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Number, distribution and neuropeptide content of rat knee joint afferents

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ABSTRACT

Retrograde tracing with Fluoro-Gold was used to identify the complete population of knee joint afferents in the lumbar dorsal root ganglia of adult female Wistar rats. There was an average of 581 ± 31 (mean ± S.D.) afferents supplying each joint. These were found distributed from L1 to L5 with the great majority localised in the L3 and L4 ganglia. Electron microscopy of the posterior articular nerve of the knee revealed an average of 103 ± 15 (mean ± S.D.) myelinated and 513 ± 39 unmyelinated axonal profiles. Since about 50–60% of the unmyelinated profiles would be expected to be sympathetic efferents, these numbers are consistent with the numbers of afferents found by Fluoro-Gold retrograde tracing and suggest that the posterior articular nerve contains about 50% of the total number of knee joint afferents in the rat. Immunohistochemistry revealed that an average of 10% of identified joint afferents expressed substance P-like immunoreactivity and that 33% expressed calcitonin gene-related peptide-like immunoreactivity.

Key words: Sensory ganglia; substance P; calcitonin gene-related peptide; Fluoro-Gold.

INTRODUCTION

There is increasing interest in the role of joint afferents both in the normal functioning of the musculoskeletal system and in the pathophysiology of joint diseases (Farfan, 1983; Cooke, 1985; Ferell et al. 1985; El-Bohy et al. 1988; Yaksh, 1988; Ellman, 1989; O'Connor et al. 1989; and see Schaible & Grubb, 1993 for review). Anatomical and physiological investigations have established that joint afferents are comprised of large calibre force, tension and pressure sensitive mechanoreceptors and small calibre myelinated and nonmyelinated nociceptors (Gardner, 1948; Freeman & Wyke, 1967; Schaible & Schmidt, 1983; Johansson et al. 1986; Heppelmann et al. 1988). Both types of neurons may be implicated in the aetiology and pathophysiology of arthritic disorders (reviewed by Kidd et al. 1990).

Considerable interest has focused on the contribution of the small diameter, peptide containing subpopulation of joint afferents to the development of the inflammatory response during the onset of synovitis or arthritis (Levine et al. 1984; Kidd et al. 1990; Mapp et al. 1990, 1994; Neugebauer et al. 1994; Sluka et al. 1994). A number of reports have described significant changes in the electrophysiological behaviour of joint afferents recorded from the dorsal roots and in the spinal cord during and after the induction of inflammatory arthritis (Coggeshall et al. 1983; Schaible & Schmidt, 1985, 1988). Corresponding studies examining the specific molecular responses of joint afferents in inflammation have been hampered by the lack of selective identification of the particular neurons of interest within the larger population of sensory neurons in the dorsal root ganglion and the dorsal horn of the spinal cord (Lembeck et al. 1981; Sluka et al. 1992; Smith et al. 1992).

A recent study of rat knee joint anatomy quantified the numbers and types of axons in posterior articular nerve of the knee (Hildebrand et al. 1991), but these authors did not attempt to determine the total number of neurons supplying the joint, nor did they attempt to identify the somata of these neurons in the dorsal root ganglia. Another group had previously reported on

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the number and location of the cell bodies of neurons supplying the rat knee joint using retrograde tracing with horseradish peroxidase and related tracers (Widenfalk & Wiberg, 1989). Their reported numbers of labelled cells, however, revealed significant inconsistencies that seemed to be mainly related to technical factors (see Discussion).

Using the retrograde tracer Fluoro-Gold (FG) to selectively identify knee joint afferents in the mouse (Salo & Tatton, 1993), we found that this tracer provided consistent and accurate identification of this subpopulation of afferent neurons in the lumbar sensory ganglia. The present paper reports the results of our initial investigations using this technique in the rat, in combination with fluorescent immunohistochemical detection of the neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP).

