From the Sections of Orthopedics and Sportsmedicine
Department of Surgical Sciences
Karolinska Institutet
Stockholm, Sweden

PEPTIDERGIC INNERVATION OF PERIARTICULAR TISSUE

- A study in the rat

Paul Ackermann

Stockholm 2001

Cover: Schematic drawing demonstrating to the right, normal nerve supply of the Achilles tendon and to the left, nerve ingrowth during healing after tendon rupture. Illustration by Eva Hall.

Published and printed by Karolinska University Press
Box 200, SE-171 77 Stockholm, Sweden

ISBN 91-7349-094-6

ABSTRACT

Painful musculo-skeletal disorders pose a tremendous burden on the healthcare system, the main reason being the limited knowledge of the underlying pathomechanisms and lack of specific treatment. Hypothetically, the peripheral nervous system in the musculo-skeletal tissues plays an important role in the regulation of pain, inflammation, vasoactivity and tissue repair. The present study in the rat was designed to explore the expression of neuropeptides in periarticular tissues under normal conditions and after injury.

A combined approach based on radioimmunoassay (RIA), immunohistochemistry (IHC) and high performance liquid chromatography (HPLC), disclosed the presence of autonomic (NPY, VIP, NA); sensory (SP, NKA, CGRP, GAL, SOM); and opioid (LE, ME, MEAP, MEAGL, N/OFQC) mediators in tendon, ligament and joint capsule. In tendon, δ-opioid receptors were also detected. The peptidergic nerve fibres occurred in abundance in the paratenon, epiligament and beneath the lining layer of the synovium, whereas the proper structures were almost devoid of nerve fibres. This suggests that the neuronal regulation of periarticular tissues highly depends on the innervation of the surrounding structures. RIA showed that the ratio of NPY (sympathetic)/ VIP (parasympathetic) was 8-times higher in ligaments than in tendons and in capsules. The ratio of SP and CGRP (nociceptive)/ GAL, MEAP, N/OFQ (anti-nociceptive) was 10-times higher in tendons and twice as high in capsules compared to ligaments. The distribution and levels in the different tissues may represent the homeostatic state of vasoactivity and nociception, and also indicate the susceptibility to physico-chemical stress.

In healing of experimental Achilles tendon rupture, nerve regeneration and neuropeptide expression at different time points (1-16 weeks) were analysed by IHC including semi-quantification. Analysis of markers for new (GAP) and mature fibres (PGP) disclosed extensive nerve fibre ingrowth into the rupture site. A peak nerve fibre expression during weeks 2-6 (regenerative phase) was followed by nerve fibre withdrawal during weeks 8-16 (remodeling phase). New nerve ingrowth is presumably a prerequisite for delivery of neuronal mediators required for tissue repair. Analysis of specific neuropeptides showed an increased number of SP and CGRP fibres one week post-rupture (inflammatory phase) around blood vessels surrounded by inflammatory cells. This complies with a nociceptive and pro-inflammatory role. During the regenerative phase, the expression of SP and CGRP peaked in the rupture site of proper tendon, seen as free sprouting nerve endings among fibroblasts and newly formed vessels, which may prove to reflect a role in cell proliferation. An initial sparse occurrence of GAL, NPY and VIP was followed by a peak expression at the early remodeling phase, i.e., around week six, which may represent modulatory effects on SP and CGRP required to end the nociceptive, inflammatory and regenerative processes.

Nociception according to hind paw withdrawal at thermal and mechanical stimuli was related to the expression of sensory neuropeptides during healing as assessed by semi-quantitative IHC. Increased expression of SP/CGRP during weeks 1-2 coincided with increased thermal sensitivity on the injured side. Conversely, increased GAL expression during weeks 4-6 coincided with decreased thermal sensitivity, and notably, also with increased mechanical sensitivity. A strong relationship between neuropeptide expression and thermal sensitivity was found by considering the combined rates of change of the three neuropeptides (SP + CGRP – GAL) during healing. The finding indicates that nociception is regulated by the speed by which the expression of potentiating and inhibitory neuropeptides is altered, rather than the absolute tissue levels.

From the present study, it seems obvious that periarticular tissues are supplied with a complex peptidergic network, which presumably takes part in maintaining tissue homeostasis, but also by its plasticity, i.e. nerve ingrowth and temporal alteration in neuropeptide expression, is capable of responding to injury. Interestingly, ingrowth of new nerves seems to be a fundamental feature of tissue repair. In a future perspective, neuronal mediators may prove to be useful in targeted pharmacotherapy and tissue engineering of painful and degenerative musculo-skeletal disorders.

LIST OF PUBLICATIONS

1. Ackermann W.P., Finn A., Ahmed M. **Sensory neuropeptidergic pattern in tendon, ligament and joint capsule. A study in the rat.** *Neuroreport* 1999 10/10: 2055-2060.

2. Ackermann W.P., Li J., Finn A., Ahmed M., Kreicbergs A. **Autonomic innervation of tendons, ligaments and joint capsules. A morphologic and quantitative study in the rat.** *Journal of Orthopaedic Research,* 2001 19/3: 372-378

3. Ackermann W.P., Spetea M., Nylander I., Ploj K., Ahmed M., Kreicbergs A. **An Opioid System in Connective Tissue: A Study of Achilles tendon in the rat.** *The Journal of Histochemistry and Cytochemistry,* 2001 49/11: 1387-1396

4. Ackermann W.P., Ahmed M., Kreicbergs A. **Early nerve regeneration after Achilles tendon rupture – a prerequisite for healing?** *Journal of Orthopaedic Research, In Press*

5. Ackermann W.P., Ahmed M., Lundeberg T., Kreicbergs A. **Sensory neuropeptide expression and nociception during Achilles tendon healing.** *Submitted*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

CGRP	calcitonin gene-related peptide
DOR	δ-opioid receptor
DYN	dynorphin B
E.g.	for example
FITC	fluorescein isothiocyanate
GAL	galanin
GAP	growth associated protein 43
HPLC	high performance liquid chromatography
IHC	immunohistochemistry
KOR	κ-opioid receptor
LE	leu-enkephalin
ME	met-enkephalin
MEAGL	met-enkephalin-arg-gly-lys
MEAP	met-enkephalin-arg-phe
MOR	μ-opioid receptor
NA	noradrenaline
NKA	neurokinin A
N/OFQ	nociceptin/orphanin FQ
NPY	neuropeptide Y
PBS	phosphate buffered saline
PGE_1	prostaglandin E1
PGP	protein gene product 9.5
RBA	radioligand binding assay
RIA	radioimmunoassay
SOM	somatostatin
SP	substance P
TH	tyrosine hydroxylase
VIP	vasoactive intestinal polypeptide

1 INTRODUCTION

1.1 BACKGROUND

Musculo-skeletal disorders pose a tremendous burden on the health care system. According to the National Research Council they affect one million workers in the US each year, costing $54 billion in compensation expenditures, lost wages and decreased productivity [12, 160]. Prominent features of musculo-skeletal disorders are pain, inflammation and degeneration. During the past several years, the incidence of overuse injuries, mostly related to work and sports, has increased considerably [69, 99]. Overuse injuries can be related to extrinsic and intrinsic factors [124]. Among extrinsic factors, repetitive movement is considered one main cause. Repetitive microtrauma of the tissues are thought to exceed the repair capability of the cells [9, 84, 125]. Intrinsic factors, some genetically determined, include e.g. obesity, extremity malalignment, joint laxity, muscle weakness and disturbed blood supply. Although some mechanical risk factors have been identified, the relationship to pain and inflammation is debatable [113] and still requires a biological explanation.

In inflammatory conditions of the locomotor apparatus pain is almost a constant feature, however, in degenerative disorders it is highly variable. Whether inflammation is a prerequisite for pain in degenerative disease is unclear. Notably, spontaneous ruptures of tendons, presumed to be caused by degeneration, are often not preceded by pain. In a study by Kannus and Jozsa, degenerative changes were found at time of surgery in 97 % of all tendon ruptures [74], whereas signs of inflammation were rare. On the whole, the relationship between pain, inflammation and degeneration of the locomotor system remains obscure, which should be attributed to the limited knowledge of neuroanatomy and pathophysiology. In particular, this applies to periarticular tissues such as ligaments, joint capsules and tendons, which exhibit a considerable variation in incidence and intensity of pain, inflammation and degeneration.

1.2 PERIARTICULAR TISSUES

1.2.1 Tendons

In chronic, painful conditions of tendons, histological studies have shown that the tissues exhibit very few signs of inflammation [11, 110]. Therefore, the classic term tendinitis has been replaced by the term tendinopathy. Localised degenerative lesions (Fig. 1) are termed tendinosis [121] to denote collagen disorganisation, increased ground substance, hypercellularity, and neovascularisation [72].

Although degeneration seems to be a prominent feature of chronic tendon conditions, this does not preclude an initial inflammatory phase [32, 73, 121]. Moreover, it appears that histological analyses of painful tendon tissues primarily have focused on the proper tendon, but overlooked the envelope, i.e. the paratenon and surrounding loose connective tissue [80]. It may prove that degeneration of tendons is related to inflammatory changes in the surrounding structures. Notably, a recent study on rats showed that application of PGE_1 to the paratenon produced degenerative like changes in the proper tendon [140].

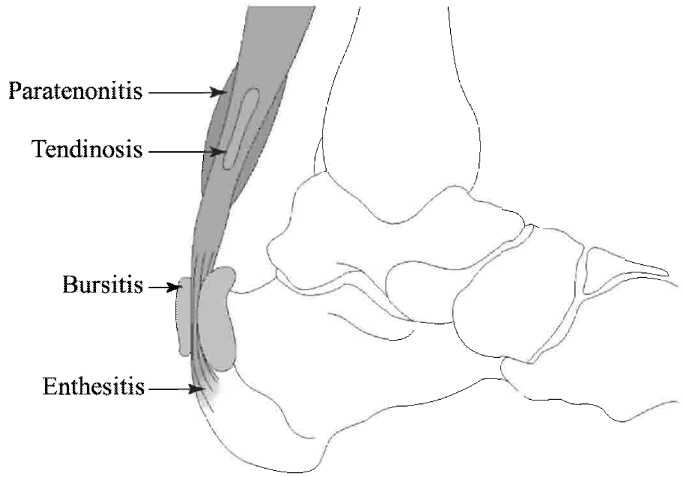


Figure 1. Lesions of the Achilles tendon.

Most tendinous structures are built up by proper tendon, paratenon and an outer layer of loose connective tissue. The proper tendon is composed of three main constituents: collagen, ground substance and cells. In degenerative conditions the ground substance, consisting of structural glycoproteins with high water binding capacity, increases leading to oedema. Conversely, the amount of collagen decreases and the fibres being the most important component for mechanical strength lose their parallel course. The latter has been attributed to loss or weakening of cross-links resulting in microtears and decreased

strength. The splitting and tear of collagen fibres have previously been claimed to be the cause of pain [77]. However, this is contradicted [77] by studies of tendon degeneration as assessed by MRI and ultrasound in painfree subjects and by reports on patients with atraumatic ruptures without preceding pain [34, 35]. In painful tendinosis, a recent report suggested that eccentric muscle exercise paradoxically can mitigate the symptoms [97].

As for cellular changes observed in tendon rupture caused by degeneration, the most plausible hypothesis pertains to impaired blood flow, hypoxia and decreased metabolic activity [72]. The predominant cellular elements of the tendon are fibroblasts (90%), while the remaining 10 % consists of chondrocytes at the bony insertion, synovial cells of the tendon surface and vascular cells (i.e. endothelial- and smooth muscle cells of arterioles). The tendon fibroblasts are responsible for the ground substance production, which is increased in response to injury and exercise. In degenerative tendons focal hypercellularity and fibroblast (tenocyte) hyperplasia are common features. Concomitantly, there is proliferation of myofibroblasts from the surrounding paratenon or loose connective tissue. The myofibroblasts produce type III collagen ("scar collagen"), which has decreased mechanical strength compared to type I collagen.

In the paratenon, there are synovial cells of three main types, called type A, B and C cells. Type A cells exhibit similarities to macrophages, e.g. a prominent Golgi apparatus, abundant digestive vacuoles, production of synovial fluid, secretion of hyaluronic acid and expression of surface antigen receptors. Type B cells have a fibroblast-like morphology and are mainly involved in synovial fluid production. Type C cells are dendritic cells that are thought to participate in autoimmune responses such as T-cell infiltration and activation. Synovial cells are suggested to play an important role in inflammatory conditions, but their involvement in degenerative processes is unclear [49]. The blood supply to tendons is received from three sites: the muscle, the bony insertion and most importantly from the paratenon. The vessels give off branches as vertical loops into the proper tendon. Degeneration is characterised by proliferation of endothelial cells as reflected by a high number of new capillaries, which may assumed to reflect a response to hypoxia. The regulation of this neovascularisation has yet to be clarified.

1.2.2 Ligaments

Clinically, pain from ligaments is less common than from tendons, which may be explained by lower mechanical load. In opposition to tendons, which exhibit striking

structural similarities to ligaments, the main clinical problem in ligamentous disorders is not related to pain and repetitive stress. Instead, it pertains to poor mechanical joint stability after traumatic injuries [161] and the risk of developing osteoarthritis [67]. The results of treatment to restore original ligament function are highly variable. It has been suggested that, apart from ligament tension after repair, restoration of blood supply is critical for healing [21]. Similar to tendons, the main blood supply to the extra-articular ligaments, such as medial collateral ligaments, originates from the surrounding tissues. However, little is known about the regulation of blood flow and mechanisms of repair.

1.2.3 Joint capsules

Pain in joint disorders is commonly attributed to inflammation causing overproduction of synovial fluid and distension of periarticular tissues. The initiating mechanisms of the overproduction of synovial fluid are essentially unclear. A wide variety of pro-inflammatory factors such as cytokines, prostaglandins and growth factors have been implicated in the pathophysiology of synovitis and pain [141].

The joint capsule is composed of an inner synovial membrane and outer fibrous layer. The synovium is generally divided into two distinct layers, the intimal lining and the sublining. The intimal lining is in direct contact with the intra-articular cavity and is responsible for the production of synovial fluid of which the main component is hyaluronic acid. The intimal lining is an avascular layer usually one or two cells deep, built up by synovial cells of type A and B – similar to the synovial layer of the paratenon. Although the intimal lining is avascular, blood flow has been suggested to be the main determinant of synovial fluid production [106, 132]. However, the exact regulation of blood flow and its relationship to synovitis and pain is still unclear.

1.3 INNERVATION

The nerves of periarticular tissues are composed of myelinated, fast transmitting Aα- and Aβ-fibres and unmyelinated, slow transmitting Aγ-, Aδ-, B- and C-fibres. The nerve endings of Aα- and Aβ-fibres are of types I-III and mediate mechanoception. The nerve endings of Aγ-, Aδ-, and C-fibres are of type IVa, so called nociceptors, mediating deep tissue pain and hyperalgesia that are characteristic features of musculo-skeletal pain. The nerve endings of B-fibres, which are autonomic, consist of type IVb fibres that are mainly localised in the walls of small arteries, arterioles, capillaries, and postcapillary veins exerting vasomotor actions.

The innervation of tendons originates from neighbouring muscular, cutaneous and peritendinous nerve trunks. From the myotendinous junction the nerve fibres cross and enter the endotenon septa. In the paratenon, nerve fibres form rich plexa and send branches that penetrate the epitenon. Most nerve fibres do not enter the tendon, but terminate as nerve endings on the surface of the tendon. As for the innervation of ligaments it seems similar to that of tendons. In the capsule nerve endings can be found in the intimal lining of the synovium, but the predominant innervation is found in the subsynovial lining, mainly as dense networks around vessels.

It is now well established that the peripheral nervous system in addition to classical functions such as nociception and vasoactivity, also participates in the regulation of a wide variety of efferent actions on cell proliferation, cytokine expression, inflammation, immune responses and hormone release. The paradoxical "efferent" role of afferent nociceptive fibres as suggested already in 1901 by Bayliss [13] is now widely recognized. So far, however, the innervation and neuronal regulation of periarticular tissues have received little attention. In particular, this applies to the specific mediators.

1.4 NEUROPEPTIDES

Neuropeptides act as chemical messengers in the central as well as peripheral nervous system. They differ from classical neurotransmitters (monoamines, acetylcholine, amino acids) in several respects. Peripheral neuropeptides are synthesized in the cell bodies and transported distally. In contrast classical neurotransmitters are synthesized in the axon terminals. The synthesis and turnover of classical transmitters are more rapid than those of neuropeptides. While classical transmitters occur in small as well as large synaptic vesicles, neuropeptides have been demonstrated in large dense-core vesicles [63]. It has been shown that neuropeptides from these vesicles are released differently from classical transmitters depending on the frequency of the action potentials. A low impulse frequency selectively activates small vesicles releasing classical transmitters, whereas higher frequencies release neuropeptides from large vesicles [65]. The large vesicles permit more than one functional neuropeptide to be processed and released together with classical transmitters [63], which offers a variety of functional interactions. Recent studies of painful tendinosis by Alfredson et al [5-8], based on microdialysis, notably, reported increased concentration of the classical transmitter glutamate, which is known to exist peripherally and to interact with the sensory neuropeptide substance P (SP) in nociception [28]. It has been shown that SP potentiates the effect of glutamate [28, 91]. The effects of

neuropeptides and classical transmitters are elicited by different receptor mechanisms. While classical transmitters act on ligand-gated ion channels, neuropeptides act by binding to specific plasma membrane receptors, called G-protein coupled receptors.

The first neuropeptide, SP, was isolated from horse brain tissue in 1931 by von Euler and Gaddum [150]. In 1978, Tatemoto and Mutt presented a method to characterize peptides, which has become widely used in the search of other neuropeptides [144]. A vast number of neuropeptides have been identified in the central and peripheral nervous system. So far, however, research on the occurrence and functions of neuropeptides in the locomotor system has been very limited.

Sensory primary afferents contain SP [64] claimed to transmit nociceptive signals [64, 86, 134]. Calcitonin gene-related peptide (CGRP) coexists with and potentiates the effect of SP [116, 156, 162]. Both SP and CGRP have also been shown to exert pro-inflammatory effects such as vasodilation and protein extravasation [20, 98, 111]. However, sensory nerve fibres also contain peptides with anti-nociceptive and anti-inflammatory effects counteracting the effects of SP and CGRP. Thus, galanin (GAL), somatostatin (SOM) as well as enkephalins, all of which occurring in primary afferents, have been shown to inhibit inflammation and nociception, the latter presumably also at the spinal level [27, 36, 56, 142, 164]. A number of studies suggest that GAL exerts its modulatory effect through inhibition of SP release [52, 164].

