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Neuronal pathways in tendon healing and tendinopathy - Update

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1. ABSTRACT (175 words)

The regulatory mechanisms involved in tendon homeostasis and repair are not fully understood. Accumulating data, however, demonstrate that the nervous system, in addition to afferent (sensory) functions, through efferent neuronal pathways plays an active role in regulating pain, inflammation, and tissue repair processes.
Thus, in normal-, healing- and tendinopathic tendons three major neuronal signalling pathways consisting of autonomic, sensory and glutamatergic neuromediators have been established. In healthy tendons, these neural elements are found in the paratenon, whereas the proper tendon is practically devoid of nerves, reflecting that normal tendon homeostasis is regulated by pro- and anti-inflammatory mediators from the tendon surroundings. During tendon repair, however, there is extensive nerve ingrowth into the tendon proper and subsequent time-dependent appearance of sensory, autonomic and glutamatergic mediators, which amplify and fine-tune inflammation and tendon regeneration. In tendinopathy, excessive and protracted sensory and glutamatergic signalling may be involved in inflammatory, painful and hypertrophic tissue reactions.
As our understanding of these processes improves, neuronal mediators may prove to be useful in the development of targeted pharmacotherapy and tissue engineering approaches to painful, degenerative and traumatic tendon disorders.

2. INTRODUCTION

Tissue repair after injury is a complex process, and usually depicted as a series of three to four overlapping sequences of molecular and cellular events (1, 2). These events are influenced by the site of the injury, age, sex, genetics, nutrition, co-morbidities, and a variety of other factors (1-4). The outcome of this coordinated series of events we label wound healing is usually tissue repair, not tissue regeneration, a limitation which can compromise the functional outcome of the healing process depending on the site of the injury. For instance, insults to internal organs such as the heart, lungs, liver, and kidneys can lead to wound healing-like fibrotic processes that can lead to deposition of scar-like material with little functional capability (5). While most soft tissues (e.g. skin, ligaments, tendons) heal via a repair process, some tissues (such as bone) can regenerate after a fracture, in part due to the nature of the bone remodeling process (3, 4).

The goal of most tissue repair in post-natal life is the restoration of the integrity of the tissue even if it is compromised. Thus, injury to the skin leads to development of granulation tissue which remodels over time, but in the meantime the scar tissue prevents loss of nutrients from the organism, and limits the potential for infection. Interestingly, tissue repair following certain types of injury to specific tissues *in utero* can lead to outcomes more resembling regeneration than the fibrotic repair discussed above. The molecular and cellular basis for these differences between post-natal healing and early *in utero* healing has been the subject of intense investigation (6, 7), and it would appear that at least some of the distinction is at the level of differences in the inflammatory response of the organism in the two environments (6, 8), with the inflammatory response *in utero* being somewhat muted and the involvement of specific cytokines/growth factors altered compared to the post-natal situation (9, 10). Whether this early *in utero* response is overt or is a consequence of the altered immune status induced by the mammalian fetal-maternal relationship during pregnancy is not yet clear. It is clear that later in gestation (e.g. equivalent to the third trimester of a human pregnancy), tissue repair following injury becomes more and more similar to post-natal responses (11).

Thus, a muted inflammatory response appears to work in favour of better healing in some circumstances. However, a compromised inflammatory response associated with diseases such as diabetes (a prevalent co-morbidity particularly in the older population with type 2 diabetes; discussed in Purkayastha and Cai, 2013 (12); Hellman et al., 2012 (13); Mirza et al., 2013 (14)), can exert a negative effect on healing, leading to chronic wounds and life-threatening complications (15). Oppositely, excessive or unregulated inflammation may result in abnormal healing, with fibrosis or disruption of the integrity of the tissue function (16). Thus, in a subset of elbow injuries involving the joint capsule (for reasons currently unknown), an abnormal, and apparently exuberant inflammation results in compromised function and joint contractures (17). Finally, “inflammation” can be induced via endogenous or exogenous mechanisms, and sometimes can be confused with tissue catabolism since some of the same mediators may be involved (18). Therefore, the key elements relate to regulation and the “Yin and Yang” of balanced responses to yield effective outcomes without loss of tissue integrity after an injurious insult (19).

As indicated in the above discussion, inflammation plays an important role in initiating and regulating tissue repair following an injury. With the exogenous inflammatory response into an injury site is an influx of circulation-based

inflammatory cells (polymorphonuclear cells, monocytes/macrophages, mast cells) with concomitant expression of a cascade of soluble inflammatory mediators, cytokines and growth factors (20). In response to this environment, the local fibroblasts and other cells in the site together with recruited exogenous mesenchymal stem cells or progenitor cells start to proliferate, migrate and differentiate to synthesize a provisional matrix. This is accompanied by neovascularization to provide blood vessels to support the metabolic activity. Subsequently, over time the matrix remodels, many of the cells in the wound site disappear or undergo apoptosis, and the neovasculature regresses. Ultimately this leaves a somewhat modified matrix compared to the original, and cells populating the matrix that are different from those in the site originally. In tissues highly adapted to perform specific mechanical functions, such as a ligament or tendon, this modified matrix (or scar) rarely functions as well as the original tissue, a situation that could lead to impaired motor performance, or joint dysfunction, including osteoarthritis and/or decreased survival in an animal in the wild.

Much of what we know about normal tissue repair/wound healing has come from studies that have focused on skin wound healing (1, 2, 21, 22), as well as the healing of other soft tissues such as tendons and ligaments. The emphasis has been on what many investigators believe to be the major contributors to outcomes, namely the most prominent cell types involved in the inflammatory responses, the quantity and quality of the matrix formed, and the regulation of the neovascularization events. Conversely, how these components are altered in compromised healing/repair due to co-morbidities such as diabetes, associated with chronic wounds in patients who are either aged and/or have compromised cardiovascular systems, or in patients taking certain drugs for other conditions, has also been the subject of intense investigation due to the impact of loss of tissue repair capabilities on survival, quality of life, and cost to the health care system.

Recently, more attention has focused on a system that has not normally been the topic of much research attention as it is not prominent in many of these tissues (e.g. skin, ligaments and tendons), and therefore the contribution of neural elements to normal tissue function, the response to injury in a variety of circumstances, and compromised tissue repair is only now starting to become the subject of more investigation. The view of the peripheral nervous system as a passive "messenger", conveying information about tissue injury to higher centres, has been superseded by the view that neural regulation is actively involved in repair processes, even if the relative contribution based on "cell" number in these tissues is small, since a regulatory system by its nature should likely be in such a situation so as not to interfere with function. Normally, connective tissues such as ligaments, tendons, menisci, and intervertebral discs are considered hyponeural, and tissues such as articular cartilage are aneural, and do not heal or repair well. However, while present at low density, neural elements can contribute to tissue regulation and response to injuries (both subclinical and overt) via release of potent neuropeptides that can modify the activity of normal fibroblast-like cells in such connective tissues (23, 24), and interact with endogenous inflammatory cells such as resident mast cells and macrophages to amplify their influence (25-27) and regulate the functioning of the microvasculature present in these tissues or occurring in response to neovascularization stimuli.

An example of such "amplification" of neural impact on normal tissue homeostasis, as well as during normal and abnormal healing is what has been termed the "Neural-Mast Cell-Myofibroblast/Fibroblast Axis" (17, 26). This postulated axis of influence has been partially characterized during skin wound healing in domestic pigs (28), flexor tendon healing in a rabbit model (29), in a rabbit model of joint injury with joint contracture formation (17) (30), and possibly in human tendinosis tissue (31). Recent in vivo (32) and in vitro studies have supported the concept using tendon cells or cells from human joint contractures (Hildebrand et al., submitted) in fibroblast-mast cell-neuropeptide collagen gel contraction studies. Thus, the neural influence could be amplified via such mechanisms and impact both normal and abnormal healing if injured tendons and other connective tissues.

While the neural elements in normal and repairing connective tissues are not present at high density, due to the complexity of different types of neural elements and their associated neuropeptides and functions, it is not yet clear how the specific contributions of unique components of the neural system are themselves regulated, and how the integrity of these components regulate outcomes of the repair process. Therefore, we present the current state-of-the-art with regard to neural influences on connective tissue healing, with a focus on tendons. Obviously, there may be site- or tissue-specific involvement of the neural system in maintenance and repair of tendons, but there may also be some generalizations that will provide clues to how the neural system contributes to normal function and repair in a tissue-independent manner.

One key point that should be emphasized is that neural influences are dynamic across the lifespan. It is known that innervation of some tissues declines with age (and may be influenced by genetics) (33, 34). It is also known that sex/gender can influence inflammatory pathways and neural impact (35). Thus, this dynamic nature may complicate interpretation of findings, but it also offers insights into regulatory points that may lead to new approaches to overcome deficits or excessive involvement in healing processes.

