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Mechanisms in Tendon Healing

Pain, Biomarkers and the Role of Mast Cells

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Abstract

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Tendon injuries and tendinopathy are common disorders, but the underlying mechanisms are not well understood. The overall aim of this thesis was to better understand the mechanisms underlying tendon healing, pain, and inflammation.

The aim of the first study was to assess biomarkers of tendon healing, including procollagen type I (PINP) and type III (PIIINP) in relation to patient outcome in 65 patients with Achilles tendon rupture (ATR). At two weeks post-ATR, PINP and PIIINP-levels were quantified using microdialysis followed by ELISA. At one-year post-ATR patient outcome was assessed using the validated Achilles tendon Total Rupture Score. We found that higher ratio of PINP and PIIINP to total protein were significantly associated with less pain but more fatigue in the affected limb.

In the second study, we applied Intermittent Pneumatic Compression (IPC) therapy for two weeks to stimulate tendon healing. The patients received either adjuvant IPC treatment or treatment-as-usual in a plaster cast without IPC. We observed that IPC therapy significantly increased PINP levels in the injured tendon, suggesting enhanced healing response.

In our third study, we investigated healing response and the role of mast cells (MCs) *in-vivo* using an ATR rat model. Three weeks postoperatively, we demonstrated an increased number of MCs and a higher proportion of degranulated MCs in the injured tendon compared to the control. We further established that MCs in the injured tendon were positive for the glutamate receptor NMDAR1.

In our final study, we assessed the effect of glutamate stimulation on *in-vitro*-derived mouse bone marrow MCs. Mast cell degranulation was quantified through β-hexosaminidase release, immunofluorescence was used to quantify NMDARs at the protein level, and RT-qPCR/ microarray was used to study the expression of NMDARs and associated genes. Glutamate induced a robust upregulation of glutamate receptors of both ionotropic and metabotropic type, both at the mRNA and at protein level. NMDAR1 co-localized with glutamate in the membrane of MCs, thereby confirming an interaction between glutamate and its receptor. Glutamate also induced expression of pro-inflammatory compounds such as IL-6 and CCL2 and transcription factors such as Egr2, Egr3 and FosB. Moreover, the NMDA-channel blocker MK-801 completely abrogated the response of MCs to glutamate, supporting a functional glutamate–glutamate receptor axis in MCs.

Together, findings presented in this dissertation reveal possible mechanisms of tendon healing in relation to pain and function, and establish a novel principle for how immune cells can communicate with nerve cells after ATR.

Keywords: Tendon Healing, Pain, Biomarkers, Mast Cells, Inflammation, Microdialysis, Neurotransmitters, Glutamate Receptors, Procollagen, Transcriptional factors

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Sammanfattning
Senskador och tendinopati är vanliga problem, men de underliggande mekanismerna otillräckligt undersökta. Målet med denna avhandling är att öka kunskapen om mekanismer under läkningen av senor, och hur dessa relaterar till smärta och inflammation.

Syftet med den första studien var att kvantifiera biomarkörer för läkning av Achillessena, genom analys av prokollagen typ I (PINP) och typ III (PIIINP) hos 65 patienter efter ruptur av Achillessenan. Två veckor efter achillesruptur kvantifierades PINP och PIIINP-nivåerna med mikrodialys följt av ELISA-analys. Ett år efter achillesruptur bedömdes patienternas upplevda besvär med användning av ett validerat formulär, Achilles Tendon Total Rupture Score. Ökad andel PINP och PIIINP av totala mängden protein vid två veckor var signifikant associerat med mindre smärta men ökad fatigue i skadat ben efter ett år.

I nästa studie utvärderade vi effekten av intermittent pneumatisk kompression (IPC) under två veckor av senans läkning efter achillesruptur. Patienterna fick antingen tilläggsbehandling med IPC eller vanlig behandling i gipsskena utan IPC. Vi kunde visa att behandling med IPC signifikant ökade nivån av PINP i den skadade senan, vilket tyder på förbättrad läkning.

I den tredje studien undersökte vi mastcellers roll under läkningen av Achillessena efter ruptur i en råttmodell. Tre veckor postoperativt visade vi ett ökat antal mastceller och en högre andel degranulerade av mastceller i den läkande senan jämfört med senan på den andra (den friska) sidan. Vi kunde också påvisa glutamatreceptorn NMDAR1 hos mastceller i den läkande senan.

I den fjärde studien bedömde vi effekten av glutamatstimulering in-vitro, på mastceller från benmärg hos mus. Degranulering av mastceller kvantifierades genom frisättning av β-hexosaminidas. För att kvantifiera NMDAR på proteinnivå använde vi immunfluorescens, och för att studera uttrycket av NMDAR och associerade gener använde vi RT-qPCR/mikroarray. Vi kunde visa att glutamat inducerar uppreglering av glutamatreceptorer av både jonotropisk och metabotropisk typ i mastceller, både på mRNA- och proteinnivå. NMDAR1 samlokaliserade med glutamat i membranet på mastceller, vilket därmed bekräftar en interaktion mellan glutamat och dess receptor. Glutamat inducerade också uttryck av pro-inflammatoriska proteiner såsom IL-6 och CCL2, samt transkriptionsfaktorer såsom Egr2, Egr3 och FosB. Dessutom upphävde NMDA-kanalblockeraren MK-801 fullständigt effekten av glutamat på mastceller, vilket talar för en funktionell betydelse av interaktionen mellan glutamat och glutamatreceptorer i mastceller.

Sammantaget visar de fynd som presenteras i denna avhandling möjliga mekanismer för läkning av Achillessena i relation till smärta och funktion och introducerar en ny princip för hur immunceller kan kommunicera med nervceller efter achillesruptur.

পড়ো তোমার প্রভুর নামে যিনি সৃষ্টি করেছেন,
Read with the name of your Lord who created,
সৃষ্টি করেছেন মানুষকে এক রক্তপিন্ড থেকে।
Created man from a blood clot.
পড়ো তোমার পালনকর্তা মহা দয়ালু,
Read, and your Lord only is the Most Beneficent,
যিনি কলমের সাহায্যে শিক্ষা দিয়েছেন,
Who taught by the pen,
শিক্ষা দিয়েছেন মানুষকে যা সে জানত না।
Taught man that which he knew not.

– Al Quraan: 96 (1-4)

To my beloved parents, family and teachers

"The reward of deeds depends upon the intentions and every person will get the reward according to what he has intended for"

-Prophet Muhammed (PBUH)

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Alim, M.A.,** Svedman, S., Edman, G., Ackermann, P.W. (2016). Procollagen markers in microdialysate can predict patient outcome after Achilles tendon rupture. *BMJ Open Sport Exerc Med*, 2(1): e000114. doi:10,1136/bmjsem-2016-000114.

II **Alim, M.A**., Arverud, D.E., Nilsson, G. Edman, G., Ackermann, P.W. (2018). Achilles tendon rupture healing is enhanced by intermittent pneumatic compression upregulating collagen type I synthesis. *Knee Surg Sports Traumatol Arthrosc,* 26:2021. doi: 10.1007/s00167-017-4621-8.

III **Alim, M.A.**, Ackermann, P.W., Eliasson, P., Blomgran, P., Kristiansson, P., Pejler, G., Peterson, M. (2017). Increased mast cell degranulation and co-localization of mast cells with the NMDA receptor-1 during healing after Achilles tendon rupture. *Cell Tissue Res*, 370(3):451-460. doi: 10.1007/s00441-017-2684-y.

IV **Alim, M.A.,** Grujic, M., Ackermann, P.W., Kristiansson, P., Peterson, M., Pejler, G. (2019). Glutamate Triggers the Expression of Functional Ionotropic and Metabotropic Glutamate Receptors in Mast Cells. *Cellular and Molecular Immunology-Nature (In revision).*

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Other contribution

Ahmed, A. S., Li, J., **Alim, M. A.**, Ahmed, M., Östenson, C. G., Salo, P. T., Hewitt, C., Hart, D.A, Ackermann, P. W. (2017). Compromised Neurotrophic and Angiogenic Regenerative Capability during Tendon Healing in a Rat Model of Type-II Diabetes. *PloS one*, 12(1), e0170748. doi:10.1371/journal.pone.0170748.