Similar to sensory fibres, autonomic fibres in the periphery also contain several mediators. Neuropeptide Y (NPY) found in sympathetic fibres has been shown to be a potent vasoconstrictor. It often co-exists with noradrenaline (NA) potentiating the vasoconstrictive action of NPY [95]. Moreover, it has recently been reported that NPY at low levels has an angiogenetic effect [51]. In parasympathetic nerve fibres, vasoactive intestinal polypeptide (VIP) has been shown to be a potent vasodilator [94]. In addition to the well established vasoactive role of VIP, other important effects have been reported over the last years. Thus, VIP has been implicated in regulating immune cells and the expression of cytokines and growth factors. Recently, a study on experimental arthritis demonstrated an anti-inflammatory effect of VIP [38]. Although, several neuropeptides have been reported to occur in more than one nerve fibre type they will in this thesis be referred to according to their original findings, e.g. sensory (SP, CGRP) and autonomic (NPY, VIP).

1.4.1 Nociception

Sensory nerve fibres respond to noxious thermal, mechanical and chemical stimuli in the periphery by SP release from C- and Aδ-fibres both centrally and peripherally. Several studies have demonstrated that SP acts as a transmitter of pain in the spinal cord [119, 133, 134]. In the periphery, release of SP leads to senzitisation of surrounding primary afferents by enhancing cellular release of prostaglandins, histamines and cytokines [131, 147]. However, recently it was shown that SP also directly stimulates nociceptor endings [81, 145]. CGRP, often colocalized with SP in unmyelinated C fibres, facilitates the release of SP and delays SP degradation in the spinal cord, thereby potentiating the nociceptive effect [83, 130, 162]. It is still unclear whether this potentiating mechanism also occurs in the periphery.

Also the autonomic nervous system has been implicated in nociception, especially after peripheral nerve injury [112]. In humans with such lesions, local injection of sympathetic transmitters increases pain [152], which can be abolished by antagonists.

There also seems to exist a peripheral endogenous anti-nociceptive system. Thus, it was recently reported that patients who received intra-articular naloxone in conjunction with arthroscopic knee surgery exhibited significantly more postoperative pain and higher supplementary analgesic consumption than patients with intravenous naloxone injections [137]. Notably, morphine has been reported to inhibit SP release from peripheral sensory nerve endings [22, 165]. The observations suggest that opioid receptors are not confined to CNS, but also occur in the periphery and that pain can be inhibited at a peripheral level [57]. Indeed, opioid receptors have been demonstrated on peripheral sensory nerve terminals of the skin [33, 136, 154].

There are two principal sources of opioid peptides in the periphery. One is the immune cells, which have been shown to contain and release opioids implicated in mitigating inflammatory pain [25, 137]. The other source is most likely the peripheral nervous system. Interestingly, a recent study showed that approximately 17% of peripheral cutaneous axons contain enkephalins presumed to be of importance in regulating pain and inflammation [27]. As for the locomotor system, the neuronal occurrence of endogenous opioids and receptors, has not been explored.

1.4.2 Tissue injury and repair

It appears that a main function of neuropeptides is to elicit and convey responses to stress and injury [62]. Neuropeptides have been shown to respond very specifically both in the central- and peripheral nervous system to peripheral inflammation and nerve injury. A number of experimental studies on arthritis have demonstrated a local increase in SP and CGRP [1, 2, 104]. After peripheral nerve lesion SP and CGRP are decreased, whereas GAL and NPY are increased in the dorsal root ganglia and spinal cord [66]. The decrease in SP returns to normal level in the spinal cord after 16 weeks [126]. The observations comply with a number of studies showing that the expression of different neuropeptides in response to external stress follows a specific temporal pattern [66]. This would seem to reflect that neuropeptides play a role in nerve repair, possibly also in repair of other tissues.

So far there is very limited data on the peripheral expression of neuropeptides after injury of musculo-skeletal tissues. Increased expression of SP and CGRP two weeks post injury has been observed in fracture- and skin healing [61, 78, 89]. As for ligament healing Grönblad et al. studied SP and CGRP expression 4 and 14 weeks after rupture and found an increase at week four and a decrease at week 14 [53]. Altogether, the temporal expression of neuropeptides indicates that they are not only involved in the acute response to injury, but also in tissue regeneration.

A neuronal role in tissue healing has been reported by a few investigators over the last years [19, 54, 117, 129]. Administration of SP or CGRP to the rat was shown to accelerate wound healing and the combination of both neuropeptides even more. In fact, healing of wounds treated with SP and CGRP was completed 10 days earlier compared to controls [76]. Conversely, capsaicin induced sensory neuropeptide depletion leads to delayed healing of corneal and skin ulcers [48, 79]. Clinically, it is well known that diabetic neuropathy is associated with increased susceptibility to skin wounds and impaired tissue healing. Complete denervation of tendons or skin leads to decreased mechanical strength and collagen content [10, 139]. Interestingly, both SP and CGRP have been shown to participate in the regulation of fibroblast and synoviocyte proliferation and angiogenesis [20, 54, 114, 168], also in the synthesis/release of cytokines and growth factors [23, 108].

2 AIMS

The present study in the rat was designed to explore the occurrence and distribution of neuropeptides in periarticular tissues under normal conditions and after injury. The overall goal was to seek a neuro-anatomical correlate to pain and the process of tissue repair.

The specific aims were:

- To explore the normal occurrence and distribution of autonomic, sensory and opioid peptides in tendon, ligament and joint capsule (papers I-III).

- To investigate the occurrence, distribution and characteristics of opioid receptors in normal tendon (paper III).

- To study nerve regeneration and ingrowth during healing of tendon rupture (paper IV).

- To analyse the temporal expression of sensory and autonomic neuropeptides during healing of tendon rupture (paper V).

- To assess nociception in relation to sensory neuropeptide expression during healing of tendon rupture (paper V).

3 MATERIALS AND METHODS

3.1 ANIMALS

Young adult male Sprague-Dawley rats (2 months old, 180-200 g) were used in all experiments. The animals were provided by a local breeder (B&K, Stockholm, Sweden) and thereafter acclimatized one week at our animal department before starting the studies to reduce stress reactions. The animal series included 148 rats. The study of normal conditions (paper I-III) included 96 rats (26 for RIA, 10 for IHC, 60 for RBA). The study on tendon rupture (paper IV, V) included 52 rats, 47 with tendon rupture and 5 with intact tendon.

The animals were housed five/cage at 21°C in a 12-hour light/dark cycle with water and pellets *ad lib* according to the Karolinska Institutet protocol. All experimental procedures were performed under deep chloral hydrate anesthesia (300 mg/kg, i.p.). At the end of the experiments the animals were euthanized either by an overdose (60 mg/kg bw) of sodium pentobarbital (Apoteksbolaget, Sweden) or decapitation under chloral hydrate anesthesia. The experiments were performed with approval from Stockholm North Animal Ethical committee.

3.1.1 Tendon Rupture

In 47 rats the right Achilles tendon was ruptured unilaterally at the mid-third of the tendon (papers IV-V). A 3-cm midline skin incision was made over the Achilles tendon, and the Achilles and plantaris tendons were dissected from the surrounding fascia. The Achilles tendon was ruptured, using a blunt instrument to tear the tendon fibres apart (Fig. 2), while the plantaris tendon was left intact. The tendon was ruptured in the midpart, about 0.75 cm from the calcaneal insertion and the myotendinous junction, respectively. The skin was closed with 5/0 non-resorbable, Polyamid 6 suture (Ethilon®II, Ethicon, Sommerville, NJ, U.S.A.). The animals were allowed to move freely postoperatively.

3.1.2 Perfusion

For immunohistochemistry, the anesthetized animals were perfused through the ascending aorta with phosphate buffered saline (PBS) followed by perfusion with Zamboni´s fixative consisting of 4% paraformaldehyde in 0.2 mol/l Sörensen phosphate buffer, pH 7.3, containing 0.2% picric acid.

3.1.3 Dissection and fixation

IHC: From the perfused animals the whole Achilles tendon (paper I-V), medial and lateral collateral ligaments (paper I-II) and knee capsule (paper I-II) were dissected and immersed in Zamboni´s fixative for two hours at room temperature. All specimens were rinsed in PBS and then soaked for at least two days in 20% sucrose in 0.1 mol/l Sörensen phosphate buffer, pH 7.2, containing sodium azide and bacitracin (Sigma Chemicals, St. Louis). The tissues were then stored at +4°C before immunohistochemical analysis (see section 3.2).

RIA and RBA: Directly after each rat was euthanized the Achilles tendon (paper I-III), medial and lateral collateral ligaments (paper I-II) and knee capsule (paper I-II) were dissected bilaterally on a cooled plate, 0°C. The sample from the left and right side were pooled into one. All dissected tissues were weighed and immediately frozen on dry ice and kept at -70°C until analysis with radioimmunoassay and radioligand binding assay (see section 3.3 and 3.5).

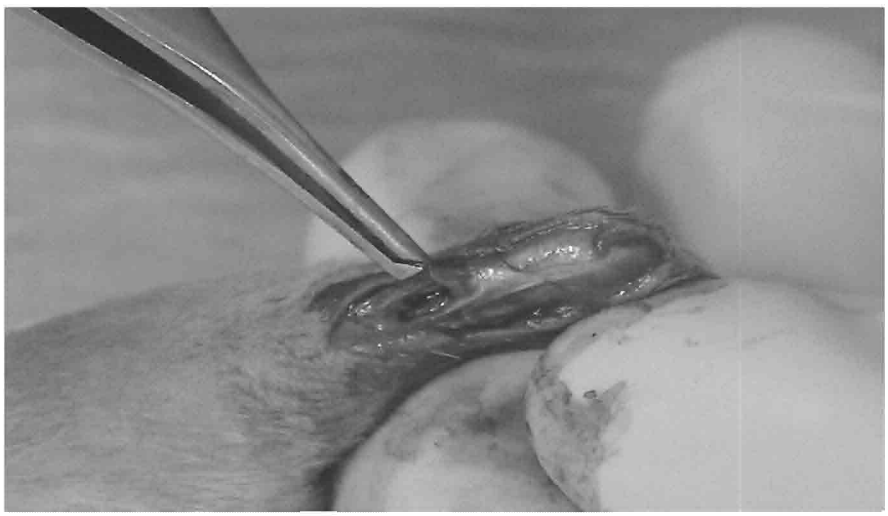


Figure 2. Tendon rupture

3.2 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) was used for *in situ* detection of the tissue antigens of interest by using labeled antibodies. The main conditions for IHC include: 1) fixation of the tissue to preserve the antigen, 2) use of well-characterized antibodies and, 3) staining and detection with low non-specific labeling.

Fixed tissues (section 3.2.1-3.2.2) were cut on a Leitz cryostat to a section thickness of 15 µm. The frozen sections were mounted directly on SuperFrost/Plus glass slides and immunostained according to the avidin-biotin system. The sections were rinsed for 2x5 min in PBS. Incubation of the sections with 10% normal goat/ horse serum in PBS for 40 minutes blocked the non-specific binding.

Subsequently, sections were incubated with primary antisera (see table 1) over night in a humid atmosphere at room temperature. After incubation with the primary antisera, the sections were rinsed in PBS (2x5 min) and then incubated for 40 min at room temperature with biotinylated polyclonal goat anti-rabbit antibodies or monoclonal horse anti-mouse antibodies (both 1:250, Vector Laboratories, Burlingame CA, USA). The monoclonal antibodies were exclusively used to detect growth-associated protein 43 (GAP). Finally, the sections were incubated for 40 minutes with a streptavidin labeled fluorochrome (see table 2).

To demonstrate specificity of the staining, the following controls were included: (a) pre-adsorption of the primary antisera with excess of homologous antigen for 12 hours at room temperature; and (b) omission of either the primary antiserum, the secondary antibody or the secondary biotinylated antibody.

A Nikon epifluorescence microscope (Eclipse E800 Yokohama, Japan) was used to examine the sections. T-Max black and white and EPL 400 color films (Kodak, Rochester) were used for photography.

3.2.1 Primary antibodies

Table 1. Characteristics of primary antibodies

Antigen	Name	Raised in	Dilution
CGRP	calcitonin gene related peptide	rabbit	1:10,000 [1]
DOR	δ-opioid receptor	”	1:10,000 [1]
DYN B	dynorphin B	”	1:10,000 [2]
GAL	galanin	”	1:5,000 [1]
GAP	growth associated protein 43	mouse	1:2,000 [3]
KOR	κ-opioid receptor	rabbit	1:10,000 [1]
LE	leu-enkephalin	”	1:20,000 [1]
ME	met-enkephalin	”	1:20,000 [1]
MEAGLE	met-enkephalin-arg-gly-lys	”	1:20,000 [1]
MEAP	met-enkephalin-arg-phe	”	1:20,000 [1]
MOR	μ-opioid receptor	”	1:10,000 [1]
N/OFQ	nociceptin/orphanin FQ	”	1:10,000 [2]
NKA	neurokinin A	”	1:5,000 [1]
NPY	neuropeptide Y	”	1:5,000 [1]
PGP 9.5	protein gene product 9.5	”	1:10,000 [4]
SOM	somatostatin	”	1:5,000 [1]
SP	substance P	”	1:10,000 [1]
TH [5]	tyrosine hydroxylase	”	1:5,000 [6]
VIP	vasoactive intestinal polypeptide	”	1:5,000 [1]

3.2.2 Fluorochromes

Table 2. Characteristics of fluorochromes

Flourochrome	Nature	Raised in	Dilution
FITC	Green color	Rabbit	1:500 [7]
Cy2™	Green color	“	1:2000 [8]
Cy3™	Red color	“	1:5000 [8]

[1] **Peninsula Laboratories, Europe, Ltd, St. Helens, UK**
[2] **A kind gift from I. Nylander, Department of Pharmaceutical Biosciences, Uppsala, Sweden**
[3] **Boehringer Mannheim Biochemicals**
[4] **Ultraclone, Cambridge, Ltd, UK**
[5] **A rate limiting enzyme in the synthesis of noradrenaline**
[6] **Chemicon International INC. Temecula, CA, USA**
[7] **Vector Laboatories Inc., Burlingame, CA, USA**
[8] **Amersham Pharmacia Biotech, UK limited**

3.2.3 Double labeling

For double staining, after completing the staining steps for the first neurotransmitter, the sections were incubated with avidin blocking solution followed by biotin blocking solution (15 minutes each). The staining for the second antibody was repeated as described for the first antibody, but with a different flourochrome. Thus, if the first antibody was visualized with a green flourochrome then a red fluorochrome was chosen for the second antibody.

In order to demonstrate the specificity of the double labeling a simultaneous single staining of each of the corresponding antibodies on consecutive tissue sections was always performed.

3.2.4 Morphology

The morphological analysis focused on nerve fibres presumed to be the predominant source of neuropeptides. It entailed comparison of the different tissues, i.e. tendon, ligament and capsule, with regard to the amount and distribution of specific neuropeptide immunoreactivities. Particular emphasis was put on the distinction between vascular and non-vascular fibres.

3.2.5 Semi-quantification

A quantitative approach was applied to assess neuropeptide immunoreactivities over time in sections of ruptured Achilles tendon (paper IV-V). The investigation included 7 groups, 5 rats in each, which were analysed at 7 time-points after tendon rupture. The study included the following 5 steps:

1) The Achilles tendons were longitudinally sectioned and the sections were numbered consecutively from the dorsal to the ventral aspect. 2) Three sections from the three levels of the tendon, ventral, middle and dorsal part, were chosen to represent the full thickness of the tendon. Staining was done simultaneously for all sections to be compared. 3) For image analysis, the first step was to save computer images from the microscopical fields (10x objective). A videocamera system (Nikon digital camera DXM 1200) was attached to the Nikon epifluorescence microscope (Eclipse E800 Yokohama, Japan) connected to a PC-computer to produce images using the software Easy Analysis (Bergström Instrument, Solna, Sweden) (Fig. 3). From each section, one image displaying the strongest immunofluorescence in the rupture site as assessed subjectively was selected. 4) The second step of the image analysis entailed quantification of all positively stained nerve

fibres, beyond a defined threshold (see below) of fluorescence intensity in each computer image. The images were analysed using a software (Easy Analysis), that denotes and considers all positively stained nerve fibres beyond a defined threshold of fluorescence intensity. The results were expressed as the fractional area occupied by positive fibres in relation to the total area. The fluorescent/total area was determined in three images in five rats at each time point, 15 images at each time point. In the image analysis, the mean coefficient of variation for two observers was 9,8 % and the mean coefficient of variation for one observer was 9,6%. 5) For statistical analysis the mean fluorescent/total area was calculated for each of the five rats at each time point.

Figure 3. Image analysis

3.3 RADIOIMMUNOASSAY

Radioimmunoassay (RIA) was used for quantifying the concentrations of neuropeptides in tissue extracts. In RIA, the antibody binds to one or more sites, called epitopes, commonly consisting of 3-6 amino acids. Therefore, specificity testing of the antiserum against other peptides to assess possible cross-reactivity is important.

3.3.1 Extraction

Fresh frozen tissues (see section 3.1.3) were boiled in 2 mol/L acetic acid, homogenized in a Polytron (15 sec), sonicated (30 sec) and centrifuged at 3000 x g for 15 min. The supernatants were lyophilized, diluted in 2 ml phosphate buffer, pH 7.4, and kept at -20°C until analysis. The neuropeptide concentrations were expressed as pmol/g wet weight tissue.

3.3.2 Assay

Neuropeptides were analysed with competitive RIA using antibodies to neuropeptide-albumin conjugates. First, neuropeptides in standards and samples were incubated for 48 hours with the antibody. This was followed by 24 hours incubation with ^{125}I-labeled neuropeptide, which competed with the samples in binding to the antibodies. ^{125}I-labeled neuropeptides bind in reverse proportions to the neuropeptide concentration in standards and samples. Subsequently, the bound and unbound fractions of the radioligand are separated using the double antibody precipitation technique. The radioactivity of the precipitates was measured in a gamma counter (1470 Wizard™, Wallac, Sweden).

3.3.3 Antibodies

Table 3. Characteristics of antibodies.

Antigen	Antiserum	Specificity	Sensitivity	Raised in	Precision
SP [11]	SP2 [1]	> 99,99 %	3 pmol/L	Rabbit	7 and 11%
CGRP [37]	CGRP8 [2]	> 99,99 %	9 pmol/ L	"	8 and 14%
GAL [29]	RatGala 4 [3]	> 99,90%	5 pmol/ L	"	6 and 8%
NPY [36]	N18405 [4]	> 99,90%	31 pmol/ L	"	7 and 12%
VIP [28]	RB311 [5]	> 99,99%	3 pmol/ L	"	6 and 7%
MEAP [7]	90:3D[II] [6]	> 99,90%	80 pmol/ L	"	N/D
DYN B [13]	113B [7]	> 99,99%	40 pmol/ L	"	N/D
N/OFQ [17]	96:2+ [8]	> 99,90%	N/D [9]	"	N/D

The antigen is specified according to the number of amino acids within brackets and the antiserum code is given by the manufacturer. Specificity considers cross-reactivity to other peptides and is denoted as 1-cross-reactivity. Sensitivity is defined by the detection limit of each neuropeptide. Precision reflects the intra- and interassay coefficients of variation. For further details see references.

