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NEURONAL MECHANISMS IN TENDON HEALING

EFFECTS OF MOBILIZATION AND IMMOBILIZATION

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Cover: Schematic drawing over an Achilles tendon rupture and the subsequent repair process, demonstrating mobilized healing (left column) and immobilized healing (right column). Illustration by Daniel Bring as inspired by artwork of Professor Aspenberg.

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To my family

As long as you put your mind to it… you have no limitations...

Mum and Dad

ABSTRACT

The incidence of tendon injuries is increasing. Tendon healing and modelling are frequently protracted, often leading to pain and impaired function so called overuse syndromes. Presumably, the sensory nervous system may contribute both to promotion of repair as well as to inhibition of healing and tissue modeling. Knowledge of the obstructive influence of systemic neuropathy, chronic inflammation and immobilization on repair and modeling as well as of how and when mobilization should be applied is vital in order to improve the healing process. This study in the rat was designed in order to further explore the local regulatory role of the sensory neuropeptides, SP and CGRP, in tendon repair and modeling.

Histological and immunohistochemical analysis over 16 weeks in a tendon rupture model detected a peak occurrence of neuronal SP/CGRP in the healing area at weeks 2-4 coinciding with a maximum increase in organized collagen. Quantitative assessments (RT-PCR) of gene expression at 1 and 2 weeks disclosed a conspicuous increase in the mRNA levels of SP- (NK_1) and CGRP-receptors (CRLR and RAMP 1) in the healing tendon at 2 weeks. The increased occurrence of sensory neuropeptides and up-regulation of their receptor expression during the maximum deposition of organized collagen suggest a regulatory role in tendon tissue proliferation. At 2 weeks a concurrent elevation in the expression of extracellular matrix (ECM) proteins (collagen type I and III, versican, decorin and biglycan), growth factors (BDNF, bFGF, IGF-1) and inflammatory mediators (COX 1, COX 2, HIF-1α) was seen.
The effects of increased physical activity on tendon healing at week 4 were assessed according to the diameter of organized collagen and the occurrence of SP/CGRP. Wheel running seemed to stimulate the rate of nerve retraction from the healing area and a subsequent decrease in the SP/CGRP occurrence. Concomitantly, an increased diameter of longitudinally organized collagen as well as an increased maturity of the repair tissue was observed.

Further investigation on the neuronal involvement during plaster-immobilized tendon healing established a decreased diameter of longitudinally organized collagen and a less mature healing area at 4 weeks post-rupture. This was paralleled with a marked decrease at week 2, in the expression of SP/CGRP-receptors to levels similar to intact controls. The findings indicate that immobilization reduces the tissue susceptibility to neuropeptide stimulation. Two weeks of immobilization also decreased the expression of ECM molecules, growth factors and inflammatory mediators, whereas these effects were not observed at week 1 - implying that a short period of immobilization does not seem detrimental to the healing process.

To investigate whether impaired healing seen in different neuropathic conditions may be due to reduced occurrence of sensory neuropeptides, capsaicin-denervated animals were assessed with respect to the development of biomechanical tissue properties during tendon healing. Lower levels of SP in the dorsal root ganglia (DRG) correlated with a decrease in transverse area, ultimate tensile strength and stress at failure in the healing Achilles tendon. Nociception, sensitivity to mechanical and thermal stimuli, after denervation correlated with the levels of SP in peripheral nerves, and was subsequently used as a surrogate for local SP occurrence. A high sensitivity to noxious stimuli at early time-points, possibly as a reflection of local SP concentrations, correlated with later improved biomechanical tissue properties, indicating that early SP occurrence is critical for the subsequent healing process.
High sensitivity to noxious stimuli preoperatively was correlated with high levels of SP and CGRP centrally as well as peripherally later during the healing process. Such findings imply that postoperative pain and also subsequent healing capacity, reflected by release of sensory neuropeptides, could be partially foreseen by preoperative assessments of pain sensitivity.
In a model of systemic chronic inflammation involving the Achilles tendon, the occurrence of sensory neuropeptides and inflammatory cells was restricted to the paratenon and bone tendinous junction, suggesting a role in paratenonitis and enthesitis. Neither nerves containing neuropeptides nor inflammatory cells were seen in the tendon proper. These findings imply that regulation of inflammation occurs in the tendon envelope and that there might be a connection to the development of tendinosis.

The results of this thesis demonstrate that the regulation of tendon repair and promotive effects of early mobilization seem closely related to a functionally intact and quickly responding peripheral sensory nervous system. The findings suggest that physical activity leading to mechanical loading of the healing tendon tissue is a prerequisite for the up-regulation of sensory neuropeptide receptors, increasing the tissue susceptibility to neuronal stimuli. Prolonged immobilization seems detrimental to the healing process. Dysregulation of the nervous system by systemic diseases such as neuropathy or chronic inflammation seems to impair tendon healing and modelling. In the future it might be possible to employ pharmacological and/or physical means to promote neuronal pathways and thereby stimulate tendon healing during normal as well as pathological conditions.

LIST OF PUBLICATIONS

I. Bring DK-I, Heidgren ML, Kreicbergs A, Ackermann PW. **Increase in sensory neuropeptides surrounding the Achilles tendon in rats with adjuvant arthritis.** *J Orthop Res.* 2005 Mar;23(2):294-301.

II. Bring DK-I, Kreicbergs A, Renstrom PA, Ackermann PW. **Physical activity modulates nerve plasticity and stimulates repair after Achilles tendon rupture.** *J Orthop Res.* 2007 Feb;25(2):164-72.

III. Bring DK-I, Reno C, Renstrom P, Salo P, Hart DA, Ackermann PW. **Joint immobilization reduces the expression of sensory neuropeptide receptors and impairs healing after tendon rupture in a rat model.** *J Orthop Res. 2009 Feb;27(2):274-280.*

IV. Bring DK-I, Reno C, Renstrom P, Salo P, Hart DA, Ackermann PW. **Prolonged immobilization compromises up-regulation of repair genes after tendon rupture in a rat model.** *Scand J Med Sci Sports. Accepted for publication.*

V. Bring DK-I, Paulson Kent, Renstrom P, Salo P, Hart DA, Ackermann PW. **Decreased substance P levels after denervation correlate with impaired tendon repair.** *Submitted to Wound Repair And Regeneration.*

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LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
CGRP	calcitonin gene-related peptide
CGRP-LI	calcitonin gene-related peptide-like immunoreactivity
COX 1	cyclooxygenase 1
COX 2	cyclooxygenase 2
CRLR	calcitonin receptor-like receptor
FCA	freund´s complete adjuvant
GAL	galanin
GAL-LI	galanin-like immunoreactivity
HIF-1α	hypoxia-inducible factor 1α
HWL	hind paw withdrawal latency
IGF-1	insulin-like growth factor 1
IHC	immunohistochemistry
IL-1β	interleukin-1β
iNOS	inducible nitric oxide synthase
NGF	nerve growth factor
NK_1	neurokinin-1 receptor
PBS	phosphate buffered saline
PGP-9.5	protein gene product -9.5
RAMP-1	receptor activity modifying protein -1
RIA	radioimmunoassay
RT-PCR	reverse transcriptase-polymerase chain reaction
SP	substance P
SP-LI	substance P-like immunoreactivity
TIMP-1	tissue inhibitor of metalloproteinase-1
TRPV1	transient receptor potential vanilloid 1-receptor
UTS	ultimate tensile strength

1 INTRODUCTION

1.1 BACKGROUND

Musculo-tendinous injuries are common and increasing in incidence both related to sports and work (1, 2). The overuse related injuries are predicted to affect 50 % of the working force in the US, according to the National Institute of Occupational Safety and Health. These so called repetitive strain injuries, the main symptom being pain, account for 60% of all reported occupational illnesses to an estimated yearly cost of 36-48 billion USD in direct worker's compensation and indirect costs.

Overall, the relationship between tendon overuse, pain and degeneration in tendinopathy is unclear. However, dysfunctional repair and modelling are generally considered to underlie the tendon pathology (3-5). Therefore more knowledge on the regulatory mechanisms of tendon repair is warranted.

Also in acute tendon ruptures degenerative changes have been found in 97% of the patients at the time of surgery, indicating that degeneration predisposes the tissue for rupture (6). Repair after tendon rupture is associated with a protracted healing process, prolonged periods of rehabilitation and sometimes a suboptimal functional outcome both after surgical and conservative treatment (7, 8). 40% of all tendon ruptures requiring surgery occur in the Achilles tendon (9). It is not uncommon that patients are unable to return to their previous level of physical activity following an Achilles tendon rupture (10).

During the past decade early mobilization and exercise have been shown to promote tissue repair after injury to the locomotor apparatus (11-13). Although, the tendon repair process and influence of early mobilization have been studied extensively, the underlying mechano-biological and cellular mechanisms still remain largely unknown. Recently, the peripheral nervous system has been demonstrated to play an important regulatory role in tissue repair and homeostasis (14-17). In tendon healing intense ingrowth of nerves expressing sensory neuropeptides, substance P and calcitonin gene-related peptide, has been demonstrated (18, 19). These neuronal mediators are known to be involved in regulating angiogenesis and tissue healing, partly by stimulating proliferation of endothelial cells and fibroblasts (20-22).

The aim of this thesis was thus to further explore the local regulatory role of the peripheral sensory nervous system in tendon repair and modeling.

1.2 TENDONS

The main function of a tendon is to transmit contractile forces from the muscles to the bones and thereby cause or resist movements around a joint. The Achilles tendon is proximally connected to the gastrocnemius and soleus muscles, while it distally attaches to the dorsal and superior surface of the tuber calcanei. The Achilles tendon is the strongest tendon in the body, and has been reported to withstand forces 12 times the bodyweight (23). The musculo-tendinous junction is irregular in shape, which increases the surface of the interface and creates a stronger connection (24). The insertion to bone, the osteotendinous junction, consists of four different layers; proper tendon tissue, fibrocartilage, mineralized fibrocartilage and bone. This transformation occurs over a distance of 1 mm (25). The collagenous fibers tend to meet the fibrocartilage border at a right angle, there hence being a change in the fiber direction shortly before it meets the cartilage. Achilles tendon ruptures usually occur in the midsubstance 3-4 cm from the calcaneal bone. Occasionally however the rupture is located at the bony attachment. The reason for the different failure modes being largely unknown. The structure of tendons allows for the storage of elastic energy. Studies have suggested that more than 30% of the required energy during jumping activities could be stored within the Achilles tendon itself (26).

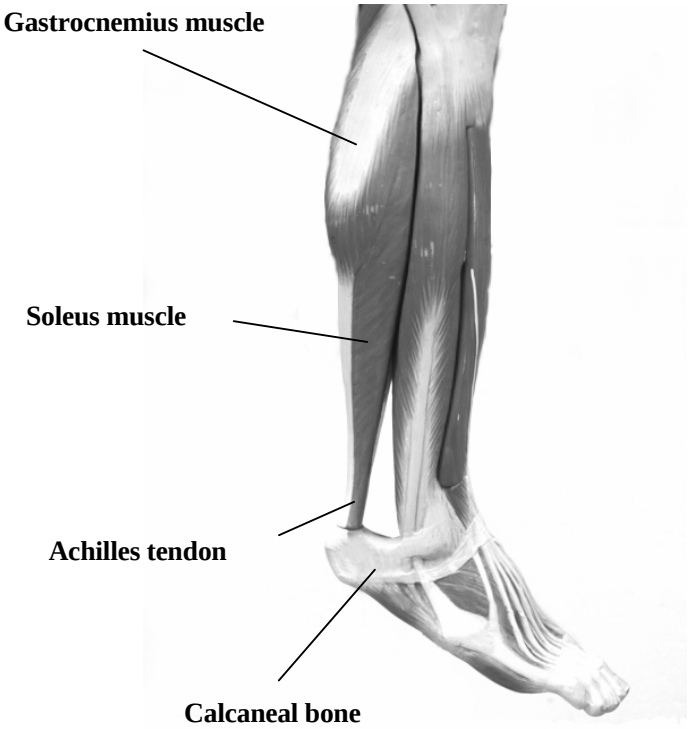

Figure 1. Lower leg (anatomical model)

The tendon can be divided into several different structural levels.

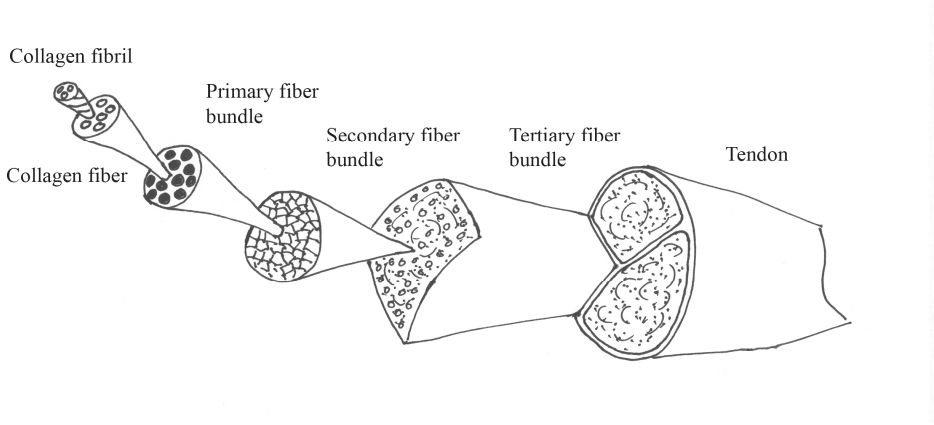

Figure 2. Tendon structure. As described by Józsa & Kannus (27).

The smallest structural unit of the tendon is tropocollagen, which forms microfibrils by fusing 5 molecules together. These subsequently unite in groups and form collagen fibrils, which are considered to be the basic load-bearing unit in the tendons and the structure around which the elongation occurs. Several fibrils join together surrounded by matrix to form collagen fibers. Groups of fibers are embedded in ground substance and surrounded by the endotenon, the so-called primary fiber bundles. The endotenon is interspersed with vessels and nerves and enfolds all larger structural levels. The primary fiber bundles are subsequently grouped together to form secondary and tertiary fiber bundles that finally comprise the tendon.

The main constituents of the tendons are the collagen fibers that are densely packed and aligned with the direction of loading. The healthy tendon consists of approximately 30% collagen, 2% elastin and 1-1.5% type III collagen embedded in the extracellular matrix consisting of about 67% water. The collagen fibers constitute 65-80% of the dry weight. While the collagen fibers withstand the load, the elastin fibers are implicated in the elastic properties of the tendon. The elastin molecule can elongate up to 70% of its length.

Fibroblasts are the most abundant cell type in tendons. They are spindle-like with long eosinophilic cytoplasmic extensions surrounding the primary fiber bundles. The fibroblasts communicate with each-other through gap junctions located at the extensions (28, 29). Their main function is to synthesize collagen, elastin, proteoglycans and glycoproteins (30).

The major collagen in adult uninjured tendons is collagen type I, which is synthesized within the fibroblasts. Following injury, collagen type III is rapidly synthesized exhibiting inferior biomechanical tissue properties compared to collagen type I. During the remodeling phase of healing, type III collagen is successively replaced by type I collagen.

The matrix (ground substance) is a gel mainly consisting of water (60-80%) in which glycoproteins and proteoglycans are important constituents. The ground substance provides both lubrication and spacing between fibers and allows gliding. The ground substance is also the medium through which nutrients and oxygen diffuse to the local cells. Three of the major proteoglycans are decorin, biglycan and versican. The structural glycoproteins decorin and biglycan resemble each-other and possibly arise from gene duplication. The function of decorin and biglycan has been described in fibrillogenesis (31), i.e. organization of the newly synthesized collagen type I molecules. Decorin and biglycan are important for the development of strength and other biomechanical properties in the mature tendon (31-33). Versican is important for the tendon to withstand compressive and tensile forces associated with loading and mobilization (34).

1.3 INNERVATION

The innervation of the Achilles tendon is derived from superficial overlying and deep tissue nerve trunks (n. Suralis) in the vicinity of the tendon (35). The majority of the nerves are situated near the musculo-tendinous junction. However, some fibers pass the musculo-tendinous junction within the septa of the endotenon. In addition, some branches also stretch from the more profoundly innervated paratenon via the epitenon to subsequently penetrate the tendon through the endotenon. The tendon proper is however under normal conditions predominantly devoid of nerve fibers.

The nerves innervating the tendon are either proprioceptive (Aα or Aβ), nociceptive (Aγ, Aδ and type C) or autonomic (type B). The myelinated and fast transmitting Aα- and Aβ-fibers terminate at type I-III receptors reacting to stretch, movement and loading respectively. The more slowly transmitting Aγ-, Aδ- and type C-fibers innervate type IVa receptors and react to different kinds of noxious stimuli. The type B

fibers are autonomic and terminate at type IVb receptors mainly localized in small vessels.

In addition to their afferent functions, it was already hypothesized in 1901 that the sensory nervous system is involved in the regulation of the peripheral tissues by the efferent release of neuronal transmitters (36).

1.4 NEUROPEPTIDES

The neuropeptide family of neuronal transmitters is active in both the central and peripheral nervous systems. They can be divided into three main subgroups; the sensory-, autonomic- and opioid-neuropeptides.

Classical neuronal transmitters (mono-amines, acetylcholine and amino acids) are either synthesized in the axonal soma, in nerve endings or are replenished by re-uptake from the synaptic cleft. However, the neuropeptides are only synthesized in the soma and thereafter transported in a central or peripheral direction (37). While the classical messengers are stored in small as well as in large diameter vesicles, the neuropeptides are only stored in outsized vesicles. They can be co-localized together with the classical transmitters as well as other neuropeptides (38). The peripherally expressed sensory neuropeptides are synthesized in the dorsal root ganglion, subsequently transported in vesicles along the microtubules and finally stored in the axon endings. The regulation of specific transmitter release is directed by the frequency of action potentials within the axon. Stimulation with a low frequency of action potentials releases small vesicles, while high frequencies are necessary in order to obtain release of the larger vesicles containing neuropeptides (39).

The sensory neuropeptides are implicated in nociception, pro-inflammation and healing. The main members of the group are substance P and calcitonin gene-related peptide, whereas galanin is usually defined as a sensory modulating peptide.

1.4.1 Substance P

This neurotransmitter, which consists of 11 amino acids and which has a molecular weight of 1348 g/mol, occurs in both the central and peripheral nervous systems. Substance P (SP) belongs to the tachykinin family of neuropeptides. It is known to be

ivolved in pain transmission (40), immunological reactions (41) and inflammation (42), as well as in healing mechanisms in different tissues (43).

