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The distribution of peptide-containing nerves in the synovia of the cat knee joint

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Summary. Synovial capsule in cats is extensively innervated by a network with axonal diameter ranging from 0.6-3 μm according to its position and neuropeptide content. Nerve markers such as Neuron Specific Enolase (NSE) and Neurofilament triplet protein (NF) could be observed only when the axonal fibre attained a critical diameter of over the 3 μm limit. The relatively thick fibres (1-3 μm) show positive immunoreactivity for Substance P (SP), 5-hydroxytryptamine (5-HT), and Vasoactive Intestinal Peptide (VIP), and seldom coreact with NSE and NF, whereas, the thinnest fibres (0.6-0.8 μm) characterized to contain either Methionine or Leucine Enkephalin (M-Enk, L-Enk) did not coreact positively with axonal markers.

We found that different anesthetics may effect variably the immunoreactivity of some neuropeptides (SP, L-Enk, 5-HT) while others (VIP, M-Enk) remained unaffected. Based on our data and the few reported ones in the pertinent literature, it is judged that urethane is the anesthetic of choice in experimental studies of neuropeptides.

Our findings of isolated positive immunoreactive cell bodies to enkephalin in synovia might suggest the presence of intrinsic relay system, where the enkephalin acts as suppressor of SP and VIP release from the sunovium nerve terminals. Such a local inter-relationship between different neuropeptide systems might have a practical role on the understanding of the pathogenesis of different arthritic processes as well as therapeutic strategy in the future.

Key words: Neuropeptides, Substance P, Protein S-100, VIP, Enkephalins, 5-hydroxytriptamine, Synovia, Cat

Introduction

Neuroanatomical studies of joints are mostly descriptive and histological studies in both human (Gardiner, 1948; Kellgren and Samuel, 1950; Ralson et al., 1960; Kennedy et al., 1982; Schutte et al., 1987) and cats (Samuel, 1952; Boyd, 1954; Clark, 1975; Clark and Burgess, 1975; Grigg and Hoffman, 1982; Grigg et al., 1982; Langford and Schmidt, 1983; Schaible and Schmidt, 1983; Rossi and Rossi, 1985; Heppelman et al., 1988) deal with the topographical distribution of nerve fibres based on the original Gairn's gold chloride impregnation technique (Gairns, 1930) or its modified version (Zimmy et al., 1985). These studies have revealed that the synovia is richly innervated by unmyelinated fine nerve fibres or plexi of fibres running between collagen fibres and blood vessels (Langford and Schmidt, 1983; Heppelmann et al., 1988). However, the exact physiological role of the various nerve fibres which are found in the synovia has so far been rarely reported (Skoglung, 1973).

A novel and useful approach in neurobiology has emerged with the application of immunocytochemistry for the identification and classification of nerve fibres according to the chemical nature of their constituents. Neuropeptides represent a very large family of biologically active chemicals which occur in all classes of neurons, including those which give rise to unmyelinated nerve fibre i.e., sensory or sympathetic efferent fibres.

The technique of immunocytochemistry has revealed that very fine sensory nerve fibres contain one or more specific neuropeptides in different organs and tissues (Polak and Bloom, 1980; Buck et al., 1982; Inagaki and Kito, 1986) as well as in the central terminal projection in the spinal cord (Gibson et al., 1981; De Groat, 1986).

The discovery of chemically coded nerve fibres has thus revolutionized studies on the innervation of various tissues and organs, as now the class of fibre (sensory, somatic or autonomic) can be recognized by its neuropeptide content and this has paved the way for new concepts in neurophysiology and therapeutic strategies.

So far reports in literature deal with pathological cases of human synovia which contain many nerve fibres demonstrated by the presence of neurofilament triplet

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protein immunoreactivity, a number of Substance-P immunoreactive fibres and occasionally Calcitonin Gene Related Peptide (CGRP) and enkephalin imnmunoreactive fibres (Grönblad et al., 1985, 1988; Gides, 1987). Synovial neuropeptide characterization in normal or experimental animal models is even scarcer (Marinozzi et al., 1988).

