Sparse substance P-like immunoreactivity in intervertebral discs

Nerve fibers and endings in the rat

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I studied rat lumbar intervertebral discs using a monoclonal antibody to substance P, which revealed immunoreactivity in the periosteum and ligaments adjacent to the intervertebral disc. Fibers containing substance P-like immunoreactivity were also found penetrating and terminating within the annulus fibrosus of both the anterior and posterior intervertebral disc. The maximum depth of penetration was 5 lamellae (annular rings) or approximately one sixth of the depth of the annulus. The terminal structures were not encapsulated (free-nerve endings) and were either branched, looped or both. The majority of fibers were varicose in appearance. Substance P-like immunoreactivity was very minor.

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The innervation of the lumbar intervertebral disc has 2 possible anatomical sources, sensory afferent and sympathetic efferent (Bogduk et al. 1981, Bogduk 1983). Retrograde labeling experiments in dogs have confirmed the presence of sensory fibers to the disc (Forsythe and Ghoshal 1984). The neuropeptide substance P is an important compound in this respect as it has been linked with both pain and neurogenic effects, such as plasma extravasation and vasodilation (Maggi and Meli 1988). I have studied the presence, proportion and relative position of SP-like immunoreactivity in nerve fibers and endings in lumbar intervertebral discs from the rat.

Material and methods

Intact L2–L3 and L4–5 intervertebral discs were excised, as previously described (McCarthy et al. 1992), from 6 2–3-month-old female Wistar rats. Immediately after removal, the tissue had a 90-min fixation in Zamboni's fixative and was cryoprotected by overnight immersion at 4 °C in a solution of 30 percent sucrose prepared in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Serial 20 µm thick transverse (2 discs, 1 from L4–5 and 1 from L2–3) or longitudinal (10 discs) cryostat sections of whole discs were made, with a 40 µm gap between adjacent sections. A total of between 30 and 80 sections was studied from each disc, depending on the direction of sectioning. A series from each disc was processed for SP-LI as follows. The substance P antibody was a monoclonal mouse antibody (Sera-Lab, England) used in a dilution of 1:800. Primary antibody was prepared along with normal swine serum (1:10) and fetal calf serum (1:10) in PBS, pH 7.4. The sections were incubated in the primary antibody solution overnight at 4 °C. Antibody reaction sites were visualized using a tetramethylrhodamine isothiocyanate conjugated (TRITC) swine anti-mouse IgG antibody (1:400, DAKO, England), prepared with normal rat serum (1:10) in PBS. The incubation time in this solution was 30 min at room temperature. Triton-x 100 (1:500) was used in all antibody solutions. The sections were studied under normal light and epifluorescence, using a BH2-DMG filter block on an Olympus (BH2) microscope. Controls in which the primary antibody was either omitted or pre-absorbed with a 10 µM substance P (Sigma, England) solution did not reveal any immunoreactivity which could be mistaken for fibers or nerve endings in the discal tissue.

As previously reported (McCarthy et al. 1991, 1992), this technique allowed for a study of the nucleus pulposus, end-plate, annulus fibrosi, as well as the adjacent ligamentous structures occasionally including periosteum, in 40 µm steps. Estimation of the presence and location of SP-LI structures was an important consideration, as sectioned material was to be used. Therefore, the following criteria were adopted. SP-LI fibers were classified as being in the annulus if they crossed over half of the first annular ring. An ending or terminal structure was counted