Exogenous application of SP actually increases vascular permeability and extravasation (44). Endogenous SP release can also stimulate angiogenesis (45). The involvement of SP in tendon healing has also been suggested (19). The effects of SP are preferentially mediated by the NK_1- ($neurokinin_1$) receptor (46), comprising 7 membrane-spanning segments, which belong to the family of G-protein coupled cell membrane receptors.

1.4.2 Calcitonin gene-related peptide

This molecule, consisting of 37 amino acids and with a molecular weight of 3789 g/mol, functions as a neurotransmitter. It appears in both the central and peripheral nervous systems. Calcitonin gene-related peptide (CGRP) is the most frequently occurring neuropeptide in primary sensory neurons (47). It often co-exists with SP in primary sensory nerves (42, 47) and is involved in pain transmission (48), immunological reactions and inflammation (42) as well as in healing mechanisms in different tissues (18).

CGRP induce vasodilation. However this effect is greatly enhanced in combination with SP (49). Interestingly, a reduced neoangiogenesis and delayed healing process is reported following inhibition of CGRP during wound repair (50).

CGRP exerts its effect through the CRLR/RAMP-1 (calcitonin receptor-like receptor/receptor activity modifying protein-1) receptor complex, i.e. the CGRP 1-receptor (51, 52). As is the case with the SP-receptor, the CGRP-receptor belongs to the G-protein coupled receptor superfamily.

1.4.3 Galanin

This neuronal mediator, which is 29 amino acids long and which has a molecular weight of 3156 g/mol, functions as a modulatory messenger often co-expressed with SP as well as CGRP (47). It has been suggested to have anti-nociceptive functions when released in the vicinity of dorsal horn neurons (53). Interestingly, galanin (GAL) has been demonstrated to exert anti-inflammatory effects and to inhibit plasma extravasation and vasoconstriction (54). This messenger has also been suggested to have anti-proliferative effects in healing tissues (54). The neuronal expression of GAL in healing tendons first occurs during the end of the proliferative phase, i.e. week 4-6 post rupture, implying a role in inhibition of the ongoing proliferation (19).

1.5 HEALING

The healing of an injured tendon is a complex process. The mechanical properties of the tendons are decided by the amount as well as the localization of the different structural proteins. There is hence a need for a well-developed regulatory system.

The healing process can be divided into three different, albeit overlapping, phases: the 1) inflammatory; 2) proliferative; and 3) remodeling phases of healing.

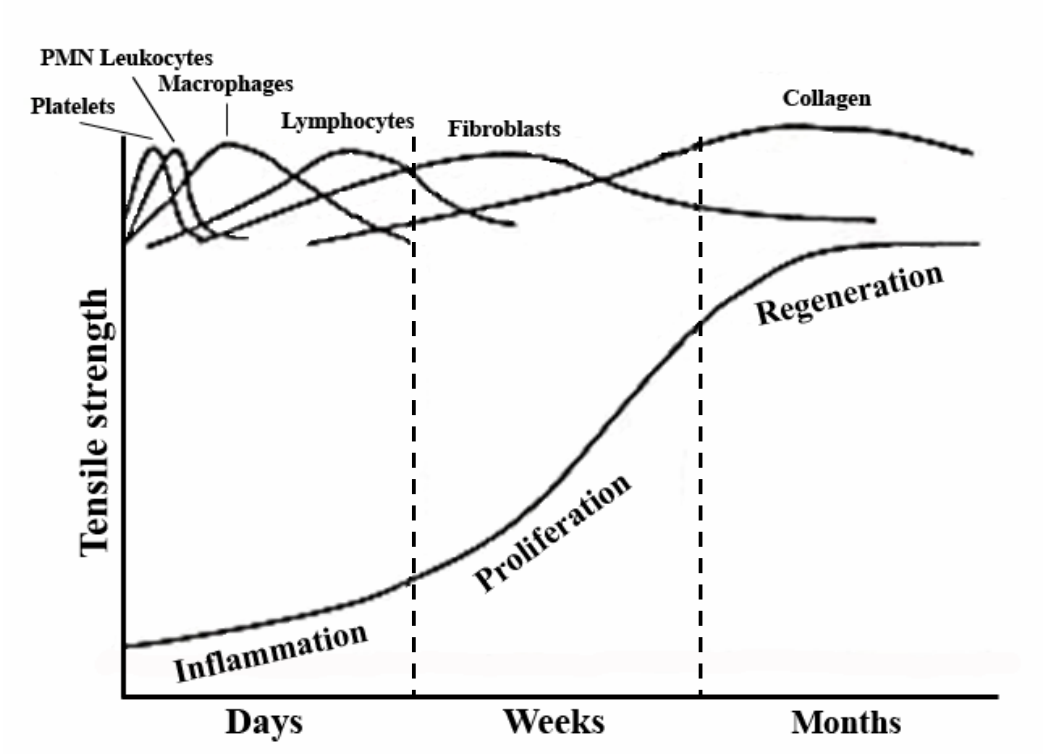

Figure 3. Extracellular matrix healing. As suggested by Gamble JG (55).

The inflammatory phase is 3-5 days long. The main task is haemostasis and the removal of damaged tissue and cells. After disruption of local blood vessels a clot is formed by fibrin and the aggregation of platelets. Stimulation with SP causes the formation of aggregates of platelets and leukocytes (42), while CGRP inhibits platelet aggregation (56). Moreover, the platelets express NK_1-receptors and an antibody-mediated blockage of this receptor inhibits the described aggregation and clot formation following activation (57, 58). Both SP and CGRP have additionally been reported to activate the arachidonate cascade in platelets, the effects of CGRP being mediated by the CRLR/RAMP 1-receptor complex (59). Whether these mechanisms are regulated by neuronal or possibly autocrine/paracrine neuropeptides is debated.

Clot formation is followed by the migration of polymorphonuclear (PMN) leukocytes into the healing site. SP and CGRP stimulate vascular aggregation of leukocytes (60,

61), while SP also increases their subsequent migration into tissues. Stimulation by SP and CGRP activates and degranulates neutrophils (62, 63). However, CGRP also seems able to inhibit the stimulatory effects of SP (64), and to reduce the accumulation of neutrophils (65). The second phase of healing, i.e. the proliferative phase, is initiated after 48-96 hours with the migration of macrophages into the healing area (3, 66). By adding SP to healing wounds an increased migration of macrophages into the injury site has been observed (67, 68). SP has also been implicated in the classical activation of macrophages and their subsequent release of pro-inflammatory cytokines (69). Interestingly, CGRP has inhibitory effects on macrophage activation (65).

The proliferative phase extends approximately between 48 hours to 6-8 weeks post-injury. The central processes are cellular proliferation and extracellular matrix synthesis (3). Macrophages are important regulators during this phase through their release of growth factors, chemotractants and proteolytic enzymes. There is also a migration of lymphocytes into the healing area (3), a process known to be stimulated by CGRP (70). SP has been reported to stimulate lymphocytes to release pro-inflammatory cytokines (69). Notably, both SP and CGRP increase fibroblast proliferation (20, 71-74). In addition to the endo- and epitenon fibroblasts migrating to the healing area, fibrocytes (bone marrow-derived leukocytes with fibroblast characteristics) have been proposed to migrate into healing wounds. As much as 30-50% of wound myofibroblasts have been suggested to originate from fibrocyte progenitors (75).

Type III collagen is the first collagen to be synthesized in order to achieve tissue integrity, although this synthesis is successively converted to type I collagen around 4 weeks post-injury (76). This conversion is at least partially driven by local hypoxia (77). During the initial two weeks fibroblasts are derived from the epitenon responsible for the major synthesis of collagen type I (78). Exogenous administration of SP in healing tendons has been shown to increase the amount of collagen and to improve the biomechanical tissue properties (15, 79). Angiogenesis in the healing area occurs in parallel with the described cellular proliferation and protein synthesis, supplying the tissue with necessary nutrients. It is noteworthy that the *in vitro* stimulation of endothelial cells with SP induces differentiation into capillary-like structures (80). It was also recently demonstrated that *in vivo* stimulation with SP injected in the paratenon could induce vascular growth into a healing tendon (20). Stimulation by CGRP induces an increased proliferation of endothelial cells (21), expressing the CGRP-receptor CRLR (81).

The remodeling phase is initiated successively around 6-8 weeks post-rupture. It is dominated by an increased organization of the structural proteins that align with the loading axis of the healing tendon. The amount of cells decreases in the healing area and protein synthesis diminishes. With increasing time from the initial injury the tissue becomes more organized and the biochemical composition slowly returns to a normal profile. Stimulation with exogenous SP increases the organization of collagen in healing tendons (20). The remodeling continues for more than a year after rupture. However, the tissue composition and biomechanical properties usually remain impaired compared to intact tendons. In fact a remaining reduction of approximately 30% of the biomechanical tissue properties has been suggested (3).

In addition to the neuronal transmitters, numerous growth factors, e.g. BDNF, NGF, bFGF and IGF-1, and pro-inflammatory mediators, e.g. HIF-1α, iNOS, COX 1 and COX 2, are involved in the different stages of healing. Several studies have reported multiple effects of the above mentioned growth factors and inflammatory mediators on the repair process from the initial inflammatory reaction through to scar remodeling (82-96) (Table 1).

Table 1. Growth factors and pro-inflammatory mediators

Local Healing Stimulatory Factors	
BDNF	Survival, growth and regeneration of sensory nerves
NGF	Survival, growth and regeneration of sensory nerves, angiogenesis, regulation of fibroblast and myofibroblasts
bFGF	Angiogenesis, fibroblast proliferation, tendon matrix synthesis, regeneration of nerves
IGF-1	Angiogenesis, fibroblast proliferation, tendon matrix synthesis, nerve elongation and branching
HIF-1α	Primary effector in the response to hypoxia after injury
iNOS	Vasodilation, vascular permeability, tendon matrix synthesis
COX 1	Stimulatory prostanoid effects during early healing
COX 2	Stimulatory prostanoid effects during early healing

1.6 MOBILIZATION AND IMMOBILIZATION

An increasing amount of data has demonstrated that mechanical stimulation or mobilization is of great importance for the properties of joint-associated connective tissue, including tendons and ligaments. Both intact and injured structures may be influenced by mechanical stimulation. The impact of immobilization, however, seems to be more pronounced than the effects of physical activity or exercise on the structure and function of tendons and ligaments.

Several studies in the literature have demonstrated the stimulatory effects of physical activity and mobilization on tendon and ligament tissue. Prolonged running yields a tendency towards an increasing cross-sectional area, an up-regulation of fibrils and an elevated oxygen uptake in the loaded tendons (97-99). Moreover, the net synthesis of collagen type I is increased during the first days after strenuous exercise (100). Interestingly, tendinopathic tendons seem to be more susceptible than normal tendons to eccentric exercise with a resulting elevated synthesis of collagen type I (101). In a study of old athletes there was a tendency towards a greater cross-sectional area of the Achilles tendon after long-term exercise compared with age-matched controls(102). Smith *et al.* have suggested, however, that tendons are more inclined to adjust with hypertrophy after physical activity during adolescence (103). The peri- and intratendinous blood flow is up-regulated during and after exercise (104), suggesting an increased local delivery of nutrients. This observation could be explained by an increased release of prostaglandin E2 and thromboxane B2 in the peri-tendinous tissue during exercise (105), a finding that could be in part an inflammatory response to mechanical loading.

The adverse effects of immobilization seem to be even more distinct than the stimulatory effects evident after training. After immobilization of knee joints for 9 weeks there was a reduced stiffness and an increased collagen turnover apparent in the collateral ligaments. No differences in collagen mass were detected, however (106). After immobilization for 15 weeks collagen fibers appeared thinner and less oriented in intact Achilles tendons (107). Immobilization has also been shown to cause an increased amount of cross-linking (108). A shorter period of immobilization (2 weeks) did not induce any differences in tendon size in an uninjured Achilles tendon (109). This was also the case when analyzing the cross-sectional area after 7 weeks of

immobilization, although the synthesis as well as the degradation of collagen was significantly elevated (110).

Considering healing of connective tissue, it is well known both clinically and experimentally that early mobilization following injuries is beneficial for the functional outcome (12, 111-113). When comparing early and intermediate mobilization versus immobilization after rupture of flexor tendons, the ultimate tensile strength load was progressively higher in the more mobilized groups (114). When comparing controlled passive mobilization with immobilization there was a stimulatory effect evident on the rate of tendon repair (13). In addition, early functional loading after rupture of Achilles tendons increases maximal tensile strength and energy absorption capacity during the early stages of healing (115).

The mechanisms underlying this mechano-biological transduction are largely unknown, but fibroblasts in culture proliferate as well as synthesize and secrete collagen in response to mechanical load and motion (116-118). Given the involvement of the peripheral sensory nervous system in several key steps in the healing process, the stimulatory effects evident after early mobilization could be mediated by the local release of sensory neuropeptides, although, this remains to be clarified. Notably, after rupture of medial collateral ligaments there were more abundant nerves expressing sensory neuropeptides in the healing area after suture of the ligament ends than after conservative treatment (119). Hypothetically, this could be interpreted as an increased neuronal ingrowth and local release of SP and CGRP during mechanically stimulated healing.

1.7 NEUROPATHY

Clinically as well as experimentally it is well known that different neuropathic conditions are associated with impaired healing processes. In diabetic patients a link between wound healing and the nervous system is apparent, as 30-50% of the patients experience neuropathy, which is the strongest predictor of diabetic wounds (120). Barker *et al* stated that all phases of wound healing are impaired in denervated tissue, the mechanisms being separate from those related to the underlying disease e.g. diabetes (17). Quattrini *et al* proposed that the peripheral nervous system is important for cellular development, vasoregulation, and leukocyte recruitment during wound

healing (121). Accordingly the finding of reduced foot skin innervation correlated with low inflammatory cell accumulation in diabetic foot ulcer healing (122). In addition, denervation leads to decreased collagen content and strength during wound healing (123). In addition, spontaneous tendon ruptures was reported in patients with a neuropathic condition (124) and a hampered tendon healing process was demonstrated in diabetic neuropathy, with reduced inflammation, decreased angiogenesis and proliferative actions (125).

Recently it was demonstrated that denervation causes a reduced angiogenesis, blood flow and ultimate tensile strength during medial collateral ligament healing (16). Stimulation of healing ligaments with exogenous SP and CGRP suggested that the main effect of SP/CGRP is related to orchestration of the repair process more than just acting as another growth factor (126). Grorud *et al* demonstrated that an induced neuropathy leading to impaired healing of ruptured ligaments was recovered after exogenous delivery of specific neuropeptides, thereby reversing the functional deficits in only 2 weeks (127).

These results clearly state the importance of the sensory nervous system in regulating tissue healing. However, whether the sensory neuropeptides SP and CGRP are also responsible for the impaired tendon healing evident in neuropathic patients remains to be elucidated.

1.8 INFLAMMATION

Inflammation is the normal reaction of the human organism to tissue damage. It can be induced by tissue overload or through the influence of microorganisms. Whether the reaction is elicited by damage or microbes, the sequence of events is essentially the same.

Acute inflammation is the body´s first line of defense and in the case of tissue damage the main task is to clear the injured area from cellular and extracellular matrix debris. The primary cellular component are PMN leukocytes (neutrophils) that phagocytose tissue debris and initiate subsequent chemotaxis of other inflammatory cells.

Chronic inflammation is characterized by the actions of macrophages and lymphocytes, which are also destructive. However, the inflammatory processes should most likely be looked upon as a continuous spectrum, with cells from the different phases being active in parallel with each-other.

Although the degenerative tendon proper does not express inflammatory characteristics as shown by normal levels of PGE_2 (128, 129), inflammation has been implicated in the development of tendon degeneration, or *tendinosis*. It is believed that excessive mechanical loading of tendons elicits inflammatory responses, degeneration and in some cases also ruptures (130). Exaggerated treadmill running induces degenerative changes in tendons (131). Repeated injection of PGE1 into the peri-tendinous tissues induces development of degenerative changes in the tendon proper (132). Fibroblast treatment with PGE2 suppresses proliferation and collagen synthesis in a dose-dependent manner (133, 134). Interestingly, mechanical loading of intact tendons and fibroblasts increases the local synthesis and release of PGE2 (105, 116). Hence the development of tendinosis could theoretically be linked to an inflammatory reaction, elicited by mechanical loading. A local inflammatory reaction might also educe neurogenic inflammation with the subsequent release of SP and CGRP, both well-known pro-inflammatory neuronal mediators. The levels of SP and CGRP have actually been shown to be increased in the paratenon in an overuse animal model (135). SP and CGRP have not only been demonstrated to increase the synthesis of COX 2 in the paratenon, but also to increase the synthesis of collagenases and IL-1β, paralleled with a depression of the synthesis of TIMP-1 (136). These are changes that could theoretically be linked to a degenerative process. However, the unequivocal involvement of sensory neuropeptides in the pathogenesis of tendon overuse injuries remains to be clarified in an appropriate animal model.

2 AIMS

This study was designed to examine the role of the peripheral sensory nervous system during promoted tendon healing (mobilization) and during compromised healing and modeling (immobilization, neuropathy and chronic inflammation).

The overall aim was to explore the local regulatory role of the sensory neuropeptides substance P (SP) and calcitonin gene-related peptide (CGRP) in connective tissue repair and modeling.

The specific aims were:

- To explore the occurrence of SP/CGRP and their receptor expression in relation to the expression of growth factors, pro-inflammatory mediators and extracellular matrix molecules during tendon healing (studies II-IV).

- To assess tendon healing and the occurrence of SP and CGRP, in relation to different levels of physical activity (studies II-III).

- To analyse effects of immobilization on the occurrence of SP/CGRP, their receptor expression, the expression of growth factors, pro-inflammatory and extracellular matrix molecules during tendon healing (studies II-IV).

- To study biomechanical tissue properties and nociception in relation to the occurrence of sensory neuropeptides during denervated tendon repair (study V).

- To investigate the occurrence of sensory neuropeptides and inflammatory cells in the tendon during chronic systemic inflammation (study I).