3. CENTRAL NEURONAL PATHWAYS

There are three major neuronal pathways by which the central nervous system regulates the inflammatory healing reflex that may be the most critical step in the repair process. The autonomic and sensory neuronal regulatory systems in combination with the glutamatergic excitatory system modulate inflammatory and trophic cellular response mechanisms through a combined release of classic nerve transmitters and so called neuropeptides (Table 1). The presence of contralateral changes in animal models of tendinopathy is highly suggestive of a key role of the central nervous system in this process (36, 37). Furthermore, recent studies have also indicated a contralateral effect during calcium deposition following injury in a murine Achilles tendon injury model (5). A mis-tuned inflammatory healing reflex by, for example, inadequate or excessive neuronal signalling can lead to deficient repair or even chronic tendon disorders (38, 39). Evidence of central pain sensitization exists in clinical studies of patients undergoing shoulder acromioplasty surgery for tendinopathy of the rotator cuff (40).

3.1. Autonomic regulation

3.1.1. Sympathetic innervation

The sympathetic nervous system regulates inflammation at local and systemic levels through the release of norepinephrine (noradrenaline) together with neuropeptide Y (NPY). Activation of the sympathetic outflow by flight-or-fight responses or pain can increase local concentrations of adrenaline and noradrenaline, capable of suppressing inflammation (38). Adrenergic and Y (NPY) receptors on immune cells allow the immune system to respond to neuromediators released from sympathetic nerves and thereby establish a neuro-immune coupling.

3.1.2. Parasympathetic innervation

The parasympathetic nervous system likewise regulates the inflammatory reflex at local and systemic levels through the combined release of acetylcholine (Ach) and the neuropeptide vasoactive intestinal polypeptide (VIP). Parasympathetic activation of the vagus nerve is called the ‘cholinergic anti-inflammatory pathway’ because it leads to mediator release in multiple organs of the body (38). Ach and VIP then interact respectively with nicotinic (ACh receptor) and VIP-receptors on tissue macrophages, which inhibit the release of pro-inflammatory cytokines (eg. TNF, IL-1, HMGB1).

3.2. Sensory regulation

Interestingly the sensory nervous system seems to act principally through release of slowly acting mediators, i.e. neuropeptides. Recent research has additionally disclosed the classical transmitter glutamate in sensory nerve fibers demonstrating an interaction between neuropeptides and classical transmitters in sensory fibers as well.

3.2.1. Sensory innervation

The paradoxical “efferent” role of afferent sensory fibers is now widely recognized (41). Sensitization of the primary afferent nociceptive nerves gives rise to altered stimulus–response coupling, leading to release of sensory neuropeptides eliciting the so-called neurogenic inflammation. The sensory neuropeptides consist of five tachykinin peptides: substance P (SP), neurokinin A (NKA), neuropeptide K, neuropeptide-g, and neurokinin B and calcitonin gene-related peptide (CGRP), which coexists with and potentiates the effect of SP (42-44). Immune cells that appear during the different phases of healing express sensory neuropeptide receptors, allowing them to be regulated (45). SP and CGRP exert pro-inflammatory effects such as vasodilation and protein extravasation (46, 47). An interesting example of the “efferent” role of afferent sensory fiber stimulation is depicted in a unilateral overuse model for Achilles tendinopathy. An increase in SP also in the unexercised control legs of an animal model points towards the involvement of descending neuronal pathways in tendinopathy development (37).

3.2.2. Opioid and opioid like signalling

The sensory nerve fibers also contain peptides with anti-inflammatory effects counteracting the effects of SP and CGRP. Thus, galanin (GAL), somatostatin (SOM) as well as opioid peptides (enkephalins, dynorphins, endomorphins), all of them occurring in primary afferents, inhibit inflammation and nociception (48-51).

3.3. Glutamatergic regulation

Accumulating data support the notion that modulation of glutamate receptors, ionotropic (NMDA, AMPA, Kainate) and metabotropic (mGlu), may have potential for targeted therapy in several persistent pain conditions, including neuropathic pain resulting from injury and/or disease of central or peripheral nerves and inflammatory or joint-related pain (e.g., rheumatoid arthritis, osteoarthritis, tendinopathy). Persistent pain is postulated to depend, at least in part, on long-term increases in synaptic efficacy of glutamatergic signalling in central nociceptive pathways, often called central sensitization (52).

AMPA receptors are present in both myelinated and unmyelinated sensory nerves of rat (GLU_{A1}) and human skin ($GLU_{A2–3}$). Increased levels of glutamate and the AMPA receptor have been shown in experimental arthritis, suggesting that enhanced glutamate release acting on peripheral AMPA receptors may contribute to the initiation of nociceptive signalling. Activation of both peripheral and central NMDA receptors as well has been implicated in pain processing (53, 54). There also appears to be an interaction between neuropeptides and glutamate. Thus, SP has in the central nervous system been demonstrated to remove a magnesium ion from the NMDA receptor, which enables glutamate to bind, thereby initiating nociceptive transmission.

Nociceptive afferents are mostly divided into two groups: peptidergic (SP-positive) and non-peptidergic, which typically express ATP-activated $P2X_3$ receptors. Kainate receptors immunoreactive to GLU_{K5} mostly display $P2X_3$-compared to SP-immunoreactivity, suggesting a greater role for the kainate receptor (GLU_{K5}) in neuropathic than inflammatory pain conditions (52).

Also, the metabotropic receptors (mGlu1 and mGlu5), which are known to potentiate the NMDA receptor excitation, are expressed in dorsal root ganglia cell bodies and peripheral terminal endings, suggesting that there are multiple loci for modulation of primary afferent sensory transmission.

4. PERIPHERAL NEURONAL PATHWAYS

The same pathways with nerve transmitters and neuropeptides occurring centrally are also found peripherally (Table 1). The presence of nerve fibers, including various neuromediators in tendon, has been demonstrated by combined quantitative and morphological assessments with an almost identical neuroanatomy of rat and human tendons (31, 55-57).

4.1. Autonomic pathways

4.1.1. Sympathetic innervation

The occurrence of sympathetic mediators has been demonstrated in tendons of both animals and humans (58-61). Noradrenaline (NA) and neuropeptide Y (NPY) were mostly observed as networks around larger blood vessels located in the loose connective tissue around the main body of the tendon (Figure 1A-B). These observations would reflect that the sympathetic tendon vasoregulation predominantly occurs in the tendon envelope, i.e. the adjacent loose connective tissue.

Adrenergic receptors have been identified on tendon cells, blood vessel walls and on nerve fibers (59, 60, 62). Moreover receptors for NPY, the Y1-receptor has been identified on tendon cells and blood vessel walls, whereas the Y2-receptor was not identified in the tendon biopsies (63). Adrenergic stimulation of tendons may be involved in cell proliferation of fibroblasts, tenocytes, endothelial cells and possibly nerve sprouting, which all are characteristics seen in tendinosis.

Elevated sympathetic activity is also known to act in an anti-inflammatory fashion by inhibiting macrophage activation and suppressing synthesis of tumor necrosis factor (TNF) and other cytokines (38). Interestingly NPY has been demonstrated as a potent immune mediator with both pro-inflammatory and anti-inflammatory actions (64, 65).

4.1.2. Parasympathetic innervation

The parasympathetic mediators acetylcholine and VIP were both identified in tendon (58, 66, 67). Nerve fibers displaying immunoreactivity to VIP have been observed as long thin varicose nerve terminals forming a "fence" in the paratenon (Figure 1C). The nerve fibers were evenly distributed between vascular structures and free nerve endings.

As opposed to sympathetic (NA/NPY) nerve terminals occurring mostly in larger vessels, VIP has predominantly been found around smaller vessels (58, 61, 68). This observation may reflect that NA and NPY (vasoconstrictive) predominantly regulate the main blood flow to the tendon proper, whereas VIP (vasodilatory) is responsible for the fine-tuning of blood flow at a microlevel (58).
The non-vascular distribution of VIP would seem to comply with an anti-inflammatory effect in the periphery (Figure 1C) (69-71). The strong anti-inflammatory role of VIP has been suggested to act through inhibition of T cell proliferation and migration (64, 71-73).

Acetylcholine (Ach) receptors have been identified on tendon cells and blood vessels (66, 67), while VIP receptors (VPAC1, VPAC2 and PAC1) have yet to be explored in tendons. Ach receptors located on tissue macrophages may be involved in the so called cholinergic anti-inflammatory pathway, inhibiting the release of pro-inflammatory cytokines (38).

Interestingly, the expression of the nicotinic receptor-alpha7, which is an essential regulator of inflammation by inhibiting tumor necrosis factor release from macrophages, has in tendon been found during early development and hence could be important during embryogenesis (74). Also, nicotinic receptors have been detected at the myotendinous junction and are suggested to promote repair by regulating cell fusion (75). The occurrence of nicotinic receptors in tendons and their function, however, need further exploration.

Overall, mapping of para-/sympathetic mediators in tendon has opened a link to increased understanding of autonomic neuro-immune modulation in tendon homeostasis.

4.2. Sensory pathways

4.2.1. Sensory innervation

The occurrence of sensory neuropeptides SP, CGRP and NKA in tendons of both animals and humans has been disclosed (Figure 2A) (Table 1) (61, 76-79). Sensory nerve fibers have been found in the tendon envelope, i.e. the paratenon, endotenon and surrounding loose connective tissue, whereas the tendon proper, notably, during normal condition is devoid of nerve fibers (Figure 3) (80). This would reflect that the neuronal regulation of tendons highly depends on the innervation of the tendon envelope.