Figure 4. High performance liquid chromatography.

[1] Brodin and Lindefors
[2] Larsson et al. 1991
[3] Theodorsson and Rugarn, 2000
[4] Theodorsson-Norheim and Hemsen, 1985
[5] The antibody is directed to the C-terminal part of the neuropeptide. Euro-Diagnostica AB, Sweden
[6] Folkesson et al. 1988
[7] Christensson-Nylander et al. 1985
[8] Ploj et al. 2000
[9] The value was not determined.

3.4 CHROMATOGRAPHY

Chromatography was used for separation of molecules according to differences in specific properties, i.e. size, charge, hydrophilicity and affinity to specific particles. The method can be used either for purification before RIA or for confirmation of extract content.

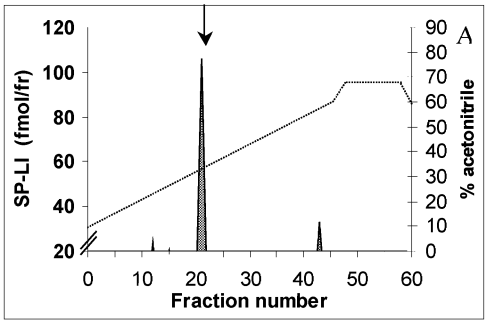
In papers I-II, reverse-phase high performance liquid chromatography (HPLC) was applied to pooled tissue extracts of tendons, ligaments and knee joint capsules, respectively (Fig. 4). A GENESIS C18 300Å, 4µ, 150 mm column was used and elution was performed with a 25 min linear gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. A GILSON UNIPOINT system software controlled two GILSON 306 HPLC pumps. A gradient of 10-60% acetonitrile was used for SP, CGRP and GAL and 100 µl of each sample was injected into the column. For VIP and NPY a gradient of 20-70% acetonitrile was used and 200 µl of each sample was injected into the column. Fractions of 1.0 ml were collected at an elution rate of 1.0 ml/min. Each fraction was lyophilized and reconstituted in 100 µl of distilled water before performing RIA. The fractions were assayed for immunoreactivity in the same tubes used for their collection.

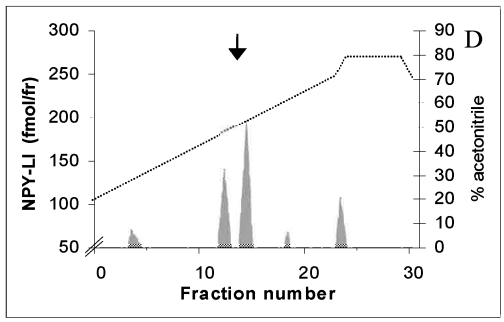
Reverse-phase HPLC analysis of tendon, ligament and capsule extracts showed distinct immunoreactive components reflecting the presence of sensory (SP, CGRP and GAL) and autonomic (NPY and VIP) peptides, respectively (Fig.5A-E). The first peak in the analysis of VIP represented VIP-fragments. The main peak of each neuropeptide eluted in the position of the corresponding synthetic peptide, which seems to corroborate that the extraction procedure applied does not affect the biochemical integrity of the neuropeptides. Hence, the quantitative results can be assumed to represent the actual peptides.

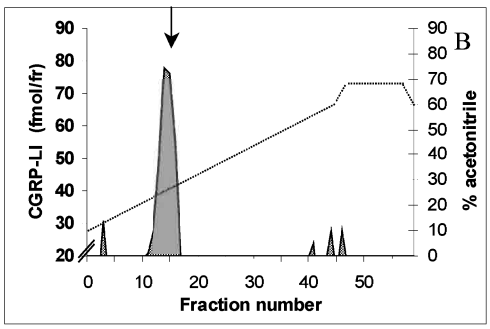
In paper III, the tissue extracts were subjected to a purification step using an ion exchange procedure prior to RIA. The method is routinely used to analyse opioid peptides in various tissue extracts and is described in detail elsewhere [31, 120]. The tissue extract was poured onto a small cation exchange column containing SP Sephadex C-25 gel (Pharmacia Diagnostics, Uppsala, Sweden). The peptides were eluted in separate fractions by stepwise elution using a series of buffers containing mixtures of pyridine and formic acid of increasing ionic strength. MEAP elutes in buffer 3 (0.35 M pyridine:0.35 M formic acid) and N/OFQ and DYN B elute in buffer 5 (1.6 M pyridine:1.6 M formic

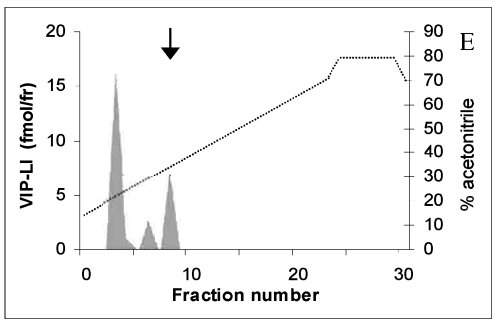
acid). The fractions were dried in a vacuum centrifuge and stored at -20°C until peptide analysis [120].

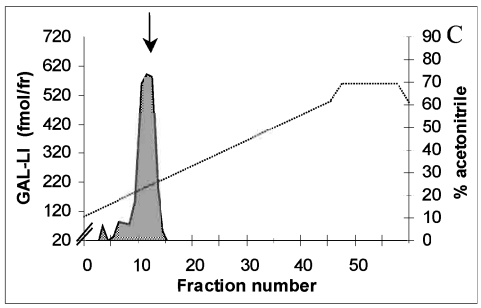
Figure 5. Reverse-phase HPLC of SP-LI **(A)**, CGRP-LI **(B),** GAL-LI **(C)** NPY-LI **(D)** and VIP-LI **(E)** in tissue-extracts. Arrows denote the elution volume of synthetic peptide. The dotted line represents the elution curve of acetonitrile.











3.5 RADIOLIGAND BINDING ASSAY

Radioligand binding assay was used to study the binding characteristics of opioid receptors in tissue homogenates using a labeled ligand.

Fresh frozen Achilles tendons (see section 3.1.3) were homogenized in 5 vol/wt of ice-cold 50 mM Tris-HCl buffer, pH 7.4 (Polytron, 2 x 30 seconds) and sonicated (15 seconds). After centrifugation at 40,000 x g for 20 minutes at 4°C, the pellets were resuspended in 30 vol/wt fresh Tris-HCl buffer and incubated at 37°C for 30 minutes in order to facilitate degradation and dissociation of endogenous opioid peptides. The centrifugation step was repeated, the final pellet was resuspended in 5 vol/wt of ice cold 50 mM Tris-HCl buffer containing 0.32 M sucrose, pH 7.4 and kept at -70^{o}C until use.

Aliquots of membrane homogenates (0.6-0.7 mg/ml protein) were incubated with [^{3}H]naloxone (54.6 Ci/mmol, New England Nuclear, Boston, MA) for 30 minutes at 25°C in a final volume of 250 µl. Reactions were terminated by rapid filtration through Whatman GF/B glass fibre filters pretreated with 0.1% polyethyleneimine (PEI) using a Brandel Cell Harvester, followed by three washings with 5 ml of ice-cold Tris-HCl buffer pH 7.4. The bound radioactivity was measured in 4 ml of Ultima Gold scintillation cocktail (Packard) using a Packard 1900 CA liquid scintillation counter. All experiments were carried out in duplicate. Non-specific binding was measured in the presence of 10 µM unlabeled naloxone. The protein concentration was determined by the method of Lowry [93]. Scatchard analysis was performed by first-order non-linear regression analysis of the data from saturation isotherms. The binding parameters dissociation constant (K_d), receptor density (B_{max}) and Hill coefficient (n_H) were calculated from these plots [135]. The values represent the mean ± s.e.m. of 3-4 independent experiments.

The binding of [^{3}H]naloxone to homogenates from Achilles tendon was specific, saturable and stereo selective. The Hill coefficient (n_H) was close to unity, 0.89 ± 0.09, indicating the non-cooperative nature of the binding process and also that [^{3}H]naloxone could interact with multiple classes with equal affinity (Fig. 6).

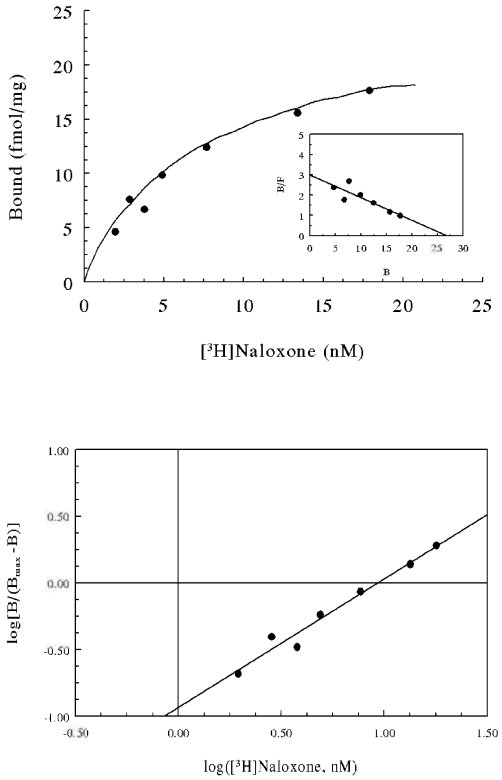


Figure 6. Saturation binding of [^{3}H]naloxone to homogenates from rat Achilles tendon. Representative saturation curve and Scatchard plot (inset) (**A**). Hill plot (**B**). B/F: bound [^{3}H]naloxone (fmol/mg protein) per free ligand (nM).

3.6 NOCICEPTIVE TESTS

Nociception during healing of Achilles tendon rupture was tested according to the behavioural reactions to noxious stimuli [71, 82]. This included both thermal and mechanical sensitivity (paper V).

3.6.1 Thermal sensitivity

The response to heat stimulation was assessed by placing the ventral surface of the right (injured side) and left (contralateral) hindpaw on the hot-plate, which maintained a temperature of 52°C [153, 166, 167]. The time (in seconds) to hindpaw withdrawal was measured and referred to as hindpaw withdrawal latency (HWL).

3.6.2 Mechanical sensitivity

The Randall Selitto Test (Ugo Basile, Type 7200, Italy) was used to assess the HWL to mechanical stimulation. A wedge-shaped pusher at a loading rate of 30 g/ s was applied to the dorsal surface of the hindpaw. The latency required to initiate the withdrawal response was assessed and expressed in seconds.

All rats were accustomed to the test procedures for 5 days to minimize stress at the actual experiments. An upper limit of 15 seconds of stimulus was set to avoid tissue damage.

3.7 STATISTICS

All variables were summarized using standard descriptive statistics (mean, median, standard deviation or standard error). Parametric methods were preferred provided that the values were approximately normally distributed. Thus, lateral differences in thermal and mechanical hyperalgesia were analysed using Student's t-test for paired observations. Relationships between ruptured and contralateral sides were expressed as Pearson's product-moment correlation coefficients. Differences in time courses for the hyperalgesia variables were determined by analysis of variance (ANOVA) for repeated measurements [159]. The distributions of the neuropeptides, however, were positively skewed (the subjects were clustered in the lower end), which made a non-parametric approach warranted. Thus, Mann-Whitney's U test was used to analyse differences between weeks and Kendall's rank correlation coefficient was used to express the relationship between CGRP and SP. All statistical tests were two-tailed and a significance level of 0.05 was applied. It should be noted that the degrees of freedom in all analyses were low with a high risk of Type II-errors, therefore accepting a false null hypothesis (no difference and/or no correlation).

4 NEUROPEPTIDES IN PERIARTICULAR TISSUE

To explore the occurrence of neuropeptides in tendons, ligaments and joint capsules both a quantitative and morphological approach was applied. RIA was employed to screen for and quantify three main classes of neuropeptides: autonomic, sensory and opioid. Immunohistochemistry was used to establish the neuronal occurrence and the distribution among different tissues of 14 neuronal mediators representing the three classes.

4.1 OCCURRENCE AND LEVELS

The RIA study focused on 2 autonomic (NPY, VIP), 3 sensory (SP, CGRP, GAL) and 3 opioid (MEAP, N/OFQ and DYN B) peptides in Achilles tendon, knee collateral ligaments and joint capsule.

4.1.1 Autonomic peptides

The RIA analysis included one sympathetic (NPY) and one parasympathetic (VIP) neuropeptide known to have opposite effects in the regulation of vasoactivity [94, 95]. Measurable concentrations (0.11-5.63 pmol/g) of the two neuropeptides were obtained in all tissues analysed. The analysis showed that NPY consistently exhibited higher concentrations than VIP in the different tissues (Fig. 7A). The concentration of NPY compared to VIP was 15-times higher in ligaments and twice as high in tendons and in capsules. This is in agreement with a previous study on ankle joints by Ahmed et al. [3]. The observation would seem to reflect that the sympathetic tonus is higher than the parasympathetic tonus in periarticular tissue. Such a difference between tissues may be assumed to be of importance for blood-flow, and hypothetically for healing capacity, possibly also for susceptibility to degeneration and injury. Notably, ligaments are more prone to rupture than tendons [161].

Most vascular nerve fibres contain multiple transmitters. Neuropeptides in contrast to other transmitters mediate more sustained changes in vascular resistance, possibly by prolonging the effect of other transmitters. Thus, NPY has been shown to potentiate the vasoconstrictive effect of NA [95]. The latter was recently reported to reduce blood flow by more than 75 % in joint capsule and collateral ligament after topical administration [101]. The high level of NPY now demonstrated in ligaments may be assumed to contribute to the strong vasoconstrictive effect of NA reported by McDougall et al. [101].

Considering the low level of VIP, it would seem that the vasoconstrictive capability is greater than the vasodilatory in periarticular tissue.

The tissue specific levels of vasoconstrictive and vasodilatory neuropeptides observed presumably represent a homeostatic state, which may be altered in response to external stress [62]. It has been suggested that impaired blood flow and hypoxic degeneration could be contributing factors in the genesis of atraumatic tendon ruptures [72]. Increased release of NPY and NA during stress and overtraining has been reported [87]. Conceivably, a combination of poor vascularisation, increased vasoconstriction and repetitive mechanical load may lead to ischemia, necrosis and rupture.

4.1.2 Sensory peptides

Among the sensory peptides screened for by RIA, SP and CGRP have nociceptive and pro-inflammatory effects, whereas GAL is known to modulate these effects. The investigation disclosed that CGRP and GAL were present in all tissues analysed, whereas SP could not be demonstrated in ligaments (Fig. 7B). The highest concentrations of SP and CGRP were found in tendons, whereas GAL essentially exhibited the same levels in the different tissues. Thus, the most conspicuous difference between the counteracting sensory neuropeptides was observed in the Achilles tendon. The relative concentrations observed may reflect a physiological balance between sensory mediators and modulators. In painful and inflammatory conditions, it may prove that there is an altered balance between these neuropeptides resulting in enhanced or perpetuated nociceptive and inflammatory responses. Several studies have demonstrated increased levels of sensory peptides in rats with experimentally induced arthritis [1, 2] and patients with rheumatoid arthritis [104] and also that SP and CGRP enhance protein extravasation [52]. The higher concentrations of SP and CGRP in tendons compared to ligaments could reflect a greater susceptibility to pain and inflammation.

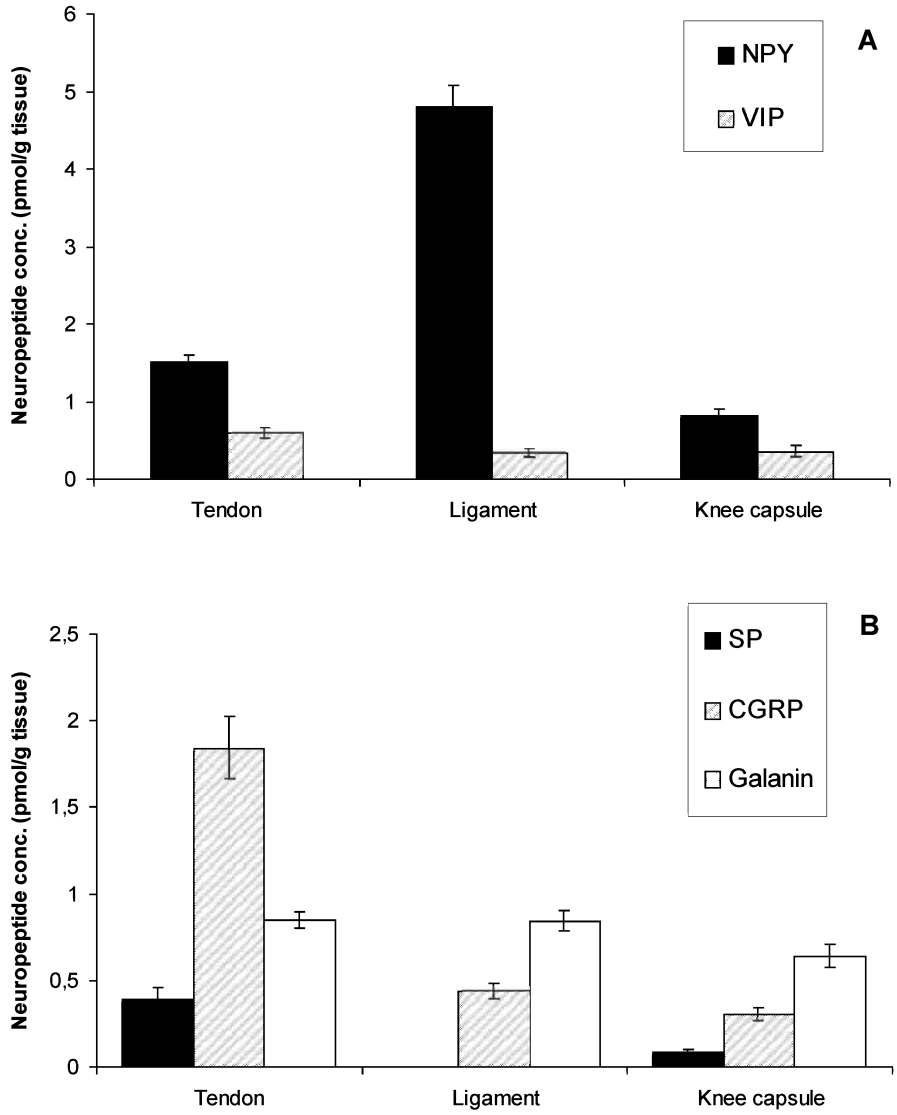


Figure 7A-B RIA tissue concentrations (pmol/g) of autonomic (NPY and VIP) (**A**) and sensory (SP, CGRP and GAL) (**B**) neuropeptides in the Achilles tendon, collateral ligaments and joint capsule of rat knee (mean ± s.e.m.).