3 MATERIALS AND METHODS

3.1 ANIMALS

Three different strains of young adult rats (approximately 2 months old) were used in the experiments. The animal series included 256 rats. Study I included 40 female Lewis rats (160-220g), which develop an acute arthritis followed by a severe chronic inflammation after immunization with Freund´s complete adjuvant (FCA). This model was used in order to study neuronal involvement in local inflammatory processes affecting periarticular connective tissue. In order to study the effects of mobilization during tendon healing, 26 male spontaneously hypertensive (SH) rats (238-297g) were used in study II. The SH rats have a strong inclination for running and are often used in studies of the physiological effects of physical activity. The Sprague-Dawley (SD) rat is one of the most commonly used laboratory animals and a majority of our studies of tendon healing used this strain. Thus, 40 rats (180-200g) in study II; 65 rats (177-300g) in study III-IV and 85 rats (115-130g) in study V were used, giving a total of 190 male SD rats.

The animals were housed at 21°C in a 12:12h light:dark cycle with water and pellets *ad lib*, according to the Karolinska Institutet´s protocol. They were housed three-to-four per cage, except in study II in which the SH rats were housed separately. All experiments were approved by the local animal ethics committee. Twenty rats in total were lost during the experiments.

3.1.1 Induction and verification of arthritis

With the purpose of inducing a local hind limb inflammation, immunization with FCA was employed. This induces a cell-mediated immunological response and causes an acute inflammatory reaction that successively develops into a chronic arthritis involving the hind limbs. Hence 0.5 mg heat-inactivated *Mycobacterium butyricum* dispersed in 100µl paraffin oil was injected subcutaneously in the base of the tail (study I) (137). Control rats were only injected with the vehicle. After 16 days, all but two of the rats injected with *M. butyricum* developed acute inflammation of the hind legs as reflected by erythema, edema and increased temperature of the periarticular tissues.

To verify and follow the development of arthritis different tests were performed. The diameter of the hind paws was measured using calipers before injection of FCA and after development of subjective signs of acute inflammation (138). The severity of the

inflammatory condition was estimated through measurements of open field locomotor activity (139), see paper I for details. Finally, weight development was measured as a general marker of health (138). The control rats remained unaffected. In order to ensure chronicity of the inflammatory reaction the animals were sacrificed on day 35 (140). Two rats were excluded from the study since no arthritis developed in these animals (study I).

3.1.2 Denervation

In order to induce a specific sensory denervation with depletion of the central and peripheral levels of SP and CGRP, the capsaicin method was used (140) which is based on the use of 8-methyl-*N*-vanillyl-6-nonenamide, or *Capsaicin*. The molecule functions through interactions with the ion channel associated transient receptor potential vanilloid 1-receptor, TRPV1, located in primary sensory neurons (141). When activated with capsaicin the receptors initiate action potentials caused by the influx of calcium, followed by a release of SP and CGRP causing presynaptic depletion. This leads to neuronal exhaustion, reducing the experience of pain and inhibiting further release of SP and CGRP. The method retains motor function and hence keeps the animals mobile.

Denervation was performed under general anesthesia using Forene® inhalation (Abbot Scandinavia AB, Sweden) 4% in O_2, 2 l/min. One group was injected with capsaicin (Sigma, USA) 50mg/kg s.c in a vehicle of 10% EtOH and 10% Tween 80 in isotonic saline for four consecutive days (study V). Depending on the protocol the capsaicin treatment induces up to 50% depletion of sensory neuropeptides (SP and CGRP) in primary sensory neurons (140). The remaining rats served as denervation controls and were subsequently injected with corresponding amounts of vehicle. To reduce respiratory symptoms caused by the peripheral neurogenic inflammation, all animals were injected with Theophylline (ACO, Sweden) 15mg/kg 15 minutes prior to each injection of capsaicin or vehicle. To ensure a sufficient wash-out period to avoid potentially toxic effects of capsaicin (142), the injections were completed seven days prior to induction of Achilles tendon rupture (143, 144) (see below). 18 rats were lost during treatment with capsaicin (study V), a number similar to studies by others (145).

3.1.3 Tendon rupture

The tendon healing model employed appears to be ideal for studies of neuronal plasticity and expression in tissue healing. Normally the proper tendon tissue is devoid of nerve fibres, but following injury a significant neuronal ingrowth and later withdrawal is evident (18). Hence this model was used and the induction of rupture was conducted as described below (study II-V).

Surgery was performed under anesthesia with injection of a mixture of ¼ Midazolam® (5mg/ml, Pharma Hameln, Germany) and ¼ Hyponym® (Janssen Pharmaceutica, Belgium) in sterile water (Fresenius Kabi AB, Sweden) (2ml/kg bw, s.c). Subsequent surgery was performed under sterile conditions. A 1-cm longitudinal incision was made in the midline of the Achilles tendon of the right hind paw. The incision penetrated the skin and the crural fascia, exposing the Achilles and plantaris tendons. The Achilles tendon was ruptured with a blunt instrument, tearing the fibres apart approximately 0.5-cm from the calcaneal insertion. The Achilles tendon was left unsutured, while the plantaris tendon was left intact, potentially serving as an internal splint. The skin was closed with 5/0 non resorbable suture material (2x2, Ethilon®II, Ethicon, USA). Postoperatively the animals were returned to their respective cages.
One rat was lost in each of studies II and III-IV during surgery.

3.1.4 Immobilization

To reduce loading and mobilization of the healing area post-rupture a group of rats had their operated leg immobilized with a padded Plaster of Paris (study II-IV) (146-148). The cast was applied from the toes up to approximately 2.5-cm above knee height. The ankle and knee were fixed at 60º and 70-80º flexion, respectively. The outer layer of the cast was covered with black pepper for protection. Inspections were performed regularly in order to assess the need for reinforcements or replacements of the casts. The immobilization was maintained until sacrifice and dissection i.e. one-to four-weeks post-rupture.

3.1.5 Free mobilization

The majority of the animals were allowed free cage activity post-rupture (study II-V) and served as normal controls to the immobilized and the high activity-groups (wheel running), respectively (studies II-IV).

Some data from the longitudinal healing study (study II) were in part presented elsewhere (18, 19), but were herein reanalysed and reevaluated.

3.1.6 Wheel running

In order to enable and verify high levels of physical activity post-rupture running wheels were employed (149). In the activity response study (study II), a group of rats had unlimited access to a running wheel mounted in each cage. Two weeks prior to surgery the running wheel was connected to a computer measuring the physical activity. The data were automatically stored twice every hour for subsequent analysis (149). The mean running distance/day was 9183 (SD 3349) meters. The rats were back running in the wheel half a day post-surgery. The rats had access to the wheel during the duration of the experiment, i.e. four weeks.

3.1.7 Dissection and tissue collection

All dissections were performed under Pentobarbitalnatrium® (60mg/ml, Apoteket, Sweden) (60mg/kg bw, i.p) anesthesia. The animals were subsequently euthanized by decapitation and exsanguinated.

3.1.7.1 Histology

In order to optimize samples for histological analysis, intra-arterial perfusion with phosphate buffered saline (PBS) was performed followed by perfusion with Zamboni´s fixative (studies I-III) consisting of 4% paraformaldehyde in 0.2 mol/l Sörensen phosphate buffer, pH 7.2 and 0.2% picric acid. The right Achilles tendon (studies I-III) and the skin from the dorsal and plantar aspects of the right hind paw (study V) were dissected and immersed in Zamboni´s fixative for at least 2h at room temperature. The samples were thereafter repeatedly rinsed in PBS. The specimens were soaked in 20% sucrose in 0.1 mol/l Sörensen phosphate buffer, pH 7.2, overnight. This procedure was repeated until all visible Zamboni´s fixative was removed. The samples were finally sectioned longitudinally at 15 μm using a Leitz cryostat. Three frozen sections from different levels, i.e. ventral, middle and dorsal parts of the tendon, were chosen to represent the tissue and mounted together on slides (Super-Frost®Plus, Menzel-Gläser, Germany). The skin from the right hind paw was sectioned perpendicularly to its surface and three sections from the dorsal and plantar surfaces, respectively, were chosen to denote the paw skin and subsequently mounted together as described above.

3.1.7.2 Radioimmunoassay

Tissues for RIA analysis were collected by bilateral dissection and pooling of Achilles tendons (study I), and by dissection of the 2nd to 6th right dorsal root ganglia (*DRG*) and the spinal cord (*SC*) corresponding to this level, i.e. innervating the hind limb (study V). The DRGs from each animal were subsequently pooled as one sample. The sampled tissue were snap frozen on dry ice and subsequently kept at -70ºC until neuropeptide extraction and protein determination.

3.1.7.3 Reverse transcriptase – Polymerase chain reaction

Before collection of samples for mRNA analyses, an attempt was made to limit possible methodological errors. Hence all surfaces and instruments were thoroughly cleaned and treated with RNAse *AWAY*® (Invitrogen™, life technologies, USA) before and after the dissection of each animal. The right Achilles tendon (study III-IV) of each animal was dissected and immediately frozen in liquid nitrogen. The tissues were kept at -70ºC until extraction of total RNA.

3.1.7.4 Mechanical testing

Collection of the Achilles tendons for mechanical analyses was performed immediately following exsanguination (study V). The samples were dissected in one piece from the right hind limb and included the distal part of the gastrocnemius muscle and posterior part of the calcaneal bone. The samples were frozen in -20ºC after dissection and subsequently kept at -70ºC until tested.

3.2 HISTOLOGY

A Nikon light and epifluorescense microscope (Eclipse E800 Yokohama, Japan) was used to analyse the sections after staining. Pictures were taken with a video camera system (Nikon, digital camera DXM 1200, Japan) for documentation and later computer-analysed. The subjective analyses were confirmed using blinded- and/or semi-quantitative analysis.

3.2.1 Morphology

The morphological analyses were focused on the occurrence and distribution of tissue inflammation (study I) and the temporal tissue maturation apparent during healing,

corresponding to the presence of inflammatory cells, fibroblasts, blood vessels and the amount and orientation of collagen bundles (study II-III).

3.2.2 Immunohistochemistry

Immunohistochemistry was conducted in order to analyse the temporal and spatial occurrence of the sensory and sensory-modulating neuropeptides, and to verify their neuronal origin. Slides were initially rinsed for 10 minutes in PBS. Incubation with 10% normal goat serum in PBS for 30 minutes blocked non-specific binding.The sections were subsequently incubated with primary antisera for protein gene product 9.5 (PGP, 1:10000, Ultraclone, UK, (studies I-II)), a general nerve cell marker, substance P (SP, 1:10000, (studies I-II and V)), calcitonin gene-related peptide (CGRP, 1:10000, (studies I-II and V)) and galanin (GAL, 1:5000,(study I)) (Peninsula Laboratories, USA) overnight in a humid atmosphere at +8C°. Following incubation with the primary antisera the sections were rinsed in PBS (3x5min) and then incubated with biotinylated polyclonal goat anti-rabbit- antibodies (1:250, Vector Laboratories, USA) for 40 minutes at room temperature. Sections were finally incubated for 40 minutes either with Cy_3- (1:5000, Amersham Biosciences, USA, (study I)) or Cy_2-conjugated avidin (1:2000, Amersham International, UK, (studies II and V)).

In order to assure the specificity of the staining the following controls were performed: (1) Preadsorbtion of the primary antisera with excess of homologous antigen (50µg/mL SP, CGRP, Peninsula Laboratories, USA) for 12h at room temperature; (2) Omission of either the primary antiserum or the secondary biotinylated antibody.

The subjective analyses were focused on the neuronal occurrence and distribution of staining for PGP, SP, CGRP and GAL, respectively in the different groups.

3.2.3 Computerized analysis

Computer aided analysis was used in order to confirm and elaborate the subjective analyses. In study II the progression of healing was assessed by measuring both the smallest diameter of new organized, parallel collagen, consistently occurring at the healing area, as well as the total tendon diameter following H&E-staining. The measurements were performed using Easy Image Measurements 2000© software (Tekno Optik AB, Sweden). Furthermore, semi-quantitative image analysis was applied to assess the fractional area, that is, the area occupied by nerves immunoreactive to PGP (study II), SP (studies I-II and V), CGRP (studies I-II and V) and GAL (study I) in relation to the total area (18). In study I two different areas of

interest were defined, i.e. paratenon (PT) and bone tendinous junction (BTJ). A picture from each section and area of interest was taken of the field exhibiting the strongest immunofluorescence. In study II, two pictures per section were taken of the healing area exhibiting the highest occurrence of immunoreactive nerve fibres. In studies I and II, three sections from each tendon were analysed. Finally, in study V two pictures were taken from each of the six sections analysed per hind limb demonstrating the most pronounced SP-positive staining in free nerve endings and perivascular nerve fibres. Perivascular CGRP-positive nerve staining was studied accordingly. The pictures were analysed using Easy Image Analysis 2000© software (Tekno Optik AB, Sweden). The program takes the area as well as the intensity of the immunological staining into account. Earlier studies have determined the mean coefficient of variation for two observers to be 9.8% and the mean coefficient of variation for one observer to be 9.6% (18).

3.3 RADIOIMMUNOASSAY

These assays were performed in order to measure the concentrations of SP, CGRP (studies I and V) and GAL (study I), respectively, in tissue extracts from the Achilles tendon (study I), DRG and SC (study V). The RIA analysis is based on antibody reaction with a high specificity for short sequences of amino acids about 3-6 in length, at one or more sites in the peptide of interest, so-called epitopes. The samples are allowed to bind to the antibody, after which a competitive binding occurs through the addition of radiolabeled peptide. The presence of the peptide is thereafter assessed using a gamma counter (1470 Wizard™, Wallac, Sweden). The readings are subsequently automatically compared against a standard curve.

The samples were extracted in 2 mol/l acetic acid, sonicated (60s) and centrifuged at 3000g for 15 minutes. The supernatants were lyophilized and kept at -70°C for storage. Before analysis the samples were dissolved in either 2 ml barbital buffer for SP, 2 ml 0.05M phosphate buffer, pH 7.4, for CGRP and GAL (study I) or in 1ml of RIA buffer (Phoenix Pharmaceuticals, Inc., USA) (study V). The samples were subsequently diluted, see papers I or V for details. Two different RIA protocol were used:

In study I ***SP-LI*** was assessed using antiserum SP2 raised in a rabbit against bovine serum albumin (BSA)-conjugated rat SP. High performance liquid chromatography (HPLC) purified rat ^{125}I-tyrosine SP was used as radioligand and rat SP was used as

standard. The detection limit was 3pmol/l (IC50=111.3 pmol/l). Intra- and inter-assay coefficients of variation were 7% and 11%, respectively. ***CGRP-LI*** was analysed using antiserum CGRP8 raised in a rabbit against BSA-conjugated rat CGRP. HPLC purified rat ^{125}I-histidyl CGRP was used as radioligand and rat CGRP as standard. The detection limit of the assay for rat CGRP was 9 pmol/l (IC50=18.75 pmol/l). Intra- and interassay coefficients of variation were 8% and 14%, respectively. ***GAL-LI*** was measured using antiserum RatGala4 raised in a rabbit against BSA-conjugated rat galanin. HPLC purified rat ^{125}I-histidyl GAL was used as radioligand and rat GAL as standard. The detection limit was 5 pmol/l (IC50=104.5 pmol/l). Intra- and inter-assay coefficients of variation were 6% and 10%, respectively. The concentrations of SP, CGRP and GAL were expressed as pmol/g wet weight tissue.

In study V ***SP-*** and ***CGRP-LI*** was assessed using commercially available rabbit anti-rat SP and CGRP RIA kits (Phoenix Pharmaceuticals, Inc., USA). The assays were performed as recommended by the supplier. The detection limit was set to 8 pmol/l (IC50 = 166.6 pmol/l) for SP, and to 22 pmol/l (IC50 = 76.9 pmol/l) for CGRP. Intraassay coefficient of variation was 3.8% for SP and 5.5% for CGRP. The concentrations of SP and CGRP were expressed as pmol/g of total protein content as assessed using the Lowry assay (see below).

3.4 PROTEIN DETERMINATION

To be able to normalize the presence of sensory neuropeptides in the RIA analyses, the total protein content in the RIA tissue extracts was analysed using the Lowry assay (study V) (150).

3.5 REVERSE TRANSCRIPTASE - POLYMERASE CHAIN REACTION

The molecular biological analyses were conducted in order to accurately measure the cellular response during repair and subsequent to different levels of mobilization (studies III-IV).

In order to extract mRNA (151), the frozen tissue samples were powdered in a Braun Micro-dismembrator (B.Braun Biotech International, USA) at liquid nitrogen temperatures, at 2600 rpm for 30 seconds and immediately treated with Trizol™ (Gibco Life Technologies, USA). The samples were subsequently, treated with chloroform (EMD Chemicals Inc., Gibbstown, NJ, USA) and 70% EtOH. The

aqueous phase was carefully collected and thereafter processed according to the column fractionation step of the RNeasy® Total RNA Kit (Qiagen, Chatsworth, CA, USA). The mRNA was eluted using molecular grade water (VWR International), quantified using Sybr Green, and stored at -70ºC until RT-PCR was performed.

All samples were reverse transcribed at the same time. The subsequent RT-PCR was performed simultaneously for each individual molecule. The PCR amplification was kept within the range of exponential progression. Glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) was used as the housekeeping gene (152-155) as levels for this gene did not vary significantly in the different groups (Bring, Reno and Hart, unpublished data). No-RT samples were analysed to ensure genomic DNA contamination was below detection limits. cDNA from tissues with high mRNA levels for the genes of interest was used as positive control. The oligonucleotide primers used for the PCR reactions are listed in Table 2. Twenty microliters of each PCR reaction were subjected to electrophoresis in a 2% agarose gel (156) that were stained with ethidium bromide. Subsequent analyses were performed using a MasterScan Interpretive Densitometer (CSPI INC., Billerica, MA) and RFLP Scanalytics software. The densitometric values obtained were corrected for background and normalized against the housekeeping gene. The data were presented either directly or expressed as % of the control values.

Re-evaluations were performed and additional aliquots of some RNA samples were re-analysed for a subset of molecules. These re-evaluations were not significantly different from the reported results.

Table 2. Oligonucleotide Primers

A summary of the oligonucleotide primers used in the RT-PCR analysis. The primers were designed using the software Primer Designer© Version 2.0. All primer sets were evaluated using GenBank data. The amplicons were sequenced to verify primer specificity. The sequence for BDNF was adopted from Ming *et al* -99 (157).