Despite the fact that most neurophysiological studies of articular function (Clark and Burgess, 1975; Grigg and Hoffman, 1982; Rossi and Rossi, 1985) as well as description of the topographical nerve distribution (Langford and Schmidt, 1983; Heppelman et al., 1988) have been performed in cats, no data on the neuropeptide innervation of feline synovia exist in literature.

The aim of the present study was to determine the extent of innervation of the feline synovial membrane using the marker NF triplet protein, Protein S-100 and Neuron Specific Enolase, and to characterize the nerves by means of their neurochemical constituents.

We shall also try to elucidate the possible effect of anesthetics on blocking neurotransmitter release, on the immunohistochemical characterization of axonal contituents, and the neurotransmitters studied.

Materials and methods

Ten adult healthy cats of both sexes (3.2 kg body weight) were anesthesized by a single intra-peritoneal injection of either 10 mg/kg ketamine hydrochloride (Parke Davis, U.K.) or 1.5 gr/kg urethane (Fluka, Switzerland) dissolved in sterile water to final concentration of 250 mg/ml. Urethane was freshly prepared. The synovial membrane and capsule of the knee joint were dissected in small segments of 10 x 5 x 2 mm in size and cut according to their longitudinal or transversal planes. These segments were immediately fixed in a 0.4% p-benzoquinone solution (Fluka, Switzerland) in Phosphate Buffered Saline (PBS) 0.1 M at pH 7.2 for 15 mins and thereafter immersed in a 10% sucrose PBS solution for 30 mins at room temperature (Pearse and Polak, 1975). The tissues were sectioned and alternatively postfixed with cold acetone for 10 mins at -20° C. The sections were cut on a cryostat at 10-12 μm thickness and dried at room temperature for 30 mins. Before immunostaining the sections were preabsorbed with normal goat serum diluted at 1:30 in PBS containing 0.01% sodium azide for 30 min at room temperature.

All primary antisera were polyclonal, raised in rabbit, against the neuropeptides, specific nerve enzymes or neurofilament triplet protein and were applied at their optimal dilutions accordingly (Table 1). The sections were incubated overnight in a humid chamber at 4° C, thereafter rinsed throughly with PBS. Afterwards the sections were incubated with the secondary goat antirabbit IgG antiserum FITC-conjugated (Biomakor, Israel) diluted at 1:16 in PBS for 45 min at RT, rinsed again in PBS and 0.05 M Tris - saline buffered solution (pH 7.8). The sections were mounted in Tris-buffer: Glycerol 1:9 (v/v) medium and viewed with a Reichert Orthoplane microscope using epiillumination system with adequate filters. In few cases the peroxidase antiperoxidase technique was applied for a better visualization of Enk-immuno-reactive cells. The dilutions of methionine and leucine subtypes were at 1:500.

The specificity of neuropeptide antisera raised at Hammersmith Hospital has previously been confirmed at the laboratory of Prof. J.M. Polak (Gibson et al., 1981). The specificity of the commercial antibody was established at our laboratory by the neutralization test consisting of incubation of control sections with primary antisera preabsorbed with an excess (40 μg/ml) of the corresponding antigen. In addition, the following controls were performed:

– positive control of tissue known to immunoreact specifically to a given antisera.

– negative controls in which the primary antisera or FITC-conjugated antisera were omitted and substituted by PBS solution.

Structures that were stained using the indirect immunofluorescent procedures (as mentioned above) but unstained in the negative control slides were considered to be specific by present standards (Sternberger, 1979). Pictures were made as soon as possible using an Agfachrome 1000 ASA film, and internegative black and white pictures were printed.

Results

Fine nerve fibres showing different neuropeptide immunoreactivity were found in the subintimal connective tissue and perivascular area of the lateral and medial synovial capsula. The nerve diameter of these fibres varied according to their location in the synovial tissue and was related to their neuropeptide constitution (Table 2). VIP-immunoreactive nerve fibres were thicker (2-3 μm) than the 5-HT and SP-immunoreactive fibres (1-2 μm), while Enk-immunoreactive fibres were the thinnest (0.6-0.8 μm). The VIP, 5-HT and SP-fibres were randomly distributed and were closely related to the blood vessels. Some of these fibres ended freely or as a network array in the synovial intima. The distribution of Enk-immunoreactivity differed considerably from the other peptides and is described below.