Thus, the abundance of vascular sensory nerve fibers detected in the surrounding loose connective tissues may reflect an important role in the regulation of blood flow to the tendon structures. Both SP and CGRP, in particular the latter, have been reported to be potent vasodilators (81). In addition, they have also been demonstrated to exert pro-inflammatory effects, for example by enhancing protein extravasation, leukocyte chemotaxis and cytokine production (46, 47). The occurrence of sensory free nerve endings unrelated to vessels predominantly seen in the paratenon (Figure 2A) suggests nociceptive, trophic and immune regulatory roles.

4.2.2. Opioid and opioid like signalling

The peripheral sensory nervous system exhibits an opioid and opioid-like source of anti-inflammatory and anti-nociceptive neuropeptides to modulate the sensory system (82-85). However, data on opioids in the peripheral nervous system and specifically tendons are quite scarce (Table 1). Notably, the existence of an opioid system in tendons has been established in the rat (86). The results clearly demonstrated the occurrence of opioid peptides (enkephalins and nociceptin) and opioid-like peptides (GAL, SOM) in the tendon (Figure 2B-C).

Thus, all four enkephalins detected (LE, ME, MEAP, MEAGL), as well as GAL and SOM, predominantly occurred in sensory C-fibers localized in the tendon envelope, i.e. the paratenon, loose connective tissue, and musculo-tendinous junction, whereas no opioids were found in the proper tendon tissue (Figure 2C). This difference in anatomical distribution might suggest that regulation of painful disorders of the tendon mainly occurs in the surrounding tissues, which, during normal conditions, also harbour the sensory and autonomic neuropeptides.
In the loose connective tissue surrounding the tendon, the opioid and opioid-like neuropeptides appear as free nerve endings around the walls of both large and small blood vessels, which may reflect involvement in both vasodilatory actions (87) and anti-inflammatory responses (Figure 2D) (88, 89). In the paratenon and the musculo-tendinous junction, the

opioid and opioid-like peptides mostly occurred in free nerve terminals without any relationship to blood vessels suggesting a paracrine or an autocrine function in the regulation of nociception (Figure 2C) (48, 90, 91). Such a regulation is probably executed in close interaction with the sensory nervous system. Thus, the release of the sensory neuropeptide SP from afferents in the cat knee joint is inhibited by intra-articular enkephalin-analogue injections (92). Whether there is an inherent balance between opioid and sensory neuropeptides under normal conditions is unknown.

The existence of an opioid system in tendons consisting of neuronal enkephalins was supported by the identification of opioid receptor analyses based on binding assays and immunohistochemistry (56). Of the three opioid receptors (DOR, KOR, MOR) studied, however, only DOR could be detected by immunohistochemistry (Figure 2C). Double staining disclosed co-existence of each of the enkephalins with DOR in the nerve fibers, in accordance with other studies suggesting that enkephalins are the main ligands for DOR (93).

The presence of peripheral opioid receptors was corroborated by receptor binding analysis, showing that tendon tissue could bind the competitive opioid receptor agonist, naloxone, in a specific and saturable way. DOR activity has been demonstrated to exert a potent inhibitory effect on SP-release (92, 94). Treatment with delta opioid agonists in the periphery elicits both anti-inflammatory and anti-nociceptive effects in models of inflammation (95). Thus, peripheral acting opioid agonists may prove effective in preventing the symtoms associated with tendinopathy (96).

Depiction of sensory and opioid pathways in the tendon envelope suggests an intricate homeostatic balance in nociception, trophic actions and immune regulation occurring in the tendon surrounding structures.

4.3. Glutamatergic regulation

Glutamate transmission, which occurs in the central nervous system, has recently also been shown to take place in the peripheral nervous system through interaction with its ionotropic (AMPA, NMDA, kainate) and metabotropic receptors (mGlu) (97).
In tendon, both the ligand glutamate and the NMDA- as well as several mGlu receptors have been identified (Table 1). Both glutamate and its receptors have been identified in nerve fibers, blood vessels and tendon cells. Likely sources of glutamate production may be nerve fibers and, in tendinopathy, the tenocytes themselves (98-100). More information is available in the section on neuronal responses to tendinopathy. Peripheral glutamatergic regulation may be involved in the maintenance of tendon strength, cell proliferation, differention and in pathological cell transformation (97, 99). Moreover, the peripheral glutamate signalling has been implicated in tendon tissue repair (101, 102).

Overall, in tendons there seems to exist similar neuronal pathways consisting of different autonomic, sensory, opioid and glutamate neuromediators as observed in other organs of the body. One important conclusion on tendon neuroanatomy is that the tendon proper during normal conditions is devoid of nerve fibers, while innervation is found in the tendon envelope, i.e. the paratenon, endotenon and surrounding loose connective tissue (Figure 3A-C).
Another vital feature of the neuronal pathways which appears is the balance between different mediators, i.e. pro- and anti-inflammatory peptides. These observations would suggest that homeostatic regulation of healthy tendon tissue is highly dependent on balanced neuro-immune-mediator modulation occurring in the tendon envelope.

5. NEURONAL RESPONSE TO TENDON INJURY

There is anatomic evidence of dynamic peripheral neuronal responses to tendon injury in a rat model of Achilles tendon rupture. Axonal sprouting and growth and a time dependent expression of neuropeptides have been found to occur during healing of Achilles tendon rupture in the rat (Figure 4-6) (80, 103). Similar reactions can be observed in other tendons and in human tendon repair as well (29, 102, 104). The study presented was conducted using immunohistochemistry including a semi-quantitative assessment focusing on the rupture site of the proper tendinous tissue. Neuronal markers for regenerating and mature fibers, ie. growth associated protein 43 (GAP) and protein gene product 9.5 (PGP), respectively, were analyzed at different time points (1 to 16 weeks) post-rupture. The temporal expressions of sensory and autonomic neuromediators were assessed at the same time points.

5.1. Inflammatory phase

In the first week post rupture, the increased occurrence of neuronal markers indicates nerve regeneration both in original nerve fibers of the tendon envelope, i.e. paratenon and surrounding loose connective tissue, and, notably, in new nerve fibers in the proper tendon tissue of the rupture site (Figure 4).

In the proper tendinous tissue, normally devoid of nerves, there was a clear GAP-immunoreactivity suggesting new nerve fiber ingrowth, which is consistent with reports on GAP levels in dorsal root ganglia after peripheral nerve injury (Figure 5) (105, 106). GAP may be involved in nerve regeneration by regulating growth cone motility and axon guidance signals (107). These observations of early nerve regeneration are in line with observations on bone, ligament and skin healing indicating that nerve ingrowth is a fundamental aspect of tissue healing (22, 108-111).

5.1.1. Autonomic regulation

At 1 week post injury, there was only weak scanty expression of sympathetic (NPY) and parasympathetic (VIP) mediators. These findings suggest suppressed anti-inflammatory effects of the autonomic nervous system during the inflammatory phase.

5.1.2. Sensory regulation

At 1 week, SP and CGRP nerve fibers were predominantly located in blood vessel walls surrounded by inflammatory cells in the loose connective tissue (Figure 8A). The findings comply with the nociceptive role of sensory neuropeptides, but also with a pro-inflammatory role. Thus, SP release enhances vasopermeability, probably to stimulate recruitment of leukocytes and cytokine production (112-115). The opioid like mediator GAL only showed weak expression, reflecting low anti-inflammatory actions (Figure 6).

5.1.3. Glutamatergic regulation

During the first week post tendon injury, microarray- followed by real-time PCR analyses demonstrated an up-regulation of glutamatergic signalling molecules involved in activation of the metabotropic glutamate receptor type 1 and the NMDA receptor (116). The localisation of the NMDA receptor on tendon cells was further evidenced by immunohistochemical staining.

It is plausible that the glutamatergic system is used to coordinate some aspects of tendon healing as has been shown in development and maintenance of bone tissue (97). An interesting observation was the fact that the expression of glutamatergic signalling molecules during tendon repair demonstrated a temporal relationship to genes involved in embryonic development (116).

5.2. Proliferative phase

From 1 to 6 weeks post rupture, there was a striking shift in neuronal occurrence from the surrounding loose connective tissue into the proper tendinous tissue. This would seem to reflect the transition of a predominantly inflammatory into a proliferative phase. The peak expression of GAP-immunoreactivity at the rupture site occurred between week 2 and 6, while that of PGP occurred somewhat later, i.e. between weeks 4 and 6 (Figure 5). The extensive ingrowth of new nerve fibers into the rupture site probably represents a neuronal involvement in tendon repair. The observed free nerve endings among fibroblasts in the tendinous tissue may reflect a stimulatory role in cell proliferation (Figure 4B) (117). The occurrence of free nerve endings around newly formed blood vessels at the rupture site suggests a role in vasoregulation, and possibly in angiogenesis (Figure 7) (118).

5.2.1. Autonomic regulation

During the proliferative phase, the occurrence of the autonomic mediators NPY and VIP was sparse until week 4. Subsequently, the expression increased to reach a peak at the end of the regenerative phase, ie. at about week 6, followed by a successive decrease.

