J. Anat. (1999) **194**, pp. 233–239, with 3 figures Printed in the United Kingdom

Quantitative analysis of the sympathetic innervation of the rat knee joint

MEL G. CATRE[1] AND PAUL T. SALO[2]

[1] *Department of Surgery, University of Toronto and* [2] *McCaig Centre for Joint Injury and Arthritis Research, Department of Surgery, University of Calgary, Canada*

(*Accepted 17 November 1998*)

ABSTRACT

Retrograde tracing with Fluoro-Gold (FG) was used to identify the complete population of knee joint sympathetic postganglionic efferents in the lumbar sympathetic chain of adult female Wistar rats. In 6 rats, the total number and distribution of FG-labelled neurons in the lumbar sympathetic chain was determined. The rat knee joint is supplied by an average of 187 ± 57 sympathetic afferents with the majority at the L3 and L4 levels. Immunohistochemistry using antibodies specific for tyrosine hydroxylase (TH), somatostatin (SS) or vasoactive intestinal polypeptide (VIP) revealed that 33% of knee joint sympathetic afferents contained TH, 42% contained VIP, and none contained somatostatin. Retrograde tracing with FG provided accurate and reproducible labelling of the joint-innervating subpopulation of sympathetic efferent neurons. This model lends itself to the further study of the molecular responses of this neuronal population in the various disorders and conditions affecting joints.

Key words: Sympathetic ganglia; tyrosine hydroxylase; vasoactive intestinal peptide.

INTRODUCTION

The role of the sympathetic nervous system in joint physiology is poorly understood, but the sympathetic fibres of the knee are known to participate in the regulation of vasomotor activity (Sato & Schaible, 1987; Ferrell & Khosbated, 1990; Karimian et al. 1995; McDougall et al. 1997) and vascular permeability (Green et al. 1991; Gibbins, 1992). Sympathetic excitatory reflexes are evoked by noxious stimuli such as forcible knee movements and intra-articular injections of prostaglandins, whereas innocuous stimuli have no effect (Jänig, 1985; Sato & Schaible, 1987).

Sympathetic contributions to acute and chronic inflammation

It has been proposed that the sympathetic nervous system contributes to the process of inflammatory arthritis (Lindh et al. 1989; Roberts, 1989; A. Lee et al. 1991). Plasma extravasation may be, in part, sympathetically mediated (Coderre et al. 1989; Green et al. 1991, 1993*a*, *b*; S. H. Lee et al. 1991). Electrical stimulation of sympathetic outflow inhibits bradykinin-induced plasma extravasation (Miao et al. 1996), yet chronic arthritis results in a prolonged abolition of vasomotor reflexes in the inflamed joint tissues (McDougall et al. 1995). This supports the idea that there are distinct functional subsets of joint-innervating sympathetic neurons, and that some of their functions are significantly changed during an inflammatory arthritis.

There is evidence that sympathetic nervous system-derived mediators enhance the severity of chronic arthritis in animal models and man (Levine et al. 1986; Coderre et al. 1990). In human patients with rheumatoid arthritis, regional sympathetic blockade effectively reduced reported pain and increased pinch strength (Levine et al. 1986).

It is also widely believed that sympathetic adrenergic activity is causally related to at least some of the pain and other sensory disturbances of reflex sympathetic dystrophy (RSD), a common sequel to

Correspondence to Dr P. T. Salo, Heritage Medical Research Building, 3330 Hospital Drive N. W., Calgary, Alberta T2N 4N1, Canada. Fax: + 001 403 270-0617; e-mail: salo@ucalgary.ca

joint injury (Schwartzman & McLellan, 1987; Roberts, 1989; Chard, 1991). However, the primacy of sympathetic involvement has not been confirmed, and the details of mechanisms by which sensory and sympathetic interactions occur are as yet poorly understood.

Despite the evidence documenting an important role of the sympathetic nervous system in the normal and pathological physiology of the joint, there has been no previous accurate description of the number and immunohistochemical characteristics of the sympathetic neurons projecting to the rat knee joint. Since the rat has been widely used as a model for the investigation of both inflammatory arthritis and reflex dystrophy, we chose to combine retrograde tracing with immunohistochemistry to characterise better this important subpopulation of neurons.