ENK

Fine immunoreactive-fibres were found running randomly as a network in the subsynovial layer (Fig. 1). Some isolated immunoreactive fibres were found in different areas of the capsule and in the central area of the articular nerve branches. We could not distinguish any difference between L- and M-Enkephalin distribution pattern. In addition positive Enk-like immunoreactivity was seen in small isolated cell bodies in the sub-intimal layer. The L-Enk immunoreactive cells were oval (Fig. 2) while the

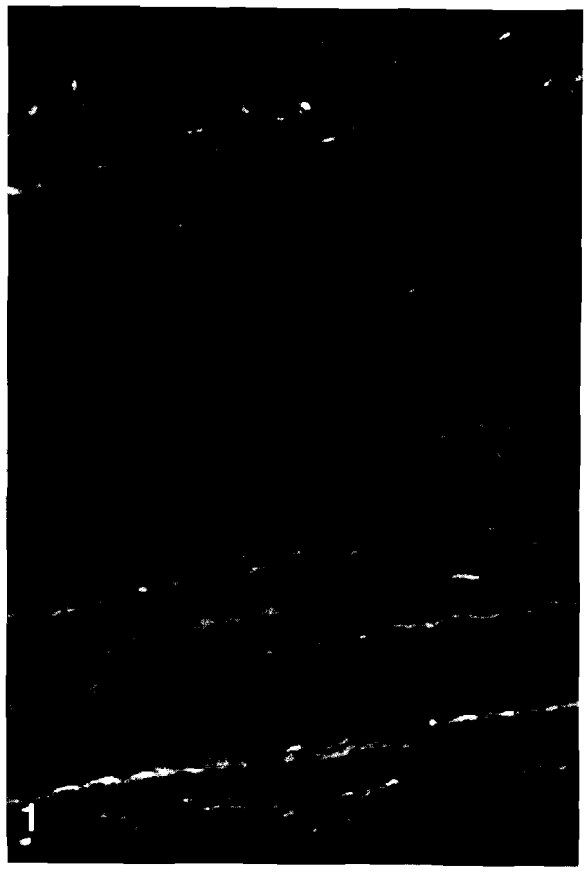

Fig. 1. Longitudinal section of synovia showing positive L-Enk fibres. The fibres in the deep layers are thicker and more crowded than in the superficial layer (upper side). × 160

Fig. 2. Immunofluorescent oval-shaped cell body and cytoplasmic extension reacting positively to L-Enk. × 400

Fig. 3. Polyhedral-shaped cell body reacting positively to M-Enk demonstrated by peroxidase antiperoxidase technique. × 630