The observations of low sympathetic (NPY) innervation would reflect that vasoconstriction is downregulated during tissue repair. The balance between the vasoconstrictive actions of NPY and the vasodilatory actions of CGRP is of decisive importance for the supply of oxygen and nutrients to the healing area. The high ratio of CGRP to NPY in the proliferative phase of healing probably represents highly perfused vessels, a necessity for tissue repair. Similar observations on the ratio CGRP to NPY have been made in studies on reinnervation of skin flaps, where CGRP immunoreactivity emerged at 2 weeks postoperatively and that of NPY 2 weeks later (119, 120). The low levels of NPY observed may also promote angiogenesis (121).

The reduced expression of parasympathetic VIP appears surprising considering the need of vasodilation during tissue repair. However, the low occurrence of VIP possibly pertains to its inhibitory action on the pro-inflammatory effects of SP and CGRP (69). Thus, the observation suggests less inhibition of SP and CGRP, which are presumed to be important regulators of early tissue repair.

5.2.2. Sensory regulation
During weeks 1 to 6, the expression of SP and CGRP peaked (Figure 6). Notably, this peak occurred at the rupture site of the proper tendon, while the sensory neuropeptide expression in the surrounding loose connective tissue declined. The latter would seem to comply with decreased pain 3-4 weeks after injury, which is substantiated by a recent microdialysis study demonstrating resolving inflammation after two weeks post tendon rupture (122). The most conspicuous finding, however, was the occurrence of SP and CGRP in free sprouting nerve endings among fibroblasts in the healing tendinous tissue (Figure 8B). The observation might reflect a stimulatory role of sensory neuropeptides on cell proliferation, as demonstrated in cultured fibroblasts (117, 123). SP and CGRP are also known to stimulate proliferation of endothelial cells (124-126). The observation of free sprouting SP- and CGRP fibers around newly formed blood vessels in the rupture site would comply with a role in angiogenesis (Figure 7). Recently, SP has also been demonstrated to exert an important role in stem cell mobilization during tissue repair (127).

The reduced occurrence of the opioid-like GAL presumably pertains to its inhibitory action on the pro-inflammatory effects of SP and CGRP (128-130).

5.2.3. Glutamatergic regulation
At two weeks post tendon injury, microdialysis followed by quantification demonstrated a 3-times up-regulation of glutamate levels in the paratenon of the healing tendon (102). Of all metabolites assessed during healing glutamate exhibited the highest elevation (102). This observation further strengthens the conception that the glutamatergic system is involved in regulating some aspects of proliferative tendon healing. Hypothetically, glutamate signalling molecules may be involved in regulating cell proliferation and differentiation as has been shown in other tissues (97).

5.3. Remodelling phase
During weeks 6 to 16 post-rupture the nerve fibers appeared to regress from the proper tendon tissue (Figure 5). While GAP-immunoreactivity almost completely disappeared during this phase, that of PGP successively returned to normal in the paratenon and surrounding loose connective tissue. The process appeared to end simultaneously with the completion of paratenon repair.

5.3.1. Autonomic regulation
Between week 4 and 6, corresponding to the transition of the proliferative into the remodeling phase, there was a dramatic increase in the expression of the autonomic neuropeptides VIP and NPY. The increase in the immunoreactivity for VIP and NPY was observed both around vessels and in free nerve endings in a "border zone" enveloping the healing tendon.

The upregulation of parasympathetic VIP may be explained by its inhibitory effect on immune cells expressing pro-inflammatoy cytokines (131). The increased occurrence of sympathetic NPY during this phase, mostly seen around vessels, should probably be attributed to its vasoconstrictive actions. Notably, increased vasoconstriction leads to a relative hypoxia, which enhances the tensile strength of the tendon by switching production of collagen from type III to type I (132).

5.3.2. Sensory regulation
Between week 4 and 6, matching the upregulation of autonomic neuropeptides, an increased occurrence of the opiod like GAL was observed both around vessels and in free nerve endings enveloping the healing tendon (Figure 6). The emergence of GAL, known to modulate the effect of sensory neuropeptides, would seem to comply with inhibition of the early inflammatory and nociceptive response to injury. Thus, GAL has been demonstrated to mitigate the proinflammatory and nociceptive effects of SP (49-51, 128).

Subsequent to the elevated expression of GAL and the autonomic neuropeptides, a significantly decreased expression of SP and CGRP followed (Figure 6). Thus, the early remodelling phase after tendon injury seems to be characterized by an increased expression of GAL, VIP and NPY, all of which are known to modulate the effects of SP and CGRP. It may well be that this modulation is required to end the nociceptive, inflammatory and reparative processes, thereby permitting entry to and maintenance of the remodelling phase.

5.3.3. Glutamatergic regulation

At six weeks post tendon injury, microdialysis followed by quantification demonstrated resolving glutamate levels as compared to the proliferative phase in the paratenon of the healing tendon (Ackermann et al. - unpublished data). The temporal expression of glutamate signalling during tendon healing seems to correspond to that of the sensory mediators. This observation may suggest that the glutamatergic system could interact with sensory mediators in regulating cell proliferation and differentiation during proliferative tendon healing.

On the whole, the observations during tendon healing clearly demonstrate the capability of the peripheral nervous system to adapt and respond to an injury. This plasticity is characterized by nerve fiber ingrowth into the rupture site, and a peak nerve fiber expression during the proliferative phase followed by nerve fiber withdrawal. Interestingly, new nerve ingrowth provides a delivery route for neuronal mediators that are required for tissue repair. Subsequently, the temporal expression of the different neuropeptides studied implies specific actions to regulate the inflammatory, proliferative and remodelling healing phases. Thus, the end effect on tissue healing will depend on the nerve fiber localization, temporal neuropeptide expression and cellular receptor expression.

6. NEURONAL CONTRIBUTIONS TO TENDINOPATHY

Interestingly, in tendinopathies with chronic pain and a failed healing response, altered neural elements have been noted in a variety of tendons from various locations (Table 2). However, the cause-and-effect relationship is currently an area under investigation in most instances for the nerve endings and neuropeptides in the pathology.

With that limitation, immunohistochemistry analysis of tissue samples does demonstrate similar patterns of innervation in tendinopathy tissue as is seen during the proliferative phase of healing after tendon injury. Thus, chronic painful tendons exhibit new ingrowth of sensory nerve fibers (Figure 9) (78, 79, 133), which is also observed during tissue proliferation in healing tendons (80). In normal tendon repair, sensory nerve ingrowth is correlated with increased nociception (103). This inflammatory phase is followed by peripherally acting autonomic and opioid-like signalling, coinciding with decreased nociception (103). Hence, the neuronal dysregulation in tendinopathy, characterized by aberrant increase of sensory nerve sprouting and a deficient autonomic and opioid-like modulation, presumably triggers pain signalling and possibly also the hyperproliferative/degenerative changes associated with tendinopathy (Figure 10). The ongoing morphologic alterations may reflect protracted or failed healing. An important point related to this concept is the role of nerves and neuropeptides during an initial response to an insult leading to the first symptoms of tendinopathy versus their role during the chronic phases of the condition, phases that can lead to overt degeneration, and, in some instances, rupture of the tendon. Some neuropeptides may serve different functions during these different “phases”, or different neuropeptides could be involved. It has been postulated that a nerve/neuropeptide-mast cell-tenocyte axis may play a role in tendinopathies and over-use syndromes (25-27), and recently it has been confirmed that higher mast cell numbers are present in human patellar tendinopathy tissue samples of patients with disease of long standing as compared to samples from more recent onset (134), and there may be a destruction of a fine equilibrium with the alterations to the neural components. Thus, some aspects of tendinopathy may be independent of such an axis, while others may involve this axis to amplify the impact on the tendon tissue.

6.1. Autonomic regulation

6.1.1. Sympathetic innervation

Chronic painful tendons exhibit a decreased occurrence of sympathetic nerve fibers, immunopositive to noradrenaline. Microscopic analysis demonstrated that sympathetic nerves related to blood vessels were distinctively decreased in patients with tendinopathy (Figure 11) (58). Computerized image analysis confirmed a 50% drop in vascular nerve fibers immunoreactive to noradrenaline in the painful tendons (58). The reduction in vasoregulatory noradrenalin suggests a reduced blood flow and a suppressed anti-nociceptive function. A recent study demonstrated that noradrenaline release leads to secretion of opioids from leukocytes (90). Similarly, patients with painful rheumatoid arthritis exhibit a decrease in vascular innervation expressing noradrenaline (135). To counteract the decreased sympathetic innervation, immune cells in the synovia from rheumatoid arthritis patients respond by upregulating noradrenaline. Likewise, in tendinopathy patients an upregulated noradrenaline production has been suggested in morphologically altered tenocytes (60, 136).

Adrenoreceptors for noradrenaline have also been identified in tendinopathy. Immunoreaction for the alpha1-adrenoreceptor has been detected in blood vessel walls, nerve fascicles and tenocytes (60, 136). Adrenegic activation of

alpha1/2-adrenoreceptors has been demonstrated to stimulate cell proliferation and differentiation (137, 138). Thus, upregulated noradrenaline in tendon cells may stimulate the alpha1/2-adrenoreceptors and contribute to tenocyte excessive cell proliferation and differentiation. In contrast, decreased vascular innervation expressing noradrenaline may be compensated by the observed increased alpha1-adrenoreceptor immunoreactions in blood vessel walls of tendinopathic patients (60, 136). More recently, alpha1-adrenoreceptor expression in tendon cells has been found to be associated with NPY expression (139), concluding a strong role of the sympathetic nervous system in peripheral tissues.