Contents

Abbreviations

AT	Achilles tendon
ATR	Achilles tendon rupture
ATRS	Achilles tendon Total Rupture Score
BMMCs	Bone marrow-derived mast cells
CGRP	Calcitonin gene related peptide
Col1a1	Collagen, type I, alpha 1
CCL2-4/7	Chemokine (C-C motif) ligand 2-4/7
DVT	Deep venous thrombosis
DAPI	4',6-Diamidino-2-Phenylindole-Dihydrochloride
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
EQ-5D	EuroQol Group's questionnaire
ERK	Extracellular signal–regulated kinase
EGR1-3	Early growth response 1-3
FAOS	Foot and Ankle Outcome Score
FGF-2	Fibroblast growth factor-2
GDP	Gross domestic product
IPC	Intermittent pneumatic compression
mGluR1-7	Metabotropic glutamate receptor type 1-7
MCs	Mast cells
MC	Mast cell
mMCP-6	Mouse mast cell protease-6
MCP-1	Monocyte chemotactic protein-1
MIP-1α/ß	Macrophage inflammatory protein 1 alfa/beta
NR4A1-3	nuclear receptor subfamily 4 group A member 1-3
NGF	Nerve growth factor
NMDAR1	N-methyl-D-aspartate receptor-1
NMDAR2A/2B	N-methyl-D-aspartate receptor type 2A/2B
PAS	Physical Activity scale
PINP	Procollagen type I N-Terminal propeptide
PIIINP	Procollagen type III N-Terminal propeptide
PNS	Peripheral nerve system
TNF-α	Tumor necrosis factor-α

1. Introduction

1.1 Background

Pain is defined as “an unpleasant sensory and emotional experience associated with actual or potential tissue injury or explained in terms of such tissue damage” by the International Association for the Study of Pain ('Classification of chronic pain. Descriptions of chronic pain syndromes and definitions of pain terms. Prepared by the International Association for the Study of Pain, Subcommittee on Taxonomy' 1986). However, there are different types of pain after tissue damage. Acute pain normally lasts less than 3 months, and is a time-limited experience that gradually resolves as the injured tissues heal. Acute pain is distinctive from chronic pain and is in general terms often perceived as more sharp than the dull ache of chronic pain (Hsu 2011). The experience of chronic pain is a subjective phenomenon ('Classification of chronic pain. Descriptions of chronic pain syndromes and definitions of pain terms. Prepared by the International Association for the Study of Pain, Subcommittee on Taxonomy' 1986) as there are no objective methods of measuring or visualizing pain. In contrast, chronic pain is defined as the pain which may persist longer than the normal time of tissue healing as often estimated more than three months after injury (Gupta et al. 2010).

Musculoskeletal pain is the most common type of pain and constitutes the majority of consultations for pain in the primary health care (Raftery et al. 2011; Lundberg 2006). Historically, the socioeconomic burden form musculoskeletal pain is substantial with inability to work according to the physical demands of the employment. Therefore, it has been anticipated that symptoms and disability may increase from soft tissues injury. However, these types of injuries may also cause some common painful disorders of the back, neck, limb and joint (e.g. osteoarthritis) (Vargas-Prada 2015).

Tendinopathy (e.g. pain from tendons) is a common type of disability and it remains a challenge for the clinicians to manage the treatment strategies (Ackermann and Renstrom 2012). The pathologies of tendon range from chronic injury to acute injury with a partial or complete tendon rupture. (Docheva et al. 2015) (Voleti, Buckley, and Soslowsky 2012). Chronic tendon injury, or tendinopathy, is the most frequently overuse tendon injury and is related to age (Li et al. 2019) and physical requirement or by sports. In the example of acute Achilles tendon rupture (ATR), the healing process is pro-

longed and the pain is variable. One year after rupture, many patients still report pain and fatigue in the affected limb (Bostick et al. 2010) (Alim et al. 2016). However, Achilles tendinopathy is exhibiting an unsuccessful healing response with messy proliferation and abnormalities of tenocytes with disruption of collagen fibers, resulting in an increase of non-collagenous matrix components (Maffulli et al. 2019). Improved modes of intervention during rehabilitation of ATR would be beneficial as would early markers that can be used to monitor the healing progress and to predict long-term functional outcome (Schwellnus 2013).

Moreover, an understanding of the molecular mechanisms involved in pain is crucial for developing mechanism-based treatment of pain. Effective treatments for tendon injuries and tendinopathy lag behind because of incomplete understanding of tendon biology and other components of the musculoskeletal system (Ackermann and Renstrom 2012). The general aim of this thesis was to investigate the molecular pathophysiology underlying tendon healing and pain mechanisms in relation to inflammation.

1.2 Tendon Basic Biology

Tendon is a crucial component of the musculoskeletal system that connects muscle to bone and transmits force for the movement (Sharma and Maffulli 2008; Nourissat, Berenbaum, and Duprez 2015). Tendon is a soft connective tissue and is predominately composed of water, which makes up 55–70% of the total tendon weight. The other major component of tendon is collagen, which represents about 60-85% of the dry weight of tendon (Kjaer 2004). In tendon architecture, fibrillar arrangement of triple-helical type I collagen molecules generates collagen fibres, which then combine to form fascicles and, ultimately, the tendon tissue (Figure 1). The type I collagen molecule mainly contains two identical α1 chains and one different α2 chain which are encoded by *col1a1* and *col1a2,* respectively (Nourissat, Berenbaum, and Duprez 2015). The collagen fibrils are the fundamental force-transmitting element of tendon tissue, and are tightly arranged within the extracellular matrix (ECM). Type I collagen and associated ECM components are produced by tenocytes (tendon cells), which are fibroblast-like cells basically found in between collagen fibres and in the surrounding of the endotenon (Figure 1) (Wu, Nerlich, and Docheva 2017). In addition to ECM molecules and collagen, other molecules like elastin, proteoglycans and glycoproteins are also involved in the tendon-specific structure (Mienaltowski and Birk 2014).

There are two main markers of collagen metabolism, procollagen type III N-terminal propeptide (PIIINP) and procollagen type I N-terminal propeptide (PINP). Both have been used in tendon and in bone tissue as early prediction markers of healing (Vestergaard, Jorgensen, et al. 2012). Procollagen type I and III are essential building blocks in all types of connective tissue, and the markers PINP and PIIINP have been used to assess collagen metabolism in intact human Achilles tendons exposed to exercise and growth factor stimulation (Vestergaard, Jorgensen, et al. 2012). The tendon also contains a range of non-collagenous proteins, which are in low amounts, but nevertheless may have important functions.

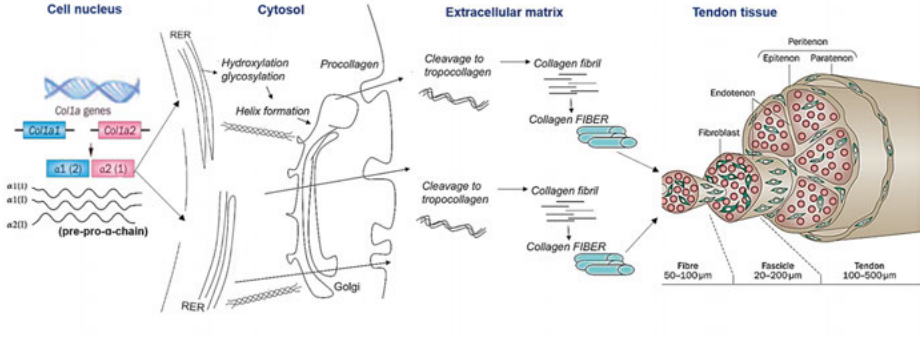

Figure 1. Tendon architecture (adapted from Nourissat et al. 2015).