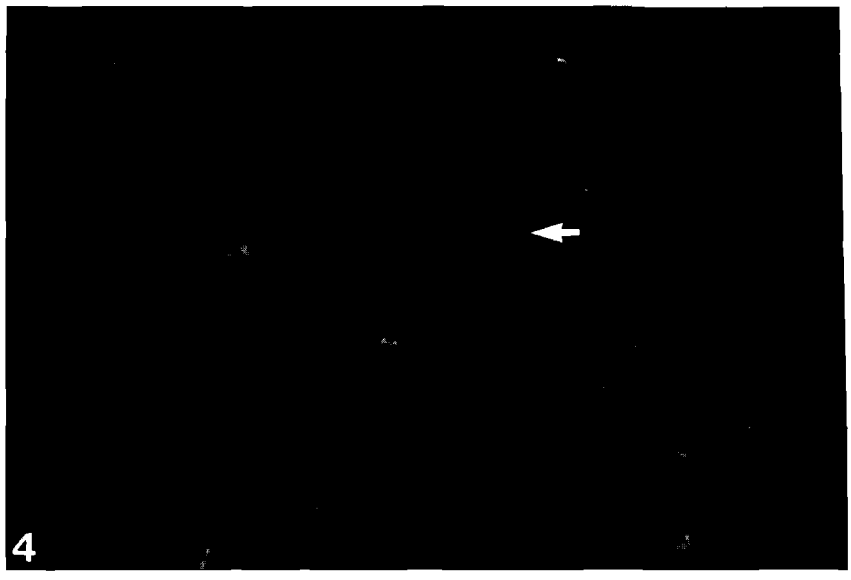

Fig. 4. Isolated 5-HT-like reacting fibres in the superficial synovia. More fibres were present in the deeper layer as well as surrounding small blood vessels (arrow). × 160

M-Enk immunoreactive cells showed a polyhedral shape (Fig. 3). These cells also showed fine connections with other fibres and cells (Fig. 3).

5-HT

Abundant serotonin immunoreactive-fibres were found randomly dispersed just beneath the synoviocyte layer making a network pattern around the small synovial blood vessels (Figs. 4, 5). A more intense reactivity and frequency was found in the endomysium of the adjacent muscles. The articular nerves showed a topographic clustering of the 5-HT fibres (Fig. 5).

VIP

VIPergic thick fibres were densely distributed in the subintimal area just beneath the synoviocytes (Fig. 6). The subsynovial connective tissue was relatively poorly innervated except in the perivascular layer. The endomysium of

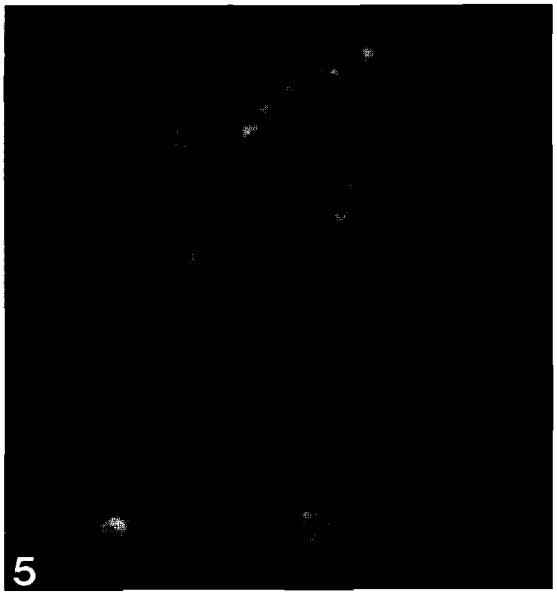

Fig. 5. One main nerve branch cross-sectioned in the deep synovia showing peripheral topographical localization of positive 5-HT- like fibres. Few fine fibres surrounding a large blood vessel (upper right side) × 160