METHODS AND MATERIALS

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 for 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.

Retrograde labelling of the knee joint sympathetic innervation was carried out in the same manner as our previously described technique for labelling joint-innervating sensory afferents (Salo & Tatton, 1993; Salo & Theriault, 1997). Briefly, a specially designed knee holder was used to position the joint for injection. Under general anaesthesia (halothane/N_2O/O_2), using sterile technique the patellar tendon was exposed by a 3–4 mm incision. With the aid of a micromanipulator, a 30 gauge Hamilton syringe was introduced obliquely through the tendon into the patellofemoral joint. Each rat had 1 knee injected with 5 μl of 2% Fluoro-Gold (FG) (Fluorochrome, Inglewood, CO) in distilled water followed after a few min by 15 μl of 0.1 M phosphate buffer containing 0.1% Fast Green dye (Sigma) both to precipitate the fluorescent tracer within the intra-articular space and to identify extravasation of the injectate from the joint (Schmued & Fallon, 1986; Salo & Theriault, 1997). After injection the needle was withdrawn, the incision was briefly irrigated with 0.9% sterile saline and the skin sutured with 5-0 nylon. Nine rats had 1 knee joint injected and 5 (designated for immunohistochemical assessment) had bilateral injections.

General histological methods

At 5–7 d after FG injection, the rats were deeply anaesthetised with intraperitoneal sodium pentobarbital and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer. After evisceration, the paravertebral sympathetic chains were dissected. The T13–L5 sympathetic ganglia were removed bilaterally and postfixed in 4% paraformaldehyde overnight. The ganglia were washed in buffer and cryoprotected in 30% sucrose overnight. Paired ganglia (left and right) were then embedded and oriented together in Tissue-tek embedding medium in 1.5 ml Eppendorf tubes, and rapidly frozen in liquid nitrogen.

Specimens were stored at −80 °C until sectioning. Blocks were mounted in a Reichert-Jung cryostat and serial 15 μm transverse sections collected onto gelatin-coated slides. After thaw-mounting and air-drying at room temperature overnight, sections were counterstained with aqueous 1% toluidine blue, dehydrated and coverslipped with Eukitt.

Method for counting labelled joint afferents

Every section through every ganglion (ranging from 100 to 200 sections per ganglion) was examined alternately under brightfield and UV epifluorescence with appropriate filters for FG (excitation: 355–425 nm, long pass filter: 460 nm) and lipofuscin autofluorescence (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 reconstruction method of Coggeshall et al. 1990; see also Salo & Theriault, 1997). Some of the larger FG-positive somal profiles could be followed over as many as 3 or 4 serial sections. Few cells exhibited significant lipofuscin autofluorescence, so that identi-