1.3 Tendon Healing and Inflammation

The initial acute stage of tendon healing includes inflammatory processes (Abate et al. 2009), and thus the term tendinitis is still used as a description. However, the chronic stage is considered as a protracted, dysregulated and maladaptive response to injury.

After injury, tissue repair normally follows three overlapping phases described as the inflammatory, proliferative and remodeling phases as seen in figure 2 (Broughton, Janis, and Attinger 2006) (Broughton, Janis, and Attinger 2006). During the inflammatory phase (within 2 weeks from injury), resident immune cells (e.g. macrophages and MCs) predominate. Different vasoactive factors and cytokines facilitate vascular leakage and the migration of leucocytes, primarily neutrophils to the inflammatory site (Langberg, Skovgaard, Karamouzis, et al. 1999; Kjaer et al. 2000; McMahon, Cafferty, and Marchand 2005). The inflammatory phase is followed by the proliferative, or repair phase (2 days-6 weeks). In this phase, fibroblasts produce collagens (e.g. procollagen type- I, PINP; and procollagen type –III, PIIINP) and extracellular matrix components (Bjorklund et al. 2011b). The proliferation phase is followed by a remodeling or maturation process (6 weeks-2 years) in which the tendon modifies its internal structure (Domeij-Arverud et al. 2015a). The molecular events during these healing phases are influenced by different factors such as, e.g., site of injury, age, sex, genes, and nutrition (Broughton, Janis, and Attinger 2006) (Broughton, Janis, and Attinger 2006) (Carter et al. 1998) (Nunamaker 1998).

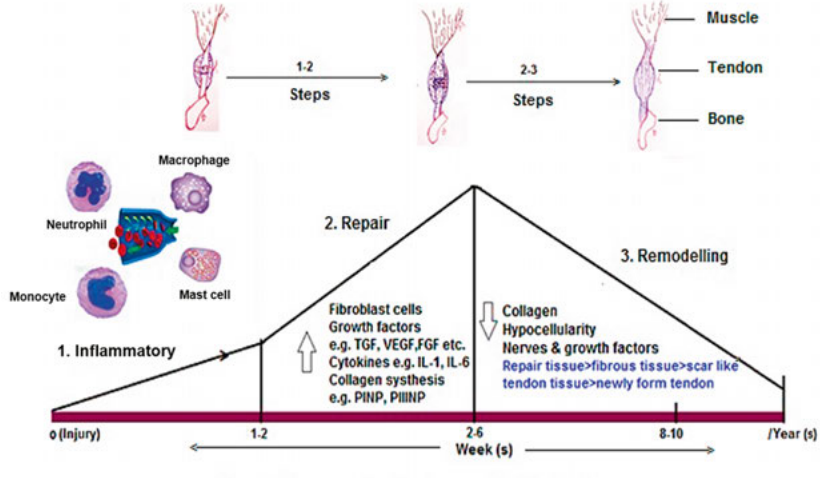

Figure 1: Three overlapping phases of tendon healing

Figure 2. Overlapping phases of tendon healing: inflammation, repair and remodeling. During the inflammatory phase, resident immune cells (e.g. macrophages and MCs) predominate. The inflammatory phase is followed by the proliferation or repair phase where fibroblasts produce collagens and extracellular matrix components. The

proliferation phase is followed by a maturation process, in which the tendon modifies its internal structure.

1.4 Role of MCs in Tendon Healing

MCs are highly granulated hematopoietic cells derived from the bone marrow. They migrate to peripheral tissues via the blood stream, where they function as fully mature cells (Chen et al. 2005; Dahlin and Hallgren 2015; Kitamura et al. 1977). Mature MCs are rich in different granular compounds such as serglycin proteoglycan, proteases (e.g. chymase, tryptase), biogenic amines (histamine, serotonin), growth factors (e.g. Vascular endothelial growth factor) and certain cytokines e.g. Tumor necrosis factor-α, (TNF-α), which are released upon degranulation and activation of MCs (see figure 3)(Galli, Nakae, and Tsai 2005; Wernersson and Pejler 2014). Potentially, several of the compounds released from activated MCs could influence the inflammatory and proliferative healing phases after tendon rupture. For example, MC-derived growth factors such as transforming growth factor beta (TGF-β) (Nakae et al. 2007) and fibroblast growth factor-2 (FGF2) could contribute to tissue remodeling processes (Maltby, Khazaie, and McNagny 2009), including stimulation of collagen synthesis (Coussens et al. 1999). Further, MC-derived vascular endothelial growth factor (VEGF) (Grutzkau et al. 1998), and nerve growth factor (NGF) may contribute to neo-angiogenesis and nerve ingrowth (Leon et al. 1994). MC proteases such as tryptase could activate protease-activated receptors on the surface of nerve cells (Akers et al. 2000) (Saito and Bunnett 2005a).

Tryptase is a MC-restricted protease that is well established as a specific marker for MCs (Castells and Schwartz 1988). MCs are in the normal state resident locally in the tendon tissue or in the loose connective tissue close to the paratenon, muscle-tendon junction or bone-tendon junction. During the tendon healing process, they may migrate to the rupture site (tendon proper) following inflammation and nerve ingrowth. MCs also reside in close proximity to the peripheral nerve endings and may be activated by different neurotransmitters (e.g. Substance P, glutamate) released from the peripheral nerves after tendon injury, Thus, activated MCs can release different inflammatory mediators such as histamine, serotonin, MC proteases (e.g. tryptase), cytokines and chemokines, that in turn may activate nearby nerves. These neuro-inflammatory responses may affect the function of the PNS, which makes them a potential target for modulating inflammation and pain.

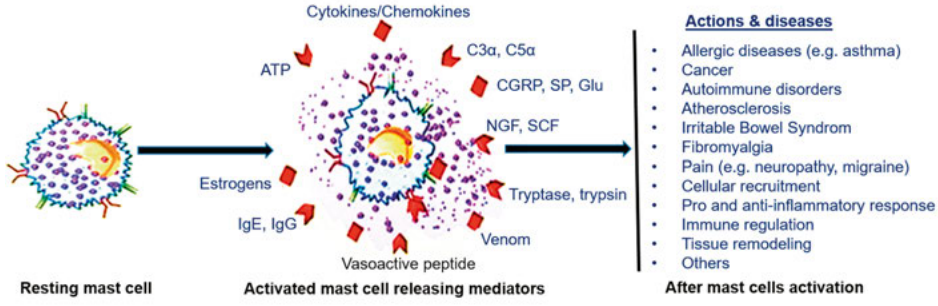

Figure 3. MC activation and release of mediators causes different actions and diseases.

Previously, MCs have also been implicated as detrimental players in several pathological settings, ranging from allergic disorders including asthma to various other types of pathologies such as cancer, autoimmune disorders and atherosclerosis (Wernersson and Pejler 2014; Anand et al. 2012). In addition, MCs have been suggested to have a number of beneficial functions, e.g., in the context of bacterial and parasite infection, as well as in wound healing (Chiu, von Hehn, and Woolf 2012; Reber et al. 2015).

1.5 Tendinopathy and Neuronal Responses

The pathological mechanisms of tendinopathy are still far from understood. In peripheral tissue, the peripheral nerve system (PNS) has a key role in regulating inflammation and pain signaling of the damaged tissue via afferent to efferent pathways (Chiu, von Hehn, and Woolf 2012). In the healthy tendon, nerve fibers are localized in the tendon sheath, so called interfibrillar matrix, whereas the tendon proper, also called intrafibrillar matrix, is devoid of nerves (Ackermann et al. 2014). During the early healing phase, extensive ingrowth of nerve fibers has been demonstrated into the tendon proper (Ackermann, Ahmed, and Kreicbergs 2002). During the repair phase there is nerve ingrowth in the tendon proper, but in the subsequent phase (Figure 2) the nerves retract back to the surrounding tendon sheath (Ackermann et al. 2003).