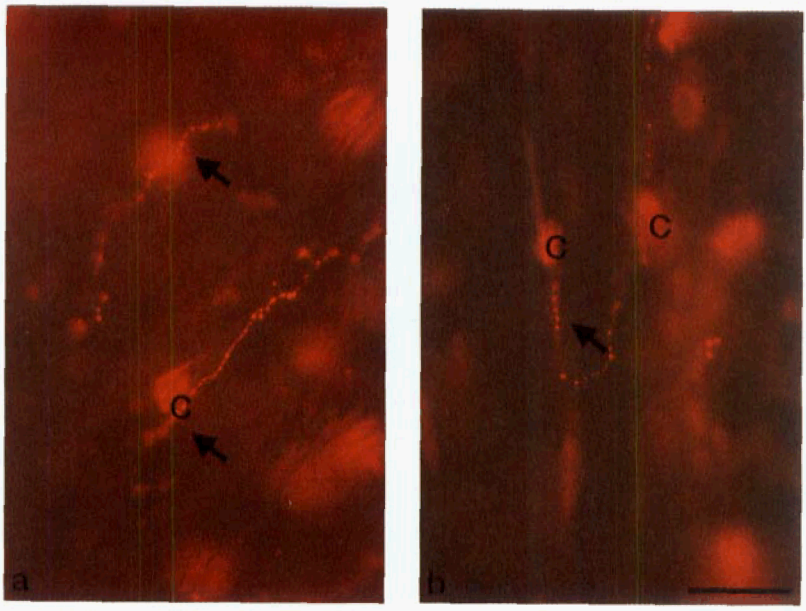

Figure 1. Photomicrographs of SP-LI fluorescent fibers and terminations in the outer annulus fibrosus. Scale bar 10 µm.
a) Varicose fibers (arrows) close to cells (C) in between the annular rings on the posterior side.
b) A contorted varicose fiber (arrow) which terminated between 2 adjacent lamellae after traversing the outer one. The terminal part of this fiber lay in close proximity to some chondrocytes (C).

when the SP-LI fibre was present in one tissue section but absent from the next adjacent section. This method was satisfactory when few fibers were present, i.e., the disc tissue, however, it became unwieldy when the amount of SP-LI increased, i.e., adjacent ligaments. In the latter case, an estimate of termination was made by judging whether or not SP-LI fibers and their branches left the section by studying the position within the tissue of the last visible part of the fibre. This was done by using high magnification (63 × objective) and judging which focal plane the fibre disappeared in with respect to the edge of the tissue.

Table 1. Estimates of SP-LI structures in and around the IVD. Fibers in complete series of tissue (n 8), L2–L3 (n 3) and L4–L5 IVDs (n 5). Mean (range)

Position	Fibers	Terminals
Anterior and anterolateral annulus	4 (1–6)	1 (0–3)
Anterior ligs. and adipose tissue	10 (6–25)	3 (1–8)[a]
Posterior and posterolateral annulus	6 (3–10)	2 (0–5)
Posterior ligs. and adipose tissue	≥20	6 (3–10)[a]
Vertebral end-plate	4 (0–6)	2 (0–3)
Nucleus and inner annulus	0	0
Vascular channels entering bone	3 (0–6)	0

Data from the remaining, incomplete, series (n 4), although not included here, did not appear to be different. It must be noted that these results come from studies of sections totalling one third of each IVD.
[a]It was not always possible to determine structures accurately (see text), usually because there were many other fibers present or the refraction of fluorescence was caused by adipose tissue.

Results

SP-LI fibers were found in all 12 intervertebral discs. Furthermore, there was no apparent difference between the overall distribution of immunoreactivity in the discs from L2–L3 and those from L4–L5. Most of the SP-LI fibers were located in the longitudinal ligamentous tissue adjacent to the disc, especially on the posterior side (Table 1). The entry point for the innervation corresponded with that of the sinuvertebral nerve, namely, the posterolateral edge. SP-LI fibers could be found around the lateral aspects of the discs, as well as in the anterior and the posterior. SP-LI fibers appeared both within the outer lamellae and around the outside, travelling from posterior to anterior, with occasional incursions into the annulus.