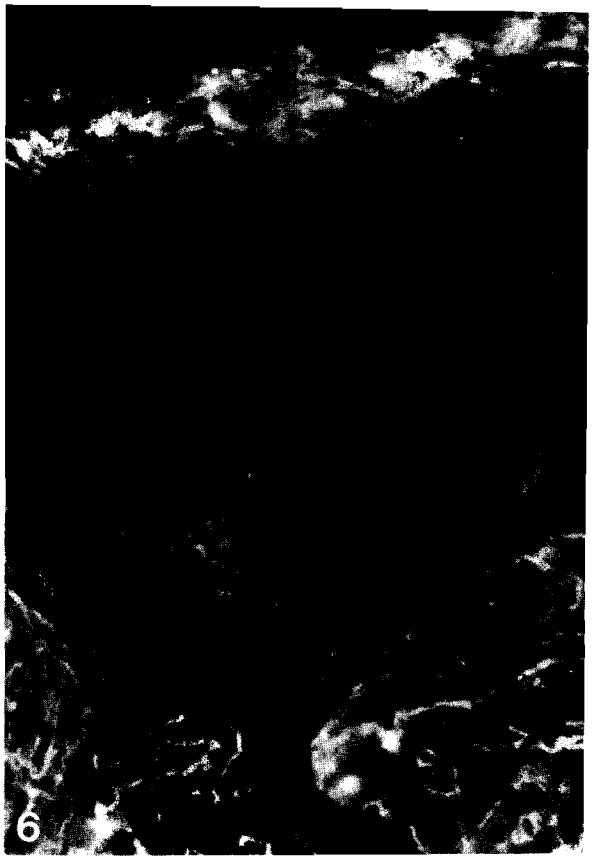

Fig. 6. Variable distribution of VIP-like fibres in the synovia. Dense distribution in the superficial area, being absent in the deeper layer of synovia, except for some perivascular positive reactivity. The endomysium of muscle fibres (lower side) also show positive immunoreactivity. × 160

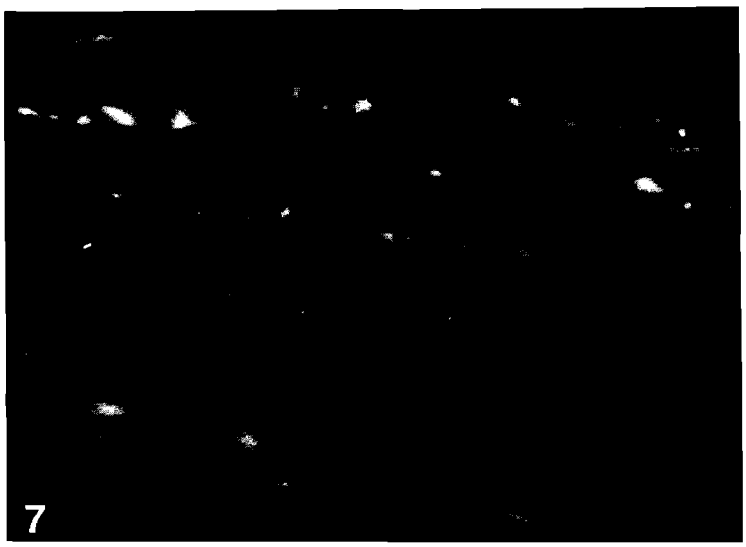

Fig. 7. Longitudinal section of SP fibres in the synovial membrane. × 160

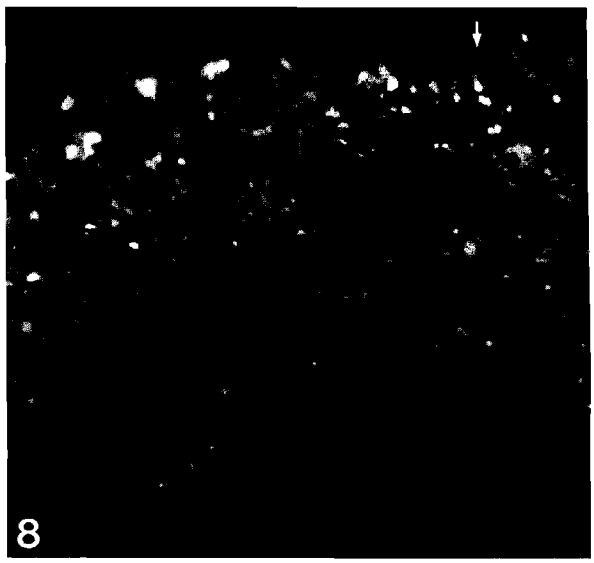

Fig. 8. Cross-section of SP-positive nerves ending in the synovial membrane. Some fibres are located in parallel between synoviocytes. × 160

subjacent muscular fibres and blood vessels showed intense VIP reactivity. The special distribution pattern of these fibres was similar to 5-HT fibres.

