A Role for Nerve Growth Factor in Arthritic Pain

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To my parents, I will be forever grateful for the sacrifices you have made to ensure a better future filled with opportunities for your daughters.

Abstract

Although chronic pain is the most common symptom of arthritis, relatively little is known about the mechanisms driving it. Upregulation of nerve growth factor (NGF) in inflammatory states has been associated with increased nociception. NGF promotes the growth and survival of sympathetic and primary sensory neurons by exerting its biological activity through its cognate receptor TrkA. Previous research has shown that, in the CNS, NGF is released from cells as a precursor (proNGF) together with the enzymes required for its conversion into the mature form (mNGF) and for its degradation. Interestingly, in a rat model of poly-arthritis, our lab demonstrated that sympathetic fibers invaded the upper dermis of the skin over the inflamed joints, a region from where they are normally absent. These ectopic fibers wrapped around peptidergic nociceptive fibers.

In this thesis, we characterized the pain-related behavior, innervation pattern of the joint synovium and skin adjacent to joint, and alterations in the NGF processing cascade in a rat model of chronic inflammation. The model was induced by an intra-articular injection of complete Freund's adjuvant (CFA) into the ankle joint. We hypothesized that the NGF processing cascade is altered during chronic inflammation and arthritis, and that these alterations contribute to pain-related behavior and cause plastic changes in peripheral innervation. Indeed, we found a hyperinnervated synovial membrane as well as sympathetic fiber sprouting into the upper dermis of the skin surrounding the joint. To examine the potential functional role of these ectopic fibers on nociceptive thresholds, we pharmacologically blocked sympathetic function using systemic Guanethidine.

Next, we investigated the NGF processing mechanisms in the periphery. We hypothesized that matrix metalloproteinase (MMP)-9 degrades mNGF in the periphery, as it was shown in the CNS. In naïve animals, we administered an MMP-2/9 inhibitor in the glabrous skin of rat hind paw and assessed the changes in pain-related behavior, innervation pattern of the skin, and alterations in the NGF processing cascade. We observed that repeated MMP-2/9 inhibitor injections caused a sensitization to both mechanical and thermal stimuli, alterations in mNGF levels and a robust sprouting of sympathetic fibers, but not of sensory fibers, into the upper dermis. This proof of principle study contributes to the validation of the NGF metabolic pathway in the periphery.

To further validate our findings, we modulated pharmacologically the endogenous mNGF levels in the mono-arthritis model. We administered α2-antiplasmin to inhibit plasmin, the key enzyme in the conversion of proNGF into mNGF. We observed that the repeated local administration of α2-antiplasmin reduced the CFA-induced allodynia and hyperalgesia, prevented the CFA-induced changes in the protein levels of mNGF and blocked the CFA-induced sprouting of sympathetic fibers into the upper dermis. Finally, we investigated the changes in TRPV1 and TrkA receptor expression in dorsal root ganglia following α2-antiplasmin administration, since inflammation is known to influence their transcription.

Together, the investigations presented in this thesis should provide a better understanding of the NGF metabolic pathway in the periphery and its alterations during chronic inflammatory states. Modulating levels of NGF via its processing mechanisms, as opposed to their drastic lowering with the use of monoclonal antibodies, may lead to novel and safer therapeutic opportunities for the treatment of pain in arthritis.

Résumé

L'arthrite est une maladie caractérisée par des douleurs articulaires causées par l'inflammation et la lésion des articulations. Bien que la douleur chronique soit le symptôme le plus fréquent associé à l'arthrite, les mécanismes sous tendant cette maladie sont encore mal connus. En condition inflammatoire, des taux élevés du facteur de croissance nerveux (NGF) sont associés à une augmentation de la sensibilité nociceptive. Le NGF promeut la croissance et la survie des neurones sympathiques et des neurones sensoriels primaires en exerçant son activité biologique par le récepteur TrkA. Des études ont montré qu'au sein du système nerveux central, le NGF est libéré sous forme de précurseur (proNGF) avec l'ensemble des enzymes requises pour sa conversion en forme mature (mNGF) et pour sa dégradation. De manière intéressante, dans un modèle de polyarthrite chez le rat, notre laboratoire a mis en évidence un bourgeonnement de fibres sympathiques dans la peau située autour de l'articulation enflammée, une zone non innervée par ces fibres en condition non pathologique. Ces fibres ectopiques se retrouvent alors autour des fibres nociceptives peptidergiques.