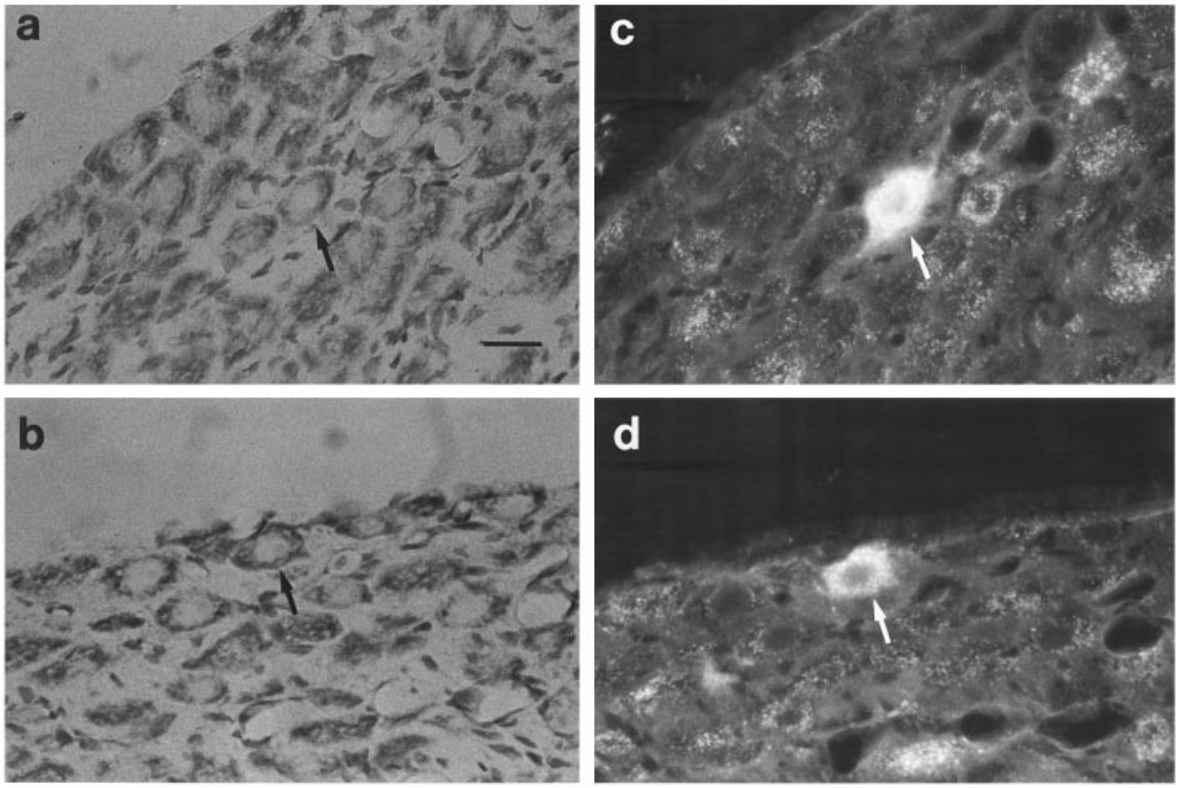

Fig. 1. Composite photomicrograph of 2 typical sections of through the L4 sympathetic ganglion, viewed with bright field (*a*, *b*) and UV epifluorescence with filters selective for FG (*c*, *d*). Joint-innervating sympathetic efferent somata are readily identifiable by bright fluorescence (arrows). Bar, 20 μm.

ficaton and counting of the labelled cells was straightforward.

Immunohistochemistry

After fixation and embedding as described above, 15 μm serial frozen sections were cut from the L3 and L4 ganglia harvested from 5 rats prelabelled by bilateral and 1 rat labelled by unilateral 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, 77% of the joint efferents would be found there. Three sets of serial sections were collected onto gelatin-chrome alum subbed slides, thaw-mounted, dried on a warming plate at 30 °C for 2 h and stored at 4 °C overnight.

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-TH (Eugene) was used at a titre of 1/2000 in 2% normal horse serum. Anti-SS (Zymed) was used at a titre of 1/1000 in 2% normal goat serum (NGS). Anti-VIP (Zymed) was used at a titre of 1/500 in 2% NGS. The following day slides were washed in 3 changes of phosphate buffer and blocked for 30 min in 10% NGS. Specific binding of anti-TH was visualised by a 1 h incubation with a biotinylated goat antirabbit IgG followed by Texas red avidin D (Vector). Anti-SS and anti-VIP binding was visualised by a rat-adsorbed biotinylated horse antimouse IgG (Vector) and Texas red avidin D. 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 took great care to ensure that the tip of the injection

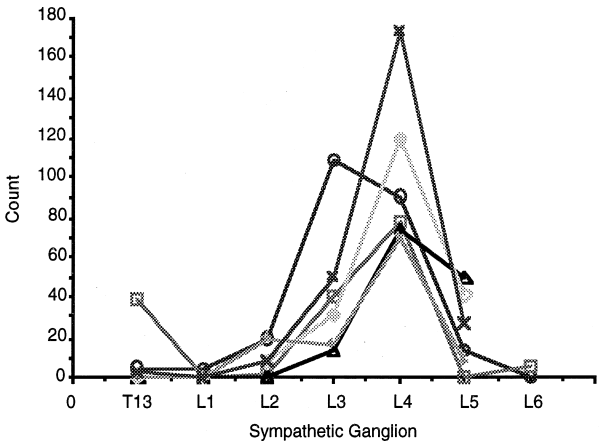

Fig. 2. Distribution of FG-labelled joint-innervating sympathetic efferent somata in the lumbar sympathetic paravertebral ganglia. Each set of joined points represents the results of a single FG injection into 1 knee joint of 1 rat. Note the concentration of sympathetic efferents in the L3, L4 and L5 ganglia.