SP

SP-immunoreactive fibres of variable diameters were scattered as a network array in the subintimal layer of synovia as well as around the small blood vessels. In longitudinal sections the various fibres extended in parallel to other connective tissue structures (Fig. 7), while in a cross section (Fig. 8), they looked like fine dots of variable sizes. Some fine nerve terminals passed through the synoviocytes towards the synovial cavity (Fig. 8). However, the

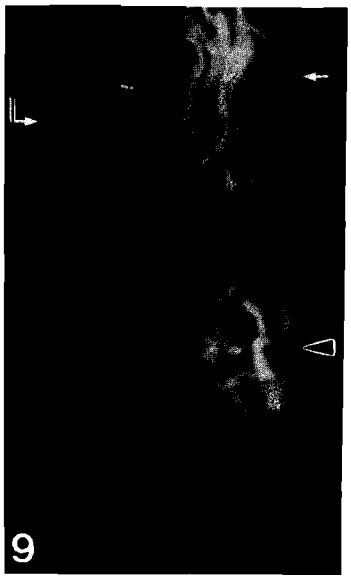

Fig. 9. NF triple protein immunoreactive fibres in the main nerve branch in the deep synovial cut obliquely (◄) and longitudinally (←). Note the negative reactivity of blood vessels (↳). × 160

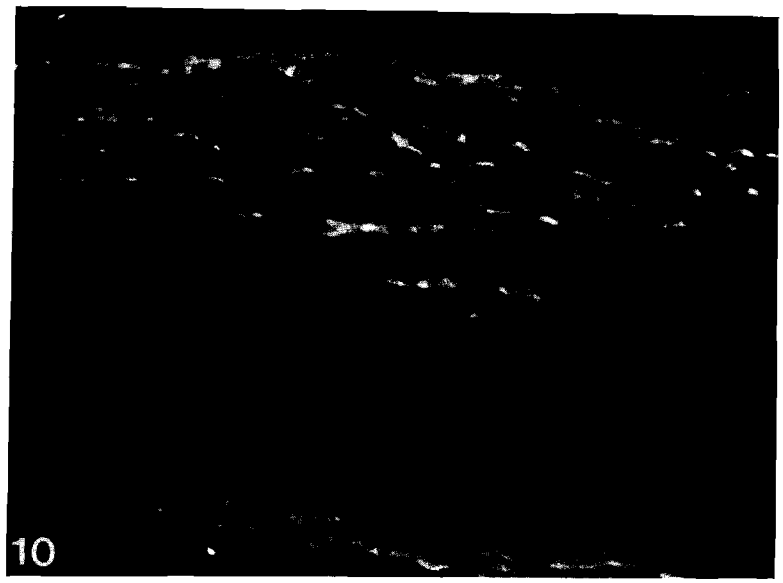

Fig. 10. NSE fibres in the superficial synovia. Note their high distribution, as compared to the specific neuropeptide immunoreacting nerves. × 160

Table 1. The origin and sources of primary antisera as referred to their optimal dilution.

Antisera to	Acronym	Origin	Dilution	Source
METHIONINE ENKEPHALIN	(M. Enk)	Synthetic	1:50	INC (USA)
LEUCINE ENKEPHALIN	(L. Enk)	Synthetic	1:50	INC (USA)
5-HYDROXY TRYPTAMINE (serotonin)	(5-HT)	Synthetic	1:750	INC (USA)
SOMATOSTATIN	(SOM)	Synthetic	1:100	INC (USA)
VASOACTIVE INTESTINAL PEPTIDE	(VIP)	Porcine	1:200	Hammersmith Hosp.
SUBSTANCE P	(SP)	Synthetic	1:200	Hammersmith Hosp.
S-100	(S-100)	Synthetic	1:300	Hammersmith Hosp.
TYROSINE HYDROXYLASE	(TH)	Synthetic	1:200	Hammersmith Hosp.
NEUROFILAMENT Triplet Protein	(NF)	Synthetic	1:400	Hammersmith Hosp.
NEURON SPECIFIC ENOLASE	(NSE)	Synthetic	1:200	Dako (Denmark)

subsynovial connective tissue contained relatively few fine SP-positive fibres. A weak immunoreactivity was also seen around the myotubes of adjacent muscles. In the present study no immunoreactivity to tyrosine hydroxylase and somatostatin could be evidenced.