Glutamate is the primary excitatory mediator of the nervous system and has been involved in various pain conditions. The glutamate receptor NMDAR1 has been identified in tendinopathy. It is a heterotetrameric complex protein (Karakas and Furukawa 2014) containing of four subunits deriving from three different protein families such as NMDAR1, NMDAR2 and NMDAR3 (Glasgow, Siegler Retchless, and Johnson 2015). However, the composition of these subunits can vary in different cell types, which affects the functional properties (Ackermann et al. 2014). In tendinopathic patients a 10-fold up-regulation of N-methyl-D-aspartate receptor-1 (NMDAR1) has

been observed in morphologically transformed tenocytes, in the endothelial and adventitial layers of neovessel walls and in presumed sprouting nerve fibers (Greve et al. 2012b). The nerve ingrowth in combination with the expression of different pain signaling pathways (neuromediator-receptor) may be a means of assessing peripheral physiological processes in pain regulation in both tendinopathy, tendon healing as well as other pain disorders. The pain signaling via PNS may be assessed by measuring glutamate-NMDAR, Substance P-NK1, CGRP-CRLR and cannabinoids-CB2 interactions (Andersson et al. 2008; Ackermann et al. 2014). Interestingly, nerve ingrowth into normally aneuronal tissue seems to be a fundamental process not only restricted to tendon but has also been observed in discs of chronic low back pain (Freemont et al. 1997).

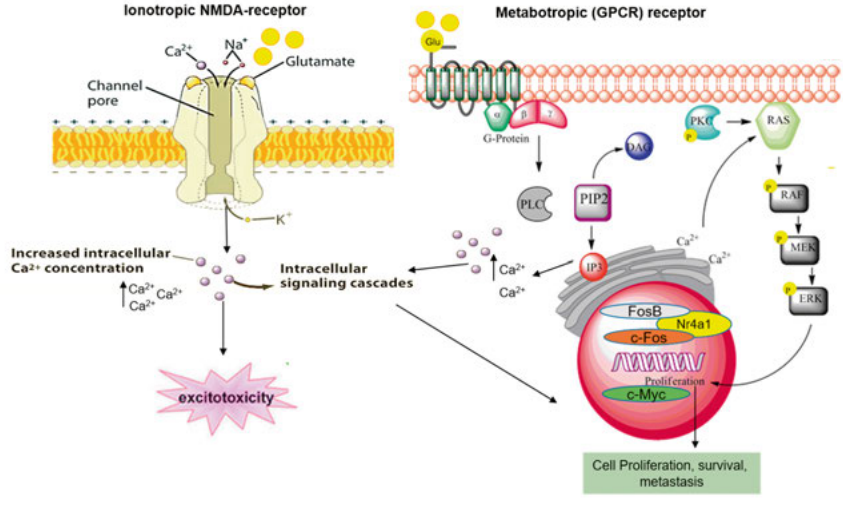

Figure 4. Proposed signal transduction pathways activated by glutamate following ionotropic NMDA-receptor and metabotropic receptor (GluR/GPCR) stimulation. (Adapted from Brian A. W. et al. 2013).

Targeting metabotropic glutamate receptors (mGluR) could represent a potential strategy for treatment of inflammation or neuroinflammation in tendinopathy. It has been demonstrated that immune cells including lymphocytes and microglia both express mGluR in response to glutamate (Boldyrev et al. 2004); (Taylor et al. 2002). In the last decades, many interesting findings have been documented to examine mGLu receptor signaling in neural and immune cells. For example, stimulation of glutamate group I metabotropic glutamate receptors induces calcium signals and c-fos gene expression in human T-cells (Miglio, Varsaldi, and Lombardi 2005). Activation of mGluR2 receptors in microglia mimics neuro-inflammation by upregulating pro-inflammatory cytokines (Pocock and Kettenmann 2007). Moreover, neuro-immune regulation

by targeting mGlu receptors is a promising concept for modulating neuro-inflammation in the periphery as well as in the CNS (Crupi, Impellizzeri, and Cuzzocrea 2019). In our study, we focused on mGlu receptors (e.g. mGluR1, mGluR2 and mGluR7) after glutamate stimulation of MCs.

Peripheral nerve endings at the site of injury can emit potent neuropeptides with the ability to modify the function of fibroblast-like cells in the healing tendon tissue (Murphy and Hart 1993). The neuropeptides can also interact with resident MCs and macrophages to affect their influence on the tissue (Hart et al. 1998). MCs reside near nerve endings and may degranulate during healing which makes them a potential target for modulating inflammation and pain. A bidirectional pathway has been suggested whereby MCs can interact with the nervous system. MC-derived mediators such as tryptase and histamine may activate peripheral nerve endings to release different neuropeptides (e.g. substance P, calcitonin gene-related peptide,). In return the subsequent release of neuropeptides can activate MCs (Le et al. 2016; Bring et al. 2009).

1.6 Interventional Approaches and Target Biomarkers

Tissue analyses of chronic painful tendons (tendinosis) in humans through immunohistochemical approaches have also demonstrated up-regulation of signal-receptor systems such as glutamate-NMDA and substance P-NK1 (Alfredson et al. 2001; Andersson et al. 2008; Bjorklund et al. 2011a). During the last decades, a method to assess the healing progression of ATR was developed by using a microdialysis technique (Bolinder, Ungerstedt, and Arner 1993), in which a small catheter is placed in the metabolically active part of the tendon, i.e. the paratenon (Greve et al. 2012a; Langberg, Skovgaard, Petersen, et al. 1999). Microdialysis of the extracellular matrix followed by the quantification of a wide variety of molecules of interest has provided reliable and usable data concerning metabolism and healing progress in many human tissues (de la Peña, Liu, and Derendorf 2000).

Intermittent Pneumatic Compression (IPC) is a mechanical compression therapy, which is administered as an outpatient treatment for leg-immobilized patients. IPC may enhance the early healing response after ATR and may be a viable and effective prophylactic treatment to prevent immobilization-induced impairments (Bring et al. 2009). In order to better understand the tendon healing process and subsequent pain regulation, we studied different tendon callus forming biomarkers (e.g. PINP, PIIINP), nerve markers (e.g. NMDA-receptors such as NMDAR1, NMDAR2A/2B, mGluR1, mGluR2, mGluR7), a MC-specific marker (e.g. tryptase), and certain cytokines (e.g. IL-6, TNF-α) and chemokines (e.g CCL2, CCL3, CCL4, CCL7).

PINP and PIIINP: PINP and PIINP are important markers for tendon callus formation. These two markers of collagen metabolism have been used in bone tissue for the early prediction of the success of interventions, e.g. for

osteoporosis treatment (Eastell et al. 1993b). Procollagen type I and III are essential building blocks in all types of connective tissue and the markers PINP and PIIINP have been used to assess collagen metabolism in intact human Achilles tendons subjected to exercise and growth factor stimulation (Vestergaard, Jørgensen, et al. 2012).

FosB and c-Fos: The Fos and Jun proteins belongs to the AP-1 transcription factor complex, and act as a central regulators for many cellular functions including cell proliferation, differentiation, and transformation (Wagner 2010). The FosB/Ap-1 complex has a key role in bone development, inflammation and tissue homoeostasis (Wagner 2010). FosB and c-Fos belong to the same family of transcription factors, and there is an alternative splice form of FosB, deltaFosB, which is a shorter variant of FosB (Eliasson et al. 2013; Ohnishi et al. 2008). The Fos genes are immediate or early genes, and can be activated in response to intracellular signaling cascades without intervening with protein synthesis (Ott et al. 2009). Previously, FosB has been shown to be mechanosensitive at both the mRNA and protein level, and in different types of cells (both in vitro and in vivo), including osteoblasts, tenocytes, periodontal ligament cells, smooth muscle cells, pulmonary epithelial cells, chondrocytes, bone marrow cells, and fibroblasts (Eliasson et al. 2013; Fitzgerald et al. 2008; Ohnishi et al. 2008). FosB can also be upregulated by different kinds of stimuli, including wounding (Shirai et al. 2001), and appears to signal mainly via Erk1/2 (Rangaswami et al. 2009). FosB has also been important for cell cycle progression in fibroblasts (Eliasson et al. 2013). During tendon healing, c-Fos and FosB might activate a general proliferative response and stimulate collagen type 1 production and perhaps also be involved in tendon cell differentiation. Therefore, further investigations are warranted.