needle was positioned precisely between the patella and the distal femur before instillation of the Fluoro-Gold and Fast Green with phosphate buffer. For this reason there were no animals rejected or excluded from the series due inadvertent injection of deeper tissue layers away from the joint. No rat was excluded from the series due to immediate extravasation of the tracer from the joint at the injection site. Unfortunately, dissection of the lumbar sympathetic chain was hampered by the small, friable nature of the ganglia and 3 rats were excluded from subsequent analysis either because of a failure to remove intact the complete set of sympathetic ganglia or because of an anatomical anomaly such as fusion of 2 ganglia in a chain. The L6 ganglion was consistently small and difficult to identify. It was examined in 2 rats and found to contain only a handful of labelled neurons. We did not examine it in subsequent rats, although this may have affected the results slightly.

Fluoro-Gold labelled somal profiles were readily identifiable in the lumbar sympathetic ganglia. Figure 1 represents photomicrographs of typical sections through an L4 sympathetic ganglion under both brightfield and UV fluorescence microscopy, respectively.

We counted 187 ± 57 (mean $\pm$ S.D.) FG-labelled cells in the sympathetic ganglia with a distribution from T13 to L6. The vast majority of labelled somata were found in the L3, L4 and L5 ganglia (Fig. 3, Table). Examination of the contralateral ganglia revealed no FG labelled neurons in rats that had unilateral joint injections.

Immunohistochemistry

One rat was excluded from this evaluation because poor quality perfusion impaired the quality of the histological sections. A few ganglia were lost due to inadvertent damage during bilateral dissection of the sympathetic chain. Figure 2 is a composite photomicrograph of typical sections stained for TH or VIP-like immunoreactivity. Counts revealed that, on average, 33% of FG-labelled profiles contained TH-like immunoreactivity and that 44% contained VIP-like immunoreactivity (Table). Somatostatin immunoreactivity was not seen in the somata of any neurons in the L3 and L4 lumbar sympathetic ganglia. Some SS-immunoreactive axonal profiles were observed, which may represent preganglionic fibres or visceral afferents, but these appeared to have no relationship with any FG-labelled neuronal somata.

Table. *Counts of FG-labelled cells in sympathetic ganglia**

Rat sympathetic chain	Series A TH/FG (%)		Series B VIP/FG (%)	
	L3	L4	L3	L4
2L	11/63 (17)	5/33 (15)	27/85 (32)	17/43 (40)
2R	11/31 (35)	—	17/63 (27)	—
3L	17/59 (29)	19/58 (33)	28/60 (47)	52/116 (45)
3R	5/15 (33)	4/9 (44)	5/10 (50)	9/12 (75)
4L	33/99 (33)	4/26 (15)	43/83 (52)	21/33 (64)
4R	—	26/89 (29)	—	35/80 (44)
5L	19/62 (31)	55/98 (56)	14/47 (30)	50/76 (66)
Totals for L3 and L4 (%)	209/642 (33)		318/708 (44)	

* Each row contains data from 1 knee joint. Each column shows the number of immunopositive profiles that are also FG-positive, divided by the total number of FG-positive profiles for each ganglion, and the fraction expressed as a percentage. The bottom row summarises the data with the total counts from all ganglia and the average percentages of FG-labelled neurons immunostained for each marker protein.