6.1.2. Parasympathetic innervation

The cholinergic innervation in tendinopathy appears to be relatively scarce. Neuronal immunoreaction to choline acetyltransferase, vesicular acetylcholine transporter and acetylcholinesterase in tendons is reported to be limited compared to other tissues investigated. However, whether cholinergic innervation in tendinopathy is significantly lower than that of healthy tendons is still unclear (66, 67, 140).

Immunohistochemical studies have identified activated markers of cellular acetylcholine production in human tendinopathy, most prominently seen in morphologically altered tenocytes (66, 67, 140). Immunoreaction to choline acetyltransferase and vesicular acetylcholine transporter has recently been detected in normal Achilles tenocytes (141). This suggests that resident tenocytes have the capability to produce acetylcholine, which is increased during tendinosis. This process implies that acetylcholine either is involved in regulating the tenocyte transformation seen in tendinopathy (142), or that acetylcholine synthesis is initiated in tenocytes in response to development of tendinopathy.

Parasympathetic muscarinic acetylcholine receptors (M2) have been identified in human tendinopathy. Thus, M2-receptors have been identified in tendon cells, blood vessel walls and nerve fibers (66, 67). The difference between normal and tendinopathic tendons consisted in an intense M2-immunostaining in morphologically altered tenocytes, which was not seen in normal tenocytes. The observations demonstrate an endogenous autocrine and/or paracrine acetylcholine and M2-receptor signalling in transformed tenocytes. M2-receptors have also been found to contribute to an increase in cell proliferation and hypercellularity, through an autocrine loop, which is replicative of the early stages of tendon healing (141).

6.2. Sensory regulation

6.2.1. Sensory innervation

Immunohistochemical and semi-quantitative assessments have clearly demonstrated that tendinopathic tendons exhibit an increased number of sensory SP-positive nerve fibers (Figure 9) (78, 79, 133). Closer analysis revealed that the nerve fibers occurred mainly as thin, varicose, sprouting nerve terminals within the tendon proper. The observation of increased ingrowth of sensory nerves into the painful tendon proper, seen as sprouting free nerve endings, possibly represents nociceptors responding to mechanical stimuli by initiating pain signalling.

The increase of the sensory neuropeptide SP in tendinopathy may, in addition to its role in nociception, reflect pro-inflammatory and trophic actions (143). Thus, SP has been found to participate in inflammatory actions such as vasodilation, plasma extravasation, and release of cytokines. SP has also been reported to stimulate proliferation of fibroblasts (143) and endothelial cells, as well as the production of transforming growth factor beta in fibroblasts. Hence, SP may well contribute to the morphologic changes observed in early tendinopathic patients, that is, tenocyte transformation, hypercellularity, and presumably neovascularization, in conjunction with mechanical loading (37, 144), and may therefore precede tendinosis.

The implications of the above mentioned effects of SP are all plausible with respect to tendon pathology since its receptor, NK-1, has been detected in tenocytes, blood vessel walls and in nerve fibers in tendinopathy (145). SP has further been shown to directly stimulate nociceptor endings in an autocrine/ paracrine manner. Similar actions could presumably occur in tendinopathy since the NK-1 receptor is present.

6.2.2. Opioid and opioid like signalling

To date there are very few publications on opioid and opioid-like signalling in tendinopathy. In line with a depressed opioid and opioid-like signalling in tendinopathy, immunohistochemical analysis detected a low occurrence of galanin (unpublished data, Ackermann *et al*.). However, a recent publication detected increased cellular expression of cannabinoid receptors in tendinopathy (146).

6.3. Glutamate pathways

Elevated interstitial levels of glutamate have also been found in tendinopathy by microdialysis (147). Furthermore, the specific localization for the increased glutamate levels has just recently been established in tendinopathic patients (100, 148). Thus, up-regulated glutamate occurrence is observed in morphologically altered tenocytes, in the endothelial and adventitial layers of blood vessel walls and in nerve fibers. Injection of glutamate has also been shown to provoke and maintain local prolonged tendon pain (149), potentially through both the ionotropic and metabotropic receptors.

One receptor for glutamate, NMDA receptor 1 (NMDAR1) has been identified in tendinopathy. Recently, both subjective and quantitative assessments demonstrated a 9-fold increased NMDAR1 occurrence in tendinopathic patients (98, 99). This finding was corroborated by a study on rat supraspinatus tendon overuse likewise demonstrating NMDAR1 upregulation (116).

Maybe the most intriguing finding regarding glutamatergic signalling is the recent report demonstrating that elevated glutamate co-existed with its up-regulated receptor NMDAR1 in nerve fibers, morphologically altered tenocytes and blood vessels (98), which may reflect cell-hyperexcitation involved in cell proliferation/differentiation. However, none of the controls exhibited neuronal co-existence of glutamate and NMDAR1 in contrast to prominent neuronal occurrence in all the painful tendons, which strongly suggests a role in pain regulation (98).

Overall, the neuronal mediator contributions to tendinopathy seem to involve an intricate regulation of cell proliferation/survival and differentiation. However, at present it is unclear to what extent this is regulated by a cellular origin of neuromediators versus a neuronal origin of neuromediators. Current and emerging data may prove that a balance in neuromediator levels is critical for tendon tissue integrity and that dysregulated neurosignalling contributes to the morphological features, i.e. cellproliferation and celltransformation, associated with tendinopathy.

7. MOLECULAR RESPONSES TO NEUROPEPTIDES AND NEURONAL INFLUENCES

Denervation impairs the healing of skin, bone, ligament and tendon in a variety of animal models (150-155). In the medial collateral ligament, a tissue with many structural and functional similarities to tendon, denervation significantly attenuates the response to injury. Scars from denervated ligaments exhibit lower blood flow, diminished angiogenesis and decreased mechanical strength when compared to their normally innervated counterparts (155).

7.1. Denervation effects on gene expression in healing ligament *in vivo*

In rabbits, denervation induced significant differences in the mRNA levels for many genes of interest in the injured MCL at two weeks post-injury (156).

7.1.1. Denervation increases mRNA levels for collagen I and III and TGF- ß1

In the denervated injury group, mRNA levels for the matrix components collagen I and III were both increased at two-weeks post-injury, in comparison to the non-denervated animals ($p \leq 0.01$). Significant differences were not detected at any of the other time points assessed.

Similarly, mRNA levels for the growth factor TGF-ß were increased at two-weeks post-injury, in comparison to non-denervated ($p \leq 0.01$) (Figure 2). VEGF and NGF mRNA levels were not significantly altered at any time points post-injury ($p > 0.01$).

7.1.2. Denervation increases mRNA levels for angiogenesis-associated matrix metalloproteinases (MMP-3, MMP-13 and uPA)

By two weeks post-injury, the mRNA level for angiogenesis-associated collagenase MMP-13 was increased almost 3-fold in the denervated group, in comparison to the non-denervated group ($p \leq 0.01$), and was 20 times the level found in normal ligaments. MMP-3 and uPA mRNA levels appeared increased in the denervated injured ligaments at 2 weeks, but these apparent differences between the experimental groups did not achieve statistical significance at any time point.

7.1.3. Denervation increases mRNA levels for MMP inhibitors (TIMP-1 and TIMP-3) and the angiogenesis inhibitor thrombospondin-1 (TSP-1)
At two weeks post-injury, mRNA levels for the angiogenic inhibitors TIMP-3 and TSP-1 in the denervated injury group were significantly increased compared to those in the non-denervated injury group ($p \leq 0.01$). Notably, mRNA levels for TSP-1 were significantly elevated in the denervated injured group at sixteen weeks post-injury ($p \leq 0.01$). Levels of mRNA for TIMP-1 were not significantly different between innervated and denervated groups at any time points.

The study demonstrated that, in a denervated MCL, mRNA levels for many angiogenic and repair-associated genes are increased during the early stages of healing. The majority of the significant differences between denervated and innervated ligaments were detected at 2 weeks following injury, where levels of six of eleven genes tested were significantly altered. Two weeks post-injury corresponds to the proliferative stage of wound healing in this ligament injury model. Levels of mRNAs for repair-associated molecules are at their highest 2-3 weeks following injury in the rabbit MCL (157).

The denervation-induced increases in mRNA levels for numerous matrix-molecule and angiogenesis-associated genes are difficult to reconcile with the previous work in the same model showing that denervated ligaments heal poorly. Based on the current findings, several possible explanations exist. Firstly, a loss of neuropeptide stimulation may substantially diminish cellular proliferation in the denervated scar, negating the effect of increased mRNA levels. Secondly, the increased levels of mRNA for TIMP-1 and TSP-1 in denervated injured ligament could have an inhibitory affect on cell function and more specifically, angiogenesis (158). Finally, the timing and regulation of expression of various interacting genes are likely very important in the production of an organized, mechanically adequate scar, and the data suggested that denervation disrupted the timing and regulation of mRNA changes for many molecules important in healing.