Over 30 percent of the SP-LI fibers which were found in close apposition to the disc entered the annular tissue. The main areas of annular innervation were the anterior and posterior edges underlying the longitudinal ligaments and associated adipose tissue (Table 1, Figure 1). Fibers and endings were found in these tissues (Figure 2). The innervation of the outer annulus was effected by what appeared to be single SP-LI fibers, which would cross over the annular lamellae before branching to send fibers parallel to the lamellae. No SP-LI was found deeper than 5 annular lamellae, which is approximately one sixth of the distance into the annulus. In addition, fibers were found penetrating the edge of the cartilage end-plate regions from the disc and adjacent ligamentous structures (Figure

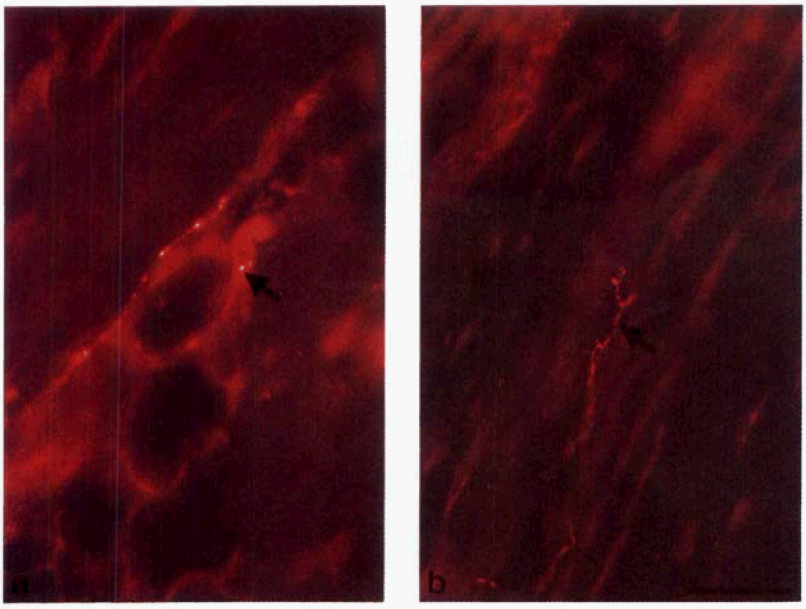

Figure 2. Photomicrographs of SP-LI fluorescent fibers in tissue adjacent to the intervertebral disc. Scale bar 10 μm.
a) 2 fibers in adipose tissue. The first (arrow) was associated with a blood vessel close to an adipose cell (A) whereas the second and more obvious fiber travelled around the lateral edge between the adipose cell and the anterior annulus.
b) A SP-LI ending within the junction between the cartilage end-plate and outer annulus fibrosus, on the anterior surface. Note the varicose appearance, more tortuous path and the small varicose protruberances (arrow) on the fiber.
Bar represents 10 μm.

2). SP-LI fibers were varicose in appearance, none were found in the nucleus pulposus or the inner annulus fibrosus (Figure 3). The majority of vascular channels entering vertebral bodies had SP-LI fibers associated with them, as reported by Bjurholm et al. (1988).

All of the terminal structures associated with the SP-LI appeared to be free-nerve endings, i.e., not encapsulated. 2 types of ending could be resolved. The most common type, in tissue adjacent to the disc, consisted of varicose fibers which branched and contorted into a series of loops (Figures 1 and 2). These endings were found especially in the longitudinal ligaments, periosteum and the junction between the cartilage endplate and annulus. The second type of ending, which was prevalent in the annular tissue, comprised a branching fibre with no contortions. In the latter case, the fibers were mainly varicose in appearance and often lined up in close juxtaposition with cells in the interlamellar spaces (Figure 1). The fine branches occasionally terminated in bulb-like enlargements similar to the endings described by Malinsky (1959). Very few obvious endings were found in each disc (Table 1). It must be emphasized that the amount of SP-LI was minimal, even though the extent of innervation was not. The overall distribution of SP-LI can be seen in the schematic representation (Figure 3).

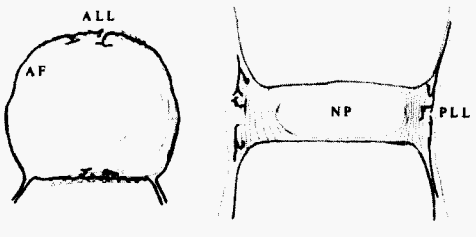

Figure 3. Schematic representations of the SP-LI found around the rat lumbar intervertebral disc. Transverse (left) and sagittal (right) sections through a hypothetical midline are outlined faintly with the innervation represented by bold lines. Nucleus pulposus (NP), annulus fibrosus (AF) and longitudinal ligaments (posterior, PLL; anterior, ALL) are indicated for orientation.