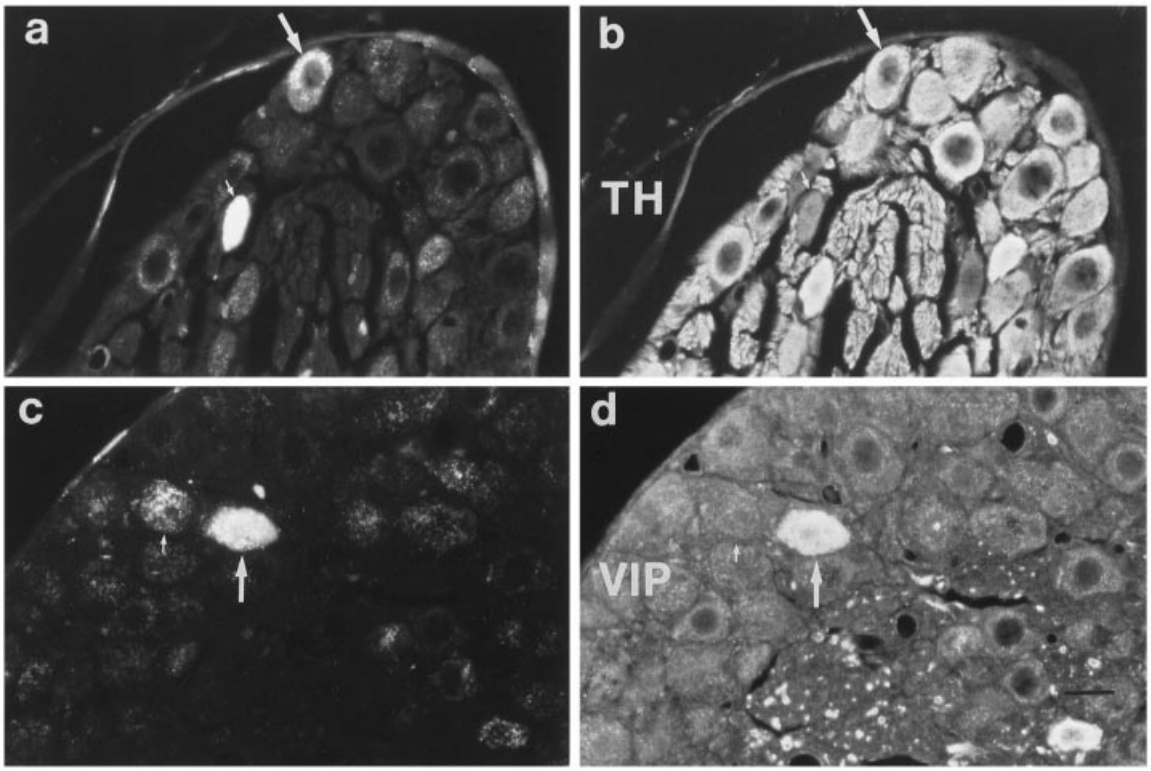

Fig. 3. Composite photomicrograph of 2 sections of FG-prelabelled L3 ganglia, immunostained for TH (*a*, *b*) or VIP (*c*, *d*). Each image features 2 FG-labelled sympathetic efferents (arrows), 1 immunostained (large arrow) and 1 not stained (small arrow). Bar, 20 μm.

DISCUSSION

Retrograde tracing, methodological considerations

There has been only a single previous reported attempt to map the sympathetic innervation of the rat knee joint. Wheat-germ agglutinin conjugated to horseradish peroxidase was used for retrograde tracing after injection into the joint (Widenfalk & Wiberg, 1989). However, this report contains a few deficiencies that may limit the reliability of the data they obtained. The injection of tracer was apparently performed free hand, without any indication whether magnification was used as an aid, or if steps were taken to detect extravasation of the tracer from the injection site. These authors actually used a variety of tracers; in some rats HRP alone was injected, in others WGA-HRP from 2 different suppliers was utilised. There was considerable variability in the time from injection to killing the animals (16–120 h). They did not state if the contralateral sympathetic chain was also examined as a control to exclude labelling from the systemic circulation.

Fluoro-Gold is now widely accepted to be a superior agent for retrograde tracing in rats (Schmued & Fallon, 1986) and produced extremely consistent results in our previously reported study of rat knee joint sensory afferents (Salo & Theriault, 1997). The use of a micromanipulator and the coinjection of Fast Green to detect extravasation greatly increased the reliability and consistency of injection.

Examining the contralateral sympathetic chain revealed 2 things. There were no FG-labelled cells found in any of the contralateral control ganglia, indicating no labelling from systemic circulation of FG and importantly, no projection of sympathetic fibres across the midline.

Number and distribution of FG-labelled sympathetic neurons

The rat knee appears to be supplied by an average of 187 ± 57 (range 116–261) sympathetic efferents. The L4 level was consistently found to contain the most FG-labelled neurons, followed by L3. This is slightly different from the distribution of rat knee joint sensory neurons, which are equally concentrated at both L3 and L4. As others have noted previously (Miao et al. 1995), we found much greater inherent variability in the anatomy of the sympathetic system than we had noted in the somatosensory ganglia.