7.2. Neuropeptide effects in tissue culture
Because of the complexity of the wound healing environment, investigators have sought to gains insights from the observation of cells or tissue specimens in culture where variables inherent to the *in vivo* environment (blood flow, mechanical loading, etc.) are controlled or eliminated. Cellular responses in the form of mRNA or protein production can be assayed in the presence or absence of specific mediators.

Neuropeptide effects on normal and injured ligament in culture
Recently it was reported that tissue cultured specimens of injured ligament respond to the addition of specific neuropeptides to the culture medium (159). In this study, mRNA levels for numerous healing-associated molecules were significantly altered by individual neuropeptides.

7.2.1. Neuropeptides downregulate expression of some growth factors
TGF-ß1 mRNA levels were significantly depressed in 2 week post-injury ligament specimens cultured with 10^{-7} M CGRP and 10^{-7} M NPY compared to untreated specimens. SP had no detectable effect on TGF-ß mRNA levels in injured ligament. TGF-ß1 mRNA levels in normal uninjured ligament and in 3 days post-injury ligament explants were not significantly affected by any of the neuropeptides employed.

The mRNA levels for bFGF were also responsive to neuropeptide exposure, with significant depression detected at 3 days post-injury with NPY, and at 2 weeks post-injury with SP and CGRP.

Similarly, the mRNA levels for VEGF were significantly depressed by CGRP and NPY only at two weeks post-injury.

7.2.2. Neuropeptides increase expression of inflammatory mediators
IL-1 mRNA levels were significantly increased by NPY and SP in the 2 week post-injury specimens. Similarly, NPY increased the mRNA levels for both TNF-α and COX-2 in 2 week post-injury specimens.

7.2.3. Neuropeptides decrease expression of matrix molecules
Type I Collagen mRNA levels were significantly depressed by SP in normal ligament specimens, by CGRP at 3 days post-injury, and by CGRP and NPY at two weeks post-injury.

Type III Collagen mRNA levels were significantly depressed by all three neuropeptides in the 2 week post-injury specimens.

Biglycan mRNA levels were significantly lowered by CGRP in both the 3 day and 2 week post-injury specimens, and by NPY in the 2 week post-injury specimens. Lumican mRNA levels were significantly lowered by NPY in uninjured specimens but were not affected by any neuropeptide in any of the injured specimens.

Thus, SP and NPY can induce increased mRNA levels for inflammatory mediators in specimens of injured ligament placed in culture at 2 weeks after injury. In contrast, all three neuropeptides tested induced significantly lower mRNA levels for several molecules associated with healing in MCL scar, including growth factors, matrix molecules, excluding lumican, and some angiogenesis-associated proteins. These data are consistent with the results of the denervation study, and support the idea that the effects of denervation are largely the result of the loss of neuropeptide stimulation. However, the results of both studies seem at odds with the previous *in vivo* data, showing that denervation impaired ligament healing in the rabbit model (155).

A number of possible explanations could account for this apparent paradox. The three neuropeptides tested promote increased blood flow and/or angiogenesis (160-164). As angiogenesis is widely accepted to be the key determinant of the outcome of wound healing, neuropeptide induced increases in blood flow and accelerated angiogenesis might be more important to the outcome of healing than the observed changes in mRNA levels for matrix molecules and growth factors (165-170).
Neuropeptides are also known to increase cellular proliferation (118, 121, 171, 172). Cells stimulated to proliferate would potentially downregulate or stop producing matrix molecules or growth factors until their proliferative phase was completed. Increased cellularity would likely subsequently lead to the formation of a larger, stronger scar in vivo, and could account for the superior healing of innervated ligaments.

Importantly, not all potentially important neuropeptides known to be present in articular tissues were tested. Vasoactive intestinal polypeptide (VIP), somatostatin (SOM) and met-enkephalin, all present in articular tissues (76, 86, 173, 174), are also likely important modulators of cellular metabolism in healing ligament. Future studies should address the influence of these mediators on healing and scar cell behavior, as the neuropeptide milieu in the healing ligament is likely very complex. Furthermore, most of the significant effects on mRNA levels were observed at two weeks post-injury. This corresponds to the late inflammatory and early proliferative phase of healing in the rabbit MCL. The way cells in the scar respond to neuropeptides appears to be time dependent, with different effects seen at different times after injury (175). Thus, testing specimens retrieved at longer intervals after ligament injury, with a broader variety of mediators, might reveal additional differences in response to neuropeptide stimulation.

Only one concentration (10^{-7} M) of each neuropeptide was tested. Physiologic levels of neuropeptides in healing ligaments or tendons are unknown. The effects of these agents may be dose-dependent and different effects might be seen at lower concentrations.
In vivo, neuropeptides are produced in a temporally and spatially regulated manner by nerve fibers in close proximity to existing or newly forming vessels (76, 176). During development, angiogenesis can be highly regulated by neuronal factors (177). It is not known whether a similar relationship is found in healing ligament, tendon, or other wounds. However, a highly localized down-regulation of matrix production would facilitate angiogenesis, which depends on MMP-mediated matrix digestion to produce a channel for proliferating endothelial cells to migrate into. Since all the neuropeptides tested to date are associated with increased blood flow and angiogenesis, the results would indicate that blood vessels and endothelium are likely the primary targets of these neuropeptides in early ligament healing (161-164, 170).

7.3 Diversity of Fibroblast Phenotypes

In recent years a number of investigations have revealed that fibroblast behavior and activity is strongly determined by genetic and developmental factors, which are retained to a considerable extent in the tissue culture environment. For example, one early study showed that when grown in three dimensional collagen gels, fibroblasts derived from cornea, tendon and dermis oriented very differently after 7 days, with corneal fibroblasts orienting into orthogonal sheets, tendon fibroblasts forming parallel bundles, and dermal fibroblasts remaining randomly oriented (178).

Much of our information about fibroblast behavior in tissue culture, particularly in the context of wound healing, comes from studies of dermal fibroblasts. Even within dermis, fibroblasts in derive from different embryonic precursors. Evidence continues to accumulate that there is considerable phenotypic diversity between fibroblasts found in different locations within a given tissue or organ (179). Thus neuropeptide effects on fibroblast behavior likely are specific to the tissue of origin, and this mandates a cautious approach to the interpretation of results derived from tissue culture based experiments.

8. HEALING RESPONSE TO ALTERED NEURONAL PATHWAYS

8.1. Inadequate neuronal signalling

Several investigations have indicated that denervation impairs the mechanical properties of both normal and injured ligament. A chemical sympathectomy with continuous systemic administration of guanethidine (40 mg/kg/day) leads to degradation of the mechanical properties of the intact medial collateral ligament (MCL) of the knee joint in rats after only ten days of treatment (180). Ligaments from treated animals had a larger cross-sectional area, a higher wet weight, a decreased modulus of elasticity and a decreased stress at failure. Some of these structural changes might be explained by the significantly increased mRNA levels for the matrix degrading enzymes MMP-13 and cathepsin K, and increased ligament blood flow induced by chemical sympathectomy.

As noted in the previous section, femoral nerve transection impairs healing of the medial collateral ligament in rabbits (155). In that study, blood flow, angiogenesis and mechanical strength of the ligament scar were all significantly decreased in denervated limbs compared to normally innervated limbs, 6 weeks after injury. Similar results have since been reported in rats, where surgical sympathectomy or femoral nerve transection each reduced failure loads of healing MCLs by 50% compared to normally innervated healing MCLs, at 2 weeks after injury (181).

One study assessed Achilles tendon healing after performing a specific sensory denervation using Spanish pepper (capsaicin), which reduced the concentrations of SP by ~60% (182). The study demonstrated that the residual SP levels after denervation correlated with the biomechanical tissue properties, i.e. transverse area, ultimate tensile strength, and stress at failure (182). Thus, higher residual SP levels after sensory neuropathy are associated with improved tensile strength and stress at failure in the healing of Achilles tendon.

Taken together these studies strongly support the idea that neuronal derived factors have a powerful influence on the structure, function and healing capacity of dense connective tissues such as ligament and tendon.

8.2. Stimulated neuronal signalling

Recent work has explored the possibility that the exogenous administration of neuropeptides or neurotrophic factors lead to improved tendon and ligament healing. Local injections of SP combined with the neutral endopeptidases thiorphan and captopril were used as a treatment for Achilles tendon rupture in rats (183, 184). This resulted in dose dependent increases in fibroblast number at the injury site between 1 and 6 weeks after injury. Histological parameters of collagen type III occurrence and collagen organization were enhanced in the SP group as compared to the control group (184). Moreover, collagen fiber orientation and angiogenesis were also improved by SP treatment (183). A second report, originating from the same laboratory utilizing the same model, showed that treatment with SP also increased stress at maximal load and work to maximal load in healing Achilles tendon, although stiffness was not improved compared to controls, and actual failure loads were not reported (185). An interesting observation from one of these studies was that the SP treated group exhibited an accelerated withdrawal of sensory nerve fibers from the injury site during healing (184). This observation may be interesting in the context of human tendinopathy with protracted, pathological sensory nerve fiber ingrowth in the tendon. It may prove that SP injections could promote sensory nerve fiber withdrawal.