METHODS

Knee joint injections

Fourteen adult female Wistar rats were obtained from Charles River (average weight 250 g). The supplier was unable to provide precise ages of the animals but indicated that they were all sexually mature and approximately 2 mo of age. All experiments were carried out in accordance with the guidelines established by the United States National Institutes of Health regarding the care and use of animals for experimental procedures. All possible efforts were made to minimise animal suffering and reduce the number of animals used.

A specially designed knee holder was employed to position the joint consistently for injection. Under general anaesthesia (halothane/nitrous oxide/oxygen), using sterile technique, a 5 mm skin incision was made over the patellar tendon and with the aid of a micromanipulator, a 30 gauge needle, connected by a short length of polyethylene tubing to a 100 μl Hamilton syringe, was introduced at an oblique angle through the tendon into the space between the patella and the groove of the distal femur. Each rat had one knee injected with 5 μl of 2% FG (Fluorochrome, Inc., Inglewood, CO) in distilled water followed after a few minutes by 15 μl of 0.1 M phosphate buffer containing 0.1% Fast Green dye (Sigma) to both precipitate the fluorescent tracer within the intra-articular space (Schmued & Fallon, 1986) and identify potential extravasation of the injectate from the joint. After injection the needle was withdrawn, the incision was briefly irrigated with 0.9% sterile saline and the skin sutured with 6–0 nylon or silk.

General histological methods

It had been previously reported that in rats, Fluoro-Gold produces a very persistent if not permanent labelling of the neurons that take it up (Schmued & Fallon, 1986). We therefore elected to wait 1 wk after FG injection before killing as a matter of convenience. At 7 d the rats were deeply anaesthetised with intraperitoneal sodium pentobarbital and perfused via the left ventricle with ice-cold normal saline followed by ice-cold fresh 4% paraformaldehyde in 0.1 M phosphate buffer (or Zamboni's fixative (Zamboni & De Martino, 1967) if designated for peptide immunohistochemistry). The spine was removed and complete laminectomies performed from T13 to S1. The pair of dorsal root ganglia at each level were removed and postfixed overnight in the same fixative. The ganglia were washed in buffer and placed in 20% sucrose overnight. The following day they were blotted dry, and each pair then suspended and oriented in Tissue-Tek embedding medium in 1.5 ml Eppendorf tubes, then rapidly frozen by immersion in liquid nitrogen.

Specimens were stored at −80 °C until sectioning. Blocks were mounted in a Reichert–Jung cryostat and serial 10 μm transverse sections collected onto gelatin-coated slides. After thaw-mounting and air-drying at room temperature overnight, sections were counterstained in aqueous 1% toluidine blue, dehydrated and coverslipped with Eukitt.

Method of counting labelled joint afferents

Every section through every ganglion (ranging from 100–200 sections per ganglion) was examined alternately under brightfield and U.V. epifluorescence with appropriate filters for FG (excitation: 355–425 nm, long pass filter: 460 nm) and lipofuscin autofluorescence (excitation: 450–490 nm, long pass filter: 515 nm, Hydén & Lindström, 1950) using a Leitz DMRB microscope with fluorescence illuminator.

The total number of FG-positive somata was counted for each ganglion as follows. The complete set of serial sections for each ganglion was examined. We mounted 20 consecutive sections on a slide, in order, in a regular grid pattern, thus enabling comparison of each section with the previous and following sections in the series. Carefully comparing each section with the previous and following section in the series allowed the labelled cellular profiles to be followed from section to section, ensuring that each labelled cell was counted only once (serial recon-