The posterior articular nerve of the rat knee joint is typically comprised of about 600 axons, of which 80% are unmyelinated (Hildebrand et al. 1991; Salo

& Theriault, 1997). Of the unmyelinated axons, neonatal lesioning with guanethidine revealed that on average, two-thirds (about 200 fibres), arose from sympathetic postganglionic efferents (Hildebrand et al. 1991). Our average count of 185 labelled sympathetic somata is consistent with this number. Had we included counts from the L6 ganglion in all our specimens, our results may have been in even closer agreement with the estimate of 200 neurons by Hildebrand et al.

Immunohistochemistry

To our knowledge, this is the first report to describe the functional immunohistochemical attributes of selectively identified joint-innervating sympathetic neurons. Interestingly, 33% of these neurons expressed tyrosine hydroxylase, a marker of catecholaminergic neurons. These fibres likely function chiefly as vasoconstrictors (Hohler et al. 1995). Surprisingly, 44% of joint efferents expressed VIP which probably signifies vasodilatory properties (Anderson et al. 1995), although VIP is also known to have other potentially important functions, including a potent stimulatory effect on cells of the immune system (Stanisz, 1994; Bellinger et al. 1996).

Although somatostatin-like immunoreactivity was seen in some axons traversing the lumbar sympathetic ganglia, we did not find any stained somata in any of the ganglia examined, including those neurons that were FG-negative. We identified no relationship of the SS+ve fibres to FG-labelled joint efferent somata.

It is concluded that this will be a useful model for the further investigation of the molecular responses of joint efferents to joint injury or arthritis.

ACKNOWLEDGEMENTS

The authors thank Ruth-Anne Seerattan for her excellent technical assistance. Funding for this research was provided by the Medical Research Council of Canada.

REFERENCES

ANDERSON CR, McALLEN RM, EDWARDS SL (1995) Nitric oxide synthase and chemical coding in sympathetic postganglionic neurons. *Neuroscience* **68**, 255–264.

BELLINGER DL, LORTON D, BROUXHON S, FELTEN S, FELTEN DL (1996) The significance of vasoactive intestinal polypeptide (VIP) in immunomodulation. *Advances in Neuroimmunology* **6**, 5–27.

CHARD M (1991) Diagnosis and management of algodystrophy. *Annals of the Rheumatic Diseases* **50**, 727–730.

CODERRE TJ, BASBAUM AI, LEVINE JD (1989) Neural control of vascular permeability: interaction between primary afferents, mast cells and sympathetic efferents. *Journal of Neurophysiology* **62**, 48–58.

CODERRE TJ, BASBAUM AI, DALLMAN MF, HELMS C, LEVINE JD (1990) Epinephrine exacerbates arthritis by an action at presynaptic α2-adrenoceptors. *Neuroscience* **34**, 521–523.

COGGESHALL RE, LA FORTE R, KLEIN CM (1990) Calibration of methods for determining numbers of dorsal root ganglion cells. *Journal of Neuroscience Methods* **35**, 187–194.

FERRELL WR, KHOSBATED A (1990) Responses of blood vessels in the rabbit knee to electrical stimulation of the joint capsule. *Journal of Physiology* **423**, 569–578.

GIBBINS IL (1992) Vasoconstrictor, vasodilator and pilomotor pathways in sympathetic ganglia of guinea-pigs. *Neuroscience* **47**, 657–672.

GREEN PG, BASBAUM AI, HELMS C, LEVINE JD (1991) Purinergic regulation of bradykinin-induced plasma extravasation and adjuvant-induced arthritis in the rat. *Proceedings of the National Academy of Sciences of the USA* **88**, 4162–4165.

GREEN PG, LUO J, HAMMOND ER, LEVINE JD (1993*a*) Trypsin enhances sympathetic neuron-dependent plasma extravasation in the rat knee joint. *Proceedings of the National Academy of Sciences of the USA* **158**, 117–119.

GREEN PG, LUO J, HELLER PH, LEVINE JD (1993*b*) Modulation of bradykinin-induced plasma extravasation in the rat knee joint by sympathetic co-transmitters. *Neuroscience Letters* **52**, 451–458.