Egr1 and Egr2 are important for tendon development; they do not appear to be specific for tendons. Both genes are also important for chondrogenesis and osteogenesis (Levi et al. 1996; Schnabel et al. 2002). It is conceivable that these transcription factors are context-dependent, and that their roles are determined by the initiating nature of injury and the cell type or tissue where the genes are activated. Egr1 and Egr2 could possibly act as master regulators of the gene response after rupture during tendon healing (Eliasson et al. 2013).

Nr4a1, Nr4a2, Nr4a3 are genes encoding members of the NR4A subfamily of nuclear hormone receptors that can bind to DNA and modulate gene expression. These proteins have been implicated in T and B lymphocyte apoptosis, and immune cell proliferation (Eliasson et al. 2013). **MCP-1** (Monocyte chemotactic protein-1, CCL2) is a member of the chemokine family. It is known that MCP-1 plays an important role in immunoregulation and inflammatory responses. MCP-1 is implicated in the pathogenesis of diseases such as psoriasis, rheumatoid arthritis and atherosclerosis (Spah 2008). **MIP-1** alpha (macrophage inflammatory protein 1 alpha) is also known as CCL3. CCL3 is produced by lymphocytes, macrophages and dendritic cells, and is

involved in the inflammatory response of blood monocytes and tissue macrophages (Maurer and von Stebut 2004). In addition, murine MIP-1 alpha can promote histamine release from mouse peritoneal MCs in a dose-dependent manner and can also activate basophils (Alam et al. 1992).

MK-801, Inhibitor/Antagonist: In order to determine which of the glutamate receptors that are involved in the signaling downstream of glutamate stimulation, we used a specific glutamate receptor antagonist. NMDAR1, NMDAR2A/2B receptor-specific antagonists may bind to NMDAR1, resulting in competitive or noncompetitive inhibition of the Glutamate/NMDAR1-R2A/R2B signaling pathway. The activation and expression of glutamate receptors and its subtypes can be blocked/reduced by the receptor antagonists like open channel blocker (e.g. MK-801). In many studies, NMDA-receptor antagonists have been added during cell culture to further reduce activation of the respective receptor (Hansen et al. 2008).

The nerve ingrowth in combination with the downstream effect of NMDAR1 and its subtype's activation may represent an important physiological mechanism during tendon healing and subsequent pain regulation. We were therefore interested to investigate the functionality of such selected biomarkers/proteins in tendon healing and pain, with the goal to outline their involvement in MC responses induced by peripheral nerve signaling. Hence, the general aim of this thesis was to increase the knowledge of the molecular mechanisms involved in tendon healing, inflammation and subsequent pain regulation after ATR.

2. Aims

The primary aim of the study was to investigate the molecular pathophysiology underlying healing mechanisms associated with pain in tendon tissue, in both rodent and human models. A secondary aim was the identification and quantification of biomarkers, which are upregulated in tendon healing of patients and rodents, and could be used for assessment of healing and for the prediction of long-term clinical outcome. The specific aims in the following studies were:

- To assess markers of tendon callus production in patients with ATR in terms of outcome, pain, and fatigue (paper I)
- To investigate whether IPC treatment can promote tendon-healing (paper II)
- To investigate the role of MCs and their relation to the NMDA receptor-1 (a glutamate receptor) during healing and pain signaling after ATR (paper III)
- To investigate the possibility that MCs can be activated by L-glutamate treatment (paper IV)

3. Investigations

3.1 Paper I

Objective

Patients who have acute ATR exhibit variable and mostly impaired long-term functional, and patient-reported outcomes. However, there is a lack of early predictive markers of long-term outcomes to facilitate the development of improved treatment methods. The purpose of this study was to assess markers of tendon callus production in patients with ATR in terms of outcome, pain, and fatigue.

Assessments

A total of 65 patients (57 men, 8 women; mean age 41±7 years) with ATR were prospectively assessed. The healing progression of ATR was assessed using microdialysis technique in which a small catheter (CMA 71; CMA Microdialysis AB, Solna, Sweden; 100 kDa molecular cut-off, 0.5 mm diameter; 30 mm in length) was placed in the metabolically active part of the tendon, i.e. the paratenon. Perfusion fluid (Macrodex) was pumped at 1.0 μL/min (CMA 107; CMA Microdialysis, Solna, Sweden) through the catheter and was finally collected in a vial (Microvial, CMA Microanalysis AB, Solna, Sweden). Samples were collected every 30 min for 2 hours. In order to assess markers of callus, production of PINP, PIIINP, and the protein content were quantified in the microdialysis dialysate. PINP and PIIINP levels were measured via a sandwich ELISA kit following the manufacturer's instructions (USCN Life Science, Inc, Houston, Texas, USA). The total protein content was assessed with the Bradford protein assay kit. Normalized procollagen levels (n-PINP and n-PIIINP) were calculated as the ratio of procollagen to total protein content. The patient's symptoms and physical activity levels were assessed using two reliable and valid scores, the Achilles tendon Total Rupture Score (ATRS), and the Physical Activity Scale (PAS). Pain and fatigue were assessed at 1 year using reliable questionnaires (Achilles tendon Total Rupture Score (ATRS)).

Results

Patients showed fatigue (about 77.6%) and pain (about 44.1%) to some extent. Interestingly, the normalized collagen production in the injured AT at 2 weeks

exhibited significant, positive correlations with the 1-year postoperative patients related outcome measures (PROMs). Thus, higher levels of n-PINP (R=0.38, p=0.016) and n-PIIINP (R=0.33, p=0.046) were significantly associated with less pain in the limb. Increased concentrations of PINP (R=−0.47, p=0.002) and PIIINP (R=−0.37, p=0.024) were related to more self-reported fatigue in the leg. The results were corroborated by multiple linear regression analyses.

Conclusions

In conclusion, this cohort study established that markers of tendon callus formation in a microdialysate from the paratenon of the healing AT seem to predict the patient-reported outcome at 1-year post-rupture. We suggest that these markers could be used as an early screening control for new interventions and novel treatment methods, and potentially to screen patients in need of specific intervention to improve the healing outcome. Optimization of the timing of assessment during the healing process might possibly improve the predictivity of the various procollagen markers for the outcome.

3.2 Paper II

Purpose and hypothesis

Adjuvant IPC during leg immobilization following ATR has been shown to reduce the risk of deep venous thrombosis. The purpose of this study was to investigate whether IPC can also promote tendon healing. Thus, we hypothesized that 2 weeks of calf IPC beneath orthosis immobilization could increase the callus production with regard to PINP and PIIINP compared to treatment-as-usual with plaster cast. A secondary aim was to investigate the long-term functional and patient-reported outcome at 3 and 12 months post-operatively.

Methods

A total of 150 people who suffered from unilateral acute ATR were post-operatively leg immobilized and prospectively randomized. Patients were divided into two groups: IPC treated (n=74) and treatment-as-usual (n=74). The IPC group received treatment to both operated and contralateral/intact limbs. The operated limb received an orthosis under immobilization and contralateral/intact limb received normal cast. Both limbs were connected to the IPC device for six hours daily during two weeks. The treatment-as-usual group received a plaster cast during two weeks. IPC (n=14) and 19 cast patients/control group (n=19) consented to undergo microdialysis at two weeks. Tendon callus production, PINP, PIIINP, and total protein content were assessed in the microdialysate followed by the enzymatic assay. During weeks 3–6, all subjects were leg-immobilized in an orthosis without IPC. Patient reported

outcome (e.g. pain) was evaluated in both groups at 3 and 12 months postoperatively using reliable questionnaires (ATRS and EQ-5D). Functional outcome was evaluated in both groups at 52 weeks postoperatively using the validated heel-rise test.