Dans ce travail de thèse, nous avons caractérisé la sensibilité nociceptive, l'innervation de l'articulation synoviale et de la peau adjacente, ainsi que les altérations de la voie métabolique du NGF, dans un modèle d'inflammation chronique chez le rat. Ce modèle est induit par une injection unique intra-articulaire de l'adjuvant complet de Freund (CFA) dans l'articulation tibiotarsienne. Nous avons posé l'hypothèse que l'inflammation chronique et l'arthrite, dans un modèle d'arthrite chez le rat, altèrent le métabolisme du NGF. Cette altération contribue alors au comportement nociceptif et cause des changements plastiques

dans l'innervation périphérique. En effet, nous avons mis en évidence que la membrane synoviale devient hyper-innervée et qu'un bourgeonnement sympathique apparait dans la partie superficielle des tissus cutanés enflammés recouvrant les articulations. Pour étudier le rôle potentiel de ces fibres sympathiques ectopiques sur la sensibilité nociceptive, nous avons utilisé une approche pharmacologique, à savoir l'administration systémique de Guanéthidine, un agent capable de détruire spécifiquement ces fibres sympathiques. Nous avons ainsi montré que cette lésion spécifique atténuait la sensibilité nociceptive chez les animaux arthritiques.

La suite de ce travail de thèse s'est concentrée sur l'étude du métabolisme du NGF au sein du système nerveux périphérique. L'hypothèse avancée est que les métalloprotéases de la matrice (MMP) -9 dégradent le mNGF en périphérie, de manière similaire à celle mise en évidence dans le système nerveux central. Ainsi, chez des rats naïfs, nous avons administré un inhibiteur des enzymes MMP-2/9 dans la peau glabre de la patte postérieure. Nous avons ensuite évalué les conséquences de ce traitement sur la sensibilité nociceptive, le patron d'innervation cutanée et l'expression des taux de NGF. Les injections répétées de l'inhibiteur des MMP-2/9 induisent une sensibilisation des réponses nociceptives mécaniques et thermiques, des modifications des taux de mNGF et un fort bourgeonnement de fibres sympathiques, mais pas de fibres sensorielles, dans le derme supérieur. Cette étude princeps valide l'implication du NGF et de son métabolisme dans le système nerveux périphérique.

Pour consolider nos résultats, nous avons cherché à moduler les niveaux de ming endogènes, dans notre modèle d'arthrite chez le rat. Nous avons ainsi utilisé l'α2-anti plasmine, inhibiteur de la plasmine, enzyme clé de conversion du prang en ming, pour réduire la production endogène de mNGF. Les injections locales répétées d'α2-antiplasmine

réduisent l'hyperalgie et l'allodynie induites par l'injection de CFA, préviennent les modifications des taux protéiques de mNGF et bloquent le bourgeonnement sympathique induit par le CFA dans le derme supérieur. Enfin, étant donné que l'inflammation est connue pour influencer la transcription des récepteurs TRPV1 et TrkA, nous avons mesuré les changements éventuels dans l'expression de ces récepteurs, dans les ganglions dorsaux rachidiens, suite au traitement par l'α2-antiplasmine.

Pour conclure, les données présentées dans cette thèse devraient permettre une meilleure compréhension de la voie métabolique du NGF en périphérie et sur ses altérations dans des états inflammatoires chroniques. Contrairement à l'utilisation d'anticorps monoclonaux induisant une baisse drastique des taux de NGF, la modulation fine de ces taux en passant par la voie métabolique du NGF pourrait mener à des possibilités thérapeutiques nouvelles, plus sures, pour le traitement de la douleur arthritique.

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Contribution of Authors

The chapters 2-4 of this doctoral thesis describe work that was used for 3 individual publications. The following statements describe the responsibility of all authors in co-authored manuscripts:

Dr. Alfredo Ribeiro-da-Silva: Principal investigator and supervisor of the doctoral thesis. For all three manuscripts, he was the focal intellectual influence, contributed significantly to the original ideas and to the writing of the manuscripts.

Dr. A. Claudio Cuello: An important collaborator in manuscripts 2 and 3. Contributed to the original ideas, offered guidance and helped in the editing of those two manuscripts.

Geraldine Longo: Designed the experimental protocols, carried out part of the behavioral experiments, performed all the immunohistochemistry and confocal microscopy experiments and most of the quantitative analysis. Geraldine did not carry out the Western blotting or zymography analysis included in the 3 manuscripts.

A more detailed description per manuscript follows below.