HILDEBRAND C, ÖCQVIST G, BRAX L, TUISKU F (1991) Anatomy of the rat knee joint and fibre composition of a major articular nerve. *Anatomical Record* **229**, 545–555.

HOHLER B, OLRY R, MAYER B, KUMMER W (1995) Nitric oxide synthase in guinea pig sympathetic ganglia: correlation with tyrosine hydroxylase and neuropeptides. *Histochemistry and Cell Biology* **104**, 21–28.

HYDÉN H, LINDSTRÖM B (1950) Microspectrographic studies on the yellow pigment in nerve cells. *Discussions of the Faraday Society* **9**, 436–441.

JÄNIG W (1985) Organization of the lumbar sympathetic outflow to skeletal muscle and skin of the cat hindlimb and tail. *Reviews of Physiology, Biochemistry and Pharmacology* **102**, 119–213.

KARIMIAN SM, McDOUGALL JJ, FERRELL WR (1995) Neuropeptidergic and autonomic control of the vasculature of the rat knee revealed by laser Doppler perfusion imaging. *Experimental Physiology* **80**, 341–348.

LEE A, CORDERRE TJ, BASBAUM AI, LEVINE JD (1991) Sympathetic neuron factors involved in bradykinin-induced plasma extravasation in the rat. *Brain Research* **557**, 146–148.

LEE S-H, WANG S-C, SEEN C-L (1991) Innervation of the knee joint in the rabbit and the Formosan rock-monkey (*Macaca cyclopis*): a retrograde HRP study. *Proceedings of the National Science Council, Republic of China*, Part B: *Life Sciences*. **15**, 171–177.

LEVINE JD, FYE K, HELLER P, BASBAUM AI (1986) Clinical responses to regional intravenous guanethidine in patients with rheumatoid arthritis. *Journal of Rheumatology* **13**, 1040–1043.

LINDH B, LUNDBERG JM, HÖKFELT T (1989) NPY-, galanin-, VIP/PHI-, CGRP- and substance P- immunoreactive neuronal subpopulation in cat autonomic and sensory ganglia and their projections. *Cell and Tissue Research* **256**, 259–273.

McDOUGALL JJ, KARIMIAN SM, FERRELL WR (1995) Prolonged alteration of vasoconstrictor and vasodilator responses in rat knee joints by adjuvant monoarthritis. *Experimental Physiology* **80**, 349–357.

McDOUGALL JJ, FERELL WR, BRAY RC (1997) Spatial variation in sympathathetic influences on the vasculature of the synovium and medial collateral ligament of the rabbit knee joint. *Journal of Physiology* **503**, 435–443.

MIAO FJ, KINNMAN E, JANIG W, LEVINE JD (1995)

Variation in the anatomy of the lumbar sympathetic chain in the rat. *Journal of the Autonomic Nervous System* **56**, 115–118.

MIAO FJ, JANIG W, LEVINE JD (1996) Role of sympathetic post-ganglionic neurons in synovial plasma extravasation induced by bradykinin. *Journal of Neurophysiology* **75**, 715–724.

ROBERTS WJ (1989) Review article. A hypothesis on the physiological basis for causalgia and related pain. *Pain* **24**, 297–311.

SALO PT, TATTON WG (1993) Age-related loss of knee joint afferents in mice. *Journal of Neuroscience Research* **35**, 664–677.

SALO PT, THERIAULT ET (1997) Number, distribution and neuropeptide content of rat knee joint afferents. *Journal of Anatomy* **190**, 515–522.

SATO Y, SCHAIBLE H-G (1987) Discharge characteristics of efferents to the knee joint of the cat. *Journal of the Autonomic Nervous System* **19**, 95–103.

SCHMUED LC, FALLON JH (1986) Fluoro-Gold: a new fluorescent retrograde axonal tracer with numerous unique properties. *Brain Research* **377**, 147–154.

SCHWARTZMAN RJ, McLELLAN TL (1987) Reflex sympathetic activity. A review. *Archives of Neurology* **44**, 555–561.

STANISZ AM (1994) Neuronal factors modulating immunity. *Neuroimmunomodulation* **1**, 217–230.

WIDENFALK B, WIBERG M (1989) Origin of sympathetic and sensory innervation of the knee joint: a retrograde axonal tracing study in the rat. *Anatomy and Embryology* **180**, 317–324.