Results

At 2 weeks post-rupture, the IPC-treated patients exhibited 69% higher levels of PINP in the ruptured Achilles tendon (AT) compared to the control group ($p = 0.001$). Interestingly, the IPC-treated contralateral, intact AT also demonstrated 49% higher concentrations of PINP compared to the non-treated intact AT of the plaster cast group ($p = 0.002$). There were no adverse events observed associated with IPC. At 3 and 12 months, no significant differences between the two treatments were observed using patient-reported and functional outcome measures.

Conclusions

IPC therapy during limb immobilization in patients with ATR seems to effectively cause upregulation of collagen type I synthesis, which may represent a way to enhance the early healing response without any adverse effects. Whether prolonged IPC application during the whole immobilization period can also lead to improved long-term clinical healing response should be further investigated. The healing process during leg immobilization in patients with ATR can be improved through adjuvant IPC therapy, which additionally prevents deep venous thrombosis.

3.3 Paper III

Aim of Investigation

The role of inflammation and the mechanisms of tendon healing after rupture have historically been a matter of controversy. The goal of this study was to investigate the role of MCs and their relation to the NMDA receptor-1 (a glutamate receptor) during healing after ATR.

Materials and Methods

Eight female Sprague Dawley rats (Taconic, Ejby, Denmark) were used (13–15 weeks old) and had their right AT transected. The mean weight of the animals was 217 gram. The animals were housed in 2 or 3 per cage,with a 12:12-h light:dark cycle and given food and water ad libitum. The skin on the right lower leg was shaved and cleaned with chlorhexidine ethanol and surgery was performed under aseptic conditions. The tendon complex was exposed through a transverse skin incision lateral to the AT. Three weeks after rupture, histological quantification of MC numbers and their state of degranulation was assessed by histochemistry. Co-localization of MC tryptase (a MC marker) and the NMDA receptor-1 was determined by immunofluorescence. The intact left AT was used as control.

Results

An increased number of MCs and a higher proportion of degranulated MCs were found in the healing compared to the intact AT. The increase of MCs was particularly prominent in the myo-tendionus junction but was also seen in the mid-tendon and bone-tendon junction. In addition, increased co-localization of MC tryptase and NMDA receptor-1 was seen in the areas of myotendinous junction, mid-tendon proper and bone tendon junction of the healing versus the intact tendon.

Conclusions

MCs of the healing tendon showed signs of activation, as evidenced by extensive degranulation in the mid-tendon and bone-tendon junction. The role of MCs during tendon healing is intriguing. Potentially, several of the compounds released from activated MCs could influence the inflammatory and proliferative healing phases after tendon rupture. Another striking finding in this study was that NMDAR1 and tryptase appear to co-localize in the healing tendon. Tryptase is a MC-restricted protease that is well established as a specific marker for MCs. Hence, the tryptase-positive cells most likely correspond to MCs and, consequently, the double tryptase:NMDAR1-positive cells may represent MCs that co-express glutamate receptors. These findings introduce a possible role for MCs in the healing phase after ATR. The present study is to our knowledge the first to document increased MC numbers in experimentally induced tendon rupture in a rat model for ATR.

3.4 Paper IV (In revision)

Aim of Investigation

In a previous study, we employed an ATR rat model and showed that MCs in the healing tendon were positive for NMDAR1. This raises the possibility that MCs can be activated by glutamate released from peripheral nerve endings.

Materials and Methods

To evaluate this hypothesis, we cultured mouse bone marrow derived MCs (BMMCs). The BMMCs were isolated from the femoral and tibia bones of C57BL/6 male mice. The mature MCs (about 4-8 weeks of culture) were stimulated with L-glutamate for different time periods (1h, 4h and 72h). Cell toxicity was monitored at each time point by performing a viability assay (PR omega-Invitrogen, Carlsbad, CA) as described (Spirkoski et al. 2012).
In order to monitor morphological changes and the state of degranulation, MCs were stained with May-Grünwald (concentrated) and with 2.5% Giemsa. In addition, MC degranulation was quantified measuring β-hexosaminidase release as described 2 (Rönnberg and Pejler 2012).

To evaluate the expression of target genes of interest at the mRNA level, we first used isolated RNA samples to perform a GeneChip™ Clariom D Mouse Array analysis (SciLab, Uppsala University). RNA quality was measured by using the Agilent 2100 Bioanalyzer system (Agilent Technologies Inc., Palo Alto, CA). We used 250 nanograms of total RNA from each sample to generate amplified and biotinylated sense-strand cDNA from the entire expressed genome according to the GeneChip™ WT PLUS Reagent Kit. Next, we screened a panel of genes from the microarray analysis to understand the comprehensive transcript expression profile. Further, this set of genes was verified by qPCR analysis. Approximately 200 ng of total RNA was used in RT-PCR for cDNA synthesis followed by iScriptTM cDNA Synthesis Kit (BioRad, CA). The primer efficiency of each primer pair was checked by the iTaqTM Universal SYBR® Green (BioRad, Hercules, CA) Supermix protocol. When a satisfactory primer efficiency (about 80-110%) and dissociation curve (slope -3.1 to -3.6) was obtained, a qPCR reaction was run in duplicates in 384-well microtiter plates (Sarstedt, Nümbrecht, Germany). The qPCR C_T values were normalized to the housekeeping gene GAPDH and calculated according to the $2^{-\Delta\Delta Ct}$ formula.

To quantify protein levels and their localization, we used immunofluorescence/confocal microscopy by following single/double staining protocols as described by Alim et al, 2017 (Alim et al. 2017). We further used ELISA to quantify IL-6, TNF-α, MCP-1/CCL2, MIP-1α/CCL3 (ThermoFisher Scientific) and MCP-3/CCL7 (eBioscience, Bender MedSystems GmbH, Vienna, Austria) following the instructions given by the respective manufacturers.

Results

We showed that glutamate induces MC degranulation, and a robust up-regulation of a panel of glutamate receptors of both ionotropic (NMDAR1, NMDAR2A/2B) and metabotropic (mGluR2, mGluR7) type at the mRNA and protein level. Quantification of the confocal staining revealed that the induction of mGlurR2 and mGlurR7 was statistically significant ($p<0.01$), whereas a trend of upregulation was seen for mGlurR1. We also showed that the glutamate receptor, NMDAR1 co-localized with glutamate in the MC membrane, which confirmed that the glutamate-glutamate receptor binding is specific. Furthermore, glutamate had extensive effects on gene expression in MCs, including an upregulation of pro-inflammatory components such as IL-6 and CCL2. Glutamate also induced an upregulation of transcription factors, including Egr2, Egr3 and, in particular, FosB. Extensive induction of FosB at the protein level was confirmed by confocal microscopy assessment. The gene array screen also indicated that glutamate induced the expression of the nuclear receptors Nr4a1 and Nr4a3, as well as the transcription factors Egr2, Egr3, FosB and cytokines/chemokines (e.g. IL-6, CCL2, and CCL7) at the mRNA level. Further analysis showed that the glutamate receptor antagonist MK-801 significantly ($p\leq0.01$) blunted the induction of Egr2, Egr3, Nr4a3 and FosB caused by glutamate. In addition, we found that MK-801 reduced the expression of tryptase (Mcpt6), a granule marker in MCs. At the protein level, MK-801 also abrogated the induction of FosB seen in glutamate-stimulated MCs. The glutamate receptor antagonist (MK-801) completely abrogated the responses of MCs to glutamate, supporting a functional glutamate:glutamate receptor axis in MCs. Finally, we provide in vivo evidence supporting a functional glutamate:glutamate receptor axis in MCs of injured tendon.