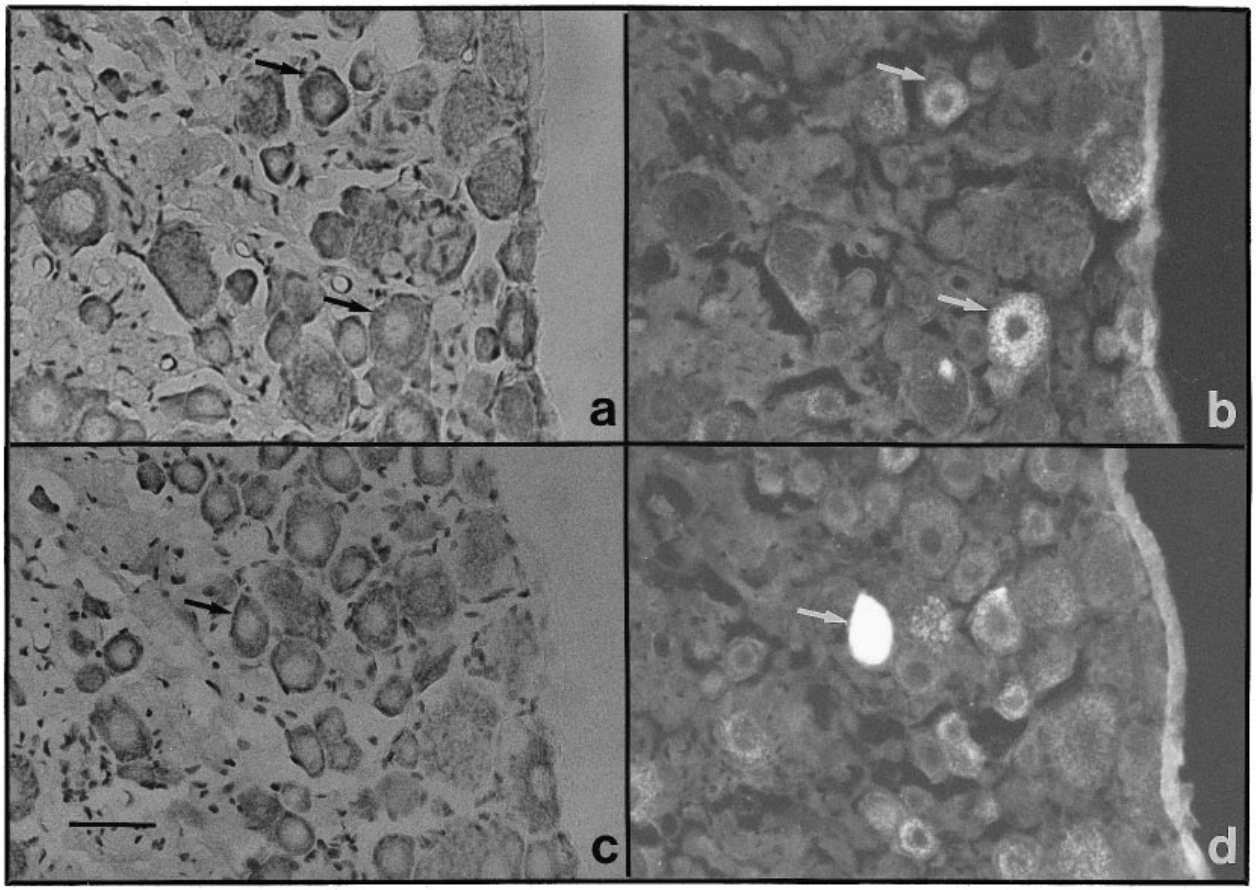

Fig. 1. Typical sections of an L3 ganglion viewed alternately under brightfield (a, c) and ultraviolet epifluorescence illumination (b, d). Arrows indicate profiles of typical Fluoro-Gold labelled knee joint afferents. Toluidine blue stain. Bar, 50 μm.

struction method of Coggeshall et al. 1990; and see Salo & Tatton, 1993). Some of the larger FG-positive somal profiles could be followed over as many as 4 or 5 serial sections.

Few cells exhibited significant lipofuscin autofluorescence, so that identificaton and counting of the labelled cells was straightforward. Somal areas of labelled profiles were measured using a computer-aided video image analysis system (MCID, Imaging Research Inc., St Catherines, ON) and size histograms plotted.