Targets	Primer Sequence		Product Size (bp)	GeneBank Accession No.
	Forward Primer	Reverse Primer		
NK_1	ggcaacgtagtggtgatatg	tacagtcacgcagatgtggt	462	NM012667
CRLR	atcgcacacttcagttggac	ctcatgcgtgctgttgttac	423	L27487
RAMP1	ggctcatcatctcttcatgg	tagagcaggatgcaatgtgg	483	NM031645
Collagen I	cagacgggagtttcacctc	gacatgtagactctttgcggc	103	J04464
Collagen III	ctgccattgctggagttg	gcagccatcctctagaac	644	AJ005395
Biglycan	gatgacttcaaaggcctcca	tcaggctcccattctcaatc	499	NM_017087
Versican	cgagactggagctactgatgg	gcttcctcagttggagacagg	480	XM_215451
Decorin	atctccgagtggtgcagtg	tgtcgtggagtcgaagctc	297	NM_24129
BDNF	cgacgtccctggctgacactttt	agtaagggcccgaacatacgattgg	491	D10938
NGF	ggtgcatagcgtaatgtcca	ttgctcctgtgagtcctgtt	372	XM_227525
IGF-1	cacttcggcctcataatacc	cctgcacttcctctacttgt	452	NM178866
bFGF	aagcggctctactgcaag	ggatccgagtttatactgcccagt	324	X07285
COX 1	atgcgcctgcagtccttcaa	catcaccaccgaatgtgctg	289	S67721
COX 2	ccttcctcctgtggctgatgac	acacctctccaccgatgacc	332	U03389
iNOS	tgagtgaggagcaggttgagga	cgggagggaagggagaataggg	326	NM_012611
HIF-1α	ccatccatgtgaccatgagg	gcaagcatcctgtactgtcc	519	NM_024359

3.6 NOCICEPTIVE TESTS

In order to evaluate the functional effects of chemical sensory denervation, the reaction of animals to thermal and mechanical noxious stimuli was measured and followed over the course of the experiment (study V).

3.6.1 Thermal hind paw withdrawal latency

As described previously (158) the Plantar Test (Ugo Basile, Type 7371, Italy) was used to assess the hind paw withdrawal latency (HWL) to thermal stimulation. In summary, the plantar surface of the righthind paw was placed on a transparent surface over an infrared light beam creating a temperature of 52ºC. The time to hind paw withdrawal was measured in seconds and referred to as the HWL to thermal stimulation.

3.6.2 Mechanical hind paw withdrawal latency

In order to assess the HWL to mechanical stimulation the Randall Selitto Test (Ugo Basile, Type 7200, Italy) was used. The right hind paw was placed on an even and elevated surface while a wedge-shaped pusher administered an increasing load on the dorsal surface. The loading rate was 30 g/s. The latency required to evoke a withdrawal response was measured and expressed in seconds and was consequently referred to as the HWL to mechanical stimulation.

3.7 MECHANICAL TESTING

The measurements of the healing Achilles tendon's mechanical properties were performed in order to evaluate the effects of the sensory denervation on tissue repair (study V).

Before testing, the Achilles tendon samples were removed from the -70ºC freezer, and defrosted wrapped in tissue paper soaked in 1% saline. The samples were kept moist using an airflow system that provided 99% relative humidity at 37ºC. The proximal end of the tendon was gripped at the musculotendinous junction in a Peltier-effect cryoclamp (Tissue Grip, GRP-TE-2250N, EnduraTEC®, USA) mounted on an EnduraTEC 450N load cell (EnduraTEC®, USA) while the distal end was gripped in a custom-made clamp that held the calcaneal bone at an angle of 60º to allow the tendon fibres to be aligned in a perfect vertical position. The tissue was carefully aligned with the load axis of the actuator in the Bose ElectroForce® 3230 Test Instrument (Bose Corporation – ElectroForce Systems Group, USA). Great care was

taken in order to assure full tissue clearance of the machine. Tendon thickness and width were measured with a digital micrometer for calculation of cross-sectional area. Finally, ultimate tensile strength (UTS) was determined by failing the tendons under constant elongation at 20 mm/min. The data were automatically stored and saved for later analyses. Failure mode was determined by a visual and tactile inspection.

3.8 STATISTICS

Standard descriptive statistics were used to summarize the variables, i.e. means, standard deviation and percentage. A p value ≤ 0.05 was considered to be statistically significant. Due to the limited sample size (n=6-21) and thus low power in the statistical analyses it is recognized that only pronounced group differences could be statistically confirmed. The majority of the data were determined to be normally distributed and thus analysed using the ANOVA test for group differences. This was followed by the Tukey´s HSD test for post-hoc analyses. The Student´s t-test for unpaired observations was used where applicable. For the skewed variables, the Kruskal-Wallis test followed by the Mann-Whitney U test for independent observations were used.

The Pearson´s correlation coefficient was used to express the relationship between normally distributed variables, while the Kendall tau_b correlation coefficient was used for skewed variables.

4 TENDON HEALING

Accumulating data suggest that the peripheral sensory nervous system plays an active role in the regulation of connective tissue healing and modeling (14-17, 159). However, the integrated neuronal mechanisms involved in tendon repair and modeling are largely unknown. Whether the peripheral sensory nervous system may contribute both to promotion of repair, by early mobilization, and to inhibited healing and tissue modeling, caused by immobilization, neuropathy or chronic inflammation, is not yet clarified. The aim of this thesis was to explore the local regulatory role of the sensory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), in tendon tissue repair, and further to assess their modulatory role in mobilization vs. immobilization, neuropathy and chronic inflammation.

4.1 FREE MOBILIZATION (STUDIES II-IV)

The aim of this study was to assess the time-dependent expression and plasticity of the sensory neuropeptides, SP and CGRP, during healing of experimental tendon rupture. Tendon samples were collected at seven time-points over a period of 16 weeks after rupture and studied morphologically and quantitatively. Rats were allowed free cage activity and were considered to represent normal healing. Histology and immunohistochemistry including semi-quantitative measurements were used to grade healing and to assess the occurrence of the sensory neuropeptides during repair. Furthermore, mRNA expression analysis was applied to explore local neuropeptide receptors, growth factors and inflammatory mediators in early tendon repair. In addition the progression of healing was assessed by analysis of the mRNA expression for extracellular matrix molecules.

4.1.1 Sensory neuropeptides and collagen deposition

In normal, non-ruptured tendons abundant neuronal immunoreactivity was evident in the paratenon, whereas the tendon proper was virtually devoid of nerve fibres. After rupture, a bimodal temporal occurrence of nerve fibres immunoreactive to SP/CGRP was noted, indicating that the sensory neuropeptides exhibit time-dependent differential effects. The first increase was observed in the tendon envelope during the inflammatory phase (weeks 0-2), where SP/CGRP were localized around blood vessels, which may reflect pro-inflammatory effects. In addition to stimulating vasoregulation and

angiogenesis, SP has recently been demonstrated as an important messenger in early repair, where it was involved in mobilizing stem stromal-like cells (160). Perivascular cells in tendons have been demonstrated as a source for precursor cells (161), indicating that our observation of early perivascular SP may also be involved in recruiting tendon stem cells.

The second peak of SP/CGRP was seen in the ruptured, normally aneuronal tendon proper during the proliferative phase (weeks 2-4) (Fig. 4A). After week 4 the nerve fibres in the tendon proper successively withdrew to the tendon envelope.

During healing the amount of new organized collagen increased with time, the most dramatic change occurring between week 2 and 4 corresponding to the proliferative phase of healing (Fig. 4B). After 4 weeks of healing, the amount of organized collagen continued to increase, but at a slower pace, reaching a maximum at the end of the experiment, i.e. week 16.

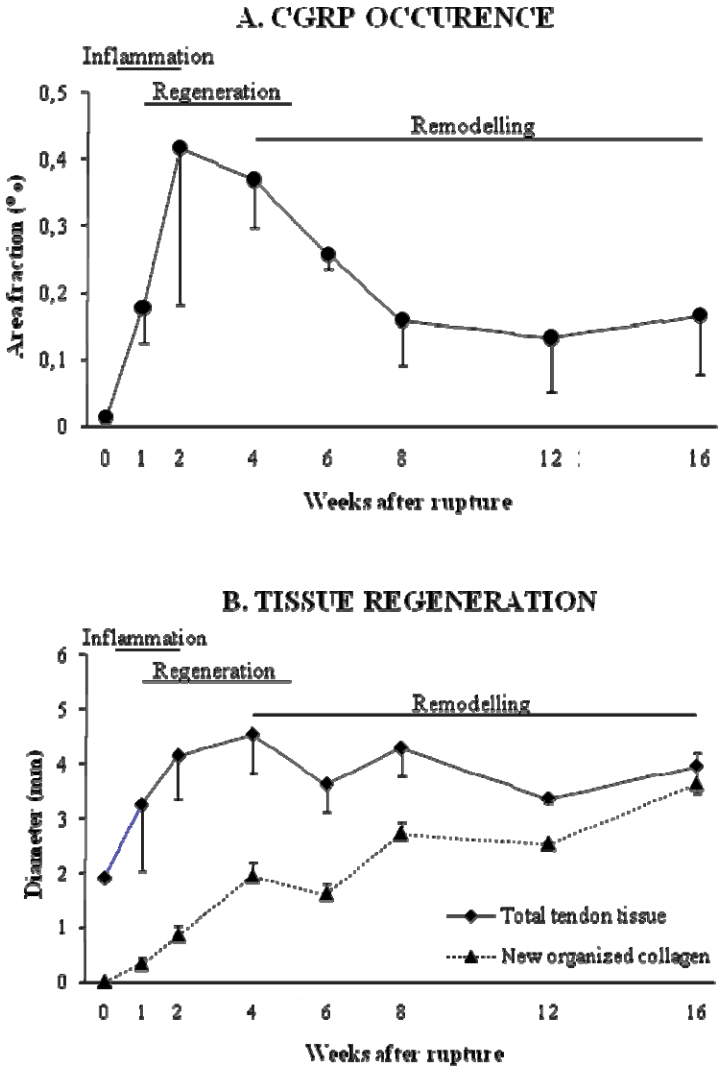

Figure 4. (A) Area occupied by nerve fibres (%) immuno-reactive to CGRP in relation to total area, of the tendon proper, in the Achilles tendon 1-16 weeks post-rupture and normal controls (week 0) (mean ± SD). (B) Mid tendon medio-lateral diameter of new organized collagen and of total tendon tissue in the healing area, in the Achilles tendon of rats 1-16 weeks post-rupture and normal controls (week 0) (mean ± SD).

The three consecutive, albeit overlapping, phases of tendon healing (the inflammatory, proliferative, and remodeling phases) exhibited clear differences in collagen formation and neuronal occurrence. The first signs of new organized collagen were noted during the inflammatory phase, week 2 post-rupture. Meanwhile, ingrowth of sensory nerve fibres from the paratenon into the tendon proper began, suggesting that inflammatory factors are involved in eliciting nerve and tissue regeneration. A peak in the presence of nerves was noted in the proliferative phase, coinciding with maximum increase in organized collagen between weeks 2 and 4. Thus the data from this analysis suggest that sensory neuropeptides are involved in the regulation of the tendon repair process.

In the ensuing remodeling phase, (that is, after 4 weeks), the successive withdrawal of sensory nerve fibres from the tendon proper was accompanied by a corresponding lower pace of new organized collagen formation. Again, this would seem to reflect a close relationship between the occurrence of sensory neuropeptides and the formation of organized collagen.

Since extensive nerve ingrowth and collagen formation occurred up to 4 weeks after rupture, subsequent analyses were conducted at 1 and 2 weeks to elucidate the underlying neuronal and molecular mechanisms involved in axonal growth and collagen deposition.

4.1.2 Sensory neuropeptide receptor expression

To establish the presence of local sensory neuropeptide receptors during tendon healing, the mRNA levels for receptors for SP (NK_1) and CGRP (CRLR and RAMP-1) were investigated. We thus demonstrated the occurrence of NK_1, CRLR and RAMP-1 in the healing and the intact control Achilles tendons by their mRNA expression in all samples analysed. The levels of mRNA for the SP- and CGRP-receptors were unchanged at 8 days post-rupture compared to before rupture (intact tendons) (Fig. 2A-C). Between 8 and 17 days there were significant increases in the presence of mRNA for NK_1, CRLR and RAMP-1 in the mobile healing group. At 17 days post-injury the expression of the receptors for the sensory neuropeptides were significantly increased. This elevated expression in sensory neuropeptide receptors occurs during the tissue proliferative phase, concomitantly with the observed increase in the presence of nerves, SP/CGRP peak occurrence and maximum collagen formation. Hence the detection of sensory neuropeptide receptors in the healing Achilles tendon denotes a functional

basis for ligand + receptor interaction in the regulation of tissue repair, e.g. through stimulation of collagen formation (20, 79). In tendons, the SP receptor NK_1 has been localized in blood vessel walls, nerve fibres and tenocytes (162-164), suggesting neuromodulatory effects at these sites. In fact, SP has been demonstrated to stimulate the proliferation of endothelial cells (angiogenesis) (20), fibroblasts (22, 165), and also stem stromal-like cells (160). The increased mRNA levels for SP and CGRP receptors after 17 days of tendon healing may therefore be assumed to indirectly represent enhanced tissue responsiveness to neuropeptides and a potential role in tissue proliferative effects.

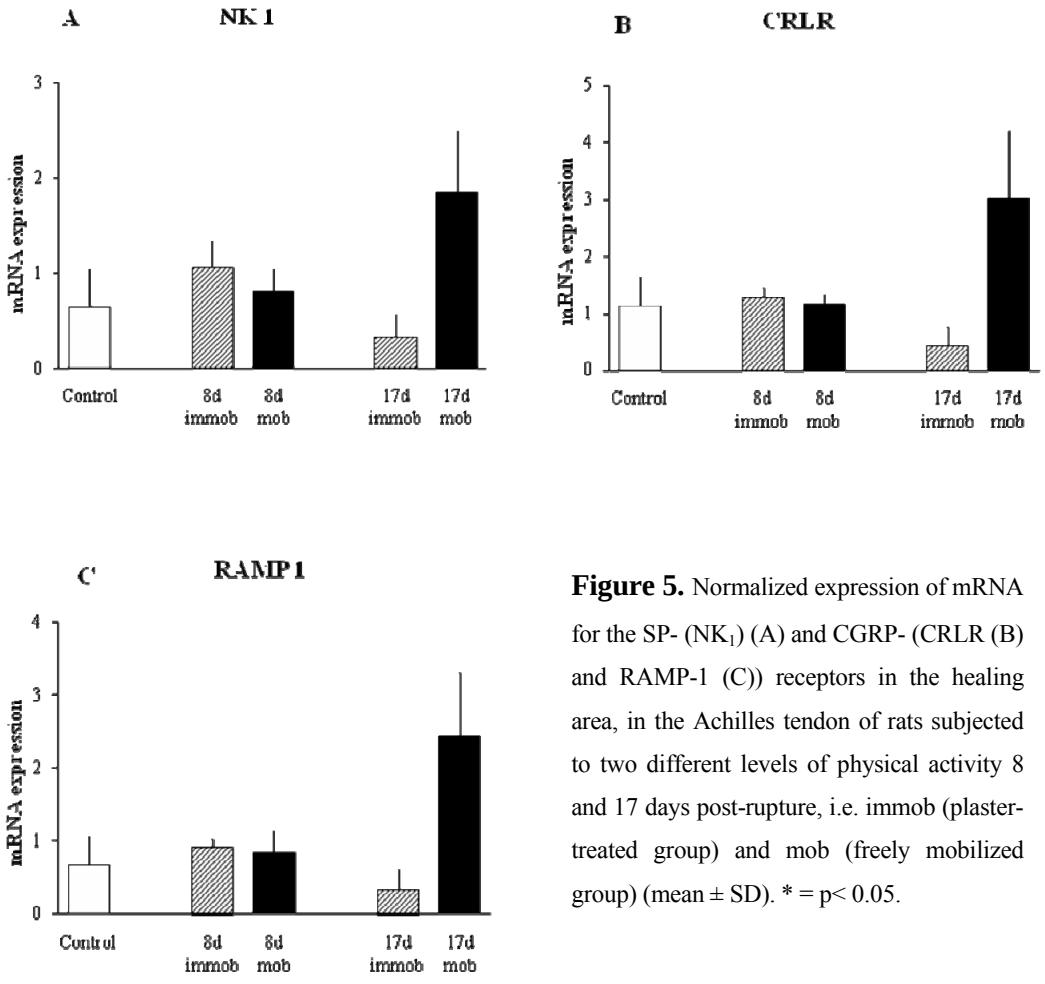

Figure 5. Normalized expression of mRNA for the SP- (NK_1) (A) and CGRP- (CRLR (B) and RAMP-1 (C)) receptors in the healing area, in the Achilles tendon of rats subjected to two different levels of physical activity 8 and 17 days post-rupture, i.e. immob (plaster-treated group) and mob (freely mobilized group) (mean ± SD). * = $p < 0.05$.

4.1.3 Growth factor expression

To investigate other local growth factors which may also be involved in tendon repair and stimulation of neuronal ingrowth, we analysed mRNA expression for nerve growth factor (NGF) and the brain-derived neurotrophic factor (BDNF), which are known to stimulate ligament repair (NGF) (83) and to regulate central and peripheral neurons related to axonal growth and neurotransmission (NGF and BDNF) (82). Morover, we analysed mRNA expression for basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1), which in addition to angiogenesis, fibroblast proliferation and tendon matrix production following injury are also involved in neuronal regulation (88-90, 166, 167).

We established the presence of BDNF, NGF, bFGF and IGF-1 by their mRNA expression in all healing and intact control Achilles tendons analysed. At 8 days levels of IGF-1 (Fig. 6D) and BDNF (Fig. 6A) mRNA were slightly elevated, while at 17 days post rupture, the growth factors BDNF (~7-fold), bFGF (~4-fold) (Fig. 6C) and IGF-1 (~2.5-fold) exhibited an increased expression in the freely mobilized group as compared to intact controls. Up-regulated bFGF and IGF-1 signaling at 17 days post-injury may contribute to our observation of increased collagen deposition and also to enhanced fibroblast proliferation during healing (168, 169). Whether the increased expression of BDNF and IGF-1 already at 8 days are involved in neuronal regeneration remains to be elucidated. The levels of NGF (Fig. 6B) mRNA were not significantly altered compared to intact controls, which is interesting since NGF is a strong promoter of wound and ligament repair (83, 85), in addition to its stimulatory effects on nerve regeneration (83, 170). The unaltered NGF expression at 8 and 17 days post-injury suggests that local cellular regulation of NGF is not a major pathway for promoting early tendon repair in freely mobilized rats. This does not preclude changes in NGF mRNA expression in the dorsal root ganglia, however, or that an altered expression of NGF receptors may occur (87), and thus can influence the NGF end-effects, since exogenous NGF has been demonstrated to promote ligament repair (83). An alternative explanation might be that the effects of NGF differ between skin, ligament and tendon healing. However, this seems less likely considering the similarities between the tissues.