4.1.3 Opioid peptides

The RIA analysis of opioids comprised two opioid peptides (MEAP, DYN B) and one opioid like peptide, N/OFQ, which are generated from three different precursors; proenkephalin, prodynorphin and pronociceptin, respectively. Among these opioid peptides MEAP and DYN B belong to classical opioid peptide families, whereas N/OFQ has only lately been discovered and characterized. [37, 105, 123]. Both opioids and the opioid like peptide have been shown to have anti-nociceptive effects [26, 107].

The analysis disclosed that both MEAP and N/OFQ occurred in all tissues analysed, but not DYN B. However, the levels were low (Table 4). A significant proportion of samples with measurable concentrations of MEAP and N/OFQ were obtained from all tissues. As for N/OFQ, however, only ligamentous tissue displayed a median concentration (0,44 pmol/g) above the detection limit. The most conspicuous finding pertained to the higher concentrations of both MEAP and N/OFQ in ligaments as compared to tendons and capsules.

This is the first observation on the occurrence of opioids including nociceptin in periarticular tissues. The results comply with a recent report demonstrating by RIA the occurrence of met-enkephalin in bone and joints by Elhassan et al. [44]using the method of Ahmed et al. (1994, 1995) for extraction and quantification of neuropeptides [1, 3, 4]. In the present investigation of periarticular tissues, only part of the samples provided measurable levels. There are two plausible reasons for this inconsistency. Apart from a low content of opioids *per se*, periartiular tissues have a high content of collagen sometimes causing difficulties in purifying the samples resulting in loss of peptides. This problem also pertained to the analysis of autonomic and sensory peptides, although it was less pronounced. Despite a somewhat high standard deviation, the results clearly demonstrate the existence of opioid peptides (enkephalin and nociceptin) in the periarticular tissues.

Table 4. Concentrations (pmol/g) of MEAP and N/OFQ in the Achilles tendon, collateral ligaments[4] and joint capsule[4] of rat knee.

	MEAP			N/OFQ		
	Tendon	Ligament	Capsule	Tendon	Ligament	Capsule
1	(4.16)[1]	<<[2]	0.10	<<	0.87	<<
2	0.27	0.78	0.04	<<	0.34	<<
3	0.09	0.74	0.08	0.14	0.98	0.06
4	N/D[3]	<<	0.06	<<	0.59	0.06
5	<<	<<	0.06	<<	0.49	<<
6	0.10	1.05	0.07	0.06	0.63	<<
7	<<	0.68	0.07	<<	0.34	0.08
8	<<	0.37	0.05	<<	<<	<<
9	0.19	0.78	0.15	0.19	0.6	0.14
10	0.06	0.51	0.10	<<	<<	<<
11	<<	0.42	0.04	<<	0.39	<<
12	0.08	0.48	0.10	0.12	0.39	0.08
Median	**0.07**	**0.50**	**0.07**	<<	**0.44**	<<
Percentage of specimens with detectable levels	60%	75%	100%	33%	83%	42%
95% confidence interval	23—97%	46—104%		2—65%	59—108%	9—74%

[1] Out of range value not included in the calculations of descriptive statistics
[2] No detectable concentration
[3] Concentration not determined.
[4] Unpublished data

The physiological role of enkephalins can be assumed to be anti-nociceptive [27, 96], anti-inflammatory [60, 85], vasodilatory [45, 109], immunosuppressive [24] and trophic [158, 169]. Notably, it has been shown that the release of SP from afferents in the cat knee joint on sciatic nerve stimuli is inhibited by intra-articular enkephalin-analogue injections [165].

As for nociceptin, the occurrence in peripheral tissues has not previously been demonstrated to our knowledge. The physiological role of nociceptin is largely unknown. In the spinal cord, low levels have been suggested to be anti-nociceptive and high levels nociceptive [26]. Recently, nociceptin was reported to reduce mechanosensitivity in the rat knee joint through inhibition of SP release [102, 103] possibly via receptor activation in the periphery.

Altogether, the present investigation shows that opioids are present in periarticular tissue. The lower concentrations of MEAP and N/OFQ in tendons and capsules compared to ligaments could reflect a greater susceptibility to pain and inflammation, in particular when considering also the high levels of SP found in tendons and capsules. Notably, pain syndromes of tendons are much more common than those of ligaments. The combined quantitative data obtained suggest that there is a balance between nociceptive and anti-nociceptive peptides under normal conditions, which may prove to be altered under pathological conditions.

4.2 TISSUE DISTRIBUTION

Further exploration of the tissues was performed by immunohistochemistry to assess the neuronal occurrence of the neuropeptides detected by RIA and also to extend the investigation to other neuropeptides representing the three main subgroups. Thus, the investigation included three autonomic (NA, NPY, VIP), five sensory (SP, CGRP, NKA, GAL, SOM) and six opioid (LE, ME, MEAP, MEAGL, DYN B, N/OFQ) mediators. The autonomic and sensory peptides were analysed in all periarticular tissues, whereas the opioid peptides only in the Achilles tendon. As for the latter tissue, also the occurrence of different opioid receptors (DOR, KOR, MOR), was investigated.

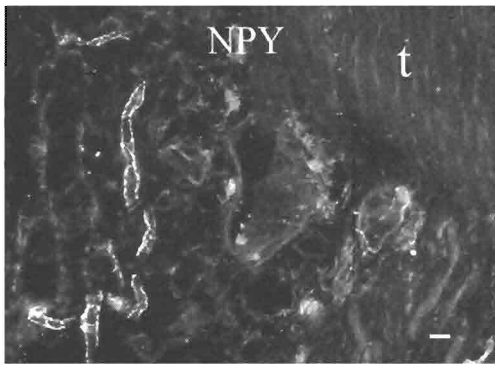
4.2.1 Autonomic peptides

Nerve fibres immunoreactive to both sympathetic (NPY, NA) and para-sympathetic (VIP) mediators were identified in the different tissues (Fig. 8). The autonomic fibres were mostly observed in the loose connective tissue around the proper tendon and ligament tissues and beneath the intimal lining layer of the synovium (see Appendix for details). In the Achilles tendon, the fibre density was higher in the proximal musculo-tendinous junction than in the distal bone insertion; no such difference in distribution could be seen in ligaments and knee capsules. Over all, the autonomic fibres were predominantly seen as networks around blood vessels. Among the three neuronal mediators, NPY was the most abundant and VIP the least.

As for the sympathetic fibres, NPY and NA immunoreactivities were almost exclusively observed as networks of varicose nerve terminals in the blood vessel walls (Fig. 8). The vascular NPY-positive fibres were abundant in the paratenon, epiligament and the sublining layer of the synovium as well as in the loose connective tissue outside these structures. Only a few weakly stained NPY fibres were found to extend from the surrounding tissues into the proper structures (tendon/ligament, synovial lining layer). NA-positive fibres were confined to the surrounding loose connective tissues mostly in large blood vessel walls, as also demonstrated in a study of human forearm muscle insertions [92]. Over all, the observations seem to reflect that the regulation of blood flow to tendons, ligaments and synovia predominantly occur in the adjacent loose connective tissue.

The parasympathetic VIP-immunoreactive fibres, albeit scarce, were mostly observed as long thin varicose nerve terminals forming a "fence" in the paratenon, epiligament and along the lining layer of the synovium (Fig. 8). The fibres appeared to be evenly distributed between vascular and non-vascular structures. As opposed to NA/NPY mostly occurring in larger vessels, VIP was predominantly found around smaller vessels, which is in line with other reports [92, 149]. This observation may reflect that NA and NPY (vasoconstrictive) predominantly regulate the main blood flow to the tissues, whereas VIP (vasodilatory) is responsible for the fine-tuning of blood flow at a microlevel. The vascular and non-vascular distribution of VIP would seem to comply with an anti-ischemic and an anti-inflammatory effect in the periphery as reported earlier [38, 50]. Notably, previous studies have suggested a strong anti-inflammatory role of VIP [41, 127] through inhibition of T cell proliferation and migration [118].

Double-staining experiments disclosed co-existence of NPY and NA, mostly in vascular fibres, whereas co-existence of NPY and VIP was seen in both vascular and non-vascular fibres. Occasionally some VIP-immunoreactivity could be seen co-localized with NA in nerve fibres of small blood vessels. The observed co-existence of the autonomic mediators presumably reflects synergistic and/or inhibitory vasoactive effects in the tissues analysed. NPY has been shown to potentiate the vasoconstrictive actions of NA [95]. NA and NPY being co-localized almost exclusively in fibres around blood vessels supports the latter notion. Conversely, the co-existence of VIP with NA or NPY would seem to represent a modulatory mechanism [90].



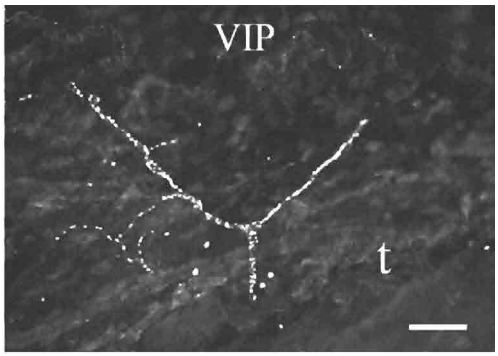


Figure 8. Immunofluorescence micrographs of two longitudinal sections through the Achilles tendon after incubation with antisera to VIP and NPY. VIP-positive nerves are arranged as a "fence", surrounding the proper tendon, of small varicosities in the paratenon. The NPY-positive fibres are arranged as nerve terminals in the vessel walls. t = tendon tissue. Bar = 50 µm.

4.2.2 Sensory peptides

Nerve fibres immunoreactive to SP, CGRP, NKA, GAL and SOM were consistently identified in the Achilles tendon, knee ligaments and capsule. The nerve fibre density and distribution in the different tissues were quite similar (see Appendix for details). Thus, the highest fibre density was observed in the surrounding tissues, i.e. the paratenon, epiligament and beneath the intimal lining layer of the synovium. The ratio of vascular:non-vascular fibres, was higher in the surrounding loose connective tissue and sublining layer than in the paratenon/ epiligament/ intimal lining layer, which exhibited a large number of non-vascular free nerve endings (Fig. 9). Overall, there was no significant difference in the tissue distribution between the specific neuropeptides. However, the occurrence of CGRP-positive fibres was clearly higher than that of the other sensory neuropeptides. The inconsistency between the observations of SP by IHC and RIA, should be attributed to methodological problems of RIA as discussed previously (section 4.1.3).

The most conspicuous findings pertained to the abundance of sensory fibres in the surrounding tissues of the structures analysed, whereas the proper structures themselves, notably, were almost devoid of nerve fibres (Fig. 9-10). This would seem to reflect that the neuronal regulation of tendons, ligaments and joint capsules highly depends on the innervation of the surrounding tissues. Thus, the abundance of immunopositive vascular fibres in the surrounding loose connective tissues may be assumed to reflect an important role in the regulation of blood flow to the proper structures. Both SP and CGRP, in particular the latter, have been reported to be potent vasodilators. [20]. In addition, they have also been demonstrated to have pro-inflammatory effects, e.g. by enhancing protein extravasation and leukocyte chemotaxis [20, 98, 111]. The occurrence of sensory free nerve endings unrelated to vessels predominantly seen in the paratenon, epiligament and intimal synovial lining suggests a nociceptive role.

The present investigation confirmed the well-known co-existence of SP and CGRP. However, each of these was also found to be co-localized with GAL and SOM. This has previously been demonstrated in dorsal root ganglia [65] and in colon [43], but not in peripheral tissues of the locomotor apparatus. The observations of multiple co-existence strengthen the notion of functional interactions between sensory neuropeptides in the periphery [65]. Thus, CGRP has been reported to enhance the nociceptive effect of SP,

whereas GAL and SOM are claimed to modulate nociception and pro-inflammation [27, 36, 56, 142, 164].

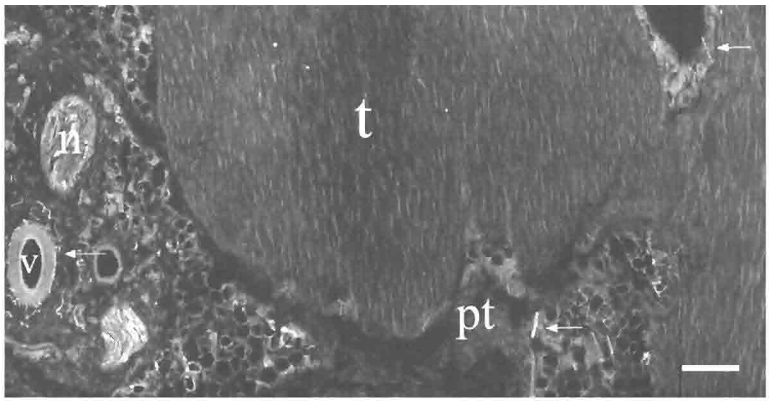


Figure 9. Overview micrographs of longitudinal sections through the Achilles tendon built up by putting together computerized images of smaller micrographs. Immunostained for SP. Arrows denote varicosities and nerve terminals. The immunoreactivity is seen in the paratenon and surrounding loose connective tissue, whereas the proper tensinous tissue, notably, is almost devoid of nerve fibres t = tendon tissue; pt = paratenon; v = blood vessel. Bar = 250 µm.

Figure 10. Schematic drawing of the innervation of a normal Achilles tendon. Symbols denote the relative amount of different peptides at three locations; paratenon, vascular and non-vascular nerve endings in the loose connective tissue. ▲ = sensory, ■ = autonomic and ● = opioid peptides.

4.2.3 Opioid peptides and receptors

4.2.3.1 Opioid peptides

The analysis showed that all enkephalins tested, LE, ME, MEAP and MEAGL, were present in peripheral nerve fibres of the Achilles tendon, whereas neither N/OFQ nor DYN B could be identified. The failure of detecting N/OFQ in immune cells and nerve fibres by IHC, but the fact that measurable concentrations were obtained by RIA should be attributed to the latter being a more sensitive assay. DYN B, however, was not identified by IHC or RIA. Its non-existence or presence in low amounts in tendon tissue under normal physiologic conditions remains to be established. Notably, it has been shown that peripheral sensory nerves immunostain for dynorphin in inflammation, but not under normal conditions [55]. LE appeared to be the most abundant opioid compared to ME, MEAP and MEAGLE in decreasing order, which may be explained by LE being the only enkephalin processed from both proenkephalin and prodynorphin [42, 88, 170].

Although, the enkephalins differed in amount, their tissue distribution displayed a similar pattern. Thus, all four enkephalins predominantly occurred in the loose connective tissue, the paratenon and musculo-tendinous junction, whereas no enkephalins were found in the proper tendon tissue. Hypothetically, this difference in anatomical distribution might suggest that regulation of painful disorders of the Achilles tendon mainly occurs in the surrounding tissues, which during normal condition also harbour the sensory and autonomic neuropeptides. In pathological conditions, however, it may prove that the anatomical distribution and amount of sensory and opioid peptides are altered so as to involve also the proper tendon.

In the loose connective tissue surrounding the tendon, the enkephalin positive fibres appeared as small varicosities around the walls of both large and small blood vessels, which may reflect involvement in both vasodilatory actions [109] and anti-inflammatory responses [60, 85]. In the paratenon (Fig. 11) and the musculo-tendinous junction the enkephalins mostly occurred as varicosities in free nerve terminals without any relationship to blood vessels suggesting a paracrine or an autocrine function in the regulation of nociception [27, 96]. Such a regulation is probably executed in interaction with the sensory nervous system. Thus, it has been shown that the release of SP from afferents in the cat knee joint is inhibited by intra-articular enkephalin-analogue injections [165]. Whether there is an inherent balance between opioid and sensory neuropeptides under normal conditions is unknown.

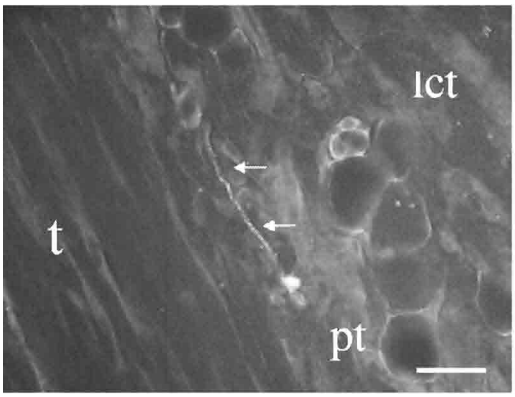


Figure 11. Immunofluorescence micrograph of a longitudinal section through the Achilles tendon showing double staining with LE- and DOR-antibodies. Arrows denote varicosities. The immunoreactivity displaying co-existence of LE and DOR is seen as free nerve endings in the paratenon. The LE-antibody is visualized with CY2™ (green) and the DOR-antibody with CY3™ (red). t = tendon tissue; pt = paratenon; lct = loose connective tissue. Bar = 50 µm.

4.2.3.2 Opioid receptors

The existence of an opioid system in connective tissues as demonstrated by neuronal immunoreactivity to enkephalins is supported by our opioid receptor analyses based on binding assays and immunohistochemistry. Of the three opioid receptors studied (DOR, KOR, MOR), only DOR could be detected by immunohistochemistry. Double staining disclosed co-existence of each of the enkephalins with DOR in the nerve fibres, which complies with other studies suggesting that enkephalins are the main ligands for DOR [40]. Thus, co-localization of enkephalins and DOR in paratenon (Fig. 11), loose connective tissue and the musculo-tendinous junction was seen in small peripheral nerve terminals, suggesting C-fibre origin. This is in agreement with other reports demonstrating that both enkephalin [27] and DOR [33, 154] are localized in unmyelinated afferent sensory axons in the skin. The major part of the enkephalins and DOR identified in the nerve fibres were found to be co-localized. The presence of peripheral opioid receptors was corroborated by the receptor binding analysis showing that tendon tissue could bind naloxone in a specific and saturable way (Fig 4). DOR activity has been shown to have a potent inhibitory effect on SP-release [58, 165]. There are several reports demonstrating that treatment with delta opioid agonists in the periphery elicit both anti-inflammatory and anti-nociceptive effects in models of inflammation [115, 143, 171]. The anti-nociceptive effects have been shown to be mediated through DOR localized on unmyelinated afferent sensory axons [171]. Presumably, the co-existence of enkephalins

and DOR in vascular nerve fibres reflects inhibition of neurogenic pro-inflammatory actions, whereas the co-existence in free nerve endings reflects inhibition of nociception. Endogenous opioids, implicated in mitigating inflammatory pain, have been reported to be released by immune cells [57, 96]. However, data on the neuronal occurrence in the periphery are sparse. The present findings suggesting a neuronal source of opioids in the periphery would seem to reflect an anti-nociceptive system in connective tissues, which may be exploited in the therapeutical setting by drugs acting selectively in the periphery.

Altogether, there seems to exist a complex neuropeptidergic network in periarticular tissues, where each structure exhibits a specific expression of neuropeptides and a specific ratio of potentiating and inhibitory mediators. The observations may be assumed to reflect unique tissue requirements under normal conditions including the susceptibility to stress. The data obtained on normal tendons, ligaments and joint capsules may be used as a neuroanatomical basis for studies of pathological conditions of the musculo-skeletal system.