Electron microscopy of the posterior articular nerve

The knee joints were retrieved from 5 rats after perfusion fixation with 4% paraformaldehyde. The popliteal fossa was carefully dissected, allowing identification and harvesting of the posterior articular nerve (PAN). These were gently stretched on a wooden toothpick and postfixed by immersion in 2.5% glutaraldehyde overnight followed by 1.5% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. The tissue was then dehydrated through a graded series of ethanols, infiltrated overnight and embedded in Epon resin with DMP-30. Polymerisation occurred over 12 h at 70 °C. Blocks were trimmed and sectioned on a Reichert–Jung Ultracut E microtome. 90 nm (gold) sections were retrieved on formvar-coated copper slot grids. Sections were then stained with 2% uranyl acetate followed by Reynolds lead citrate. Electron micrographs were obtained using a JEOL 100CXII electron microscope.

Immunohistochemistry

After fixation and embedding as described above, 10 μm serial frozen sections were cut from the L3 and L4 DRGs harvested from 8 rats prelabelled by intra-articular FG injection as described above. We elected to use only the L3 and L4 ganglia for the immunohistochemistry because we had found that on average, 88% of the joint afferents would be found there. One rat had to be excluded from this series because of a technical problem; a majority of the sections failed to remain adherent to the slides. Initially, we collected sections alternately into 2 series (a and b), one to be used for SP immunohistochemistry and the other for CGRP immunohistochemistry. This proved to be

Table 1. *Counts of FG labelled joint afferents in the lumbar ganglia*

Rat	L1	L2	L3	L4	L5	Totals
RJ1	10	17	294	258	32	611
RJ2	23	24	260	303	2	612
RJ3	12	8	305	207	34	566
RJ4	0	21	234	246	38	539
RJ5	13	27	199	254	85	578
Mean (±S.D.)	11.6	19.4	258	254	38	581 (±31)

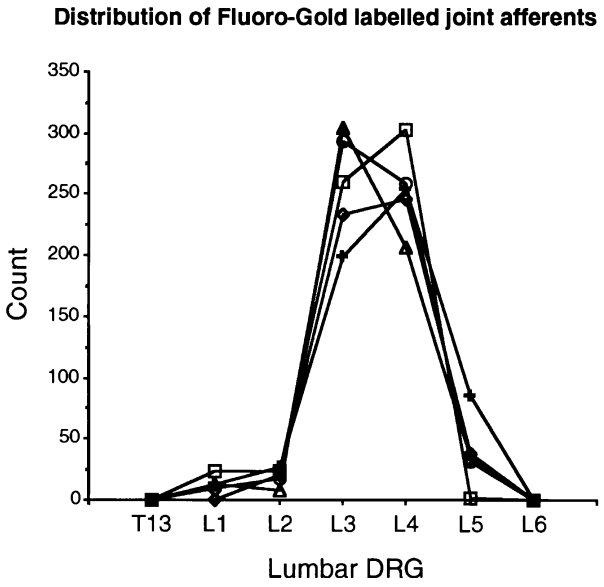

Fig. 2. Distributions of Fluoro-Gold labelled knee joint afferents in the lumbar dorsal root ganglia. Each set of joined points represents the results of 1 knee joint injection in 1 animal.