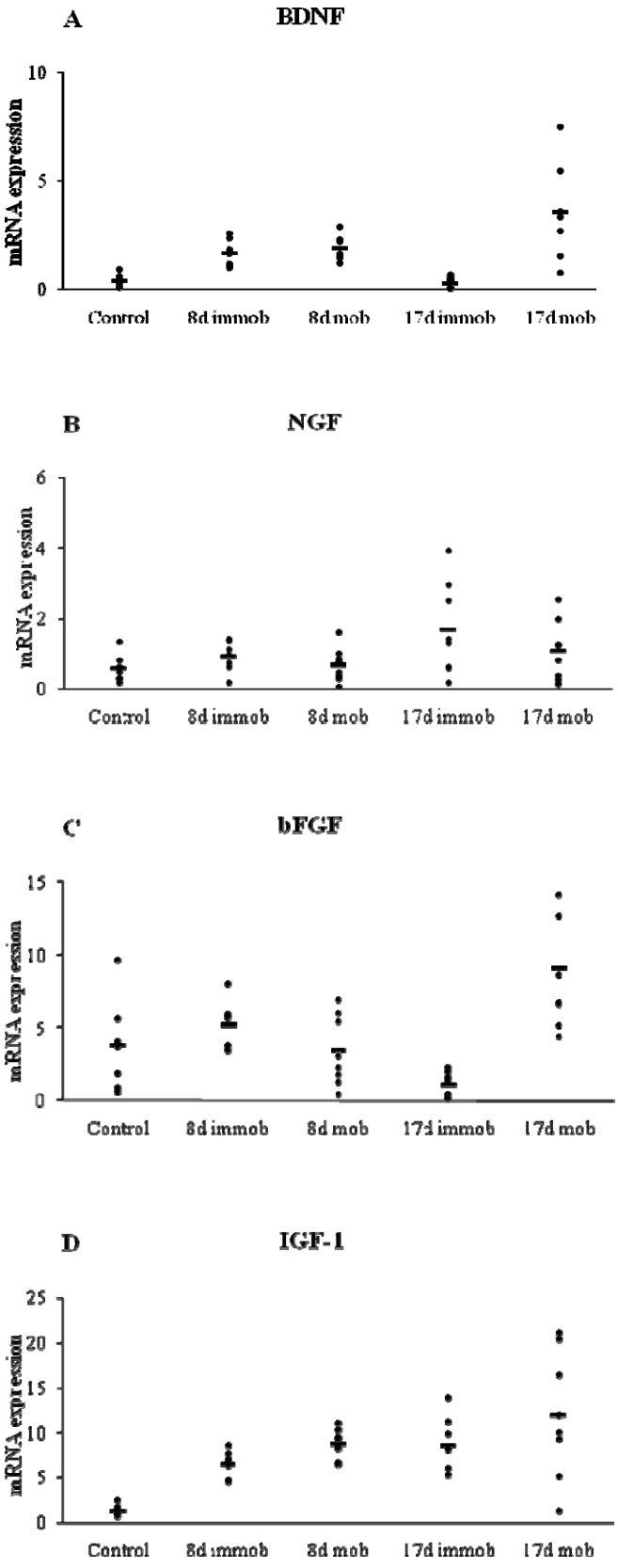

Figure 6. Normalized expression of mRNA for the growth factors BDNF (A), NGF (B), bFGF (C) and IGF-1 in the healing area, in the Achilles tendon of rats subjected to two different levels of physical activity 8 and 17 days post-rupture, i.e. immob (plaster-treated group) and mob (freely mobilized group) (mean ± SD). * = $p < 0.05$.

4.1.4 Pro-inflammatory mediator expression

Inflammatory mediators are fundamental molecular agents in repair processes and are believed to stimulate healing in the inflammatory phase (91). We thus analysed the mRNA expression for four different inflammatory factors; cyclooxygenase 1 and 2 (COX 1 and 2), inducible nitric oxide synthase (iNOS) and hypoxia-inducible factor-1α (HIF-1α), which are also believed to enhance tendon repair from the inflammatory healing phase through to scar remodeling (91, 93-95).

We were able to measure detectable mRNA levels for all the pro-inflammatory substances assessed (COX 1, COX 2, iNOS, HIF-1α) in the tendon tissue samples analysed. At 8 days post-rupture the expressions of both HIF-1α (Fig. 7D) and COX 2 (Fig. 7B) mRNA were increased in the mobilized tendons compared to the controls, whereas notably the mRNA levels for COX 1 (Fig. 7A) and iNOS (Fig. 7C) did not change significantly. At 17 days post-rupture the mRNA levels for COX 1 (~1.5-fold), COX 2 (~16-fold) and HIF-1α (~6.5-fold) were all higher in the mobilized group than in the controls, while iNOS still did not display any significant differences.

The increases in mRNA levels for COX 1 and 2 at 17 days of mobilization post injury presumably represent augmented prostanoid effects on blood vessels, nerve endings and inflammatory cells, since cyclooxygenases convert arachidonic acid into various pro-inflammatory and healing stimulatory molecules (92, 171). The elevation in mRNA levels for HIF-1α may reflect effects on vessel ingrowth since HIF-1α is a promoter of angiogenesis (172, 173). Interestingly, HIF-1α is up-regulated in mast cells that are exposed to SP (174). The present data suggest that the up-regulated COX 1-2 and HIF-1α mRNA expressions are involved in the tendon repair process.

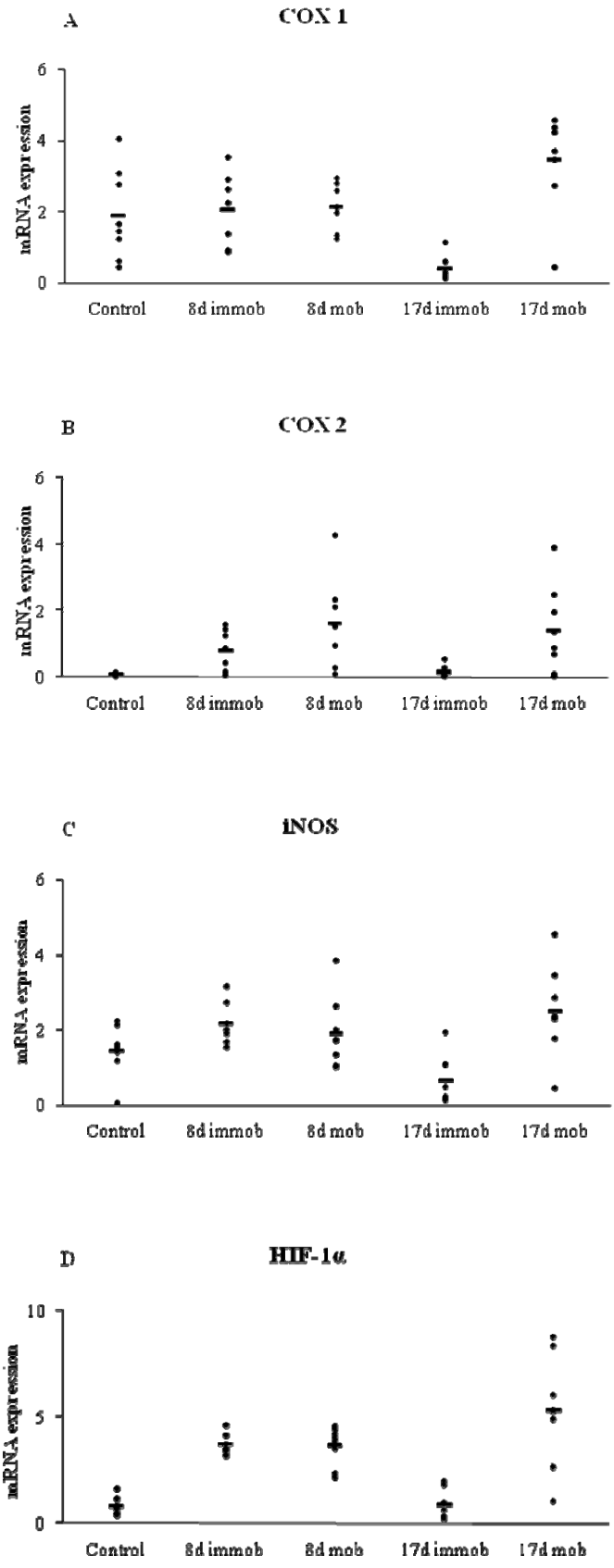

Figure 7. Normalized expression of mRNA for the pro-inflammatory mediators COX 1 (A), COX 2 (B), iNOS (C) and HIF-1α in the healing area, in the Achilles tendon of rats subjected to two different levels of physical activity 8 and 17 days post-rupture, i.e. immob (plaster-treated group) and mob (freely mobilized group) (mean ± SD). * = $p < 0.05$.

4.1.5 Extracellular matrix molecule expression

Five major structural extracellular matrix (ECM) molecules were analysed during early tendon healing. mRNA levels for collagen types I and III were assessed as the major structural proteins in tendon reformation. Morover, the expression of important linking proteoglycans in collagen fibrillogenesis, versican, decorin and biglycan, were analysed. At 8 days post-rupture, the mRNA levels for collagen type III and biglycan were increased compared to intact controls (Table 3). From 8 to 17 days post-rupture the mRNA levels for collagen types I and III, versican, decorin and biglycan increased even further. The increases at 17 days in mRNA levels for decorin (~2.5-fold) were similar to those for collagen I (~2.5-fold), while the increases in biglycan (~14-fold of intact control values) were similar to those for collagen III (~11-fold). Based on these findings we speculate that decorin is more involved in collagen I organization and that biglycan may be more related to collagen III formation. Both decorin and biglycan have been described in fibrillogenesis (31), while decorin also has been implicated in allowing inter-fibrillar sliding of collagen type I that occurs during loading (33, 175). Notably, exogenous SP administration has been demonstrated to increase collagen fibril organization, which concords with our observations that the peak expression of sensory neuropeptides in the healing area was temporally and spatially associated with the collagen formation. It is noteworthy that the synthesis of versican is highly regulated by pro-inflammatory growth factors such as transforming growth factor-β (176), which is released in response to SP stimulation (177).

Table 3. Extracellular matrix mRNA expression

Normalized expression of mRNA for the assessed tendon extracellular matrix molecules in the healing area, in the Achilles tendon of rats subjected to two different levels of physical activity 8 and 17 days post-rupture, i.e. immob (plaster-treated group) and mob (freely mobilized group) (mean ± SD).

	Extracellular Matrix (ECM)				
	Ctrl	**8d immob**	**8d mob**	**17d immob**	**17d mob**
Collagen I	1.35 SD 0.37	2.37 SD 0.45	2.93 SD 0.62	0.61 SD 0.44	4.62 SD 2.27
Collagen III	1.28 SD 1.3	6.96 SD 1.81	6.34 SD 3.58	1.46 SD 1.31	15.34 SD 3.80
Versican	1.17 SD 0.95	3.29 SD 0.91	2.38 SD 1.26	0.63 SD 0.39	4.94 SD 2.21
Decorin	1.99 SD 0.58	3.90 SD 0.71	3.82 SD 0.86	0.94 SD 0.69	7.17 SD 3.03
Biglycan	0.64 SD 0.27	5.55 SD 0.77	5.93 SD 0.83	1.34 SD 0.99	9.36 SD 4.62

Taken together, there seems to exist an early neuronal plasticity in tendon healing, with a maximum nerve and sensory neuropeptide occurrence at 4 weeks post-injury, which is combined with a local increase in the expression of sensory neuropeptide receptors. This neuronal plasticity is temporally and spatially related to local collagen synthesis and formation. Whether the sensory neuropeptides and their receptors exert direct effects on collagen synthesis or/and act indirectly via activation of cells that increase the observed expression of growth factors and inflammatory mediators remains to be elucidated.

5 PROMOTED TENDON REPAIR

5.1 PHYSICAL ACTIVITY (STUDY II)

The previous results from the longitudinal study on tendon healing in rats with free cage activity demonstrated that the maximum nerve ingrowth and organized collagen formation occurred up to 4 weeks post-tendon rupture. Since early mobilization and exercise have been shown to promote tissue repair after injury to the musculoskeletal system (11, 13), this study was designed to examine the effects of different levels of physical activity at 4 weeks post-rupture. In an attempt to appreciate the temporal effects of the physical activity, e.g. mechanical stimuli, the results were subsequently compared with the results of the previous longitudinal study. Two different levels of physical activity were studied: free cage mobilization was considered to represent the *normal healing condition*, while a group with free access to a running wheel was considered the *intervention group*.

Histology and immunohistochemistry including semi-quantitative measurements were used to grade healing according to the diameter of new organized collagen and to assess the occurrence of the sensory neuropeptides (SP,CGRP) in relation to different levels of physical activity.

5.1.1 Sensory neuropeptide occurrence

Looking at the effects of mobilization after 4 weeks of healing, a higher activity level post-rupture generally resulted in a lower density of sensory nerve fibres within the tendon proper, indicating an earlier retraction of nerve fibres from the tendon proper back to the tendon envelope (Fig. 8B, 8D, 8F). Specifically, the neuronal immunoreactivity to SP/CGRP and PGP (a general nerve marker) at week 4 was less abundant in the tendon proper in the wheel-running group compared to in the freely mobilized group. In the wheel-running group only a few SP/CGRP-positive nerves were apparent in the tendon close to the musculo-tendinous junction, whereas in the less active groups they were abundant along the tendon proper. Conversely, the wheel-running group exhibited a higher occurrence of nerve fibres in the tendon envelope. The neuronal character of CGRP- and SP-immunoreactivity was confirmed by positive staining with PGP.

The morphological observations were confirmed by computerized semi-quantitative assessment. Analysis at week 4 of the two groups disclosed a significant difference of 53% less CGRP in the wheel-running group ($p < 0.05$). There was also a decrease in

the occurrence of PGP, although this was not significant. In contrast, SP immunoreactivity was too low to permit a valid semi-quantitative analysis.

Although the decreases in PGP were not significant in the running group, this does not preclude an earlier withdrawal of sensory nerve fibres from the healing area since CGRP immunoreactivity was significantly reduced. The reduced levels of CGRP in the running group may also imply a decreased neurogenic inflammation.

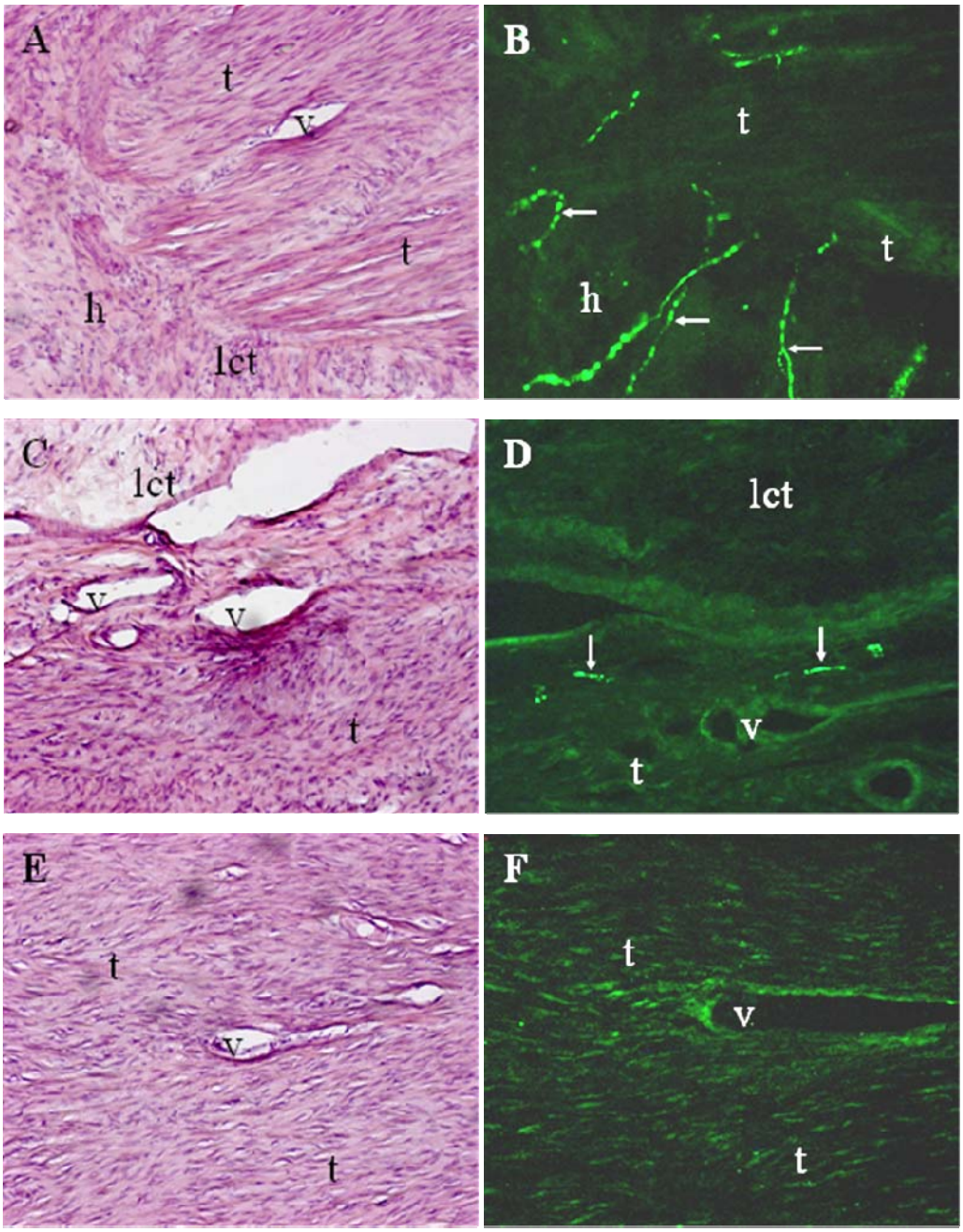

Figure 8. Hematoxylin-eosin (A, C, E) and immunofluorescence (CGRP) (B, D, F) micrographs of longitudinal consecutive sections through the Achilles tendon of rats subjected to three different levels of physical activity 4 weeks post-rupture, that is, plaster-treated group (A-B, immob), freely mobilized group (C-D, mob) and wheel-running group (E-F, run). Arrows denotes varicosities and nerve terminals.
t = tendon tissue; v = vessels; lct = loose connective tissue; h = healing area

5.1.2 Histological healing

The histological analysis at week 4 post-rupture demonstrated that higher physical activity was associated with a more mature repair tissue as reflected by fewer inflammatory cells, well differentiated fibroblasts and longitudinal organization of collagen (Fig. 8A, 8C, 8E). Histological analysis revealed the greatest number of new organized parallel collagen fibres in the ruptured tendons in the most physically active group. This finding was verified by computerized analysis. Thus the mid tendon medio-lateral diameter of longitudinally organized, parallel collagen at the rupture site in the wheel-running group was 48% greater than that in the freely mobilized group ($p < 0.05$).