Discussion

The findings reported here extend those of Weinstein et al. (1988). Those authors also showed that SP in dog dorsal root ganglia does change after discography. In my study, SP-LI fibers were found throughout the outer annulus fibrosus, with no immunoreactivity in the nucleus pulposus. By far the greatest proportion of SP-LI was located in the ligamentous and adipose tissues adjacent to the disc, which shows a similarity to findings in human tissue (Korkala et al. 1985).

Many of the SP-LI fibers which circumnavigate the intervertebral disc entered the edges of the annulus fibrosus and later exited again into the adjacent ligaments. It is, therefore, easy to mistake fibers travelling in the disc from those terminating there, if one only takes occasional, and proportionally small samples,

such as in the usual human study. It may be that such en passant fibers, which appear varicose, can release their contents at the varicosities. However, no proof of this is available.

SP-LI fibers were also found entering the annulus, not only in the anterior region, as previously reported (Coppes et al. 1990), but also in the posterior and more lateral regions. The endings were branched, varicose and apparently associated with cells within the annular tissue. The structures which most closely resembled those endings described by Coppes et al. (1990), namely, spin-like, all had a varicose distribution of staining and a convoluted path. The fibers forming these terminations appeared close to cell bodies within the matrix of the annulus and outer edges of the cartilage end-plate.

In absolute terms, there are few SP-LI fibers within the rat lumbar discs. However, fibers of any type are scarce within this tissue (Roofe 1940, Malinsky 1959, Guilliot et al. 1988, Weinstein et al. 1988, McCarthy et al. 1992). The types of fibers described in this study would have thin, or even no myelination on their peripheral axons (McCarthy and Lawson 1989). However, as was shown previously, there are myelinated fibers in the tissue adjacent to the disc which do not enter the annulus in the same areas as the SP-LI fibers described here (McCarthy et al. 1992). Instead, it would appear that myelinated fibers are related to the presence of more complex endings in the adjacent ligaments (Roofe 1940, Malinsky 1959, Yoshizawa et al. 1980, Guilliot et al. 1988, McCarthy et al. 1991, McCarthy et al. 1992). Therefore, one may assume that the SP-LI fibers in the rat lumbar intervertebral disc are C-fiber/unmyelinated afferents.

Sympathetic post-ganglionic and sensory afferents are the only nerve fibers whose presence is anatomically expected in the lumbar disc (Bogduk et al. 1981, Bogduk 1983, Forsythe and Ghoshal 1984, Kojima et al. 1990, McCarthy et al. 1992). Of these 2 innervations, at least in the rat, SP-LI is only found in the soma and fibers of sensory afferent neurons (Lee et al. 1985, Ju et al. 1987). In fact, the interest in innervation of the discs by SP-LI fibers stems from its possible relationship to both nociception and neurogenic effects (Weinstein et al. 1988, Grönblad et al. 1991). The majority of studies which have produced the basis for this relationship, however, have been performed in animals (Duggan et al. 1988, Holzer 1988, Maggi and Meli 1988). Therefore, although it may be valid to hypothesize that in some animal studies the finding of primary afferent fibers containing SP-LI would, indeed, indicate nociception as a possible option, a flaw with studies of SP-LI in human disc is that there is little direct basis for suggesting any such relationship. On saying this, however, one must consider the evidence from discogram studies in humans (Antti-Poika et al. 1990) which have demonstrated that pain on injection requires some area of weakness between outer annular or peridiscal tissue and the injection site. The limiting factor in any speculation is the small absolute number of SP-LI fibers found within the annular tissue. This must reduce their potential effectiveness to signal pain, in which case they must be considered a small part of such a mechanism. Furthermore, if one considers the possibility that these fibers have an efferent capacity, then their effects must be either extremely specific, or capable of acting at extremely low concentrations. As such, the role of the SP-LI innervation may, or may not, reflect its proportion.

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