5 HEALING OF RUPTURED TENDON

Having established the normal occurrence of neuropeptides in periarticular tissues, further analysis aimed at studying the expression of neuropeptides during healing of tendinous tissue. Besides cytokines and growth factors, neuronal mediators in the periphery have been suggested to participate in tissue repair [19, 54, 117, 129].

5.1 NEURONAL PLASTICITY

Nerve regeneration during healing of Achilles tendon rupture in the rat was studied by immunohistochemistry including a semi-quantitative assessment focusing on the rupture site of the proper tendinous tissue. Neuronal markers for regenerating and mature fibres, i.e. growth associated protein 43 (GAP) and protein gene product 9.5 (PGP), respectively, were analysed at different time points (1-16 weeks) post-rupture. The morphologic distribution and expression of the neuronal markers over time were analysed in relation to the three different phases of tissue healing, the inflammatory, the regenerative/proliferative and the remodeling phase.

5.1.1 New nerve fibre ingrowth

In the first week post rupture, corresponding to the transition from the inflammatory to the regenerative phase, there was increased neuronal immunoreactivity to PGP and GAP both in original nerve fibres of the surrounding loose connective tissue and, notably, in new nerve fibres in the proper tendon tissue of the rupture site (Table 5). In general, PGP positive fibres were predominantly observed in the surrounding loose connective tissue, whereas GAP fibres mainly occurred in the rupture site of the proper tendon. While GAP appeared mostly unrelated to vessels, PGP was predominantly seen around vessels. The vascular distribution of PGP in the loose connective tissue may be assumed to reflect the vasoregulatory role of the peripheral nervous system, but possibly also a proinflammatory role. In the proper tendinous tissue, normally GAP-negative, there was a clear GAP-immunoreactivity suggesting new nerve fibre ingrowth (Table 5), which is consistent with reports on GAP levels in dorsal root ganglia after peripheral nerve injury [17, 30, 146]. GAP has been suggested to be involved in nerve regeneration by regulating growth cone motility and axon guidance signals [46, 47]. Our observations of early nerve ingrowth are in line with observations on fracture and skin healing [61, 78, 89] indicating that this process is a fundamental feature of tissue healing.

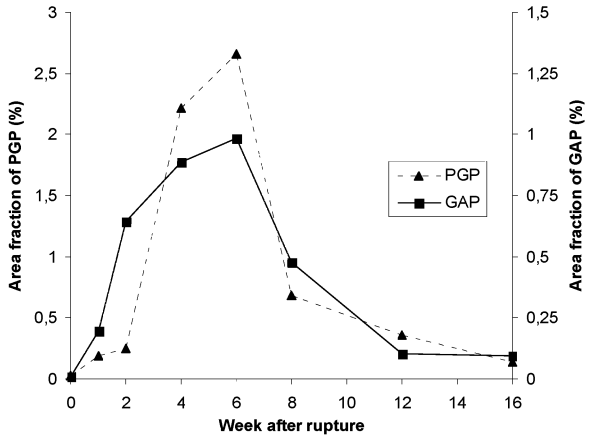


Figure 12. Area occupied by nerve fibres (%) immunoreactive to GAP and PGP in relation to total area, in the mid third of the tendon, over 16 weeks post rupture (mean±s.e.m.).

Table 5. Areal expression of GAP and PGP in relation to total tissue area (%), in the mid third of the tendon, at different time-points after rupture. Values represent means ± s.e.m. (n=5, except week 6 where n=3). Differences between the weeks are calculated with the Mann-Whitney U test.

	GAP			PGP		
Week	Area fraction (%)	Weeks	P-Value	Area fraction (%)	Weeks	P-Value
0	0,009 ± 0,004			0,022 ± 0,006		
1	0,197 ± 0,059	0 vs. 1	0,009	0,186 ± 0,056	0 vs. 1	0,009
2	0,643 ± 0,254			0,248 ± 0,063		
4	0,886 ± 0,384			2,214 ± 1,619		
6	0,984 ± 0,188	1 vs. 6	0,025	2,662 ± 0,839	1 vs. 6	0,025
8	0,478 ± 0,281			0,680 ± 0,241		
12	0,104 ± 0,030			0,357 ± 0,112		
16	0,095 ± 0,046	6 vs. 16	0,025	0,132 ± 0,038	6 vs. 16	0,034

5.1.2 Nerve fibre peak expression

From one to six weeks post rupture of Achilles tendon, there was a striking shift in neuronal immunoreactivity from the surrounding loose connective tissue into the proper tendinous tissue (Fig. 13). This would seem to reflect the transition of a predominantly inflammatory into a regenerative phase. The peak expression of GAP-immunoreactivity in the rupture site occurred between weeks two and six, while that of PGP occurred somewhat later, i.e. between weeks four and six (Fig. 12, Table 5). The earlier peak of GAP should be explained by its trophic role in regulating growth cone formation. Analogously, the extensive ingrowth of GAP- and PGP-fibres into the rupture site probably represents a neuronal involvement in repair of tendon. The observed free nerve endings among fibroblasts in the tendinous tissue may reflect a stimulatory role in cell proliferation [100, 114]. The occurrence of free nerve endings around newly formed blood vessels in the rupture site suggests a role in vasoregulation, possibly also in angiogenesis [54].

5.1.3 Nerve fibre withdrawal

During week 6 to 16 post rupture (remodeling phase) the expression of the neuronal markers showed a significant decrease in the rupture site (Fig. 12, Table 5). The nerve fibres appeared to withdraw from the area. While GAP-immunoreactivity almost completely disappeared during this phase, that of PGP successively returned to normal in the paratenon and surrounding loose connective tissue. The process appeared to end simultaneously with the completion of paratenon repair.

The observations during tendon healing clearly demonstrate the capability of the peripheral nervous system to respond and adapt to injury. This plasticity is characterized by nerve fibre ingrowth into the rupture site, a peak nerve fibre expression during the regenerative phase followed by nerve fibre withdrawal.

The experimental model employed appears to be highly appropriate and relevant for studies of the role of the peripheral nervous system in tissue repair. To our knowledge, no similar studies of repair are based on tissues normally devoid of a neuronal supply. The ingrowth and later withdrawal of nerve fibres in the proper Achilles tendon after rupture convincingly reflect that a neuronal supply is fundamental in the repair process. Although, it may be argued that new nerve fibre ingrowth after injury merely represents a secondary phenomenon to neovascularisation, this is contradicted by the abundant occurrence of free

Figure 13. Schematic drawing of nerve ingrowth during healing after Achilles tendon rupture.

sprouting nerve endings unrelated to vessels in the healing area. Presumably, new nerve ingrowth provides a delivery system for neuronal mediators that are required for tissue repair.

5.2 NEUROPEPTIDERGIC EXPRESSION

The extensive nerve regeneration and ingrowth observed in tendon rupture prompted an immunohistological analysis of the specific neuropeptides involved. Thus, the temporal expression of different neuropeptides presumed to exert trophic actions was examined. The study included sensory (SP, CGRP), sensory modulating (GAL), and autonomic (NPY, VIP) mediators.

The analysis including semi-quantification demonstrated an early (week 1) expression of SP and CGRP positive fibres both in the loose connective and the rupture site of the proper tendon (see appendix Table 7). The expression peaked during the regenerative phase. In contrast, GAL, NPY and VIP positive fibres first emerged at the end of the regenerative phase, weeks 4-6 (Table 7), most evident in the border zone between loose connective tissue and proper tendon.

5.2.1 Inflammatory phase

One week post-rupture, corresponding to the end of the inflammatory phase, SP and CGRP fibres were predominantly located in blood vessel walls surrounded by inflammatory cells in the loose connective tissue. Only a weak and scanty expression of GAL, NPY and VIP was observed. The findings comply with the nociceptive role of sensory neuropeptides, but also with a pro-inflammatory role. Thus, SP release has been shown to enhance vasopermeability [128] and to stimulate recruitment of leukocytes [18, 29, 122].

5.2.2 Regenerative phase

During weeks one to six, the expression of SP and CGRP peaked (Fig. 15). Notably, this peak occurred in the rupture site of proper tendon, while the neuronal immunoreactivity in the surrounding loose connective tissue declined. The latter would seem to comply with decreased pain 3-4 weeks after injury. The most conspicuous finding, however, was the occurrence of SP and CGRP in free sprouting nerve endings among fibroblasts in the healing tendinous tissue (Fig. 14). The observation might reflect a stimulatory role of

sensory neuropeptides on proliferation as has been demonstrated in cultured fibroblasts [114, 168]. SP and CGRP are also known to stimulate proliferation of endothelial cells [54, 114, 172]. The observation of free sprouting SP- and CGRP fibres around newly formed blood vessels in the rupture site would seem to comply with a role in angiogenesis (Fig. 14).

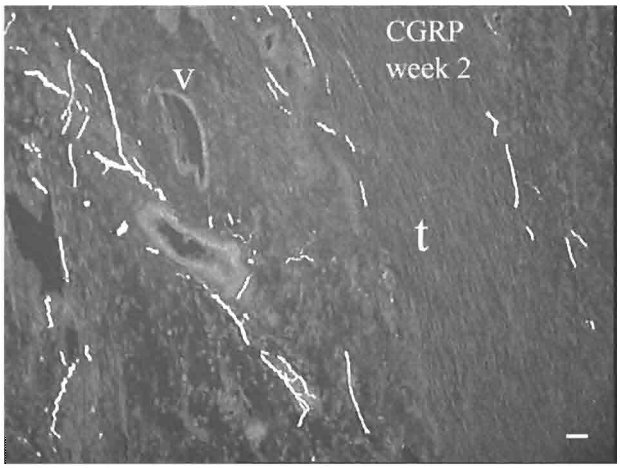


Figure 14. Immunofluorescence micrograph of longitudinal sections through healing Achilles tendon 2 weeks post rupture after immunostaining for CGRP. Nerve fibres immunoreactive to CGRP are seen in the healing tendinous tissue around newly formed vessels and as free nerve endings. t = tendon tissue; v = blood vessel. Bar = 50 μm.

During the regenerative phase the occurrence of NPY, VIP and GAL positive fibres was sparse till week four. Subsequently, the expression increased to reach a peak at the end of the regenerative phase, i.e., around week six, followed by a successive decrease (Fig. 15). This pattern in temporal expression was assessed morphologically and semi-quantitatively for GAL (Table 7), whereas the assessment of NPY and VIP was confined to subjective morphology. Over all, the occurrence of NPY, VIP and GAL was clearly lower than that of the sensory neuropeptides during the first weeks, in particular at the rupture site of the proper tendon

As for NPY, the observations would seem to reflect that vasoconstriction is down regulated during tissue repair. Notably, low levels of NPY have also been suggested to promote angiogenesis [173]. The low expression of VIP appears surprising considering

the need of vasodilation during tissue repair. Given the predominant occurrence of VIP around small sized vessels under normal conditions, where, hypothetically, it regulates the fine-tuning of vasoactivity, the demand of this function is probably low in the early phase of tissue repair. Another explanation for the low occurrence of VIP pertains to its inhibitory action on the pro-inflammatory effects of SP and CGRP as demonstrated by Delgado et al. [38, 39]. Thus, the observation may represent less inhibition of SP and CGRP, which are presumed to be important regulators of early tissue repair [48, 76, 79, 151, 155].

As for blood flow during tissue repair, it may be assumed that the balance between the vasodilatory actions of CGRP and the vasoconstrictory actions of NPY is of decisive importance for the supply of oxygen and nutrients. The high ratio of CGRP to NPY during the regenerative phase of healing probably represents highly perfused vessels, a necessity for tissue regeneration. Similar observations on the ratio CGRP to NPY have been made in studies on reinnervation of skin flaps, where CGRP immunoreactivity emerged at two weeks postoperatively and that of NPY two weeks later [75].

5.2.3 Remodeling phase

Between weeks four and six, corresponding to the transition of the regenerative into the remodeling phase, there was a dramatic increase in the expression of GAL, VIP and NPY, followed by decreased expression of SP and CGRP (Fig. 15). The increase in the immunoreactivity of GAL, VIP and NPY was observed both around vessels and in free nerve endings in a "border zone" enveloping the healing tendon. The emergence of GAL, known to modulate the effect of sensory neuropeptides, would seem to comply with inhibition of the early inflammatory and nociceptive response to injury. Thus, GAL has been demonstrated to mitigate the proinflammatory and nociceptive effects of SP [36, 56, 68, 164]. Similarly, the increased occurrence of VIP may be explained by its inhibitory effect on immune cells expressing pro-inflammatoy cytokines [38]. The increased occurrence of NPY during this phase mostly seen around vessels should probably be attributed to its well-known vasoconstrictive actions, while its low occurrence in the early regenerative phase may prove to reflect an angiogenic role [173]. Notably, increased vasoconstriction leads to a relative hypoxia, which is known to enhance the tensile strength of the tendon by switching production of collagen from type III to type I [138].

Over all, it seems that the early remodeling phase after tendon injury is characterised by an increased expression of GAL, VIP and NPY, all of which are known to modulate the effects of SP and CGRP. It may prove that this modulation is required to end the nociceptive, inflammatory and regenerative processes, thereby permitting entry to and maintenance of the remodeling phase. On the whole, it seems that the temporal expression of the different neuropeptides studied complies with specific actions in injury, pain and regeneration. Whether neuropeptides mainly act directly on the target tissues or indirectly by regulating the release of cytokines, growth factors and chemokines from immune cells is unclear. Given the latter mechanisms it still remains to clarify whether the effects on these cells pertain to chemotaxis, differentiation and/or function.

5.2.4 Bilateral response

Analysis of the intact contra-lateral tendons (controls), failed to demonstrate immunoreactivity to the neuropeptides within the proper tendon tissue at any time point. However, the surrounding paratenon and loose connective tissue exhibited a clear immunoreactivity to all neuropeptides tested, which notably changed over time. The temporal changes in immunoreactivity on the intact side appeared similar to those seen on the injured side, although less pronounced. This may reflect that neuronal responses to injury are mediated partly by central pathways. This observation is supported by previous studies [14, 15] on contralateral neuropeptidergic and nociceptive responses to injury. As early as 1974 Hirsch, in a thesis on tensile properties during tendon healing, noted that during healing also the contralateral tendon to the injured side exhibited increased tensile strength [59]. This notion may be assumed to reflect trophic effects of a bilateral neuronal response to injury, possibly mediated through central pathways.

The involvement of both peripheral and central mechanisms in response to peripheral injury has been reported from studies of peripheral nerve lesions showing a similar pattern of neuropeptidergic change peripherally and centrally [66]. However, these reports focusing on changes in the spinal cord concluded that there was a decrease in SP and CGRP expression in the first four weeks after peripheral nerve lesion and, notably, an increase in GAL at week one [70, 126, 148]. Comparison to data from the present study on tendon rupture disclosed that the opposite seemed to have occurred at each time point, although it is questionable whether meaningful conclusions about neuronal mechanisms after injury can be drawn by comparing a peripheral nerve lesion with a tendon rupture. Nonetheless, assuming that comparisons can be made, the increase in SP and CGRP at the

rupture site of the tendons during the first weeks as well as the decrease in GAL seem to indicate that there is an inverse relationship between the neuropeptidergic expression centrally and peripherally. Hypothetically, the observations may reflect that there is a synchronised expression of neuropeptides centrally and peripherally in response to peripheral injury.

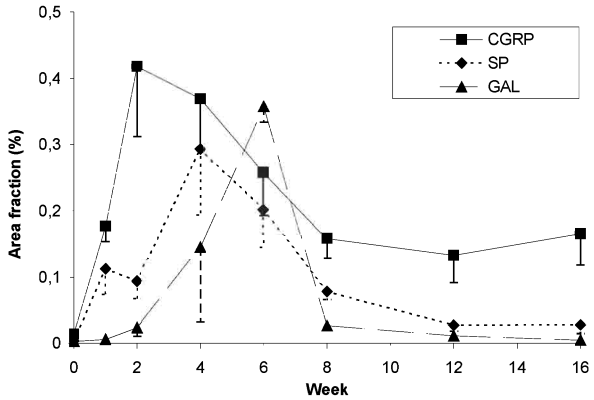


Figure 15. Area occupied by nerve fibres (%) immunoreactive to SP, CGRP and GAL in relation to total area, in the mid third of the tendon, over 16 weeks post rupture (mean±s.e.m.).

5.3 NOCICEPTION

After injury, pain and early tissue repair seem to be closely linked to each other suggesting a synchronized regulation. However, little is known about the expression of specific neuronal mediators in relation to nociception during tissue repair. In the present investigation of ruptured Achilles tendons, sensory neuropeptides and nociception were analysed in two separate experimental groups. In one group, the occurrence of SP, CGRP and GAL at different time points during tendon healing was assessed by semi-quantitative immunohistochemistry (Table 7). In the other group, nociception was analysed by measuring the hindpaw withdrawal latencies (HWL) to thermal and mechanical stimuli (Fig. 16). The results from the injured leg were compared to those of the same leg before rupture (baseline). Decreased HWL is referred to as increased thermal / mechanical sensitivity (more pain) and increased HWL is referred to as decreased thermal / mechanical sensitivity (less pain).

5.3.1 Thermal stimulation

Increased expression of SP/CGRP during weeks 1-2 coincided with increased thermal sensitivity on the injured side (Fig. 15-16). Conversely, increased GAL expression during weeks 4-6 coincided with decreased thermal sensitivity (Fig. 15-16). Subsequently, during weeks 6-8, the expression of all three mediators decreased to almost normal levels, which was accompanied by a decrease in thermal sensitivity to baseline values. Although the analysis of neuropeptides and nociception was based on two separate groups of rats, the results appear to support a nociceptive role of SP and CGRP and an anti-nociceptive role of GAL. Thus, nociception to thermal stimulus was related over time to the peripheral expression of these partly counteracting neuropeptides.

5.3.2 Mechanical stimulation

No relationship between the expression of SP/CGRP and mechanical sensitivity could be demonstrated. However, the observed increase in GAL expression during weeks 4-6 was found to coincide with increased mechanical sensitivity, which is in contrast to the decreased thermal sensitivity observed during the same period (Fig. 15-16). The results suggest that SP and CGRP do not regulate mechanosensitivity in the periphery [81]. This, however, does not preclude an involvement of these mediators centrally. Whether the increased mechanical sensitivity observed between week four to six should be attributed to the increased expression of GAL in the periphery or a decreased expression centrally has yet to be determined. The latter explanation is supported by reports on decreased

GAL expression in the spinal cord six weeks after peripheral nerve lesion [148], which is known to increase mechanical nociception [163]. However, it is questionable whether meaningful conclusions about neuronal mechanisms after injury can be drawn by comparing a peripheral nerve lesion with a tendon rupture.