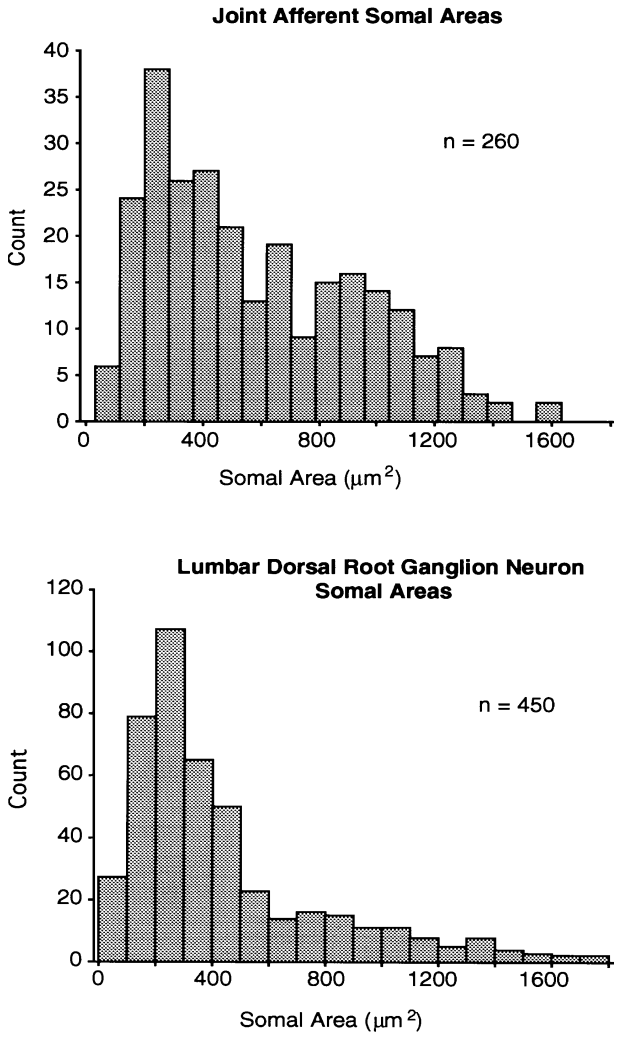

Fig. 3. Distribution of somal areas of FG labelled knee joint afferents. Sizes were distributed across the broad range of sizes found in the larger population of dorsal root ganglion neurons.

somewhat cumbersome, so for the remainder of the specimens, a single series of sections was collected.

Sections were collected onto gelatin-chrome-alum subbed slides, thaw-mounted and dried on a warming plate at 30 °C for 2 h. The detailed immunohistochemistry protocol for visualisation of the neuropeptides has been previously reported (Theriault et al. 1993). Briefly, after washing in 3 changes of phosphate buffer and blocking with normal goat serum for 30 min at room temperature, primary incubation was performed overnight in humidity chambers at 4 °C. Anti-SP (Incstar) was used at a titre of 1/2000 in 2% normal horse serum. Anti-CGRP (Cambridge Research Biochemicals) was used at a titre of 1/3000 in 2% normal goat serum. The following day slides were washed in 3 changes of phosphate buffer and blocked for 30 min in 10% normal goat serum. Specific binding of anti-CGRP was visualised by a 1 h incubation with a biotinylated goat antirabbit IgG followed by Texas red avidin D (Vector). Anti-SP binding was visualised by a rat-adsorbed biotinylated horse antimouse IgG (Vector) and Texas red avidin D. The slides were rinsed in phosphate buffered saline and coverslipped with Mowiol. Slides were stored at 4 °C in the dark for no more than 1 wk prior to viewing. Every 4th section was viewed using the Leitz DMRB fluorescence microscope, alternating filters appropriate for FG (excitation: 355–425 nm, long pass filter: 460 nm) or Texas red (excitation: 515–560 nm, long pass filter: 580 nm). We chose to examine every 4th section in order to minimise the possibility of double counting a labelled cell. On each section viewed, the total number of FG-labelled profiles was counted, then the number of FG-labelled profiles that also contained Texas red immunofluorescence was also determined (Fig. 3).

RESULTS

Fluoro-Gold injection of rat knees

A total of 14 rats underwent knee joint injection. We were very careful to ensure that the tip of the injection needle was positioned precisely between the patella and the distal femur before instillation of the Fluoro-Gold and Fast Green and phosphate buffer. For this reason there were no animals rejected or excluded from the series due to inadvertent injection of deeper tissue layers away from the joint. One rat was excluded from the series due to immediate extravasation of some of the tracer from the joint at the injection site.