The mean diameter of new organized collagen in the freely mobilized group from this study of activity response proved to be the same as in the corresponding group from the earlier longitudinal study at week 4, i.e. 2.1 ± 0.8 and 1.9 ± 0.3 mm, respectively. To assess the impact of physical activity on the pace of tendon healing, the outcome of the wheel running group at 4 weeks was compared with the results obtained on the mobilized rats at the different time-points of the longitudinal study. The mean diameter of the new organized collagen in the wheel-running group at 4 weeks corresponded to that evident at 14 weeks in the longitudinal study without wheel-running (Fig. 9).

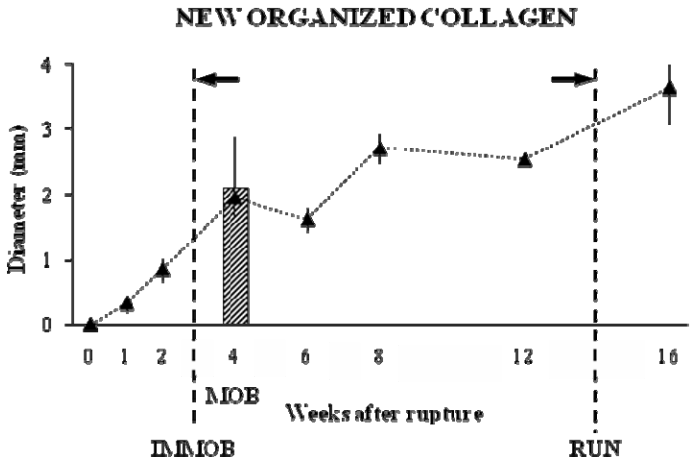

Figure 9. Mid tendon medio-lateral diameter of new organized collagen in the healing area, in the Achilles tendon of rats 1-16 weeks post-rupture and in the Achilles tendon of rats subjected to three different levels of physical activity 4 weeks post rupture, that is, immob (plaster-treated group), mob (freely mobilized group) and run (wheel-running group) (mean ± SD). The freely mobilized group (mob) from the activity response study was comparable with the longitudinal group at week 4 post-rupture with regard to the amount of new organized collagen. When extrapolating the degree of maturation in the two extreme groups healing in the plaster-treated group (immob) corresponded roughly to healing at week 3 in the longitudinal study (left arrow and dotted line), while the wheel-running group (run) corresponded to healing at week 14 (right arrow and dotted line).

The accelerated tissue repair associated with increased physical activity was also reflected by the findings for sensory neuropeptides. Their occurrence in the tendon proper was lower at week 4 in the most active group, inversely matching the tissue maturation. Although this could be attributed to physical activity inhibiting new nerve in growth in general, this seems less likely given the number of studies demonstrating that sensory neuropeptides increase the proliferation of fibroblasts and endothelial cells (20-22). Therefore, from these findings we hypothesize that a high degree of physical activity results both in an earlier nerve ingrowth and retraction of nerve fibres from the tendon proper, suggesting concurrence with the elevated expression of receptors for sensory neuropeptides. The retraction of nerve fibres already by 4 weeks would appear to reflect a more mature stage of healing compared to that noted in the freely moving rats in the longitudinal study, in which retraction did not occur until between weeks 8 to 16. Taken together, it would appear that increased physical activity affects the rate of neuronal plasticity and thereby promotes tendon healing. However, this conclusion is based merely on correlations and need to be verified by further studies.

6 IMPAIRED TENDON REPAIR AND MODELING

Having concluded that the peripheral nervous system has a role in mediating the effects of physical activity on enhancing tendon healing, further studies were aimed at examining the possible impeding effects of immobilization, neuropathic conditions and chronic inflammation on tendon healing and modeling. The analyses intended to elucidate possible inhibiting neuronal mechanisms.

6.1 IMMOBILIZATION (STUDY II-IV)

This study aimed at examining the effects of tendon immobilization, via a Plaster of Paris leg cast, on healing and the occurrence of sensory neuropeptides. Free cage mobilization was considered to represent the normal healing condition. Comparisons were also made between the immobilized group and a group with free access to a running wheel, as described above. The study was conducted up to 4 weeks post-rupture. Histology and immunohistochemistry including semi-quantitative measurements were used to grade healing and to assess the occurrence of SP and CGRP.

6.1.1 Sensory neuropeptide occurrence

At 4 weeks post-tendon rupture, the immunohistochemical analysis indicated that the presence of immunopositive nerve fibres in the healing area was dependent on the level of physical activity, or mobilization, during the healing process. Thus the immobilized, plaster cast-treated group exhibited a higher presence of nerves positive for PGP/SP/CGRP. These differences were even more pronounced when comparing the two extreme groups, with the most prominent divergence being the presence of nerves along the whole tendon length in the immobilized group. The nerves in the running group were limited to the proximal part of the tendon.

Computerized semi-quantitative assessments were applied in order to verify the subjective analysis. Neither the CGRP nor PGP immunoreactivities displayed any significant differences between the immobilized and the mobilized groups. When comparing the two extreme groups at week 4, the CGRP occurrence was 57% ($p< 0.05$) and that of PGP 55% lower in the proper tendon of the wheel-running group compared to the immobilized group ($p<0.05$). The immunoreactivity to SP was too low to measure using the current methodology. The results from this study thus demonstrated

a higher sensory nerve fibre occurrence at 4 week post-rupture after immobilization. Whether this finding suggests a delayed retraction of sensory nerves following immobilization or/and an increased sensory nerve ingrowth remains to be clarified. Moreover, it had yet to be demonstrated if these increases in sensory neuropeptides had any functional implications, i.e. a parallel expression of their receptors, in the immobilized tendon. Thus, assessment of mRNA levels was used to study the effect of immobilization compared to mobilization on the expression of local regulatory neuropeptide receptors, growth factors and inflammatory mediators at 1 and 2 weeks of tendon repair. Furthermore, the effect on the healing process was assessed by analysis of mRNA levels for extracellular matrix molecules.

6.1.2 Sensory neuropeptide receptor expression

mRNA levels for receptors for SP (NK_1) and CGRP (CRLR and RAMP-1) were studied at 8 and 17 days post-rupture to examine the effect of immobilization. The levels of mRNA for the SP and CGRP receptors were comparable at 8 days post-rupture in the immobilized healing group to both the intact tendon control group and to the mobilized group (Fig. 5A-C). Hence it appears that 1 week of immobilization does not overtly alter the sensitivity of the healing tissue to sensory neuropeptide regulation. Between 8 and 17 days however, while there were increases in the values in the mobilized group, the levels of NK_1, CRLR and RAMP-1 mRNA in the immobilized group remained constant or even decreased, reaching levels similar to those for the intact tendon control group ($p>0.05$) (Fig. 5A-C). Thus at 17 days post-rupture there were pronounced differences in the potential expression of the receptors for SP as well as for CGRP between the immobilized and mobilized groups. Therefore, two weeks of immobilization appears to lead to a down-regulation of SP and CGRP receptor mRNA levels, which are up-regulated 3-4-fold at this time-point during mobilized healing. Such down-regulation of sensory neuropeptide receptors may possibly reflect impaired stimulation of cell proliferation, vasoregulation and neovascularization, which are all well documented effects of SP and CGRP (20-22, 178, 179).

6.1.3 Growth factor expression

Since we established increased mRNA levels for the local growth factors BDNF, IGF-1 and bFGF during healing, and noted that those for NGF were unchanged, further analyses were aimed at assessing mRNA levels during immobilization.

Overall, 8 days of plaster-treated immobilization post-injury did not lead to any significant differences in growth factor mRNA expression, whereas by 17 days of immobilization post-injury, significant changes in expression levels were noted when compared to the freely mobilized group (Fig. 6A-D). Thus at 17 days post-rupture, while exhibiting increased expression in the mobilized group, the BDNF and bFGF mRNA levels in the immobilized specimens were equal to or even less than control values. The two other growth factors examined, NGF and IGF-1, were not significantly influenced by immobilization. IGF-1 mRNA levels at 17 days were up-regulated in both the mobilized and immobilized groups as compared to controls, suggesting enhancement of fibroblast mitogenesis and tendon matrix production (168, 169) even after immobilization. The NGF and IGF-1 genes, uninfluenced by immobilization, may provide more fundamental functions in the repair process and may thus not be influenced by mechanical factors.

The expression of the growth factors analysed, likewise as for the expression of the sensory neuropeptide receptors, exhibited no differences between the immobilized and freely mobilized groups at 8 days post-rupture. These findings support the hypothesis that a short period of immobilization does not overtly hamper the healing process, but it is unlikely that these time points can be directly translated to healing human tendons. The optimal time of immobilization is likely specific to particular species and tendons. Interestingly, it has been demonstrated that a shorter period of immobilization, i.e. 2-5 days, after muscle injury in the rat leads to a less pronounced scar formation and signs of a more distinct regeneration in the healing area (180). Hence a short period of immobilization after injury might be beneficial for functional outcome.

However, 2 weeks of immobilization post-tendon injury resulted in a significant down-regulation of bFGF mRNA, which may suggest an explanation for decreases in angiogenesis, fibroblast proliferation and tendon matrix production (88-90). Moreover, immobilization also led to reductions in the expression of BDNF that is an important factor for axonal growth.

6.1.4 Pro-inflammatory mediator expression

The mRNA levels for the pro-inflammatory substances identified to be increased during tendon healing (COX 1, COX 2 and HIF-1α) or were unchanged (iNOS) were analysed following immobilization.

The data revealed that 8 days of immobilization post-injury was not associated with any significant differences in mRNA levels for inflammatory mediators compared to

levels in the mobilization group (Fig. 7A-D). However, at 8 days the mRNA levels for HIF-1α had increased in both the immobilized and mobilized group, suggesting that HIF-1α also has an important role in angiogenesis during immobilization. COX 1, COX 2 and iNOS mRNA levels did not differ from the control group at 8 days post-rupture. At 17 days of immobilization post-injury significantly lower levels of mRNA for all inflammatory mediators analysed were recorded compared to the mobilization group, although obvious increases for COX 2 in the mobilized group did not reach statistical significance.

The reduced expression of various inflammatory mediators by 2 weeks of immobilization may thus reflect an obstruction of numerous pathways in the healing process.

6.1.5 Extracellular matrix molecule expression

The mRNA levels for the extracellular matrix (ECM) molecules, collagen types I and III, versican, decorin and biglycan, that were determined to be increased during tendon healing, were analysed after immobilization.

At 8 days post-rupture, the mRNA levels for all but one of the ECM molecules assessed were unchanged or increased to the same extent in the immobilized and mobilized groups (Table 3). Interestingly, at 8 days versican was the only ECM molecule that only exhibited elevated mRNA levels in the immobilized group.

From 8 to 17 days post-rupture, the mRNA levels for all ECM molecules assessed decreased significantly in the immobilized group, while they increased significantly in the mobilized group (Table 3). Thus mRNA levels for the ECM molecules at 17 days were significantly lower in the immobilized group than in the mobilized group and did not differ from the intact control group.

These results imply that the initial ECM molecule response is mainly unchanged after 1 week of immobilization, in concordance with the mRNA data of sensory neuropeptides receptors, and that a negative, possibly stress-shielding effect of immobilization is only evident after 2 weeks post-injury. In this model it is evident that 2 weeks of immobilization led to detrimental effects on the subsequent production of matrix proteins. This conclusion was confirmed by histological grading of the healing tissues.

6.1.6 Histological healing

The histological analysis was performed in order to follow the development of healing at 2 and 4 weeks in the immobilized, mobilized and running rats, respectively. The analysis demonstrated that the rate of healing was closely related to the degree of mobilization, whereas immobilization significantly hampered the process of tissue maturation. Hence the immobilized groups displayed more inflammatory cells, less blood vessels and fibroblasts and a decreased structural organization with disarrayed collagen fibres.

At 14 days the immobilized group displayed fewer small diameter capillaries in the healing area as compared to the mobilized group. Moreover, the tissue structure was less organized in the immobilized group. Within the healing tendons of the immobilized group there were also hypocellular areas of disorganized collagen, i.e. collagen fibres diverging from the functional axis. At 28 days the differences in structural organization between the two groups were even more evident, with an increasing area of highly disorganized collagen lacking longitudinal orientation in the immobilized group, while the mobilized group exhibited an increasing collagen organization. In addition, the immobilized group exhibited fibroblasts in arrays surrounding large diameter collagen bundles, exhibiting a rounded phenotype, in contrast to the freely mobilized group where the fibroblasts were more abundant, spool-shaped and oriented along the loading axis. The data clearly suggest structural impairment in tendon healing already after 2 weeks of immobilization, which is additionally aggravated at 4 weeks of immobilization.

The amount of new organized parallel collagen fibres in the healing tendons was approximated by computerized analysis 4 weeks post-tendon rupture. Thus the mid tendon medio-lateral diameter of longitudinal parallel collagen fibres at the rupture site was lower in the immobilized compared to the mobilized group, although these values did not reach significance. Interestingly, when comparing the diameters at the rupture sites of the immobilized and the wheel-running group, the diameter was significantly smaller in the immobilized, plaster-treated group ($p < 0.05$) (Fig. 10). These data also indicated linearity between the degree of mobilization and the diameter of organized collagen, implying stimulation of the healing process.

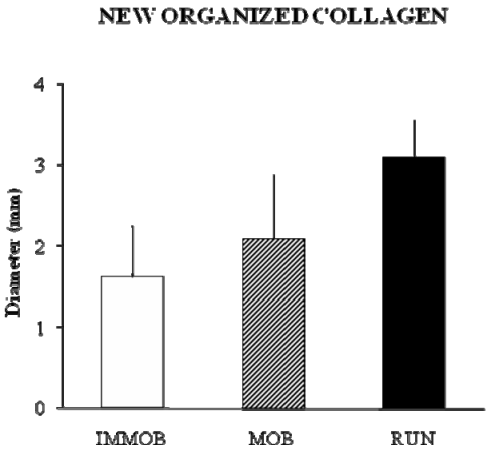

Figure 10. Mid tendon medio-lateral diameter of new organized collagen in the healing area, in the Achilles tendon of rats subjected to three different levels of physical activity 4 weeks post-rupture, that is, immob (plaster-treated group), mob (freely mobilized group) and run (wheel-running group) (mean ± SD). * = $p < 0.05$.

The histological data strengthened and confirmed the molecular findings, demonstrating hampered tissue repair already after 2 weeks of immobilization as compared to in the mobilized groups, which demonstrated up-regulated expression of the sensory neuropeptide receptors as well as the growth factors, inflammatory mediators and ECM molecules analysed. The present findings would also appear to reflect a potentially close temporal relationship between the mRNA levels for SP and CGRP receptors, fibroblast proliferation/differentiation and the formation of longitudinally organized collagen and extracellular matrix proteins during the healing process.

6.2 NEUROPATHY (STUDY V)

Since the previous results demonstrated that sensory neuropeptides and their receptors are associated with the tendon healing process, the impaired healing in different neuropathic conditions may be a result of a reduced local presence of sensory neuropeptides. The present studies were thus conducted in order to investigate whether a decreased expression of SP and CGRP during Achilles tendon healing would influence the development of biomechanical tissue properties and nociception.

The model for chemical denervation applied herein, utilizing capsaicin to induce a selective sensory denervation, has been widely used (140, 144, 181-183). The advantage of a chemical sensory denervation over nerve transection is that it does not directly influence the motor control, i.e. there are no apparent confounding effects due to changed mechanical stimulation of the healing area.

6.2.1 Sensory neuropeptide levels

Radioimmunoassay (RIA) was used in order to determine the concentrations of SP and CGRP in tendon healing following denervation at their segmental production sites, in the lumbar dorsal root ganglia (DRG) and at the central neuronal projection site, in the spinal cord (SC) at levels corresponding to the innervation of the Achilles tendon.

6.2.1.1 SP and CGRP levels in the DRG

The levels of SP/CGRP in the denervated, operated (DOp)-group were at 1 week 62/37% and at 4 weeks post rupture 64/36% lower ($p<0.05$), respectively compared to the corresponding levels of the operated (Op)-group. These changes are in agreement with previously published studies (140). At 8 weeks the SP/CGRP levels had increased in the DOp-group so that there were no significant differences between the groups, implying that the synthesis of the sensory neuropeptides recovers after approximately 8 weeks post-denervation and tendon rupture.

6.2.1.2 SP and CGRP levels in the spinal cord

SP concentrations in the DOp-group were at 1 week 56%, at 4 weeks 30% and at 8 weeks 54% lower, compared to the Op-group, but these differences were not statistically significant due to animal-to-animal variation ($p>0.05$). In contrast, CGRP levels in the DOp-group were 67%, 54%, and 68% lower at weeks 1, 4, and 8, respectively ($p<0.05$) when compared to the corresponding levels in the Op-group. The values at 8 weeks may reflect that denervation subsides more slowly in the CNS compared to in the DRG.

6.2.2 Sensory neuropeptide occurrence

Immunohistochemical analyses were performed in order to assess the peripheral occurrence at the healing site of SP and CGRP following denervation. The tissue selected for analyses was the subcutaneous tissue harvested close to the ruptured Achilles tendon. The subjective analysis revealed that the neuropeptides, SP and CGRP, were depleted in small free nerve endings and perivascular nerve structures of the DOp-group compared to in those of the Op-group. However, there was considerable variability in SP/CGRP density between different animals and thus the semi-quantification only resulted in a significant difference at week 1, with a lower perivascular CGRP occurrence in the DOp-group as compared to in the Op-group. The

high inter-individual variability in the response to denervation prompted later intra-individual correlation analyses.