5.3.3 Rate of change in neuropeptide expression

The relationship observed between neuropeptide expression and nociception over time became even more evident when considering the rates of change (slope) in expression instead of the absolute values (Fig. 17). Thus, thermal sensitivity was closely related to the rate of change in SP and CGRP expression during weeks 1-4 and inversely related to the rate of change in GAL expression from week four to eight (Fig. 17). As for mechanical sensitivity, there was no relationship to the rate of change in SP-CGRP expression, but clearly so when considering the rate of change in GAL up to week six (Fig. 15). At week eight, the rate of change in GAL expression decreased without a concomitant decrease in mechanical sensitivity. The results suggest that the rate of change in peripheral GAL expression is related to both thermal (inversely) and mechanical (directly) sensitivity, whereas that of SP and CGRP only to thermal sensitivity. Admittedly, the relationship for each of the three neuropeptides was confined to certain time intervals of the whole experimental period. Most importantly, the analysis of individual neuropeptides does not elucidate the significance of their interactions in producing a net nociceptive effect at each time point. Therefore a combined approach was applied to obtain an estimate of the net nociceptive effect over the whole experimental period.

Given the well-known fact that CGRP potentiates the nociceptive effect of SP, the rates of change in expression of the two neuropeptides were added into one rate. This was considered to provide a more relevant variable to be related to nociception than each of the rates. Subsequently, the rate of change in GAL expression representing anti-nociception was calculated and subtracted from that of SP plus CGRP. The resultant variable obtained by combining the three rates (SP+CGRP-GAL) was then related to the results of nociceptive tests. As can be seen in (Fig. 17B) the combined rate of change in neuropeptide expression was strongly related to thermal nociception over time. However, no such relationship could be demonstrated for mechanical nociception. Reports showing that CGRP potentiates [157, 162] and GAL inhibits the nociceptive effect of SP [16, 36, 164] seem to comply with our findings. Altogether, the results seem to indicate that the

nociceptive response after injury is determined by the combined effect of several mediators representing potentiating and inhibitory mechanisms. Moreover, the absolute tissue levels of neuropeptides are probably of less importance than the rate of change over time.

“THERE ARE NO GAINS WITHOUT PAINS”

Stevenson 1952

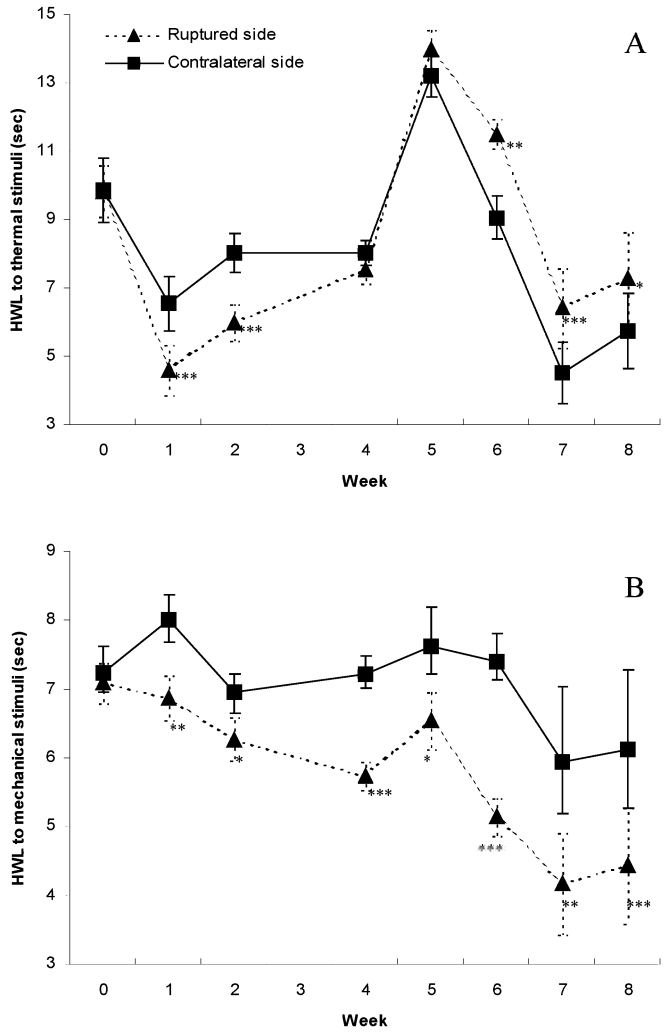


Figure 16. Time course of tendon rupture induced thermal (**A**) and mechanical (**B**) hyperalgesia measured by hindpaw withdrawal latencies (HWL). Each symbol represents the mean ± S.E.M. (n=12). The difference between groups was determined by two-way analysis of variance (ANOVA). Asterisks represent significant differences between the ruptured and contralateral side (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

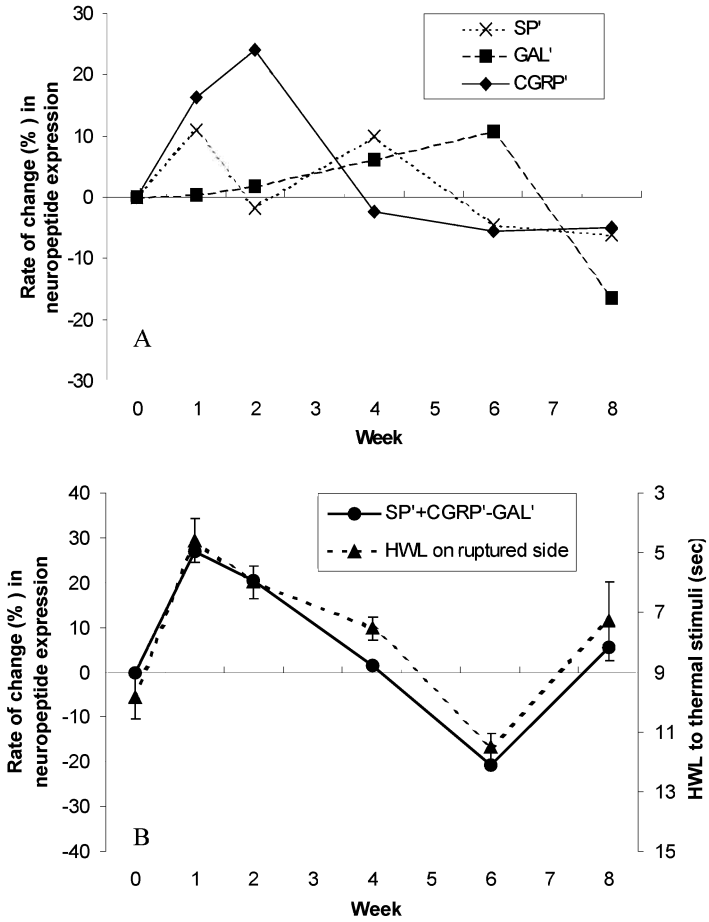


Figure 17. The individual rate of change in expression of SP, CGRP and GAL (**A**). The combined rate of change in expression of all three neuropeptides, according to SP plus CGRP minus GAL, shown together with thermal nociception (**B**) over 8 weeks post rupture.

6 SUMMARY AND CONCLUSIONS

The aim of the present thesis was to explore the occurrence and distribution of neuropeptides in periarticular tissues in the rat and to assess their expression during healing of tendon rupture. Hypothetically, the peripheral nervous system in the musculo-skeletal tissues plays an important role in the regulation of pain, inflammation, vasoactivity, and also tissue repair.

The normal occurrence of neuropeptides in tendons, ligaments and joint capsules was studied both quantitatively (RIA) and morphologically (IHC). RIA was applied to screen for and quantify three main classes of neuropeptides: autonomic, sensory and opioid mediators. The analysis disclosed that autonomic (NPY, VIP), sensory (SP, CGRP, GAL), opioid (MEAP) and opioidlike (N/OFQ) peptides were present in all tissues analysed, except for DYN B and, notably, SP in ligaments. The concentration of the sympathetic peptide NPY compared to the parasympathetic peptide VIP was 8-times higher in ligaments and than in tendons and capsules. The differences noted may reflect a higher sympathetic tonus, which may be of importance for blood-flow, and, presumably, also for healing capacity. The concentrations of the sensory peptides SP and CGRP were highest in tendons and lowest in ligaments, whereas the sensory modulatory peptide GAL essentially exhibited the same levels in the different tissues. The higher concentration of SP and CGRP in tendons and capsules compared to ligaments under normal conditions may reflect a greater susceptibility to pain and inflammation. This may explain why pain syndromes of tendons and capsules are more common than those of ligaments. Notably, both MEAP and N/OFQ exhibited lower concentrations in tendons and capsules than in ligaments. These findings would seem to comply with tendons and capsules being clinically more prone to painful disorders.

Altogether, the analysis of periarticular tissues clearly demonstrates the occurrence of autonomic, sensory and opioid peptides presumed to be involved in nociception, inflammation and vasoactivity. The relative levels in the different tissues may be assumed to represent the homeostasis of different functions and, possibly the susceptibility to external stress.

Further exploration of the tissues was performed by IHC to assess the neuronal occurrence of the neuropeptides detected by RIA and also to extend the investigation to

the other known neuropeptides representing sensory, autonomic and opioid mediators. The autonomic and sensory peptides were analysed in all three periarticular tissues. Additionally, opioid peptides and receptors were analyzed in the Achilles tendon. The IHC analysis essentially confirmed the RIA results. It disclosed the neuronal occurrence of autonomic (NA, NPY, VIP), sensory (SP, CGRP, NKA, GAL, SOM) and opioid (LE, ME, MEAP, MEAGL) mediators. In tendon, the delta opioid receptor was identified, but not receptors of kappa and mu type. The most conspicuous finding was the abundance of peptidergic nerve fibres in the surrounding tissues, i.e. the paratenon, epiligament and beneath the lining layer of the synovium as opposed to the proper structures, which, notably, were almost devoid of nerve fibres. This would seem to reflect that the neuronal regulation of tendons, ligaments and joint capsules depends on the innervation of the surrounding tissues.

The autonomic fibres were predominantly seen as networks around blood-vessels, while the sensory and opioid positive fibres including the opioid receptors were observed both around vessels and as free nerve endings. The vascular distribution of autonomic and sensory fibres supports a role in vasoregulation, possibly also inflammation. The occurrence of sensory peptides in free nerve endings suggests a role in nociception, whereas that of opioid peptides including their receptors presumably reflects an anti-nociceptive role.

Having established the normal occurrence and distribution of neuropeptides in periarticular tissues, further investigations aimed at analysing nerve regeneration and neuropeptide expression during healing of experimental Achilles tendon rupture. Tendon rupture in the mid-portion between musculo-tendinous junction and bony insertion was achieved by tearing with a blunt instrument. Tendon samples were retrieved at different time points (1-16 weeks) post-rupture. Nerve regeneration and nerve distribution was analysed by IHC including semi-quantification of neuronal markers for new and mature fibres; growth associated protein 43 (GAP) and protein gene product 9.5 (PGP), respectively. The semi-quantitative analysis focused on the rupture site of the proper tendinous tissue.

In the first week post rupture, corresponding to the inflammatory phase, there was increased neuronal immunoreactivity to PGP and GAP in the surrounding loose connective tissue. From one to six weeks post rupture (the regenerative phase), there was

a striking shift in neuronal immunoreactivity from the surrounding loose connective tissue to the proper tendinous tissue, notably, normally devoid of nerve fibres. The peak expression of GAP-immunoreactivity in the rupture site occurred between weeks two and six, while that of PGP was seen somewhat later, between weeks four and six. During weeks 8 to 16 post rupture (remodeling phase), the expression of the neuronal markers showed a significant decrease in the rupture site. The nerve fibres appeared to withdraw from the area. While GAP-immunoreactivity almost completely disappeared during this phase, that of PGP successively returned to normal in the paratenon and surrounding loose connective tissue. The observations during tendon healing clearly demonstrate the capability of the peripheral nervous system to respond and adapt to injury. This plasticity is characterized by nerve fibre ingrowth into the rupture site, a peak nerve fibre expression during the regenerative phase followed by nerve fibre withdrawal during the remodeling phase.

The experimental model employed appears to be highly appropriate and relevant for studies of the role of the peripheral nervous system in tissue repair. To our knowledge, no similar studies of repair are based on tissues normally devoid of a neuronal supply. The ingrowth and later withdrawal of nerve fibres in the proper Achilles tendon after rupture convincingly reflect that a neuronal supply is fundamental for tissue healing. Presumably, new nerve ingrowth is a prerequisite for delivery of neuronal mediators required for tissue repair.

The extensive nerve ingrowth observed during tendon healing prompted an investigation of the temporal expression of specific neuropeptides. This entailed morphological and semi-quantitative analyses of sensory (SP, CGRP), sensory modulating (GAL), and autonomic (NPY, VIP) neuropeptides by IHC. One week post-rupture (inflammatory phase), SP and CGRP fibres were predominantly located in blood vessel walls surrounded by inflammatory cells in the loose connective tissue. During week two to six (regenerative phase), the expression of SP and CGRP peaked. Notably, this peak occurred in the rupture site of proper tendon, seen as free sprouting nerve endings among fibroblasts in the healing tendinous tissue. Up to week four the occurrence of GAL, NPY and VIP was sparse. Subsequently, the expression increased to reach a peak at the end of the regenerative phase, around week six, followed by a successive decrease, also of SP and CGRP till the end of the experiment (week 16). The early appearance of SP and CGRP in tissues surrounding the tendon would seem to comply with a nociceptive and pro-

inflammatory role, whereas the later occurrence in the rupture site may prove to reflect a role in cell proliferation. The emergence of GAL, VIP and NPY, in the early remodeling phase, besides regulation of vasoactivity, may represent modulatory effects on SP and CGRP. It may prove that this modulation is required to switch from the nociceptive, inflammatory and regenerative processes to the remodeling phase.

The expression of sensory neuropeptides observed during tissue repair called for an investigation of the nociceptive response after injury. The investigation was based on two separate experimental groups analysed for sensory neuropeptides and hindpaw withdrawal latencies (HWL), respectively. The latter entailed both thermal and mechanical sensitivity at different time points during tendon healing, which were related to the temporal expression of SP, CGRP and GAL as assessed by semi-quantitative IHC.

Increased expression of SP/CGRP during weeks 1-2 coincided with increased thermal sensitivity on the injured side, whereas no relationship between the expression of SP/CGRP and mechanical sensitivity could be demonstrated. The observed increase in GAL expression during weeks 4-6, however, was found to coincide with increased mechanical sensitivity and notably, with decreased thermal sensitivity during the same period. Whether the increased mechanical sensitivity observed should be attributed to increased expression of GAL peripherally or decreased expression centrally has yet to be determined. The relationship between neuropeptide expression and nociception over time became even more evident when considering the rates of change (slope) in neuropeptide expression instead of the absolute values. However, the best relationship to nociception (thermal sensitivity) was found by combining the rates of change of the three neuropeptides, SP + CGRP – GAL. This was based on the assumption that CGRP potentiates the effect of SP, whereas GAL acts inhibitory. The finding indicates that nociception is regulated by the speed by which the expression of potentiating and inhibitory neuropeptides is altered, rather than the absolute tissue levels.

The relevance of the present study pertains to the possibility of intervening in different efferent mechanisms of the peripheral nervous system, which presumably are implicated in a wide variety of functions such as vasoactivity, nociception, inflammatory responses and regeneration. Specific neuronal intervention, however, presupposes that the innervation of the musculo-skeletal system according to specific mediators is clarified. From the present study, it seems obvious that periarticular tissues are supplied with a

complex peptidergic network, which presumably takes part in maintaining tissue homeostasis, but also by its plasticity, i.e. nerve ingrowth and temporal alteration in neuropeptide expression, is capable of responding to injury. Interestingly, ingrowth of new nerves seems to be a fundamental feature of tissue repair.

Whether neuronal mediators could be utilised in tissue engineering has so far not been explored. As for pharmacotherapy, it may prove that this can be targeted to specific neuronal mediators to enhance or inhibit their effects in painful and inflammatory conditions of the musculo-skeletal system.

7 ACKNOWLEDGEMENTS

The author wishes to express his sincerest gratitude to all involved in the journey leading to the completion of this thesis, for their support in so many ways. In particular I would like to thank:

Professor Andris Kreicbergs, head of the orthopedic laboratory, for your more than generous giving of time, care, professional genius and guidance of scientific education. For creating a most inspiring scientific climate I would like to give you my deepest appreciation.

Associate Professor Mahmood Ahmed, my supervisor, for your energetic and inspiring support in planning and getting me started, for teaching me the "golden" principles of immunohistochemistry and radioimmunoassay and for providing such a great atmosphere to our group.

Professor Per Renström, my co-supervisor and head of the sports unit, for your enthusiastic back up, for introducing me to sports medicine and the wonderful spirit that you form in the sports medicine group.

Professor Thomas Lundeberg, head of the RIA-laboratory, for your brilliant ideas contributing to new perspectives in this thesis.

Tony Li, Tashfeen Ahmad, my roommates, for your always-friendly help regarding no matter what, for the everlasting memories from our enjoyable conference trips and endless discussions, for the chocolate breaks that made it possible to work also late hours.

Daniel Bring, Jonas Bergström, Henrik Lundblad, Greta Åberg, Gustav Rydelius, Martin Andersson, my research colleagues in our lab for all fruitful discussions.

Ann-Britt Wikström, Anja Finn, Ingrid Vedin, Liss Garberg, Barbro Granberg and Anna-Lena Kastman, Annika Kling for excellent technical assistance but even more for creating a pleasant working atmosphere.

Former and present colleagues: Mariana Spetea, Joakim Carleson, Indre Bilevicute-Ljungar, Katarina Jansson, Lena Heffler.

Everyone working at the M3-building for making a nice working atmosphere.

Everyone in the section for sports medicine for creating such a great spirit.

Eva Hall, Anders Vigant, Philip Waldenström, Thomas Hach for excellent contributions in artwork. Gunnar Edman for professional statistical considerations.

All my friends for being there and sharing my thoughts and encouraging my ideas.

My mother and father for supporting me in all aspects of life.

Marie-Louise, for your endless love, understanding and support.