Number, distribution and sizes of joint afferent somata

Intense FG labelling was found in DRG somata of all diameters. A montage of photomicrographs of a typical section through an L3 DRG is presented in Figure 1. FG-labelled cells were distributed in an extremely consistent manner over 4 or 5 segments, with the maximum numbers always found at either L3 or L4. The somal distributions through the lumbar ganglia are shown in Table 1 and Figure 2. In the 5 rats that had the complete set of lumbar ganglia examined, we found 581 ± 31 (mean $\pm$ S.D.) labelled afferents per knee joint injection. Somal size measurements were broadly distributed across the full range of sizes found in the rat dorsal root ganglion (Fig. 3). Notably, the joint afferent subpopulation contained a much larger proportion of large diameter somata than is found in the general population of undifferentiated sensory afferents.

Electron microscopy of the posterior articular nerve

Electron micrographs of cross sections of 5 PANs were obtained (Fig. 4), and we counted 616 ± 33 (mean $\pm$ S.D.) axonal profiles per PAN. Of these, 103 ± 15 were myelinated and 513 ± 39 were unmyelinated axons.

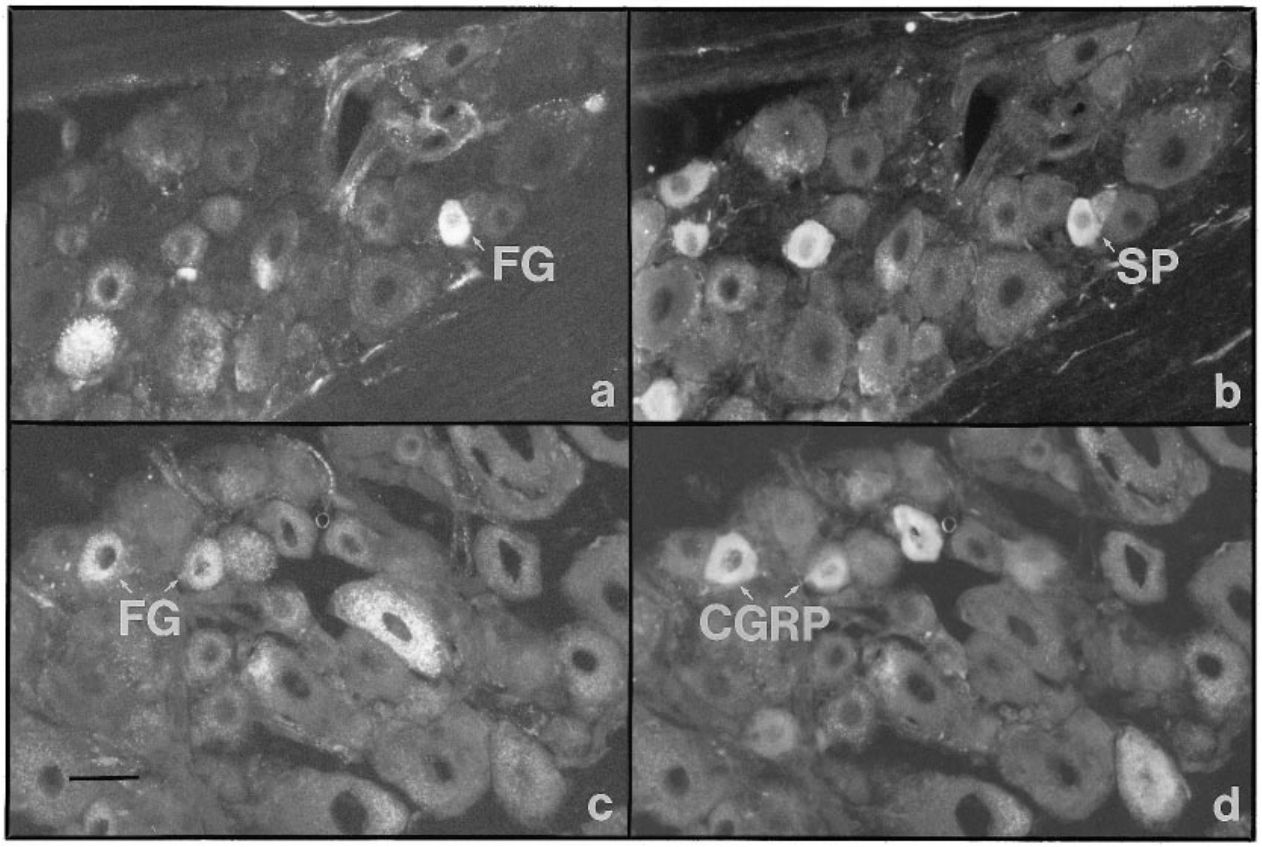

Fig. 4. Combined Fluoro-Gold labelling and fluorescent immunohistochemistry. The two sections shown were each photographed alternately with filters appropropriate for FG (left side of figure), then Texas red (right side of figure). The section shown in panels a and b (above) was incubated with anti-SP antibody. The section shown in panels c and d (below) was incubated with anti-CGRP antibody. Double labelled cells are indicated with arrows. Bar, 50 μm.

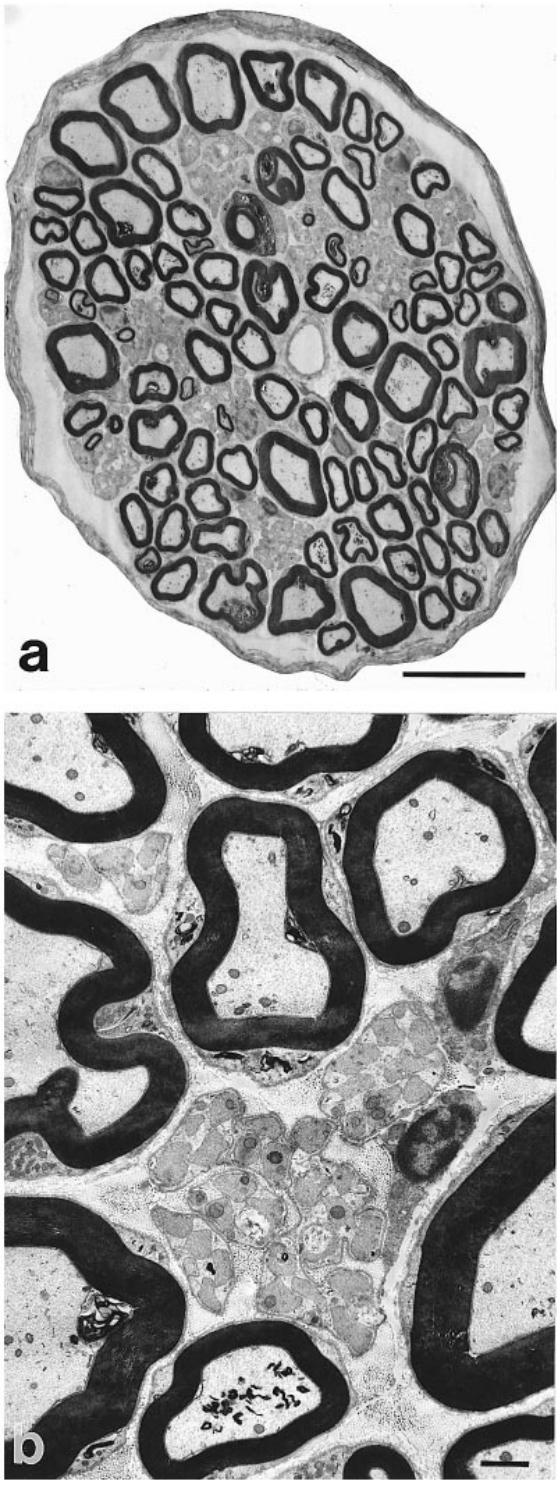

Fig. 5. (a) Low power electron micrograph of a cross section of the PAN. Myelinated axon profiles are readily identified. Bar, 10 μm. (b) Higher power electron micrograph of the same section seen in Fig. 5. Unmyelinated axons can be seen in Remak bundles. Bar, 1 μm.