Another recent study assessed the effect of exogenous neuropeptide administration on the healing of denervated ligaments in rats (181). The aim was to restore or improve the healing responses of the medial collateral ligament in joints partially denervated by femoral nerve transection or surgical sympathectomy. In this study SP, VIP and NPY all improved the healing of denervated MCLs. In the case of SP and VIP treatment, several mechanical properties, including failure load and failure stress were reported to be higher than those of intact, uninjured MCL. Treatment with SP, VIP and NPY also significantly improved the histological appearance of healing MCLs. Treated ligaments showed

more organized scars and the appearance of increased matrix production. Interestingly, CGRP did not improve any of the measured histological or mechanical parameters of MCL healing in this model.
Another approach to the augmentation of neuronal factors in wound healing has been the use of nerve growth factor (NGF). NGF was initially described and characterized as a trophic factor for specific neuronal populations in the peripheral nervous system (186). Subsequent studies have shown that NGF displays an extended spectrum of biological functions (138), and promotes skin wound healing in both rodents and humans (187-189). NGF can promote ligament healing in rats (190). In this study, NGF was continuously administered to the injury site for 7 days via an implanted mini-osmotic pump. At 7 days after injury, the fractional area occupied by blood vessels was increased, indicating that angiogenesis was promoted by NGF. By 14 days, in addition to increased vascular density, an increase in nerve fiber density was noted in the NGF treated specimens, although no differences in the mechanical properties of the ligament were detected. By 42 days after injury, nerve fiber and blood vessel densities were progressively increased, and scar mechanical properties were also significantly improved in the NGF group. Thus, early exposure of injured tissues to exogenous NGF can lead to improved mechanical outcomes, a critical factor for structurally important tissues such as tendons and ligaments. Once optimized, this approach could have broad clinical implications. In this context it is interesting to notice that blockage of NGF with humanised monoclonal antibodies, eg. Tanezumab, in stage 3 clinical trials provides much more effective pain relief than traditional therapy with NSAIDs (191). However, balancing the inhibition of NGF-mediated pain and NGF-mediated regulation of tissue metabolism and repair will be a turning point whether NGF-targeted therapies will ever reach the patients in need (192).

Tendon repair is reported to be promoted also by physical activity, which recently was shown to be linked to accelerated neuronal plasticity (193, 194). Morover, it has been demonstrated that physical activity and training leads to increased levels of various neuromediators, including SP and CGRP, which may be involved in regulating the healing response (193, 195). Interestingly, not only the neuromediator ligands are influenced by physical activity, but maybe more importantly, so are the neuromediator receptors. Thus, mRNA-levels for the SP- and CGRP-receptors are in mobilized tendons significantly increased at 17 days post tendon injury compared to immobilized controls (194). It may prove that enhanced tendon repair after physical activity is related to an increased peripheral sensitivity to sensory neuropeptide stimulation, implying an up-regulation of sensory neuropeptide receptors. Whether the sensory neuropeptide receptors elicit different responses or affect different cell types depending on loading conditions after injury has yet to be investigated.
Other physical means of stimulating tendon repair include intermittent pneumatic compression, which applied 1 hour daily for two weeks post tendon injury induced a substantially elevated occurrence of SP by 110% and CGRP by 47% and simultaneously increased the fibroblast density by 53% and vessel density by 64% (196). Moreover, it was recently demonstrated that intermittent pneumatic compression could promote tendon repair in immobilized condition by increasing the biomechanical tissue properties; maximum force increased 65%, energy increased 168% and tendon length increased 25% (197). These results demonstrate that the compression treatment may have a substantial clinical impact, considering that immobilization is the basic principle in the treatment of ruptured tendons. In fact, clinical translation of the preclinical data has demonstrated that adjuvant intermittent pneumatic compression applied at least 6 hours daily for two weeks post human Achilles tendon rupture upregulated the concentration of local essential metabolites in the paratenon of the healing tendon (102). The metabolite exhibiting the largest elevation, 1.5-times, after adjuvant compression treatment was glutamate (102).

On the whole, our data indicate several interesting pharmacological and physical strategies of promoting neuronal pathways that could be further developed and employed to enhance tendon repair.

9. PERSPECTIVE

From the above discussions, it should be apparent that the neural contributions to normal tissue function, repair, and in some instances, pathology, support the contention that these neural regulatory components play essential, but as yet incompletely defined roles in mechanically active tissues such as tendons. While not present at high density, the neural elements are part of a highly complex integrative system that likely serves multiple roles in such tissues, contributing to homeostasis, proprioception and proper functioning of the various tissues in a joint to ensure that it works as a smooth machine (e.g. the joint as a mechanical organ concept). It is also likely that there are some aspects in common between the repair functions that such neural elements play in different tissues. However, considerable more investigation is needed to provide clarity with regard to the dynamic aspects of their role(s), and how neural elements are modulated by genetics, aging, and sex and co-morbidities. Some of this complexity likely serves fundamental roles

in repair and restoration of function, but it is clear also from the work of Salo *et al* (33, 34, 198) that innervation of normal joint tissues can decline with age in both rats and mice, and thus elevate risk for degenerative joint disease. Whether similar changes occur in tendons and ligaments throughout the body during aging and following events such as menopause, remains to be delineated. Similarly, how such changes impact the repair process in tendons and ligaments, as well as other connective tissues, also remain to be clarified.

In particular, how impaired neural contributions resulting from aging, co-morbidities such as diabetes or genetic variables impact tissue repair will be critical to elucidate. Conversely, excessive or aberrant neural involvement in healing could also be detrimental to functional outcomes. Clearly, the key missing piece to the puzzle is "what regulates the regulators?" and how does our understanding of the neural responses interface with the other elements of the host response to injury. Hopefully a raised awareness of neural contributions to effective tissue repair will stimulate increased research in this area.

From the above discussion, nerves certainly play an essential role in response to injury and healing following an injury. This conclusion is reinforced from preclinical animal studies and from patient populations. The hierarchy of influences on healing is most certainly complex, with redundant systems operative to ensure a reasonable outcome even if the system as a whole is compromised by disease or aging. Interestingly, neural influences likely rank high in such a hierarchy based on what is known regarding connective tissue homeostasis, breakdown and healing in spinal cord injured people or preclinical models (199). Thus, spinal cord injured individuals exhibit alterations in these processes below the level of the injury, but retain regulatory control above the level of the injury. While likely multifactorial, this is evidence for the importance of the neural system in regulation.

While nerves are likely central to regulation, one new area of research could also provide additional insights into chronic conditions such as tendinosis or other chronic abnormal "healing" situations where the responder cells (e.g. fibroblasts, myofibroblasts, and others) become altered. Thus, understanding the impact of epigenetics on responsiveness to regulatory systems such as nerves is currently emerging. Unlike genetics, which is the genome everyone inherits, epigenetic processes occur during life and result in modifications to the genome which in turn affect regulation of gene expression and function ( ). Thus, in conditions of chronic stress of various sorts (200), conditions of chronic exposure to environmental factors (201-203), and due to chronic stimulation, such as in rheumatoid pannus (204, 205) as examples, DNA can be modified by methylation or derivatization affecting outcomes. Whether such changes can occur with some regularity in tendinosis, or abnormal healing situations in tendons, ligaments and other connective tissues, or even be facilitated by neural influences, remains to be determined. However, it is likely that such alterations may relate to the neural regulation of tissue responses, and thus be relevant to better understanding both normal and "deviant" healing processes.

In summary, considerable progress has been made on several of the "fronts" discussed above, to document the involvement and potential involvement of innervation to regulate and modulate repair of tendons and related connective tissues. It is also clear that further progress in this area will require a multi- or trans-disciplinary approach, bringing together molecular and cell biologists with neuroscientists and neurophysiologists, biomedical engineers, and clinician-scientists to advance the field. The field is certainly poised to make significant advances, and such advances may well be translated into new interventions to treat specific patient populations via the basic information generated, as well as identify those at risk (via genomic and epigenomic screening and other approaches) for loss of neural contributions to maintaining connective tissue function and integrity.

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Figure legends

Figure 1. A-C. Immunofluorescence micrographs of longitudinal sections through the Achilles tendon after incubation with antisera to Noradrenaline (NA) (A), NPY (B) and VIP (C). NA-positive fibers are mainly found as nerve terminals in outer layers of the blood vessel walls. The NPY-positive fibers are arranged as nerve terminals in the vessel walls. VIP-positive nerves are arranged as a "fence", surrounding the proper tendon, of small varicosities in the paratenon. t = tendon tissue; Pt = paratenon; Bar = 50 µm. Reproduced with permission from (58).

Figure 2. A-D. Immunofluorescence micrographs of longitudinal sections through the Achilles tendon after double staining (co-localization) with antisera to SP and CGRP (A), SP and GAL (B) LE and DOR (**C**) and incubation with antisera to ME (**D**). A co-existence of SP and CGRP is seen in nerve fibers localised in the paratenon (A), indicating possible pro-inflammatory actions. Moreover, SP is also co-localised with GAL (B), which may reflect anti-inflammatory actions. The immunoreactivity displaying co-existence of LE and DOR is seen as free nerve endings in the paratenon (C), which indicates a potential peripheral anti-nociceptive system. ME immunoreaction is localised in a vessel wall (D). t = tendon tissue; Pt = paratenon; Bar = 50 µm. Reproduced with permission from (76, 86).