The use of two types of anesthesia for the surgical procedure has revealed major variability in the identification of neuropeptides in the synovia (Table 2). The SP immunofluorescence was relatively decreased whereas L-Enk was better visualized under ketalar anesthesia. The neurotransmitters 5-HT, as well as S-100 were better visualized by urethane anesthesia. However, VIP, M-Enk and cytoskeletal NF were not affected by the type of anesthesia used. Axonal neurofilaments were found only in the main nerve branches (Fig. 9), but rarely observed in the fine nerve synovial extension. Similar topographical localization was noted for protein S-100. NSE showed relatively dense immunoreactivity corresponding to the thick nerve fibres in the synovial membrane (Fig. 10).

Discussion

The present immunohistochemical study of the cat synovial membrane innervation has revealed abundant

Table 2. The anesthetic effect on immunoreactivity of neuropeptides and cytoskeletal proteins in the cat synovia.

ANTISERA	NERVE DIAMETER (μM)	ANESTHETIC	
		KETALAR	URETHANE
SP	1-2	±	+
L-Enk	0.6-0.8	+	±
M-Enk	0.6-0.8	+	+
VIP	2-3	+	+
5-HT	1-2	–	+
S100	3-4	–	+
NF	3-4	+	+
NSE	3-4	+	+

+ positive immunoreactivity; ± weak positive immunoreactivity;
– negative immunoreactivity.

fine nerve terminals about 1 μm in diameter with positive immunoreactivity to various neurotransmitters. The majority of fibres run in the longitudinal plane parallel to the blood vessels, fibrous stroma and muscle fibres. Therefore, in transverse sections the nerve fibres appear as dotted or fragmented fluorescent signals except for Enk-immunoreactive-like fibres.

Positive NF reactivity was observed in large nerve branches (3-4 μm), but was absent in fine terminals (1 μm). This finding can be attributed to the axonal size which may be a limiting factor in identification of this cytoskeleton protein (Friede and Samorajski, 1970). Therefore, colocalization of NF and neuropeptides in very fine nerve fibres in the synovial membrane could be considered as incongruous. Similarly, the absence of other nerve markers such as NSE and S-100 in the fine nerve fibres can also be explained in the same way.

The presence of peptide immunoreactive nerves in the synovia suggests a complex reciprocal information exchange with the central nervous system. In fact the presence of some putative neuropeptides in the spinal cord have already been described (Buck et al., 1982; Mense, 1986).

VIPergic nerves were found as free terminals in synovial intima or surrounding the blood vessels. This peptide was suggested to participate in the hemodynamic regulation in the central nervous system and gut (Said, 1982), as well as salivary, pancreas and thyroid glands (Bourder et al., 1988).

VIP which have been localized in the dorsal root ganglia and spinal cord as well as in the unmyelinated nerve fibres are considered to be involved in the modulation of inflammatory processes (Levine et al., 1985). VIP is known to possess a vasodilatory and bronchodilatatory effect, and therefore, is capable of antagonizing the 5-HT effect (Landberg and Saria, 1987).

The present study has revealed the presence of 5-HT immunoreactive fibres randomly distributed as free terminals in synovial membrane or as a network around the blood vessels. A similar distribution pattern has been described in cerebral blood vessels (Inagaki and Kito, 1986) which has shown a decrease in their fibre density and size toward the periphery. The role of 5-HT as a chemical mediator for vasodilatation in the early phases of inflammatory response is now well established (Fantone and Ward, 1988).

The presence of Enk-immunoreactive fibres in the synovia may also be linked to the intrinsic modulation of pain signals. The presence of immunoreactive cell bodies to enkephalin is suggestive of the presence of an intrinsic ganglionic relay system. It has been reported that endogenously-released Enk may act in an inhibitory manner to depress the release of excitatory transmitters such as SP and VIP (De Groat, 1986).

SP fibres are found in various organs with a rich sensorial innervation (Polak and Bloom, 1980) and originate in the sensory ganglia; such as the substantia gelatinosa of the spinal cord (Gibson et al., 1981; Inagaki and Kito, 1986). The present findings showed that SP nerve fibres surround the synovial blood vessels or randomly run in the synovial intima, ending freely between the synoviocytes. A similar distribution of SP fibres throughout epithelial cells of rat urinary bladder have also been reported (Yokokawa et al., 1985).