6.2.3 Sensitivity to mechanical and thermal stimulation

In order to verify denervation and to relate nociception to central and peripheral levels of SP and CGRP the mechanical and thermal sensitivities to noxious stimuli was assessed. Both denervation and surgery resulted in significant differences in hind paw withdrawal latency (HWL) between the groups and over time ($p<0.001$).

6.2.3.1 Mechanical Sensitivity

After completion of the denervation, but prior to tendon surgery, mechanical sensitivity declined as evidenced by significantly increased HWL compared to both the operated- and normal control groups ($p<0.05$), strengthening the concept of a successful denervation (Fig. 11A).

At 1 week post-tendon rupture, i.e. during the inflammatory phase of healing, the mechanical sensitivity had increased more in the DOp-group than in the Op-group, and was notably not different from the two control groups. This equality in mechanical sensitivity remained until 6 weeks post-tendon rupture, through the proliferative phase of healing. Interestingly, at 8 weeks post-tendon rupture, during the remodeling phase of healing, there was again a significantly decreased mechanical sensitivity in the DOp-group, as compared to in the control groups ($p<0.05$). This latter finding implies that the chemical denervation remains functional, regarding nociception, until 8 weeks post-tendon rupture even though the synthesis of the sensory neuropeptides had returned to normal in the dorsal root ganglion. The decreases in nociception at 8 weeks are presumably related to the centrally observed lower levels of sensory neuropeptides in the spinal cord in the denervated group.

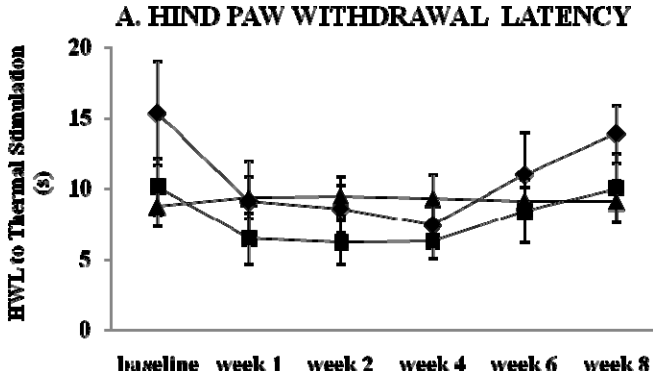

Figure 11A. Time course of the development of sensitivity to mechanical stimulation measured by hind paw withdrawal latency (HWL) (mean ± SD). * = $p< 0.05$.

6.2.3.2 Thermal Sensitivity

When analyzing the sensitivity to thermal stimuli after denervation and surgery, at 1 through 4 weeks post tendon rupture, there were also no detectable differences between the DOp- and Op-groups (Fig. 11B).

The non-significant differences in nociception between the groups at 1-4 weeks post-rupture may reflect that the surgical trauma mobilizes sufficient local nociceptive mediators to eliminate the central effects of denervation, as implicated by the lower levels of sensory neuropeptides in the spinal cord. This also suggests that nociception is more dependent on peripheral mediators during the initial phases following a tissue trauma.

After 6 weeks post-rupture the differences between the denervated and operated control groups had reoccurred and there was again a significantly decreased thermal sensitivity in the DOp-group ($p<0.05$). These results concord with the measurements of mechanical sensitivity.

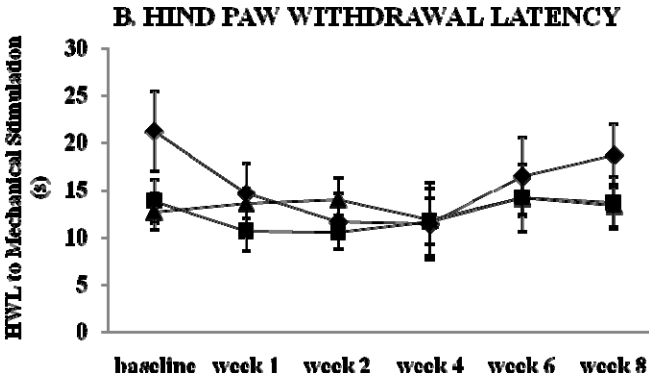

Figure 11B. Time course of the development of sensitivity to thermal stimulation measured by hind paw withdrawal latency (HWL) (mean ± SD). * = $p< 0.05$.

6.2.4 Biomechanical tissue properties

To evaluate the effects of denervation on the healing process, the development of biomechanical tissue properties was followed over the course of the experiment. The transverse area of the tendon at week 1 post-rupture was significantly smaller in the DOp-group as compared to in the Op-group ($p< 0.05$) (see Appendix Table 4), this likely being explained by a more pronounced inflammatory reaction in the Op-group with an intact nervous system. During the subsequent healing process, the difference in transverse area decreased and became non-significant.

The ultimate tensile strength (UTS) at week 1 post-tendon rupture exhibited equal values in the DOp- [mean 33 Newtons (N)] and the Op-group [mean 32 N] (see Appendix Table 4). At week 4 however, the mean UTS in the DOp-group [mean 52 N] was 20% lower than corresponding values in the Op-group [mean 61 N], although the high variability in UTS values and the different failure modes of the tendons resulted in the differences being non-significant. Interestingly, at 8 weeks all samples in the DOp-group ruptured in the midsubstance of the tendon at a higher mean UTS [mean 82 N] than did the Op-group [mean 52 N] ($p< 0.05$), where all except one sample failed at the bone-tendinous junction. The differences in the distribution of the failure modes were significant, and denote an altered homeostasis or adaptive process in the different groups (see Appendix Table 5). This observation implies that intact innervation, while improving the healing of the tendon midsubstance, promotes bone remodeling/resorption at the tendon-bone insertion site. (184, 185). Since the tendon-bone complex failed in the operated, non-denervated animals, it is not possible to comment on the UTS of the tendon mid-substance in the non-denervated group.

Based on these findings we hypothesize that in neurologically intact animals, tendon rupture induces a significant regional inflammation involving neurogenic factors which in addition to improving healing of the mid-substance rupture also weakens the bony insertion site, most likely by stimulating bone resorption. In fact, SP is known to stimulate bone resorption (186-188), and we have observed that chronically inflamed tendons exhibit an increased presence of nerves expressing SP and CGRP at the bone-tendinous junction (study I).

Stress at failure at week 1, although not significant, tended to be higher in the DOp- (3.6 N/mm^2) compared to in the Op-group (2.7 N/mm^2) ($p= 0.13$) (see Appendix Table 4), likely due to a smaller transverse area. At week 4 there were no differences detected between the groups, while at 8 weeks stress at failure, although not significant, again tended to be higher in the DOp group [mean 4.9 N/mm^2] as compared to in the Op-group [mean 3.4 N/mm^2] ($p= 0.12$). The differences in stress detected at week 8 may most likely be explained by the observed differences in failure mode.

6.2.5 Correlation analysis

Due to high individual animal-to-animal variability, both in the normal levels of neuropeptides detected in the tissues and in the subsequent response to chemical denervation, correlation analyses were performed on individual rats for neuropeptide levels, nociception and biomechanical tissue properties.

6.2.5.1 Correlation of SP and CGRP levels with biomechanical tissue properties

Notably, only the concentration of SP demonstrated correlations with the biomechanical tissue properties. Thus lower SP levels in the dorsal root ganglia of the DOp-group correlated with decreased transverse area, UTS and stress at failure (r^2=0.39, p=0.036; r^2=0.53, p=0.005; r^2=0.43, p=0.023, respectively). This may reflect the possibility that SP levels in the DRG accurately also depict the extent of denervation at the peripheral local levels. Thus we speculate that local SP levels influence ultimate tensile strength and stress at failure. In fact, SP is known to stimulate proliferation of fibroblasts (20, 22, 165), and other studies have reported that exogenous local administration of SP promotes tendon and ligament repair (15, 127).

6.2.5.2 Correlation of nociception with peripheral levels of SP

Interestingly, high sensitivity to thermal and mechanical stimuli at weeks 2 and 4 was related to subsequent increased occurrence of SP at week 4 in the subcutaneous tissue. (see Appendix Table 6).

6.2.5.3 Correlation of SP levels at the healing site with biomechanical tissue properties

At the biomechanical level, the healing tissue properties in the late proliferative phase of healing at week 4 should most likely depend on synthesis of structural proteins from week 1 up to this time point, an outcome that could be related to the early neuropeptide levels. We established a good correlation between mechanical and thermal sensitivity and peripheral local SP levels, consistent with other studies (19). The sensitivity to noxious stimuli was hence used as a surrogate for the early peripheral SP levels, in accordance with the fact that peripheral sensitization of nerves is mediated by SP (19, 189).

Interestingly, increased thermal sensitivity at week 2 had a strong correlation with increased UTS and stress at failure at 4 weeks in the DOp-group (see Appendix Table 7). Moreover, thermal sensitivity at weeks 1 and 4 also had a tendency to correlate with UTS and stress at failure at week 4. In addition, in the DOp-group mechanical sensitivity at week 1 showed a tendency to correlate with UTS and demonstrated a correlation with stress at failure at 4 weeks. These observations may reflect that the peripheral SP levels at week 2 are the most critical for optimizing the subsequent biomechanical properties of the healing tissues at week 4.

6.2.5.4 Correlation of preoperative nociception and SP/CGRP occurrence during healing

The findings from the present report demonstrated that high sensitivity to noxious stimuli preoperatively was correlated with high levels of SP and CGRP centrally as well as peripherally later at weeks 1, 4 and 8 during the healing process (see Appendix Table 8). Such findings imply that the post-operative pain (190) and also subsequent healing capacity could be partially foreseen by preoperative assessments of sensitivity to noxious stimuli. In the future, pre-operative nociceptive tests could potentially have an impact on the need for administration of analgesics as well as on the intensity and timing of rehabilitation after injury.

The present results both substantiate the conception of an essential function for the sensory neuropeptides SP and CGRP during tendon tissue repair, and add new information to our understanding of their roles. Whether pharmacological or physical means of promoting neuronal pathways, i.e. optimizing the expression of sensory neuropeptides and their corresponding receptors, could be developed and employed following injury specifically in neuropathic patients has yet to be explored.

6.3 CHRONIC INFLAMMATION (STUDY I)

Based on the findings that peripheral neuropeptides appear to influence tendon repair, the present study was aimed at analyzing whether chronic pathological conditions involving the tendon could be related to changes in sensory neuropeptide occurrence.
The current study employed a model of systemic chronic inflammation (adjuvant arthritis) and pain to analyse the occurrence of sensory neuropeptides in the Achilles tendon. The neuropeptides in the tendon were quantified using radioimmunoassay (RIA) and thereafter their specific localizations were determined by immunohistochemistry. These results were then related to the occurrence of inflammatory cells as analysed by histological examination.

6.3.1 Sensory neuropeptide levels

After the development of chronic arthritis the concentrations of both SP and CGRP were increased in the Achilles tendons compared to in control samples. This is in agreement with other studies examining at the tissue in chronic arthritic joints (140,

191). The relative increase in concentration for SP was 21.8% ($p < 0.05$) and for CGRP 71.1% ($p < 0.05$). The concentration of galanin (GAL), a sensory modulating peptide, proved to be unchanged in the Achilles tendons of the arthritic group compared to the controls. These results suggest increased pro-inflammatory actions of SP/CGRP such as vasodilation, plasma extravasation, release of cytokines and stimulation of metalloproteinases (192-194), while anti-inflammatory GAL (195-197) did not seem to be activated in the arthritic group.

6.3.2 Sensory neuropeptide occurrence

Overall, the subjective immunohistochemical assessments confirmed the RIA analyses and also provided information regarding neuropeptide distribution in the different regions of the Achilles tendon. The most pronounced differences in immunoreactivity to SP/CGRP between the arthritic group and the controls were observed in the bone tendinous junction, which in arthritic rats exhibited an increased number of free nerve endings positive for SP/ CGRP and notably even GAL surrounding the fibrocartilage. The second region of the Achilles tendon demonstrating marked differences in neuropeptide presence between the groups was the paratenon. SP/CGRP-positive nerve fibres in the form of thin, varicose, non-vascular nerve terminals were more abundant in the paratenon of the arthritic rats compared to in that of the controls. GAL-immunoreactive nerve fibres appeared unchanged, i.e. equal in the paratenon of the arthritic rats compared to controls.

Notably, in other regions of the Achilles tendon there were no clear differences between the groups. In the proper tendinous tissue, we observed no SP/CGRP/GAL-positive nerve fibres in the arthritic rats or in the controls. The neuronal localization of the staining was confirmed by positive immunoreactivity for PGP, a general nerve marker.

Semi-quantitative image analysis of SP, CGRP and GAL expression confirmed the spatial differences in neuropeptide occurrence as assessed subjectively. Thus in the bone tendinous junction of the arthritic rats there were significant increases in the occurrence of all of the three neuropeptides examined SP, CGRP and GAL compared to in the controls. In the paratenon of the arthritic rats, there was also a significant increase in SP and CGRP compared to the controls. The occurrence of GAL in the paratenon, however, proved to be equal in both groups.

These results imply that sensory neuropeptide occurrence in the healing tendon proper exhibits proliferative actions, while in the tendon surroundings, i.e. paratenon

and the bone tendinous junction they possibly exhibit pro-inflammatory actions. Moreover, experimental studies reveal that CGRP potentiates the nociceptive and inflammatory effects of SP (198, 199). The increased concentration of SP and CGRP observed in the Achilles tendon envelope in arthritic rats may reflect the nociceptive and inflammatory actions in enthesitis and paratenonitis. We speculate that this sensory neuropeptide up-regulation in the tendon envelope underlies not only the symptoms of arthritic tendinopathy, but also the development of overuse tendinopathy, but this needs to be tested in an appropriate tendinopathy animal model.

6.3.3 Histological inflammation

The Achilles tendons in rats with adjuvant arthritis displayed a severe inflammatory reaction, although limited to the envelope. The surrounding paratenon and the bone tendinous junction were edematous and flooded with inflammatory cells in the arthritic tendons as compared to the controls. The proper tendinous tissue, however, did not show any signs of inflammation and did not differ significantly from the controls. This is in agreement with the finding of an increased presence of sensory neuropeptides in the envelope of the inflamed tendons. The pathological changes thus appeared to be limited to the surrounding tissues rather than the tendon proper.

These results suggest that the patophysiology of the painful tendon originates in the tendon envelope, and it could be speculated that the changes in neuronal expression could be involved in the development of Achilles tendinopathy. A pilot study demonstrated that repeated tendon loading increased the expression of SP and CGRP in the paratenon (135). In fact, it has been demonstrated that SP and CGRP stimulation of the paratenon (136) increases the synthesis of both COX-2 and IL-1β, leading to inflammation with production of e.g. PGE1. In addition, it has been reported that repetitive injections of PGE1 administered to the paratendinous tissue induces degenerative changes in the tendon proper (132).

7 SUMMARY AND CONCLUSIONS

The aim of the present thesis was to assess the contributory role of the peripheral nervous system in tendon healing, and to further explore the potential of neuronal mechanisms to promote or hamper repair processes and tissue modelling. Hypothetically, specific neuronal mediators such as the sensory neuropeptides SP and CGRP released in the injured area during tendon healing may have a regulatory role in the repair process. The stimulating effects of mobilization during repair as well as the impaired healing and tissue modelling after immobilization, neuropathic conditions and chronic inflammation are suggested to be modulated by the local sensory nervous system.

Neuronal involvement in tendon healing with respect to collagen organization was explored morphologically in a longitudinal study. The occurrence of nerve fibres and sensory neuropeptides (SP, CGRP) during the different phases of healing were studied by IHC analysis over a period of 16 weeks post-injury in a rat tendon rupture model with freely-moving animals. The following studies on repair in the healing tendon midsubstance are based on tissues normally almost devoid of neuronal supply. Yet from week 1 post rupture there was a striking shift in neuronal immunoreactivity from the surrounding structures into the rupture site of the proper tendon, presumably as a prerequisite for delivery of neuronal mediators required for tissue repair. During weeks 2-4 (proliferative phase) IHC accordingly disclosed a peak in the occurrence of neuronal SP/CGRP in the healing area that coincided with maximum increase in new organized collagen. Thus the data from this analysis suggest that sensory neuropeptides are involved in the regulation of collagen formation and organization in the tendon repair process.

To further establish the presence of local sensory neuropeptide receptors and local cellular regulatory factors involved in axonal growth and extracellular matrix production, quantitative assessments (PCR) of gene expression at 1 and 2 weeks were performed. The PCR analysis demonstrated a conspicuous increase in the mRNA expression of SP-receptor (NK_1) and CGRP receptors (CRLR and RAMP) in the healing tendon at 2 weeks post-rupture. The detection of sensory neuropeptide receptors in the healing Achilles tendon denotes a functional basis, ligand + receptor expression, for the regulation of tissue repair, e.g. by stimulation of collagen formation.

Interestingly, the peak expression of the neuronal mediators and their receptors at week 2 was temporally matched by an increased presence of collagen types I and III and the linking proteins versican, decorin and biglycan. Hence at 2 weeks the mRNA levels for biglycan (~14-fold of intact control values) were similar to those for collagen III (~11-fold), while the increases for decorin (~2.5-fold) were similar to those for collagen I (~2.5-fold). We hypothesize that the sensory neuropeptides and their receptors in the healing area are temporally and spatially associated with the collagen deposition and involved in the increased collagen fibril organization during tendon healing.

To further assess stimulatory factors involved in repair, mRNA expression analyses of growth factors and pro-inflammatory mediators known to have a regulatory role in repair were conducted at 1 and 2 weeks post-rupture. Two weeks of healing induced significant increases in the mRNA levels of the growth factors BDNF (~7-fold), bFGF (~4-fold) and IGF-1 (~2.5-fold) and the inflammatory mediators COX 1 (~1.5-fold), COX 2 (~16-fold) and HIF-1α (~6.5-fold) as compared to intact controls. The results suggest stimulation of fibroblast proliferation (bFGF, IGF-1, COX 1-2), angiogenesis (bFGF, IGF-1, HIF-1 α, COX 1-2), and nerve regeneration (BDNF, IGF-1, COX 1-2) involved in regulating the tendon repair process. However, the unaltered tissue levels of the nerve growth factor and iNOS suggest that these are not major local cellular regulatory pathways for promoting early tendon repair in freely mobilized rats.