Figure. 3. A-C. Overview micrographs of longitudinal sections through the Achilles tendon built up by putting together computerized images of smaller micrographs. Incubation with antisera to general nerve marker PGP. Micrographs depict the proximal half of the Achilles tendon at increasing magnification in figures (A-C). Arrows denote varicosities and nerve terminals. The typical vascular localization of NPY is depicted in (B), whereas the free nerve endings are typical localization of SP (C). The immunoreactivity is seen in the paratenon and surrounding loose connective tissue, whereas the proper tendinous tissue, notably, is almost devoid of nerve fibers pt = paratenon. Reproduced with permission from (80).

Figure 4. A-B. Overview micrographs of longitudinal sections through the Achilles tendon two weeks post rupture built up by putting together computerized images of smaller micrographs. Incubation with antisera to a nerve growth marker, GAP-43. Micrographs depict the proximal half of the Achilles tendon at increasing magnification in figures (**A-B**). Arrows denote varicosities and nerve terminals. The GAP-positive fibers, indicating new nerve fiber ingrowth, are abundantly observed in the healing proper tendon tissue. Reproduced with permission from (80).

Figure 5. Area occupied by nerve fibers (%) immunoreactive to GAP and PGP in relation to total area, in the mid third of the tendon, over 16 weeks post rupture (mean±s.e.m.). Reproduced with permission from (80).

Figure 6. Area occupied by nerve fibers (%) immunoreactive to SP, CGRP and GAL in relation to total area, in the mid third of the tendon, over 16 weeks post rupture (mean±s.e.m.). Reproduced with permission from (103).

Figure 7. A-H. Photomontage of typical high power images of immunohistochemically stained nerve fibers (arrows) at different time points after medial collateral ligament injury in the rabbit. Scale bar = 20 microns At two weeks CGRP fibers appear very fine in the early scar. At 6 weeks there is the appearance of CGRP-immunoreactive growth cone like structures (large arrow head) and sprouting as well as a predominantly perivascular distribution of fibers. Fourteen weeks post-injury there are fewer CGRP-immunoreactive fibres in the scar with most found again in the epiligament. All SP positive profiles were found to be perivascular at 2 weeks after injury. Similar to the appearance at 2 weeks, SP positive profiles were only found associated with proliferating vessels within the scar at later time points. The letter v indicates a vessel lumen. Scale bar at bottom right = 20 microns and applies to the entire montage. Typical image of NPY containing fibres associated with small arterial vessels in a 6 week post-injury scar. The letter v indicates a vessel lumen. Six weeks post-injury, staining for the marker PGP 9.5, which stains all nerve fibres, revealed a pattern that combined aspects of the other markers, with most fibres in a perivascular location, and some free in the scar matrix. Reproduced with permission from (78).

Figure 8. A-B. Immunofluorescence micrograph of longitudinal sections through healing Achilles tendon 1- (A) and 2- (B) weeks post rupture after incubation with antisera to CGRP. Nerve fibers immunoreactive to CGRP at week 1 are seen as vascular and free nerve endings in the loose connective tissue (A). At week 2, CGRP- immunoreactivity occurs mainly in the healing tendinous tissue as sprouting free nerve fibers (B). v = blood vessel; lct = loose connective tissue; t = proper tendon tissue; Bar = 50 μm. Reproduced with permission from (103).

Figure 9. A-B. Immunofluorescence micrographs of longitudinal sections of healthy Achilles tendon (A) and tendinosis tissue (B) after immunostaining for SP. Arrows denote varicosities and nerve terminals. The micrograph illustrates SP-positive nerve fibres in close vicinity to a proliferated vessel (B). v = blood vessel. Bar = 50 μm. Reproduced with permission from (79).

Figure 10. A-B. Hematoxylin and eosin micrographs of longitudinal sections through the patellar tendon of healthy control (A) and painful tendinopathy (B). Arrows denote tenocytes. The healthy tendon is homogeneous, with organized parallel collagen structure and thin, elongated tenocytes (A). The tendinopathy, on the other hand, is marked by collagen disorganization, increased cell count, activated tenocytes, and vascular ingrowth in the tendon proper (B). V = blood vessel. Bar = 50 μm. Reproduced with permission from (79).

Figure 11. A-B. Immunofluorescence micrographs of longitudinal sections through the patellar tendon of healthy control (A) and painful tendinopathy (B) stained for TH (a marker for noradrenaline). Arrows denotes nerve fibers. In the healthy tendon, a strong relation is seen between blood vessels and TH positive nerves (A). In painful tendinopathy, a decreased number of TH positive nerves, which are blood vessel related is seen. V = blood vessel. Bar = 50 μm. Reproduced with permission from (79).

Table 1. Neuronal pathways in tendons

PATHWAY	SIGNALLING	MEDIATOR	RECEPTOR	ACTIONS
Autonomic	Sympathetic	Noradrenaline Neuropeptide Y	alpha-,beta-adrenoceptors Y1, Y2[1],Y3[2]	Pro-(anti)-inflammatory
	Parasympathetic	Acetylcholine VIP	Nicotinic, muscarinic VPAC1-2[2], PAC1[2]	Anti-inflammatory
Sensory	Sensory	Substance P CGRP Neurokinin A NKB*, NPK*, NPG*	Neurokinin 1 CRLR, RAMP-1 Neurokinin 2[2] Neurokinin 3[2]	Pro-inflammatory
	Opioid	Enkephalins: LE, ME, MEAP Dynorphins: DYN B[1] Endomorphins[1] Nociceptin[1] Opioid like: Galanin Opioid like: Somatostatin	delta-opiod receptor kappa-opiod receptor[1] mu-opiod receptor[1] N/OFQ receptor[2] Galanin receptor 1-3[2] Somatostatin receptor 1-5[2]	Anti-inflammatory
Excitatory	Glutamatergic	Glutamate	NMDA mGluR1[1], mGluR5-7 AMPA[2] Kainate[2]	Cell-proliferative

[1] Not detected in tendon [2] Not yet assessed in tendon, VIP = vasoactive intestinal polypeptide, VPAC = Vasoactive intestinal peptide receptor, PAC1 = Pituitary adenylate cyclase-activating polypeptide type I receptor, CGRP = calcitonin gene-related peptide, CRLR = calcitonin receptor-like receptor,RAMP-1 = receptor activity-modifying protein 1, NKB = neurokinin B, NPK = neuropeptide K, NPG =neuropeptide-g, LE = leucine enkephalin, ME = Methionine-enkephalin, MEAP = methionine-enkephalin-arginine-phenylalanine N/OFQ = Nociceptin/orphanin FQ peptide, NMDA = N-methyl-D-aspartate receptor, mGluR = metabotropic glutamate receptors, AMPA = alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

Table 2. Neuronal alterations in tendinopathy

PATHWAY	SIGNALLING	MEDIATOR	RECEPTOR	ACTIONS
Autonomic	Sympathetic	Noradrenaline ↓	alpha-,beta-adrenoceptors ↑	Cell proliferation / differentiation
		Neuropeptide Y[2]	Y1 ↑	Cell proliferation / differentiation
	Parasympathetic	Acetylcholine ↑	Nicotinic[2],muscarinic ↑	Cell proliferation / differentiation
		VIP[2]	VPAC1-2[2], PAC1[2]	Cell proliferation / differentiation
Sensory	Sensory	Substance P ↑	Neurokinin 1 ↑	Tenocyte / endothelial cell proliferation, MMP-3 ↑
		CGRP[2]	CRLR[2], RAMP-1[2]	
		Neurokinin A[2]	Neurokinin 2[2]	
	Opioid	Enkephalins[2]	delta-opiod receptor[2]	Cell proliferation / differentiation
		Cannabinoids	Cannabinoid receptor 1 ↑	
		Galanin[2]	Galanin receptor 1-3[2]	
		Somatostatin[2]	Somatostatin receptor 1-5[2]	
Excitatory	Glutamatergic	Glutamate ↑	NMDA ↑	Cell proliferation / differentiation
			Phosfo-NMDA1 ↑	
			mGluR5 ↑	
			mGluR6-7 →	

[2] Not yet assessed in tendinopathy, VIP = vasoactive intestinal polypeptide, VPAC = Vasoactive intestinal peptide receptor, PAC1 = Pituitary adenylate cyclase-activating polypeptide type I receptor, CGRP = calcitonin gene-related peptide, CRLR = calcitonin receptor-like receptor,RAMP-1 = receptor activity-modifying protein 1, NKB = neurokinin B, NPK = neuropeptide K, NPG =neuropeptide-g, LE = leucine enkephalin, ME = Methionine-enkephalin, MEAP = methionine-enkephalin-arginine-phenylalanine N/OFQ = Nociceptin/orphanin FQ peptide, NMDA = N-methyl-D-aspartate receptor, mGluR = metabotropic glutamate receptors, AMPA = alfa-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor.

Running title: "Neurosignalling in tendons"