Conclusions

These findings introduce the possibility that glutamate signaling can have a functional impact on peripheral MC populations, thereby contributing to the regulation of inflammation caused by activation of peripheral nerve signaling.

4. Discussion

4.1 Paper I

In this paper, we established that tendon callus forming biomarkers, procollagen type I (PINP) and III (PIIINP), and total protein content in microdialysate of the injured Achilles tendons at 2 weeks can predict the patient-reported outcome such as pain and fatigue at 1-year postoperatively. We demonstrated that higher levels of normalized procollagen, PINP was associated to less experience of pain and patient's self-reported fatigue This supports our assumption that procollagen markers can predict patient-related outcome, especially pain and fatigue after ATR. The positive correlation of the normalized level of PINP in the injured AT with the patient reported outcome advocates that collagen type I is an essential protein in the healing process. However, we also found that higher levels of PINP in the injured AT as a single predictor was related to greater fatigue in the affected limb. In fact, at week 2 of AT healing, fibroblast-like cells (tenocytes) can synthesize PINP and PIIINP and help contribute to the arrangement of loose connective tissue at the healing site (Carlsson et al. 2011). Likewise, in bone healing, both PINP and PIIINP have been documented as markers of bone formation in response to osteoporosis treatments (Eastell et al. 1993a). Moreover, some other studies have demonstrated that PINP also can be used as a bone turnover marker for the prediction of fracture risk and monitoring of osteoporosis treatment (Naylor and Eastell 2012) (Glendenning 2011). Accordingly, high levels of PINP have been associated with ageing and bone loss (Eastell et al. 1993a; Olsson et al. 2014). Therefore, similar to bone loss, we can speculate that the higher levels of PINP and PIIINP detected in our study could indicate a degenerative condition, such as tendinopathy. This might explain the negative relationship to the patient related outcome measures. Moreover, the positive association between the normalized PINP and PIIINP at 2 -week post injury and the patient-reported outcome suggests that qualitative and pure collagen type I and III synthesis is important for the tendon healing. However, if the patients have earlier tendon degeneration/tendinosis, i.e. tendinopathy, this may affect the actual production of procollagen I and III in relation to total protein levels This finding suggests that procollagen markers could be used as an early prognostic biomarker for new interventions and novel treatment methods, and potentially to screen patients in need of specific intervention to improve the healing outcome.

4.2 Paper II

In this paper, we wanted to investigate if IPC therapy is effective for the early healing response in ATR patients at 2 weeks post-operatively. We established that 2 weeks of IPC therapy during lower limb immobilization in patients with ATR could significantly upregulate the healing marker, PINP. However, the most innovative step of this study is to use the IPC treatment under leg immobilization in an orthosis, which led to elevated production of PINP in the treatment group. It has been demonstrated that adjuvant IPC treatment is an effective method to reduce the risk of DVT during leg immobilization of ATR patients at 2 weeks post-operatively (Domeij-Arverud et al. 2015b) (E. et al. 2013). In this paper, we confirmed that IPC treatment increased the production of PINP level with about 70% in the injured tendon compared to the plaster cast group. These observations suggest an improved repair response in the IPC group, since PINP is a marker of type I collagen turnover, which is the strongest and most abundant building block in the Achilles tendon as well in other tendon tissues (Wu, Nerlich, and Docheva 2017; Rittie 2017). Leg immobilization reduces blood flow and leads to reduced organized collagen diameter and occurrence. However, two weeks of IPC treatment under immobilization increases blood circulation and enhances such biochemical and morphological properties after ATR in rats. (Schizas et al. 2010). The contralateral, intact AT of the plaster cast group did not exhibit any upregulation of PINP as compared to healing tendon, suggesting that leg immobilization may inhibit collagen type I production. In earlier studies it has also been demonstrated that 2 weeks of leg immobilization impairs proliferative repair after ATR, with up to 80% loss in tensile strength attributed to reduced collagen type I production (Bring et al. 2009; Schizas et al. 2010). Consequently, the experimentally induced increase in levels of PINP in the healing IPC-treated compared to the contralateral intact AT, suggests that IPC treatment is effective to counteract impairments of the healing process associated with leg immobilization.

In this study we also demonstrated that an elevation of PIIINP could indicate the synthesis of collagen type III, which is an unorganized “scar” collagen produced in the initial phase ,i.e., in the first 2 weeks of healing process. In contrast, the higher level of PINP in the IPC group may reflect the synthesis of the strong, high-quality and organized collagen type I. This indicates that type I pure collagen is essential for the progressed healing response after 2 weeks. Moreover, a recent study demonstrated that rat Achilles tendon healing is accelerated by an additional supply of collagen type I, resulting in improved tear resistance (Muller et al. 2016). Therefore, the upregulated PINP in the healing AT of the IPC group is presumably reflective of a whole cascade of activated proliferative healing mechanisms.

Accordingly, IPC has also been demonstrated to promote neurovascular ingrowth, fibroblast proliferation, and to increase the supply of sensory neuropeptides to the healing connective tissue (Bring et al. 2009; Schizas et al. 2010).

4.3 Paper III

In this study, we discovered a novel aspect of MCs and their possible role in tendon healing in the context of AT rupture in a rat model, as well as in communication with nerves. We established that MCs are found more abundantly in the injured tendon as compared to the intact AT. Previously, a clinical study based on samples derived from patients suffering from AT rupture has provided limited support for an increase in MC numbers during healing (Scott et al. 2008) as well as in tendinopathy (Dean et al. 2016). In addition, an increased number of MCs has been suggested after rabbit deep flexor tendon repair (Berglund et al. 2010) and in the tendinopathy seen in the calcaneal tendon overuse rat model (Pingel et al. 2013). In this paper we documented an increased number of MCs in the injured rat Achilles tendon 3-weeks post rupture. A major finding of this investigation was that MCs of the injured tendon showed signs of activation, as evidenced by extensive degranulation. MC activation was prominent in all investigated areas of the healing tendon, i.e., MTJ, mid-tendon and BTJ, suggesting that tendon healing is associated with widespread activation of MCs. The consequence(s) of MC activation in the context of tendon healing and possibly tendinopathy development is intriguing. When MCs are activated to degranulate, they release a wide panel of preformed mediators from their secretory granules (Wernersson and Pejler 2014; Espinosa and Valitutti 2018). These mediators include a panel of MC-restricted proteases such as tryptase, chymase and CPA3 (Pejler et al. 2010) as well as bioactive amines (e.g., histamine, serotonin, dopamine), proteoglycans, cytokines, growth factors (e.g., TGF-beta, NGF, FGF2, VEGF) and lysosomal hydrolases (Wernersson and Pejler 2014). Several of these compounds could potentially influence the inflammatory and proliferative healing phases after tendon rupture. In addition, MC-derived growth factors such as TGF-beta (Nakae et al. 2007) and FGF2 could contribute to tissue remodeling processes, including stimulation of collagen synthesis (Coussens et al. 1999; Maltby, Khazaie, and McNagny 2009). Furthermore, MC-derived VEGF (Grutzkau et al. 1998) and NGF may contribute to neo-angiogenesis and nerve ingrowth (Leon et al. 1994) and MC proteases such as tryptase could activate protease-activated receptors on the surface of nerve cells (Akers et al. 2000) (Saito and Bunnett 2005b).

In this paper, we also observed that NMDAR1 and MC tryptase co-localize in the injured tendon. According to our knowledge, it has not been shown pre-

viously that MCs can express such receptors, although there is limited evidence that MCs may respond to glutamate receptor antagonists (Hamasato et al. 2013). Nevertheless, it is known that various other types of immune cells, e.g., neutrophils, can express various glutamate receptors (Hamasato et al. 2013). Thus, our present study introduces the MC as a novel cell type potentially having the ability to respond to glutamate. Therefore, we may speculate that MCs expressing NMDAR1 can respond to glutamate released from afferent nerve endings, possibly inducing MC activation and in this way providing an additional amplifying loop to maintain MC activation and continuous nerve cell engagement.

