP for this purpose, immunohistochemical techniques developed recently provide even more sensitive, and less problematic techniques for studying peripheral nerve axons. In particular, immunofluorescence for protein gene product (PGP) 9.5 antibody has been used successfully to examine peripheral innervation, including detailed examination of the morphology of corpuscular sensory endings (e.g. Ramieri et al. 1992; Castano et al. 1995; Hilleges et al. 1995; Navarro et al. 1995). Moreover, a significant advantage of using antibody PGP 9.5 is its in vitro application, which permits use with human tissue, in contrast to HRP histochemistry. Immunohistochemical analysis of cruciate ligament innervation using antibody PGP 9.5 is likely to reveal further insights to the sensory innervation of articular tissue, and may resolve some of the questions raised in the present study regarding the morphology of cruciate ligament sensory receptors. However, a few recent studies of human ACL using immunohistochemistry have not greatly increased our understanding of the numbers and types of receptor endings (Rivard et al. 1993; Aune et al. 1996).

Axons and sensory endings in the cranial cruciate ligament and posterior joint capsule of the knee were visualised using anterogradely transported HRP. Dense filling of axons and nerve terminals was achieved in the best cases, reflecting transport of HRP in massive amounts to knee tissues. In addition reaction product was present primarily within neural elements and not between axons at 36 h after injection. We presume that nearly all neural elements of the knee which are fed by the sciatic nerve were labelled.

The labelling in the posterior capsule displayed features consistent with the morphological characteristics of endings defined using other methods (cf. Freeman & Wyke, 1967). The small ovoid and spray-shaped endings that were densely labelled suggest Ruffini endings (type 1; Freeman & Wyke, 1967) assuming that the dense labelling over the entire area of the presumptive ending reflects multiple arborisations of the axon into terminal endings. The small ovoid endings with a dense central core suggest pacinian corpuscles (type II; Freeman & Wyke, 1967). The faint 'cloud' of reaction surrounding the dense central core may reflect diffusion of HRP from its in vivo locations. Aldskogius et al. (1986) reported similar observations for heavily labelled cutaneous sensory endings, which were attributed to 'leakage' (exocytosis) of HRP into the skin. Incomplete fixation of the HRP prior to the incubation reaction also may contribute to diffusion of HRP.

The 2 types of endings we identified in CCL appeared to differ from those of the posterior capsule. The ovoid endings were similar in shape and size to the type II endings in the posterior capsule, although we never observed a central core on any section that would reflect a single axon termination. Also, these endings in the CCL always had a dense, homogeneous accumulation of reaction product within a sharply defined border. This homogeneous labelling may reflect pacinian corpuscles with multiple central cores, as reported for the human ACL and cat CCL (Sjölander et al. 1989; Haus & Halata, 1990). HRP migration may have further obscured the central core(s) expected for the terminal endings of pacinian corpuscles. Alternatively, these endings may be type I endings (Ruffini corpuscles) as reported previously using gold chloride staining in human ACL (Haus & Halata, 1990), cat CCL (Sjölander et al. 1989) and human fibular collateral ligament (de Avila et al. 1989). The homogeneous accumulation of reaction product is consistent with dense terminal axonal arborisations that characterise Ruffini-like endings. The smooth border between the labelled region and surrounding tissue suggests a thin capsule, which also is consistent with Ruffini-like endings in ligament (de Avila et al. 1989; Haus & Halata, 1990).

The large unevenly filled regions in CCL were much larger than any endings of the posterior capsule, measuring up to 1500 μm in length. These presumed endings were never observed in the posterior capsule. They exhibited compartments of reaction product in a swirling fashion with well-defined internal borders, all housed within an apparent capsule. These endings bear the greatest similarity to previously identified Golgi tendon organ-like endings of ligaments (type III; Freeman & Wyke, 1967). Moreover, they were found in quantities (0–4 per ligament) similar to previous reports of Golgi tendon organ-like endings (Skoglund, 1956; Freeman & Wyke, 1967; Koch et al. 1995). However, Golgi tendon organ-like endings reported in ligament using other means typically were 500–700 μm in maximum extent (Skoglund, 1956; Freeman & Wyke, 1967; Sjölander et al. 1989; Koch et al. 1995). The large endings we observed may be a

larger type I ending, or a series of type I endings. Indeed, Haus & Halata (1990) reported that type I and type III endings in human ligament differed only in size, noting similar morphological attributes on examination with electron microscopy. However, type I endings of the posterior capsule apparently are not encapsulated.

The location of receptors throughout the CCL in its sagittal plane, and in the subsynovial layers and between collagen fascicles, generally agrees with other recent reports (Sjölander et al. 1989; Haus & Halata, 1990). This contrasts with earlier studies that identified only a few larger, Golgi-tendon like organs applied to the surface of the ligament at its ends (Skoglund, 1956; Freeman & Wyke, 1967). Sensory fibres in human ACL were recently identified using antisera for PGP 9.5 (Aune et al. 1996). Single fibres were found mostly in the loose connective tissue while smaller fibres were found in association with vasculature; fewer fibres were found between the dense regions of collagen fascicles.

The 5 to 17 ovoid receptors per ligament that we observed are more than the one-to-several endings reported by many other investigators (see Introduction). This may reflect the increased sensitivity of anterograde HRP labelling versus staining with gold or silver salts. Indeed, our counts likely underestimate sensory endings. Although we injected all DRGs known to supply the afferents of the sciatic nerve, the cruciate ligaments may receive contributions from branches of the femoral nerve (Kennedy et al. 1982). Also, the insertion of the CCL into bone has been identified as a preferred region for sensory endings (Sjölander et al. 1989; Gomez-Barrena et al. 1992). We were unsuccessful in our attempts to examine the bone-ligament interface after decalcifying the bone with EDTA, in contrast to a previous report (Marfurt & Turner, 1983). After decalcification we could not produce a reaction product, suggesting that the HRP was no longer enzymatically active.

The neuron-specific labelling with WGA–HRP offers a significant advantage over previously used staining techniques in which differentiation of neural and nonneural elements was difficult. Gold and silver stains often stain connective tissue and vascular elements (de Avila et al. 1989; Bancroft & Stevens, 1990; Koch et al. 1995). It also was not difficult with serial sections to trace HRP reaction product in neural structures throughout the tissue.

Although this technique provides some benefits over other techniques, ligament and articular capsule present special problems for HRP histochemistry. One key to effective HRP histochemistry is achieving adequate fixation for tissue preservation and enzyme localisation without excessive loss of HRP activity from the fixative (Mesulam, 1982). Insufficient tissue preservation may have led to disintegration of the joint capsule and synovia of the CCL during our initial attempts at reacting the HRP with TMB and peroxide. Attempts to fix the tissue better (i.e., increasing perfusion durations, postperfusion fixation) virtually eliminated reaction product, probably by depressing HRP enzymatic activity, and were abandoned. The capsular tissue and delicate synovia of the ligament were preserved when the tissue slices were dried on glass slides prior to the incubation reaction, although this process may depress the sensitivity of the reaction (Mesulam, 1982).

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