Peptide immunohistochemistry

FG proved to be fully compatible with fluorescent immunohistochemistry in this model. Figure 5 is a composite photomicrograph of typical sections stained for SP and CGRP-like immunoreactivity. Counts revealed that 10% of FG-labelled profiles contained SP-like immunoreactivity and that 33% contained CGRP-like immunoreactivity (Table 2).

DISCUSSION

Retrograde tracing methodologies

Intra-articular knee joint injection with FG resulted in the labelling of a consistent number of afferent neurons with a characteristic distribution in the lumbar DRGs. A similar previous study using horseradish peroxidase and wheat germ agglutinin-conjugated horseradish peroxidase as the tracing agent revealed highly variable counts of the total numbers of joint afferents ranging from 64 to 1067 (Widenfalk & Wiberg, 1989). Examination of their data showed that the total counts seemed to vary considerably depending on the tracer used and also as a function of the time between injection and killing, presumably because of intracellular degradation of the enzyme tracer. The distribution of labelled neurons throughout the lumbar ganglia also was quite variable, suggesting difficulties with reproducibility of the manual injections of the rather small rat knee joint. They reported that their rats had 12 thoracic ganglia and 5 lumbar ganglia. They did not report which strain of rats they used. Our rats were found to have 13 thoracic vertebrae and 6 lumbar vertebrae, in agreement with the reported typical rat anatomy (Hebel & Stromberg, 1986). It is generally conceded that FG is a superior tracer to horseradish peroxidase and its conjugates, in many ways, including its higher affinity for neurons, its persistence in the cell soma for prolonged periods after labelling and, importantly, the fact that it does not label axons of passage (Schmued & Fallon, 1986; Theriault & Tator, 1994).

Numbers of knee joint afferents

The number of neurons counted was little more than twice the average total number of neurons we found to supply the mouse knee joint using the same methods (Salo & Tatton, 1993). The number of axons counted in the PAN was very similar to the numbers reported by Hildebrand et al. (1991). They showed that two-thirds of the unmyelinated profiles in the PAN were sympathetic efferent fibres. Assuming that this proportion is similar in all rats, it would suggest that in our rats the PAN contained an average total of

Table 2. *Counts of SP and CGRP-labelled joint afferents*

Rat	Ganglion	Total FG positive profiles	FG and SP double-labelled profiles (%)	FG and CGRP double-labelled profiles (%)
RJP1	L4 series a	103	8 (7.8)	
RJP1	L4 series b	142		52 (37.7)
RJP3	L4 series a	335	48 (14.3)	
RJP3	L4 series b	485		160 (33)
RJP4	L3 series a	42	4 (9.5)	
RJP4	L3 series b	223		74 (33.1)
RJP4	L4	61		21 (34.4)
RJP5	L3	78		27 (34.6)
RJP5	L4	137		39 (28.5)
RJP6	L4	429	34 (7.9)	
RJP7	L3	124	10 (8.6)	
RJP7	L4	246	24 (9.8)	
RJP8	L3	97	10 (10.3)	
RJP8	L4	229	22 (9.6)	
Average %			9.9	33.2

about 171 unmyelinated afferents and therefore an average total of about 274 afferent axons, accounting for about 50 % of the total afferent supply of the knee joint.

Immunohistochemistry

Another advantageous characteristic of FG is its compatibility with fluorescent immunohistochemistry. The proportion of neurons containing SP-like immunoreactivity was somewhat different from that reported in the cat by Hanesch et al. (1991) using a similar methodology. They reported that in the cat about 15 % of knee joint afferents contained SP-like immunoreactivity as compared with the 10 % we found in the rat. This most likely reflects species differences. Interestingly, the 33 % of rat knee joint afferent neurons containing CGRP-like immunoreactivity was virtually identical to the 33.5 % of cat knee joint afferents reported by Hanesch et al. (1991). Since SP and CGRP are colocalised in a majority of peptidergic neurons, our results indicate that under normal circumstances only one third of the joint afferent population contains these proinflammatory neuromodulators. Using this method of selectively identifying knee joint afferents it will now be possible to determine selected molecular responses of these identified neurons to joint inflammation or injury.

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