8 REFERENCES

1. Ahmed, M., Bjurholm, A., Schultzberg, M., Theodorsson, E. & Kreicbergs, A. Increased levels of substance P and calcitonin gene-related peptide in rat adjuvant arthritis. A combined immunohistochemical and radioimmunoassay analysis. *Arthritis Rheum* **38**, 699-709 (1995).
2. Ahmed, M. *et al.*. Capsaicin effects on substance P and CGRP in rat adjuvant arthritis. *Regul Pept* **55**, 85-102 (1995).
3. Ahmed, M., Bjurholm, A., Srinivasan, G.R., Theodorsson, E. & Kreicbergs, A. Extraction of neuropeptides from joint tissue for quantitation by radioimmunoassay. A study in the rat. *Peptides* **15**, 317-322 (1994).
4. Ahmed, M., Srinivasan, G.R., Theodorsson, E., Bjurholm, A. & Kreicbergs, A. Extraction and quantitation of neuropeptides in bone by radioimmunoassay. *Regul Pept* **51**, 179-188 (1994).
5. Alfredson, H. *et al.*. Glutamate NMDAR1 receptors localised to nerves in human Achilles tendons. Implications for treatment? *Knee Surg Sports Traumatol Arthrosc* **9**, 123-126 (2001).
6. Alfredson, H., Forsgren, S., Thorsen, K. & Lorentzon, R. In vivo microdialysis and immunohistochemical analyses of tendon tissue demonstrated high amounts of free glutamate and glutamate NMDAR1 receptors, but no signs of inflammation, in Jumper's knee. *J Orthop Res* **19**, 881-886 (2001).
7. Alfredson, H., Ljung, B.O., Thorsen, K. & Lorentzon, R. In vivo investigation of ECRB tendons with microdialysis technique--no signs of inflammation but high amounts of glutamate in tennis elbow. *Acta Orthop Scand* **71**, 475-479 (2000).
8. Alfredson, H., Thorsen, K. & Lorentzon, R. In situ microdialysis in tendon tissue: high levels of glutamate, but not prostaglandin E2 in chronic Achilles tendon pain. *Knee Surg Sports Traumatol Arthrosc* **7**, 378-381 (1999).
9. Archambault, J.M., Wiley, J.P. & Bray, R.C. Exercise loading of tendons and the development of overuse injuries. A review of current literature. *Sports Med* **20**, 77-89 (1995).
10. Aspenberg, P. & Forslund, C. Bone morphogenetic proteins and tendon repair. *Scand J Med Sci Sports* **10**, 372-375 (2000).
11. Åstrom, M. Chronic Achilles tendinopathy. A survey of surgical and histopathologic findings. *Clin Orthop* **316**, 151-164 (1995).
12. Barnard, P. Musculoskeletal disorders and workplace factors: a critical review of epidemiologic evidence for work-related musculoskeletal disorders of the neck, upper extremity, and low back. *US Department of Health and Human Services (National Institute of Occupational Safety and Health)* **A99**, 97-628 (1997).
13. Bayliss, W.M. On the origin from the spinal cord of the vasodilator fibres of the hind limb and on the nature of these fibres. *J Physiol (Lond)* **26**, 173-209 (1901).
14. Bileviciute, I., Lundeberg, T., Ekblom, A. & Theodorsson, E. Bilateral changes of substance P-, neurokinin A-, calcitonin gene-related peptide- and neuropeptide Y-like immunoreactivity in rat knee joint synovial fluid during acute monoarthritis. *Neurosci Lett* **153**, 37-40 (1993).

15. Bileviciute-Ljungar, I. & Lundeberg, T. Contralateral treatment with xylocaine reduces nociceptive behaviour in mononeuropathic rats. *Neuroreport* **11**, 291-295 (2000).
16. Blakeman, K.H. *et al.*. Mice over-expressing galanin have elevated heat nociceptive threshold. *Neuroreport* **12**, 423-425 (2001).
17. Bolden, D.A., Sternini, C. & Kruger, L. GAP-43 mRNA and calcitonin gene-related peptide mRNA expression in sensory neurons are increased following sympathectomy. *Brain Res Bull* **42**, 39-50 (1997).
18. Bowden, J.J. *et al.*. Direct observation of substance P-induced internalization of neurokinin 1 (NK1) receptors at sites of inflammation. *Proc Natl Acad Sci U S A* **91**, 8964-8968 (1994).
19. Brain, S.D. Sensory neuropeptides: their role in inflammation and wound healing. *Immunopharmacology* **37**, 133-152 (1997).
20. Brain, S.D., Williams, T.J., Tippins, J.R., Morris, H.R. & MacIntyre, I. Calcitonin gene-related peptide is a potent vasodilator. *Nature* **313**, 54-56 (1985).
21. Bray, R.C., Butterwick, D.J., Doschak, M.R. & Tyberg, J.V. Coloured microsphere assessment of blood flow to knee ligaments in adult rabbits: effects of injury. *J Orthop Res* **14**, 618-625 (1996).
22. Brodin, E., Gazelius, B., Panopoulos, P. & Olgart, L. Morphine inhibits substance P release from peripheral sensory nerve endings. *Acta Physiol Scand* **117**, 567-570 (1983).
23. Broome, C.S. & Miyan, J.A. Neuropeptide control of bone marrow neutrophil production. A key axis for neuroimmunomodulation. *Ann N Y Acad Sci* **917**, 424-434 (2000).
24. Brown, S.L. & Van Epps, D.E. Suppression of T lymphocyte chemotactic factor production by the opioid peptides beta-endorphin and met-enkephalin. *J Immunol* **134**, 3384-3390 (1985).
25. Cabot, P.J. Immune-derived opioids and peripheral antinociception. *Clin Exp Pharmacol Physiol* **28**, 230-232 (2001).
26. Calo', G., Guerrini, R., Rizzi, A., Salvadori, S. & Regoli, D. Pharmacology of nociceptin and its receptor: a novel therapeutic target. *Br J Pharmacol* **129**, 1261-1283 (2000).
27. Carlton, S.M. & Coggeshall, R.E. Immunohistochemical localization of enkephalin in peripheral sensory axons in the rat. *Neurosci Lett* **221**, 121-124 (1997).
28. Carlton, S.M., Zhou, S. & Coggeshall, R.E. Evidence for the interaction of glutamate and NK1 receptors in the periphery. *Brain Res* **790**, 160-169 (1998).
29. Carolan, E.J. & Casale, T.B. Effects of neuropeptides on neutrophil migration through noncellular and endothelial barriers. *J Allergy Clin Immunol* **92**, 589-598 (1993).
30. Chong, M.S. *et al.*. GAP-43 expression in primary sensory neurons following central axotomy. *J Neurosci* **14**, 4375-4384 (1994).
31. Christensson-Nylander, I., Nyberg, F., Ragnarsson, U. & Terenius, L. A general procedure for analysis of proenkephalin B derived opioid peptides. *Regul Pept* **11**, 65-76 (1985).
32. Clancy Jr, W.G. Achilles tendonitis in runners: a report of five cases. *Am J Sports Med* **4**, 46-57 (1976).

33. Coggeshall, R.E., Zhou, S. & Carlton, S.M. Opioid receptors on peripheral sensory axons. *Brain Res* **764**, 126-132 (1997).
34. Cook, J.L., Khan, K.M., Kiss, Z.S., Purdam, C.R. & Griffiths, L. Prospective imaging study of asymptomatic patellar tendinopathy in elite junior basketball players. *J Ultrasound Med* **19**, 473-479 (2000).
35. Cook, J.L., Khan, K.M., Kiss, Z.S., Purdam, C.R. & Griffiths, L. Patellar tendinopathy in junior basketball players: a controlled clinical and ultrasonographic study of 268 patellar tendons in players aged 14-18 years. *J Ultrasound Med* **19**, 473-479 (2000).
36. Cridland, R.A. & Henry, J.L. Effects of intrathecal administration of neuropeptides on a spinal nociceptive reflex in the rat: VIP, galanin, CGRP, TRH, somatostatin and angiotensin II. *Neuropeptides* **11**, 23-32 (1988).
37. Darland, T., Heinricher, M.M. & Grandy, D.K. Orphanin FQ/nociceptin: a role in pain and analgesia, but so much more. *Trends Neurosci* **21**, 215-221 (1998).
38. Delgado, M., Abad, C., Martinez, C., Leceta, J. & Gomariz, R.P. Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease. *Nat Med* **7**, 563-568 (2001).
39. Delgado, M., Abad, C., Martinez, C., Leceta, J. & Gomariz, R.P. VIP: a very important protein in arthritis. *Nat Med* **7**, 563-568 (2001).
40. Dhawan, B.N. *et al.*. International Union of Pharmacology. XII. Classification of opioid receptors. *Pharmacol Rev* **48**, 567-592 (1996).
41. Dickinson, T., Mitchell, R., Robberecht, P. & Fleetwood-Walker, S.M. The role of VIP/PACAP receptor subtypes in spinal somatosensory processing in rats with an experimental peripheral mononeuropathy. *Neuropharmacology* **38**, 167-180 (1999).
42. Dores, R.M., McDonald, L.K., Steveson, T.C. & Sei, C.A. The molecular evolution of neuropeptides: prospects for the '90s. *Brain Behav Evol* **36**, 80-99 (1990).
43. Ekblad, E., Ekman, R., Hakanson, R. & Sundler, F. Projections of peptide-containing neurons in rat colon. *Neuroscience* **27**, 655-674 (1988).
44. Elhassan, A.M., Lindgren, J.U., Hultenby, K., Bergstrom, J. & Adem, A. Methionine-enkephalin in bone and joint tissues. *J Bone Miner Res* **13**, 88-95 (1998).
45. Florez, J. & Mediavilla, A. Respiratory and cardiovascular effects of met-enkephalin applied to the ventral surface of the brain stem. *Brain Res* **138**, 585-900 (1977).
46. Frey, D., Laux, T., Xu, L., Schneider, C. & Caroni, P. Shared and unique roles of CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomical plasticity. *J Cell Biol* **149**, 1443-1454 (2000).
47. Gagliardini, V., Dusart, I. & Fankhauser, C. Absence of GAP-43 can protect neurons from death. *Molecular and Cellular Neurosciences* **16**, 27-33 (2000).
48. Gallar, J., Pozo, M.A., Rebollo, I. & Belmonte, C. Effects of capsaicin on corneal wound healing. *Invest Ophthalmol Vis Sci* **31**, 1968-1974 (1990).
49. Gause, A. Role of B cells in the pathogenesis of rheumatoid arthritis: potential implications for treatment. *BioDrugs* **15**, 73-79 (2001).
50. Gherardini, G. *et al.*. Venous ulcers: improved healing by iontophoretic administration of calcitonin gene-related peptide and vasoactive intestinal polypeptide. *Plast Reconstr Surg* **101**, 90-93 (1998).

51. Grant, D.S. & Zukowska, Z. Revascularization of ischemic tissues with SIKVAV and neuropeptide Y (NPY) [In Process Citation]. *Adv Exp Med Biol* **476**, 139-154 (2000).
52. Green, P.G., Basbaum, A.I. & Levine, J.D. Sensory neuropeptide interactions in the production of plasma extravasation in the rat. *Neuroscience* **50**, 745-749 (1992).
53. Grönblad, M., Korkala, O., Konttinen, Y.T., Kuokkanen, H. & Liesi, P. Immunoreactive neuropeptides in nerves in ligamentous tissue. An experimental neuroimmunohistochemical study. *Clin Orthop* 291-296 (1991).
54. Haegerstrand, A., Dalsgaard, C.J., Jonzon, B., Larsson, O. & Nilsson, J. Calcitonin gene-related peptide stimulates proliferation of human endothelial cells. *Proc Natl Acad Sci U S A* **87**, 3299-3303 (1990).
55. Hassan, A.H., Pzewlocki, R., Herz, A. & Stein, C. Dynorphin, a preferential ligand for kappa-opioid receptors, is present in nerve fibers and immune cells within inflamed tissue of the rat. *Neurosci Lett* **140**, 85-88 (1992).
56. Heppelmann, B., Just, S. & Pawlak, M. Galanin influences the mechanosensitivity of sensory endings in the rat knee joint. *European Journal of Neuroscience* **12**, 1567-1572 (2000).
57. Herz, A. Role of immune processes in peripheral opioid analgesia. *Adv Exp Med Biol* **373**, 193-199 (1995).
58. Hirota, N. *et al.*. Met-enkephalin and morphine but not dynorphin inhibit noxious stimuli-induced release of substance P from rabbit dorsal horn in situ. *Neuropharmacology* **24**, 567-570 (1985).
59. Hirsch, G. Tensile properties during tendon healing. *Acta ortopaedica scandinavica.* Supp. 153, (Munksgaard, Copenhagen, 1974).
60. Hong, Y. & Abbott, F.V. Peripheral opioid modulation of pain and inflammation in the formalin test. *Eur J Pharmacol* **277**, 21-28 (1995).
61. Hukkanen, M. *et al.*. Rapid proliferation of calcitonin gene-related peptide-immunoreactive nerves during healing of rat tibial fracture suggests neural involvement in bone growth and remodeling. *Neuroscience* **54**, 969-979 (1993).
62. Hökfelt, T. *et al.*. Neuropeptides--an overview. *Neuropharmacology* **39**, 1337-1356 (2000).
63. Hökfelt, T., Johansson, O., Ljungdahl, A., Lundberg, J.M. & Schultzberg, M. Peptidergic neurones. *Nature* **284**, 515-521 (1980).
64. Hökfelt, T., Kellerth, J.O., Nilsson, G. & Pernow, B. Substance p: localization in the central nervous system and in some primary sensory neurons. *Science* **190**, 889-890 (1975).
65. Hökfelt, T. *et al.*. Coexistence of peptides with classical neurotransmitters. *Experientia* **43**, 768-780 (1987).
66. Hökfelt, T., Zhang, X. & Wiesenfeld-Hallin, Z. Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* **17**, 22-30 (1994).
67. Jacobsen, K. Osteoarthrosis following insufficiency of the cruciate ligaments in man. A clinical study. *Acta Orthop Scand* **48**, 520-526 (1977).
68. Jancso, G., Santha, P., Horvath, V. & Pierau, F. Inhibitory neurogenic modulation of histamine-induced cutaneous plasma extravasation in the pigeon. *Regul Pept* **95**, 75-80 (2000).

69. Jarvinen, T.A. *et al.*. Work-related upper extremity musculoskeletal disorders. *Curr Opin Rheumatol* **13**, 150-155 (2001).
70. Jessell, T., Tsunoo, A., Kanazawa, I. & Otsuka, M. Substance P: depletion in the dorsal horn of rat spinal cord after section of the peripheral processes of primary sensory neurons. *Brain Res* **168**, 247-259 (1979).
71. Jourdan, D., Ardid, D. & Eschalier, A. *Pharmacol Res* **43**, 103-110 (2001).
72. Jozsa, L. & Kannus, P. Pathophysiology of tendon overuse injuries. In *Human Tendons*. 178-184 (Human Kinetics, Champaign, IL, US, 1997).
73. Kannus, P. Etiology and pathophysiology of chronic tendon disorders in sports. *Scand J Med Sci Sports* **7**, 78-85 (1997).
74. Kannus, P. & Jozsa, L. Histopathological changes preceding spontaneous rupture of a tendon. A controlled study of 891 patients. *J Bone Joint Surg Am* **73**, 1507-1525 (1991).
75. Karanth, S.S., Dhital, S., Springall, D.R. & Polak, J.M. Reinnervation and neuropeptides in mouse skin flaps. *J Auton Nerv Syst* **31**, 127-134 (1990).
76. Khalil, Z. & Helme, R. Sensory peptides as neuromodulators of wound healing in aged rats. *J Gerontol* **51**, B354-B361 (1996).
77. Khan, K.M., Cook, J.L., Maffulli, N. & Kannus, P. Where is the pain coming from in tendinopathy? It may be biochemical, not only structural, in origin. *Br J Sports Med* **34**, 81-83 (2000).
78. Kishimoto, S. The regeneration of substance P-containing nerve fibers in the process of burn wound healing in the guinea pig skin. *J Invest Dermatol* **83**, 219-223 (1984).
79. Ko, F. *et al.*. Toxic effects of capsaicin on keratinocytes and fibroblasts. *J Burn Care Rehabil* **19**, 409-413 (1998).
80. Kvist, M. Achilles tendon injuries in athletes. *Sports Med* **18**, 173-201 (1994).
81. Laird, J.M. Role of central and peripheral tachykinin NK1 receptors in capsaicin-induced pain and hyperalgesia in mice. *Pain* **90**, 97-103 (2001).
82. Le Bars, D., Gozariu, M. & Cadden, S.W. Evaluation de la douleur aigue chez l'animal d'experience. Premiere partie. *Ann Fr Anesth Reanim* **20**, 347-365 (2001).
83. Le Greves, P., Nyberg, F., Terenius, L. & Hokfelt, T. Calcitonin gene-related peptide is a potent inhibitor of substance P degradation. *Eur J Pharmacol* **115**, 309-311 (1985).
84. Leadbetter, W.B. Cell-matrix response in tendon injury. *Clin Sports Med* **11**, 533-578 (1992).
85. Lembeck, F., Donnerer, J. & Bartho, L. Inhibition of neurogenic vasodilation and plasma extravasation by substance P antagonists, somatostatin and [D-Met2, Pro5]enkephalinamide. *Eur J Pharmacol* **85**, 171-176 (1982).
86. Lembeck, F., Folkers, K. & Donnerer, J. Analgesic effect of antagonists of substance P. *Biochem Biophys Res Commun* **103**, 1318-1321 (1981).
87. Levenson, C.W. & Moore, J.B. Response of rat adrenal neuropeptide Y and tyrosine hydroxylase mRNA to acute stress is enhanced by long-term voluntary exercise. *Neurosci Lett* **242**, 177-179 (1998).
88. Lewis, R.V. *et al.*. An about 50,000-dalton protein in adrenal medulla: a common precursor of [Met]- and [Leu]enkephalin. *Science* **208**, 1459-1461 (1980).
89. Li, J., Ahmad, T., Spetea, M., Ahmed, M. & Kreicbergs, A. Bone reinnervation after fracture: a study in the rat. *J Bone Miner Res* **16**, 1505-1510 (2001).