4.4 Paper IV (In revision)

Based on our previous *in-vivo* data, we hypothesized that glutamate might have an impact on MC activation, and we therefore evaluated this possibility by employing an *in-vitro* mouse model. Interestingly, we demonstrate that glutamate induces the expression of a panel of glutamate receptors on the MC surface. Notably, these findings provide evidence for a functional glutamate: glutamate receptor axis expressed by MCs. Another interesting observation was that the glutamate receptor induction was transient, being substantially weakened at 4h after glutamate stimulation. Hence, this indicates that the functional impact of the glutamate:glutamate receptor axis expressed by MCs might be most pronounced in the early stages after nerve cell discharge. Overall, this is in general agreement with the role of MCs in other types of settings, e.g. allergic reactions and in the defense against envenomation, where MCs mostly contribute at early stages, through mechanisms associated with rapid degranulation and actions of released granule compounds (Metz et al. 2006). Beside glutamate receptors, we also show that exposure of MCs to glutamate activates the expression of a number of other genes. These included several inflammatory compounds, and it is thus possible that signaling through a glutamate:glutamate receptor axis in MCs can contribute to the inflammatory reaction that accompanies tendinopathy and other similar conditions. It is also plausible that cytokines/chemokines released through such a mechanism can contribute, either directly or indirectly, to pain signaling in such settings. Importantly, the induction of these genes was completely abrogated in the presence of a broad-range glutamate receptor antagonist, indicating that the induction of these genes was a direct consequence of signaling mediated by the glutamate:glutamate receptor axis.

Among the genes found to be upregulated by glutamate was FosB, which was upregulated at a higher amplitude than most other glutamate-induced genes in MCs. FosB is a transcription factor which dimerizes with Jun family members, thereby forming AP-1 complexes that are implicated in a large variety of settings such as cell proliferation, differentiation and apoptosis

(Uluckan et al. 2015). In particular, FosB has been implicated in drug addiction (Nestler 2015), but there are also reports suggesting that FosB can have an functional impact on neurogenesis (Manning et al. 2019), osteoblastoma (Fittall et al. 2018), regulation of COX-2 expression (Cervantes-Madrid, Nagi, and Asting Gustafsson 2017), TGFβ1 signaling (Barrett, Millena, and Khan 2017) and regulation of complement receptor expression (Nomaru et al. 2014). Elevated levels of FosB expression have additionally been seen in a tendinopathy model (Eliasson et al. 2013). In the latter study it was also seen that Egr2 was upregulated in the injured tendon (Hammerman, Aspenberg, and Eliasson 2014), in line with our observed upregulation of Egr2 in glutamate-stimulated MCs. Hence, we may suggest a plausible scenario where high levels of glutamate receptor expression is induced after initial exposure of MCs to glutamate released from neurons, followed by induction of FosB expression as a consequence of signaling events downstream of glutamate:glutamate receptor interaction. The downstream consequences of FosB upregulation most likely include activated transcription of target genes. Possibly, FosB expression could thereby contribute to the induction of gene expression seen in glutamate-stimulated MCs. In agreement with this, a previous report suggested that Nrf1 in complex with FosB activates TNF expression in a murine MC-like cell line (Novotny et al. 1998).

Altogether, this study has identified a novel axis by which neurons potentially can interact with the immune system. Future initiatives will be taken to evaluate if the glutamate:glutamate receptor axis expressed by MCs potentially can be exploited for therapeutic purposes to ameliorate inflammatory/pain responses following tissue injury.

5. Concluding Remarks

In conclusion, higher levels of procollagens (PINP, PIIINP) in the healing AT are associated with the tendon repair process. These tendon callus-forming markers can also be used as early predictive markers for long term functional outcome such as pain and fatigue. IPC treatment enhances the tendon healing process by upregulating type-I collagen synthesis. An increased number of MCs and a higher proportion of degranulated MCs were found in the healing tendon compared to the intact tendon. This indicates MC activation contributing to tissue healing. Co-localization of MCs and glutamate receptors (NMDAR1) in the healing tendon may have a functional impact on the communication between peripheral MCs and nerve cells in the tendon after rupture (Fig. 5). Glutamate signaling can activate MCs, thereby contributing to inflammation, tendon healing and subsequent pain regulation.

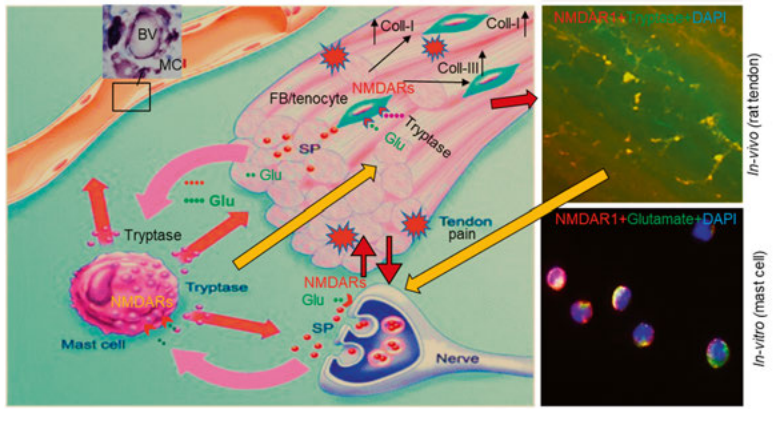

Figure 5. A possible mechanism of tendon healing, inflammation and pain regulation after ATR rupture**.** After tendon injury, peripheral nerve endings can release potent neuropeptides, e.g. glutamate (green dots), SP (red dots) that may activate MCs or fibroblast-like cells (tenocytes) via glutamate receptors, e.g. NMDAR1 (red arrowhead). Activated tenocytes can proliferate and increase the type-I and type-III collagen synthesis during tendon healing. Activated MCs can release proteases, e.g. tryptase (pink dots) which may have a functional impact on tendon cells or may activate nearby nerves via NMDARs for pain regulation (red stars). MCs can also self-activate through the NMDAR1 receptors on its surface by released proteases (autocrine pathway). MCs can also affect other tissues by mediating the angiogenic process. From our recent findings (right panel), the co-localization of NMDAR1-MC tryptase (in-

vivo), NMDAR1-glutamate (in-vitro) and increased procollagens levels in healing tendon, may support these mechanistic pathways. Abbreviations: Glu=Glutamate; SP=Substance P; FB=Fibroblast; Coll-I, Col-III= collagen type-I and type-III; BV=blood vessel, MC=mast cell. This picture is modified and adapted from (Christensen, Alfredson, and Andersson 2015) but original art by Gustav Andersson.

6. Future Perspective

These studies have raised some intriguing questions and thoughts which need to be further investigated:

- Quantitative analysis of target biomarkers expression in MC knock out models: As a possible extension of the project, it would be of great interest to evaluate the effects of tendon rupture in MC-deficient mice as well as in mouse strains lacking individual MC mediators such as chymase and tryptase. For example, it will be of large interest to assess whether MC deficiency will affect the expression of those genes that we found were induced in MCs by glutamate stimulation

- Furthermore, more samples from microdialysis or tissue/biopsy of human Achilles tendon (ATR or tendinopathy patients) could also be assessed for different inflammatory markers (e.g. histamine/proteases, cytokine-chemokine profiles) or neuronal markers (e.g. glutamate, PGP 9.5, SP) to better understand the biology of human tendon healing and tendinopathy

- In addition, it would be of interest to investigate the impact of additional signalling pathways, like calcium dependent calmodulin, ERK1/2 signalling, caspases and inflammasomes

Further experimental studies are also needed to in detail evaluate the role of a MC-nerve cell loop during tendon healing, inflammation and pain responses.

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