Since the peptidergic nerve terminals do not form synapses, it is suggested that the bioactive compound is released from the nerve terminals into the joint cavity. In fact, increased SP levels have been reported in the arthritic joint fluids (Levine et al., 1984), whereas SP infusion into rat knee joints does aggravate the severity of adjuvant-induced arthritis (Levine et al., 1984, 1985).

It appears that SP nerve terminals are not only primarily conductors of nociceptive information to spinal cord as previously thought (Inagaki and Kito, 1986), but that they possess other roles such as

antidromic activation (Levine et al., 1984). Therefore SP may play a role in the inflammatory response acting either via its vasodilatatory effect (Skrabenek and Powell, 1980) or indirectly by stimulation of histamine release from the synovial mast cells (Iverson, 1985). Recently, it was shown that synovial mast cells in experimentally-induced arthritis in rats, contain SP and CGRP (Hukkanen et al., 1990). Intra-articular administration of exogenous SP induces a tissue inflammatory response such as vasodilatation, increased permeability with macrophage stimulation (Bar Shavit et al., 1980) and mast cell degranulation (Payan et al., 1984). All these might well explain the enhanced severity of experimental arthritis response, as observed by Levine et al. (1984).

It is known that inflammatory conditions such as rheumatoid-arthritis (RA) may cause the release of additional mediators such as prostaglandins which in turn will facilitate the release of other neurotransmitters from the nerve endings (Grönblad et al., 1988).

Recently, immunocytochemical studies of human synovial neuropeptides revealed a decrease in SP and Enk-positive nerve fibres in synovia of RA patients as compared to the normal and osteoarthritic patients (Grönblad et al., 1988). Contradictory findings were reported by Levine et al. (1984, 1985), according to whom increased density of SP positive fibres is a contributing factor to RA in rats. Such a discrepancy may be explained by the possible effect of anesthesia on the visualisation of certain neurotransmitters. Anesthetic compounds can variably affect the synthesis or release of neurotransmitters as well as the transport of biological precursors in the nerve system, (Barkai, 1978). Therefore, they may interfere with electrophysiological recording (Millar et al., 1989). It was reported that anesthesia may also alter the concentration of synaptic vesicles of nerve endings of the cat carotid body (Morgan et al., 1981) or decrease the muscular hypersensitivity in rat motor end plates (Blunt and Vrboba, 1975). Pentobarbital anesthesia was found to reduce the 5-HT turnover in rabbit brain, whereas halloperidol seems to increase it (Barkai, 1978). However, the type of anesthesia does not affect the VIP effect on vascular conductance (Bouder et al., 1988). The present study has revealed that some neuropeptide reactivity was decreased in cat synovia when ketalar was applied, whereas urethane anesthesia often preserved localization of neuropeptides, such as 5-HT and SP, in the fine nerve fibres. Recently it was reported that urethane-anesthesized cats offered remarkable visually-evoked potentials (Millar et al., 1989). Therefore, it is suggested that urethane is the anesthetic of choice in experimental studies of neuropeptides. Moreover, the choice of fixation technique may also have an impact on the visualization and characterization of neuropeptide antigens in tissues. In our study the identification of Enk and VIP neuropeptides was possibly due to the use of Benzoquinone solution (BQS) fixation which is known to better preserve some of the neuropeptide antigenicity (Pearse and Polak, 1975). Formaldehyde fixation is considered to destroy the immunoreactivity site of some neuropeptides; especially of VIP at the C-terminal mid-region area (Said, 1982).

In conclusion, it is suggested that characterization of neuropeptides in peripheral tissues, especially synovia, has to take into account the type of anesthesia, the topographic localization in relation to the main nerve branches of synovia, the type of fixative solution and the plane of section of histological specimens.

Further characterization of neuropeptides has to be performed in normal as well as experimental animal models in the future for a better understanding of the possible neurotransmitter impairment, function and distribution in human joint disorders.

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