Altogether, in tendon healing there appears to exist an early neuronal plasticity, a capacity to respond and adapt to injury, reflected by a maximum nerve ingrowth and sensory peptide expression at 4 weeks post-injury, paralleled with a local increase in peptide receptor expression. This neuronal plasticity is temporally and spatially related to the local collagen synthesis and deposition. The present data seem to imply a regulatory role of the peripheral nervous system on extracellular matrix (ECM) synthesis, either through direct effects on fibroblast proliferation or/and indirectly via activation of inflammatory cells responding to peptide stimulation and causing synthesis and release of growth factors and pro-inflammatory mediators.

Considering the peak nerve ingrowth and collagen formation occurring up to 4 weeks post-rupture, this time point was chosen to assess the occurrence of the sensory neuropeptides SP/ CGRP and the amount of organized collagen in relation to different levels of physical activity. Free cage mobilization was compared to free access to a running wheel.

Neuronal immunoreactivity to SP/CGRP at week 4 was less abundant in the tendon proper of the wheel-running group and limited to the area close to the musculo-tendinous junction. This was contrary to the freely mobilized group, which showed immunopositive staining further along the tendon proper. Computerized semi-quantitative assessment disclosed a 53% decreased occurrence of CGRP paralleled with a 40% larger diameter of longitudinally organized, parallel collagen at the rupture site in the wheel-running group. The results suggest that physical activity increases the rate of sensory nerve regeneration (ingrowth and retraction), which may be related to the enhancement of tendon healing. In fact, the wheel running group exhibited a more mature repair tissue as reflected by fewer inflammatory cells, well differentiated fibroblasts and a greater amount of new organized, parallel collagen fibres. Notably, the diameter of new organized collagen in the wheel-running group at 4 weeks corresponded to that observed at 14 weeks in the longitudinal study. Hence the higher degree of physical activity appeared to have accelerated the healing process corresponding to 10 weeks of repair, which seems highly associated to the rate of sensory nerve regeneration.

Given a role of the peripheral nervous system in mediating the stimulatory effects of physical activity in the repair process, further analyses were aimed at examining the neuronal involvement in the inhibitory effects of immobilization on tendon healing. The effects of immobilization after application of a Plastic of Paris leg cast, restricting movement and mechanical loading of the healing area, were assessed by IHC at 4 weeks post-rupture compared to a group allowed free cage mobilization and a group with free access to a running-wheel.

IHC clearly revealed showed a higher presence of nerves positive to the nerve marker PGP and to SP/CGRP longitudinally along the tendon in the immobilized group at 4 weeks post-rupture. Semi-quantitative assessment disclosed a 57% and 55% higher occurrence of CGRP and PGP, respectively, in the proper tendon of the immobilized compared to the wheel-running group, whereas no significant differences between the immobilized and mobilized groups were detected. These findings suggest a delayed retraction of sensory nerves after immobilization or/and a promoted retraction in the wheel-running group.

To study if the higher occurrence of sensory neuropeptides after immobilization was matched with a corresponding receptor expression, the mRNA levels for SP and CGRP-receptors were analysed by PCR at 1 and 2 weeks post-rupture. Furthermore,

the principal effects of immobilization on the healing process were assessed by analysis of the mRNA levels for growth factors and inflammatory mediators in relation to extracellular matrix molecules at 1 and 2 weeks.

mRNA expression analyses revealed no detectable differences in the expression of receptors for SP (NK_1) and CGRP (RAMP) between the immobilized and mobilized groups at 1 week post-rupture. Hence, it appears that one week of immobilization does not inhibit the susceptibility of the healing tissue to sensory neuropeptide regulation. However, at 2 weeks the SP/CGRP receptor levels in the immobilized group decreased to levels similar to intact controls, while those of the mobilized group were up-regulated 3-4-fold at this time-point. This seems to reflect that prolonged immobilization leads to a down-regulation of SP and CGRP receptor mRNA levels. A down-regulation of receptor expression could entail reduced tissue susceptibility to neuropeptide regulation, such as cell proliferation, vasoregulation and neovascularization.

Interestingly, the decrease in neuropeptide receptors first observed after 2 weeks of immobilization was paralleled with reduced mRNA levels for growth factors, inflammatory mediators and extracellular matrix molecules. Thus at week 2 the mRNA levels for the growth factors (BDNF, bFGF), inflammatory mediators (COX 1, HIF-1α, iNOS) and all ECM molecules assessed (collagen type I, III, versican, decorin, biglycan) had decreased significantly in the immobilized group as compared to the mobilized controls. These results are in line with the observations on sensory neuropeptide receptors that the impeding effects of immobilization are evident at first after two weeks post injury. The reduced mRNA levels demonstrated here may reflect that immobilization obstructs numerous pathways involved in the wound healing process such as fibroblast proliferation (bFGF, COX 1), angiogenesis (bFGF, HIF-1 α, iNOS, COX 1), and nerve regeneration (BDNF, IGF-1, COX 1). IGF-1 mRNA levels at 2 weeks, however, were up-regulated to the same extent in both the immobilized and mobilized groups, suggesting enhancement of fibroblast mitogenesis and tendon matrix production even after immobilization. The IGF-1 gene, uninfluenced by immobilization, may provide a basic function not influenced by mechanical factors in the repair process.

To confirm structural effects of immobilization, histological analyses were performed at 2 and 4 weeks in immobilized, mobilized and running rats. The histological data confirmed the mRNA expression analyses by displaying more inflammatory cells, fewer blood vessels and fibroblasts and disarrayed collagen fibres, reflecting structural

disorganization, in the immobilized group. The structural impairment was aggravated with time. Consequently, the diameter of organized collagen at the rupture site was significantly smaller in the immobilized rats compared to the wheel-running group at 4 weeks post-rupture. The data indicate linearity between the degree of mobilization and the diameter of organized collagen. Thus the histological data strengthened the molecular findings demonstrating hampered tissue repair already after 2 weeks of immobilization.

Altogether, the present findings may reflect a close relationship between the mRNA levels for SP and CGRP receptors and the expression of ECM proteins, growth factors, inflammatory mediators and the formation and deposition of collagen during the healing process.

Given that the peripheral sensory nervous system is involved in tendon repair, the impaired healing evident in different neuropathic conditions may be a result of a reduced local presence of sensory neuropeptides. Hence by applying a method for chemical denervation utilizing capsaicin, the effects of a decreased presence of SP and CGRP during 8 weeks of Achilles tendon healing were analysed. RIA was used to examine the concentrations of SP and CGRP in the spinal cord and in the dorsal root ganglia (DRG) at levels innervating the Achilles tendon, while IHC analysis was performed to assess the local occurrence of sensory neuropeptides at the healing site.

Chemical denervation caused significantly reduced concentrations of SP/CGRP in the DRG at 1 and 4 weeks post-rupture and of CGRP at 1,4 and 8 weeks in the spinal cord, which may reflect that denervation subsides more slowly in the spinal cord compared to the dorsal root ganglia. IHC strengthened the RIA analysis, indicating that SP/CGRP were almost depleted in the local peripheral tissues at early time points following denervation.

The nociceptive response, sensitivity to mechanical and thermal stimuli, following denervation was assessed over the course of the experiment. The results of this analysis strengthened the concept of a successful denervation and moreover suggested that early pain sensitivity after injury is related to local peripheral mechanisms, while nociception during later phases of healing seems to be dependent on the spinal expression of neuropeptides.

To directly evaluate the effects of denervation on the healing process the development of biomechanical tissue properties was followed over the course of the experiment. Due

to high animal-animal variation in the response to denervation intra-individual correlation analyses were performed.

The most striking finding was that lower SP levels in the DRG after denervation correlated with the decreased transverse area, UTS and stress at failure. These findings suggest that the individual SP levels during healing are important for the development of the biomechanical tissue properties. Presumably, the SP levels in the DRG most accurately depict the extent of denervation, also at the peripheral level.

Considering that biomechanical tissue properties likely depend on the synthesis of structural proteins during earlier time-points of repair, we used the sensitivity to noxious stimuli, which correlated to the peripheral, local levels of SP, as a surrogate measure of early SP levels. Increased sensitivity to noxious stimuli at earlier time-points correlated with increased UTS and stress at failure at 4 weeks after denervation. These observations may reflect that the earlier neuronal activities, i.e. at week 2, are the most critical for optimizing the subsequent biomechanical properties of the healing tissues. This conclusion is in agreement with our findings at weeks 2-4 of the peak occurrence of nerves expressing sensory neuropeptides and the increase in the expression of sensory neuropeptide receptors.

Interestingly, high sensitivity to noxious stimuli preoperatively was correlated with high levels of SP and CGRP centrally as well as peripherally later during the healing process. Such findings imply that the post-operative pain and also subsequent healing capacity, i.e. release of sensory neuropeptides, could be partially foreseen by preoperative assessments of sensitivity to noxious stimuli.

At 8 weeks, all samples in the denervated group ruptured in the healing midsubstance of the tendon at higher mean UTS than did the operated control group, where all except one sample failed at the bone-tendinous junction. The differences in the distribution of the failure modes were significant, and denote an altered tissue homeostasis or adaptive process in the different groups. This observation implies that intact innervation, while improving healing of the tendon midsubstance, promotes bone remodelling/resorption at the tendon-bone insertion site. We have in fact disclosed that the bone-tendinous junction exhibits an increased presence of nerves expressing SP and CGRP in the chronically inflamed tendon.

Considering that the peripheral sensory nervous system modulates tendon repair we employed a model of systemic inflammation to analyse the occurrence of sensory neuropeptides during a chronic pathological condition involving the Achilles tendon.

Using RIA a significantly higher occurrence of SP and CGRP was established in the tendons with chronic inflammation, while the presence of GAL, however, remained unchanged. Semi-quantitative immunohistochemistry applied to different regions of the tendon in arthritic rats disclosed an increased occurrence of SP and CGRP positive nerve fibres in the paratenon and bone tendinous junction. The occurrence of inflammatory cells was also limited to the surrounding tissues, i.e. paratenon and bone tendinous junction. Notably, neither nerve fibres containing neuropeptides nor inflammatory cells were recorded in the tendon proper, suggesting that regulation of inflammation occurs in the tendon envelope. These results are interesting considering that sensory neuropeptide occurrence in the healing tendon proper exhibits proliferative actions, while SP/CGRP presence in tendon surroundings, i.e. paratenon and bone tendinous junction, possibly has more pro-inflammatory actions. The observed SP and CGRP upregulation in the paratenon and bony insertion suggests a pathogenic role in paratenonitis and enthesitis often evident in patients with rheumatoid arthritis. Whether these phase-dependent changes in sensory peptides could be involved in the development of overuse tendinosis needs to be tested in an appropriate overuse tendinopathy animal model.

The results of the study demonstrate that the peripheral nervous system is involved in regulating tendon repair, one pathway possibly being mediation of mechano-biological transduction via transformation of early mobilization, i.e. mechanical loading, into increased expression of growth factors, matrix proteins and improved tissue function. Physical activity seems to be one prerequisite for the up-regulation of sensory neuropeptide receptors, thus activating the tissue susceptibility to neuronal stimuli. Dysregulation of the sensory nervous system due to systemic diseases such as neuropathy or chronic inflammation seems to hamper tendon repair and impair tendon modelling.

Whether pharmacological or/and physical means of promoting neuronal reparative pathways could be developed and employed in tendon injury and disease has yet to be explored.

8 CLINICAL CONSIDERATIONS

The present thesis strengthens the concept that early mobilization following tendon rupture enhances healing. However, a shorter period of immobilization, i.e. 1 week in this model, does not seem to inhibit the healing process and could possibly be beneficial in order to permit the inflammation to subside and initiate the subsequent proliferative phase of healing. The same biological principle of applying a short immobilization period following surgery is presumably applicable also to the healing human tendon, however, notable differences in healing between humans and rats make it difficult to specify specific intervention time-points.

Conversely, prolonged immobilization associated with stress-shielding effects on the tendon leads to impaired scar formation and delayed healing. This was apparent by a vast reduction in the expression of the majority of the repair-promoting mediators and structural proteins analysed. Whether specific biomarkers of the repair process or specific imaging modalities could be employed to determine the appropriate time-point and degree of mobilization remains to be investigated.

The current findings present an experimental basis for development of early and potentially also more aggressive rehabilitation programs after periarticular connective tissue injury.

The regulation of tendon repair and promoting effects of early mobilization seem closely related to a functionally intact and quickly responding peripheral nervous system. This may explain why patients with different neuropathic conditions (i.e. diabetes) exhibit impaired and prolonged healing. Clinically, also in a genetically diverse healthy population, it would be of great importance to identify patients at risk for compromised or disturbed healing. Hence the findings of strong correlations between preoperative nociception and the subsequent central and peripheral levels of sensory neuropeptides suggest a way to potentially foresee the patients´ healing potential and post-operative pain. This could theoretically identify patients at risk of disturbed healing, which in the future may receive pharmacological or physical means for enhanced healing. Clinical application of this experimental method could potentially identify patients at risk and the results of this study may lead to the development of new pharmacological or/and physical therapies for enhanced healing.

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11 APPENDIX

Table 4.
Biomechanical tissue properties (area, ultimate tensile strength (UTS) and stress) in the healing Achilles tendon of the denervated and surgically treated- (DOp-), operated control- (Op-) and normal control- (Cntrl-) groups, at 1, 4 and 8 weeks post tendon rupture. The values are expressed as mean ± SD.

	Biomechanical Properties						
	Cntrl	**DOp – 1w**	**Op – 1w**	**DOp – 4w**	**Op – 4w**	**DOp – 8w**	**Op – 8w**
Area (mm^2)	1.8 ± 0.6	9.4 ± 1.7	12.7 ± 2.7	13.8 ± 1.3	15.3 ± 2.5	17.0 ± 1.9	16.7 ± 5.3
UTS (N)	41.2 ± 6.5	32.9 ± 9.7	32.5 ± 6.7	51.5 ± 17.1	61.0 ± 6.9	82.4 ± 19.9	51.9 ± 13.8
Stress (N/mm^2)	24.2 ± 6.1	3.6 ± 1.1	2.7 ± 0.8	3.7 ± 1.2	4.1 ± 0.7	4.9 ± 1.4	3.4 ± 1.6

Table 5.
Different failure modes (muscle tendinous junction (MTJ), mid substance (MS) and bone tendinous junction (BTJ)) in the healing Achilles tendon of the denervated and surgically treated- (DOp-), operated control- (Op-) and normal control- (Cntrl-) groups, at 1, 4 and 8 weeks post tendon rupture.

	Failure Mode						
	Cntrl	**DOp – 1w**	**Op – 1w**	**DOp – 4w**	**Op – 4w**	**DOp – 8w**	**Op – 8w**
MTJ	0	0	2	3	0	0	1
MS	1	6	4	2	3	6	0
BTJ	5	0	0	2	3	0	5
Tot	6	6	6	7	6	6	6

Table 6.

Correlations between mechanical and thermal sensitivity (hind paw withdrawal latency (HWL)) at 2 and 4 weeks and the peripheral local subcutaneous levels of substance P (SP) at 4 weeks post tendon rupture, as assessed by immunohistochemistry, in the denervated and surgically treated- (DOp-) and the operated control- (Op-) groups. Only tendencies and significant correlations are presented.

Mechanical and Thermal Sensitivity – Correlations with Peripheral Subcutaneous Levels of SP

	DOp – w4	Op – w4
Mechanical w2	$r^2 = -0.86$, $p = 0.003$	
Mechanical w4	$r^2 = -0.67$, $p = 0.048$	$r^2 = -0.65$, $p = 0.041$
Thermal w2	$r^2 = -0.68$, $p = 0.044$	$r^2 = -0.56$, $p = 0.090$
Thermal w4	$r^2 = -0.80$, $p = 0.010$	

Table 7.

Correlations between mechanical and thermal sensitivity in the hind limb (hind paw withdrawal latency (HWL)) and the biomechanical tissue properties (ultimate tensile strength (UTS) and stress) in the healing Achilles tendon in the denervated and surgically treated- (DOp-), the operated control- (Op-) and normal control- (Cntrl-) groups, at 1, 4 and 8 weeks post tendon rupture. Only tendencies and significant correlations are presented. A high sensitivity for noxious stimuli early on in the healing process correlated with a subsequent increase in the biomechanical tissue properties at a later time point.

Mechanical and Thermal Sensitivity – Correlations with Biomechanical Tissue Properties

	DOp – w4		DOp – w8	
	UTS (N)	**Stress (N/mm^2)**	**UTS (N)**	**Stress (N/mm^2)**
Mechanical w1	$r^2 = -0.55$, $p = 0.091$	$r^2 = -0.65$, $p = 0.046$		
Mechanical w8			$r^2 = -0.60$, $p = 0.091$	
Thermal Baseline				$r^2 = -0.60$, $p = 0.091$
Thermal w1	$r^2 = -0.59$, $p = 0.068$			
Thermal w2	$r^2 = -0.89$, $p = 0.006$	$r^2 = -0.78$, $p = 0.015$		$r^2 = -0.60$, $p = 0.091$
Thermal w4	$r^2 = -0.62$, $p = 0.051$	$r^2 = -0.52$, $p = 0.099$		

Table 8.

Correlations between preoperative mechanical and thermal sensitivity in the hind limb (hind paw withdrawal latency (HWL)) and the central (spinal cord (SC)) and peripheral (dorsal root ganglion (DRG) and subcutaneous and perivascular tissue) levels of substance P (SP) and calcitonin gene-related peptide (CGRP), as assessed by radioimmunoassay (DRG and SC) and immunohistochemistry (subcutaneous and perivascular tissue), in the denervated and surgically treated- (DOp-), the operated control- (Op-) and normal control- (Cntrl-) groups. Only the significant correlations are presented.

	Preoperative Mechanical and Thermal Sensitivity – Correlations with SP and CGRP				
	SP DRG	**SP SUBCUTANEOUS**	**CGRP DRG**	**CGRP SC**	**CGRP PERIVASCULAR**
Mechanical baseline (R)	$r^2 = -0.22$, p= 0.014			$r^2 = -0.40$, p= 0.000	$r^2 = -0.32$, p= 0.006
Mechanical baseline (L)	$r^2 = -0.37$, p= 0.000		$r^2 = -0.27$, p= 0.002	$r^2 = -0.45$, p= 0.000	$r^2 = -0.35$, p= 0.003
Thermal baseline (R)	$r^2 = -0.23$, p= 0.010			$r^2 = -0.34$, p= 0.000	$r^2 = -0.38$, p= 0.001
Thermal baseline (L)	$r^2 = -0.30$, p= 0.001	$r^2 = -0.19$, p= 0.028	$r^2 = -0.184$, p= 0.028	$r^2 = -0.38$, p= 0.000	$r^2 = -0.36$, p= 0.002