90. Li, Q., Johansson, H. & Grimelius, L. Innervation of human adrenal gland and adrenal cortical lesions. *Virchows Arch* **435**, 580-589 (1999).
91. Liu, X., Andre, D. & Puizillout, J.J. Substance P post-synaptically potentiates glutamate-induced currents in dorsal vagal neurons. *Brain Res* **804**, 95-104 (1998).
92. Ljung, B.O., Forsgren, S. & Friden, J. Sympathetic and sensory innervations are heterogeneously distributed in relation to the blood vessels at the extensor carpi radialis brevis muscle origin of man. *Cells Tissues Organs* **165**, 45-54 (1999).
93. Lowry, O., Rosebrough, N., Farr, A.L. & Randall, R.J. Protein measurement with the folin reagent. *J Biol Chem* **193**, 265-275 (1951).
94. Lundberg, J.M., Anggard, A., Fahrenkrug, J., Hokfelt, T. & Mutt, V. Vasoactive intestinal polypeptide in cholinergic neurons of exocrine glands: functional significance of coexisting transmitters for vasodilation and secretion. *Proc Natl Acad Sci U S A* **77**, 1651-1655 (1980).
95. Lundberg, J.M. & Hokfelt, T. Multiple co-existence of peptides and classical transmitters in peripheral autonomic and sensory neurons--functional and pharmacological implications. *Prog Brain Res* **68**, 241-262 (1986).
96. Machelska, H. & Stein, C. Pain control by immune-derived opioids. *Clin Exp Pharmacol Physiol* **27**, 533-536 (2000).
97. Mafi, N., Lorentzon, R. & Alfredson, H. Superior short-term results with eccentric calf muscle training compared to concentric training in a randomized prospective multicenter study on patients with chronic Achilles tendinosis. *Knee Surg Sports Traumatol Arthrosc* **9**, 42-47 (2001).
98. Maggi, C.A. Tachykinins and calcitonin gene-related peptide (CGRP) as co-transmitters released from peripheral endings of sensory nerves. *Prog Neurobiol* **45**, 1-98 (1995).
99. Mani, L. & Gerr, F. Work-related upper extremity musculo-skeletal disorders. *Prim Care* **27**, 845-864 (2000).
100. McCarthy, K.D. & Partlow, L.M. Neuronal stimulation of (3H)thymidine incorporation by primary cultures of highly purified non-neuronal cells. *Brain Res* **114**, 415-426 (1976).
101. McDougall, J.J., Ferrell, W.R. & Bray, R.C. Spatial variation in sympathetic influences on the vasculature of the synovium and medial collateral ligament of the rabbit knee joint. *J Physiol (Lond)* **503**, 435-443 (1997).
102. McDougall, J.J., Hanesch, U., Pawlak, M. & Schmidt, R.F. Participation of NK1 receptors in nociceptin-induced modulation of rat knee joint mechanosensitivity. *Exp Brain Res* **137**, 249-253 (2001).
103. McDougall, J.J., Pawlak, M., Hanesch, U. & Schmidt, R.F. Peripheral modulation of rat knee joint afferent mechanosensitivity by nociceptin/orphanin FQ. *Neurosci Lett* **288**, 123-126 (2000).
104. Menkes, C.J. *et al.*. Substance P levels in the synovium and synovial fluid from patients with rheumatoid arthritis and osteoarthritis. *J Rheumatol* **20**, 714-717 (1993).
105. Meunier, J.C. *et al.*. Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature* **377**, 532-535 (1995).
106. Miao, F.J. Role of sympathetic postganglionic neurons in synovial plasma extravasation induced by bradykinin. *J Neurophysiol* **75**, 715-724 (1996).
107. Millan, M.J. Multiple opioid systems and pain. *Pain* **27**, 303-347 (1986).

108. Monneret, G., Pachot, A., Laroche, B., Picollet, J. & Bienvenu, J. Procalcitonin and calcitonin gene-related peptide decrease LPS-induced TNF production by human circulating blood cells. *Cytokine* **12**, 762-764 (2000).
109. Moore, R.H. & Dowling, D.A. Effects of enkephalins on perfusion pressure in isolated hindlimb preparations. *Life Sci* **31**, 1559-1566 (1982).
110. Movin, T. Tendon pathology in long-standing Achillodynia. Biopsy findings in 40 patients. *Acta Orthop Scand* **68**, 170-175 (1997).
111. Nakamura-Craig, M. & Smith, T.W. Substance P and peripheral inflammatory hyperalgesia. *Pain* **38**, 91-98 (1989).
112. Nathan, P.W. Pain and the sympathetic system. *J Auton Nerv Syst* **7**, 363-370 (1983).
113. Neely, F.G. Biomechanical risk factors for exercise-related lower limb injuries. *Sports Med* **26**, 395-413 (1998).
114. Nilsson, J., von Euler, A.M. & Dalsgaard, C.J. Stimulation of connective tissue cell growth by substance P and substance K. *Nature* **315**, 61-63 (1985).
115. Nozaki-Taguchi, N. & Yamamoto, T. Involvement of nitric oxide in peripheral antinociception mediated by kappa- and delta-opioid receptors. *Anesth Analg* **87**, 388-393 (1998).
116. Oku, R. *et al.*. Calcitonin gene-related peptide promotes mechanical nociception by potentiating release of substance P from the spinal dorsal horn in rats. *Brain Res* **403**, 350-354 (1987).
117. Onuoha, G.N. & Alpar, E.K. Levels of vasodilators (SP, CGRP) and vasoconstrictor (NPY) peptides in early human burns. *Eur J Clin Invest* **31**, 253-257 (2001).
118. Ottaway, C.A. & Greenberg, G.R. Interaction of vasoactive intestinal peptide with mouse lymphocytes: specific binding and the modulation of mitogen responses. *J Immunol* **132**, 417-423 (1984).
119. Piercey, M.F., Dobry, P.J., Schroeder, L.A. & Einspahr, F.J. Behavioral evidence that substance P may be a spinal cord sensory neurotransmitter. *Brain Res* **210**, 407-412 (1981).
120. Ploj, K., Roman, E., Gustavsson, L. & Nylander, I. Basal levels and alcohol-induced changes in nociceptin/orphanin FQ, dynorphin, and enkephalin levels in C57BL/6J mice. *Brain Res Bull* **53**, 219-226 (2000).
121. Puddu, G., Ippolito, E. & Postacchini, F. A classification of Achilles tendon disease. *Am J Sports Med* **4**, 145-150 (1976).
122. Quinlan, K.L. *et al.*. Neuropeptide regulation of human dermal microvascular endothelial cell ICAM-1 expression and function. *Am J Physiol* **275**, C1580-C1590 (1998).
123. Reinscheid, R.K. *et al.*. Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science* **270**, 792-794 (1995).
124. Renström, P. & Johnson, R.J. Overuse injuries in sports. A review. *Sports Med* **2**, 316-333 (1985).
125. Renström, P. & Kannus, P. Prevention of sports injuries. In *Sports medicine*. (ed Strauss, R.H.) 307-329 (Saunders, Philadelphia, US, 1991).
126. Robertson, B., Schulte, G., Elde, R. & Grant, G. Effects of sciatic nerve injuries on delta -opioid receptor and substance P immunoreactivities in the superficial dorsal horn of the rat. *European Journal of Pain : EJP* **3**, 115-129 (1999).

127. Said, S.I. In Pro-Inflammatory and Anti-Inflammatory Peptides. (ed Said, S.I.)(Marcel Dekker, 1998).
128. Saria, A., Lundberg, J.M., Skofitsch, G. & Lembeck, F. Vascular protein linkage in various tissue induced by substance P, capsaicin, bradykinin, serotonin, histamine and by antigen challenge. *Naunyn Schmiedebergs Arch Pharmacol* **324**, 212-218 (1983).
129. Schaffer, M., Beiter, T., Becker, H.D. & Hunt, T.K. Neuropeptides: mediators of inflammation and tissue repair? *Arch Surg* **133**, 1107-1116 (1998).
130. Schaible, H.G. On the role of tachykinins and calcitonin gene-related peptide in the spinal mechanisms of nociception and in the induction and maintenance of inflammation-evoked hyperexcitability in spinal cord neurons (with special reference to nociception in joints). *Prog Brain Res* **113**, 423-441 (1996).
131. Schaible, H.G. & Grubb, B.D. Afferent and spinal mechanisms of joint pain. *Pain* **55**, 5-54 (1993).
132. Simkin, P.A. Synovial perfusion in the human knee: a methodologic analysis. *Semin Arthritis Rheum* **25**, 56-66 (1995).
133. Snider, R.M. *et al.*. A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* **251**, 435-437 (1991).
134. Snijdelaar, D.G., Dirksen, R., Slappendel, R. & Crul, B.J. Substance P. *European Journal of Pain : EJP* **4**, 121-135 (2000).
135. Spetea, M. *et al.*. Affinity profiles of novel delta-receptor selective benzofuran derivatives of non-peptide opioids. *Neurochem Res* **23**, 1211-1216 (1998).
136. Stein, C. *et al.*. Local opioid receptors mediating antinociception in inflammation: endogenous ligands. *Prog Clin Biol Res* **328**, 425-427 (1990).
137. Stein, C., Hassan, A.H., Lehrberger, K., Giefing, J. & Yassouridis, A. Local analgesic effect of endogenous opioid peptides. *Lancet* **342**, 321-324 (1993).
138. Steinbrech, D.S. *et al.*. Fibroblast response to hypoxia: the relationship between angiogenesis and matrix regulation. *J Surg Res* **84**, 127-133 (1999).
139. Stelnicki, E.J. *et al.*. Nerve dependency in scarless fetal wound healing. *Plast Reconstr Surg* **105**, 140-147 (2000).
140. Sullo, A., Maffulli, N., Capasso, G. & Testa, V. The effects of prolonged peritendinous administration of PGE_1 to the rat Achilles tendon: a possible animal model of chronic Achilles tendinopathy. *Journal of Orthopaedic Science* **6**, 349-357 (2001).
141. Szekanecz, Z. Update on synovitis. *Current rheumatology reports* **3**, 53-63 (2001).
142. Szolcsßnyi, J., Helyes, Z., Oroszi, G., NTmeth, J. & PintTr, E. Release of somatostatin and its role in the mediation of the anti-inflammatory effect induced by antidromic stimulation of sensory fibres of rat sciatic nerve. *Br J Pharmacol* **123**, 936-942 (1998).
143. Taiwo, Y.O. & Levine, J.D. Kappa- and delta-opioids block sympathetically dependent hyperalgesia. *J Neurosci* **11**, 928-932 (1991).
144. Tatemoto, K. & Mutt, V. Chemical determination of polypeptide hormones. *Proc Natl Acad Sci U S A* **75**, 4115-4119 (1978).
145. Ueda, H. In vivo molecular signal transduction of peripheral mechanisms of pain. *Jpn J Pharmacol* **79**, 263-268 (1999).

146. Van der Zee, C.E. *et al.*. Expression of growth-associated protein B-50 (GAP43) in dorsal root ganglia and sciatic nerve during regenerative sprouting. *J Neurosci* **9**, 3505-3512 (1989).
147. Vasko, M.R., Campbell, W.B. & Waite, K.J. Prostaglandin E2 enhances bradykinin-stimulated release of neuropeptides from rat sensory neurons in culture. *J Neurosci* **14**, 4987-4997 (1994).
148. Villar, M.J. *et al.*. Neuropeptide expression in rat dorsal root ganglion cells and spinal cord after peripheral nerve injury with special reference to galanin. *Neuroscience* **33**, 587-604 (1989).
149. von During, M., Fricke, B. & Dahlmann, A. Topography and distribution of nerve fibers in the posterior longitudinal ligament of the rat: an immunocytochemical and electron-microscopical study. *Cell Tissue Res* **281**, 325-338 (1995).
150. von Euler, U.S. & Gaddum, J.H. An unidentified depressor substance in certain tissue extracts. *J Physiol (Lond)* **72**, 74-87 (1931).
151. Wagner, U. *et al.*. Galanin and somatostatin inhibition of substance P-induced airway mucus secretion in the rat. *Neuropeptides* **28**, 59-64 (1995).
152. Wallin, G., Torebjörk, H. & Hallin, R. Preliminary observations on the patho-physiology of hyperalgesia in the causalgic pain syndrome. In *Sensory function of the skin in primates*. (ed Zotterman, Y.) 489-499 (Pergamon, Oxford, UK, 1976).
153. Wang, D., Lundeberg, T. & Yu, L.C. Antinociceptive role of galanin in periaqueductal grey of rats with experimentally induced mononeuropathy. *Neuroscience* **96**, 767-771 (2000).
154. Wenk, H.N. & Honda, C.N. Immunohistochemical localization of delta opioid receptors in peripheral tissues. *J Comp Neurol* **408**, 567-579 (1999).
155. Wiesenfeld-Hallin, Z., Bartfai, T. & Hokfelt, T. Galanin in sensory neurons in the spinal cord. *Front Neuroendocrinol* **13**, 319-343 (1992).
156. Wiesenfeld-Hallin, Z. *et al.*. Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. *Neurosci Lett* **52**, 199-204 (1984).
157. Wiesenfeld-Hallin, Z. *et al.*. Immunoreactive calcitonin gene-related peptide and substance P coexist in sensory neurons to the spinal cord and interact in spinal behavioral responses of the rat. *Neurosci Lett* **52**, 199-204 (1984).
158. Willson, N.J. *et al.*. Effects of methadone hydrochloride on the growth of organotypic cerebellar cultures prepared from methadone-tolerant and control rats. *J Pharmacol Exp Ther* **199**, 368-374 (1976).
159. Winer, B.J. (ed) *Statistical Principles in Experimental Design*. (McGraw-Hill, New York, US, 1962).
160. Winnick, E. *Reuters health* (2001).
161. Woo, S.L.Y. *et al.*. Anatomy, biology, and biomechanics of tendon and ligament. In *Ortopaedic basic science. Biology and biomechanics of the musculoskeletal system*. (eds Buckwalter, J., Einhorn, T. & Simon, S.) 24, 581-616 (American Academy of Ortopaedic Surgeons, Rosemont, US, 2000).
162. Woolf, C. & Wiesenfeld-Hallin, Z. Substance P and calcitonin gene-related peptide synergistically modulate the gain of the nociceptive flexor withdrawal reflex in the rat. *Neurosci Lett* **66**, 226-230 (1986).

163. Xu, S., Zhang, Y., Lundeberg, T. & Yu, L. Effects of galanin on wide-dynamic range neuron activity in the spinal dorsal horn of rats with sciatic nerve ligation. *Regul Pept* **95**, 19-23 (2000).
164. Xu, X.J. *et al.*. Spantide II, a novel tachykinin antagonist, and galanin inhibit plasma extravasation induced by antidromic C-fiber stimulation in rat hindpaw. *Neuroscience* **42**, 731-737 (1991).
165. Yaksh, T.L. Substance P release from knee joint afferent terminals: modulation by opioids. *Brain Res* **458**, 319-324 (1988).
166. Yu, L.C., Hansson, P., Brodda-Jansen, G., Theodorsson, E. & Lundeberg, T. Intrathecal CGRP8-37-induced bilateral increase in hindpaw withdrawal latency in rats with unilateral inflammation. *Br J Pharmacol* **117**, 43-50 (1996).
167. Yu, L.C., Hansson, P. & Lundeberg, T. The calcitonin gene-related peptide antagonist CGRP8-37 increases the latency to withdrawal responses bilaterally in rats with unilateral experimental mononeuropathy, an effect reversed by naloxone. *Neuroscience* **71**, 523-531 (1996).
168. Yule, K.A. & White, S.R. Migration of 3T3 and lung fibroblasts in response to calcitonin gene-related peptide and bombesin. *Exp Lung Res* **25**, 261-273 (1999).
169. Zagon, I.S. & McLaughlin, P.J. Identification of opioid peptides regulating proliferation of neurons and glia in the developing nervous system. *Brain Res* **542**, 318-323 (1991).
170. Zamir, N., Weber, E., Palkovits, M. & Brownstein, M. Differential processing of prodynorphin and proenkephalin in specific regions of the rat brain. *Proc Natl Acad Sci U S A* **81**, 6886-6889 (1984).
171. Zhou, L., Zhang, Q., Stein, C. & Schafer, M. Contribution of opioid receptors on primary afferent versus sympathetic neurons to peripheral opioid analgesia. *J Pharmacol Exp Ther* **286**, 1000-1006 (1998).
172. Ziche, M. *et al.*. Substance P stimulates neovascularization in vivo and proliferation of cultured endothelial cells. *Microvasc Res* **40**, 264-278 (1990).
173. Zukowska-Grojec, Z. *et al.*. Neuropeptide Y: a novel angiogenic factor from the sympathetic nerves and endothelium. *Circ Res* **83**, 187-195 (1998).

9 APPENDIX

Table 6. Morphological assessment of neuropeptide occurrence in the Achilles tendon, medial collateral ligament and synovium of the knee joint 4 = abundant; 3 = frequent; 2 = moderate; 1= sporadic; 0 = absent; F = mostly free nerve fibres; VA = mostly perivascular nerve fibres; N/D = not determined; T = tendon proper; PT = Paratenon = paratenon; LCT = loose connective tissue; L = ligament proper; EL = epiligament; IL = intimal lining; SL = sublining

	TENDON			LIGAMENT		SYNOVIUM	
Peptides	**T**	**PT**	**LCT**	**L**	**EL**	**IL**	**SL**
Sensory							
SP	1	3	3	1	2	2	2
	F	F	VA	F	F	F	VA
CGRP	1	4	4	1	3	3	3
	F	F	VA	F	F	F	VA
NKA	1	2	2	1	1	1	2
	F	F	VA	F	F	F	VA
GAL	0	2	2	0	2	1	2
		F	VA		F	F	VA
SOM	0	2	2	0	2	1	2
		F	VA		F	F	VA
Autonomic							
NPY	1	2	4	1	3	1	3
	F	VA	VA	F	VA	F	VA
VIP	1	3	2	1	3	1	2
	F	F	VA	F	F	F	VA
TH	0	1	3	0	1	0	3
		VA	VA		VA		VA
Opioid							
LE	0	2	2	N/D	N/D	N/D	N/D
		F	VA				
ME	0	2	2	N/D	N/D	N/D	N/D
		F	VA				
MEAP	0	1	2	N/D	N/D	N/D	N/D
		F	VA				
MEAGLE	0	1	2	N/D	N/D	N/D	N/D
		F	VA				

Table 7. Significance of differences between levels of CGRP, above the diagonal, SP below the diagonal, and GAL in the table below, from week 0 (baseline) to week 16, as analysed by two-tailed Mann-Whitney's U-test. All differences are calculated as the earlier week minus the later, i.e. an increase in the level is represented by a minus sign. *p< .05, ** p< .01

SP \ CGRP	Week 0[1]	Week 1[1]	Week 2[1]	Week 4[2]	Week 6[3]	Week 8[1]	Week 12[2]	Week 16[4]
Week 0	-	-2.61**	-2.61**	-2.45*	-2.24*	-2.61**	-2.45*	-2.45*
Week 1	-2.61**	-	-2.19*	-2.45*	-1.94	-0.52	1.23	-0.49
Week 2	-2.61**	-0.10	-	0.00	-0.45	-2.19*	-1.96*	-1.96*
Week 4	-2.61**	-1.57	-1.78	-	-1.77	-2.45*	-2.31*	-2.02*
Week 6	-2.24*	-1.04	-1.64	-0.45	-	-2.24*	-2.12*	-1.06
Week 8	-2.61**	-0.52	-0.10	2.19*	-1.94	-	-0.49	-0.49
Week 12	-2.61**	-1.36	-1.57	-2.40*	2.24*	-1.98*	-	-0.58
Week 16	1.64	-1.64	-1.64	-2.24*	-1.96*	-1.34	-0.75	-

\ GAL	Week 0[1]	Week 1[1]	Week 2[1]	Week 4[1]	Week 6[3]	Week 8[1]	Week 12[1]	Week 16[4]
Week 0	-	-0.31	-0.94	-2,61**	-2,24*	-2,61**	-2,40*	-1,47
Week 1		-	-0,73	-2,19*	-2,24*	-2,40*	-1,57	-0,98
Week 2			-	-0,94	-2,24*	-0,52	-0,10	-0,49
Week 4				-	-1,34	-0,52	-1,57*	-1,96*
Week 6					-	-2,24*	-2,24*	-2,12*
Week 8						-	-1,36	-2,21*
Week 12							-	-1,23
Week 16								-

[1] n= 5 for GAL, CGRP and SP.
[2] n= 4 for CGRP and n= 5 for substance P.
[3] n= 3 for GAL, CGRP and SP.
[4] n= 4 for GAL, CGRP and n= 3 for substance P.