Manuscript 1

Sympathetic fiber sprouting in inflamed joints and adjacent skin contributes to pain related-behavior in arthritis

G. Longo*, M. Osikowicz* and A. Ribeiro-da-Silva

Journal of Neuroscience (2013) 33(24): 10066-10074

** Both authors contributed equally to the manuscript*

Geraldine Longo: Designed the experimental protocols, carried out part of the time dependent behavioral experiment and the entire Guanethidine study, planned and performed all the immunohistochemistry and confocal microscopy experiments and performed most of the quantitative analysis. Geraldine wrote the first version of the manuscript.

Dr. Maria Osikowicz: Contributed to the design of experimental protocols, performed part of the time dependent behavioral experiment, and carried out the western blotting experiment. Maria contributed to the editing of the manuscript.

Manuscript 2

Inhibition of endogenous NGF degradation induces mechanical allodynia and thermal hyperalgesia in rats

M. Osikowicz*, **G. Longo***, S. Allard, A.C. Cuello and A. Ribeiro-da-Silva

Molecular Pain (2013) 9;37

** Both authors contributed equally to the manuscript*

Geraldine Longo: Collaborated jointly with Maria Osikowicz in the design of the dose-response MMP2/9 inhibitor drug administration protocols, planned and performed all the immunohistochemistry and confocal microscopy experiments and the quantitative analysis. Geraldine contributed in the writing and editing of the manuscript.

Dr. Maria Osikowicz: Design of experimental protocols, performed part of the behavioral experiments, and carried out the western blotting and zymography experiments. Maria wrote the first version of Manuscripts 2.

Dr. Simon Allard: Helped design all experimental protocols described in manuscript 2 and contributed to the writing and editing of that manuscript.

Manuscript 3

Local administration of α2-antiplasmin alleviates allodynia and hyperalgesia in a rat model of inflammatory arthritis

G. Longo*, M. Osikowicz*, H. Liu, A.C. Cuello and A. Ribeiro-da-Silva

(To be submitted)

** Both authors contributed equally to the manuscript*

Geraldine Longo: Collaborated jointly with Maria Osikowicz in the design of the α2-antiplasmin drug administration protocols, planned and performed all the immunohistochemistry and confocal microscopy experiments and the quantitative analyses. Geraldine contributed significantly to the writing and editing of the manuscript.

Dr. Maria Osikowicz: Design of drug administration protocols, performed part of the behavioral experiments, and carried out the western blotting experiments. Maria contributed to the writing of the first version of Manuscripts 3.

Hao Liu: Collaborated as a research project course student in Manuscript 3. Processed part of tissue for immunohistochemistry, acquired the micrographs and quantified the TRPV1 and TrkAin the dorsal root ganglia component of this manuscript.

"Do not think of yourself, think of others. Think of the future that awaits you, think about what you can do. Above all, don’t fear difficult moments. The best comes from them"

— Rita Levi-Montalcini

Chapter 1

General Introduction

1.1 What is Arthritis?

Imagine feeling shooting pain while twisting the cap off a bottle of water, or being a thriving athlete where playing sports has to be put to an end due to the excruciating joint pain. These are examples of what people afflicted with some form of arthritis are suffering on a daily basis, and whether it is a hobby or going about your daily activities, your quality of life would be entirely compromised. The term arthritis comes from the Greek words "arthro" meaning joint and "itis" meaning inflammation, and is used to refer to over 100 rheumatic disorders. However, their common denominator is inevitably joint and musculoskeletal pain, which is why these conditions are classified together. Approximately 4.6 million, or one in six, Canadians aged 15 years and older are afflicted with arthritis (O'Donnell et al., 2011). Moreover, 70 percent of people afflicted with rheumatoid arthritis (RA) are women. Among all causes of disability in Canada, arthritis ranks first for women and second for men (O'Donnell et al., 2011). Alas, the disease causes a hefty financial burden to society, costing the Canadian economy $6.4 billion annually.

1.2 Different Types of Arthritis

As the two most common forms of arthritis are osteoarthritis (OA) and RA (Figure 1), we will further expand exclusively on these types of arthritis in the following sections.

However, in brief, since there are numerous related conditions, we also refer here to other forms of arthritis. For instance, gout is an acute condition caused by uric acid crystals that collect in the joints. This results in a flare up lasting days or weeks, causing the rapid development of severe joint pain and swelling (Schlesinger, 2007). Another type is psoriatic

arthritis, a type of inflammatory arthritis that appears in the skin. Many different symptomatic patterns exist, but when its spread is symmetrical, it closely resembles RA (Tam and Geier, 2004). Lastly, another form of inflammatory arthritis is Reactive arthritis, which refers to pain, stiffness, redness or swelling in a joint resulting from a previous bacterial infection. Problems may be in the joints only or involve other body systems such as the skin, muscles or tendons (Bykerk, 2013).

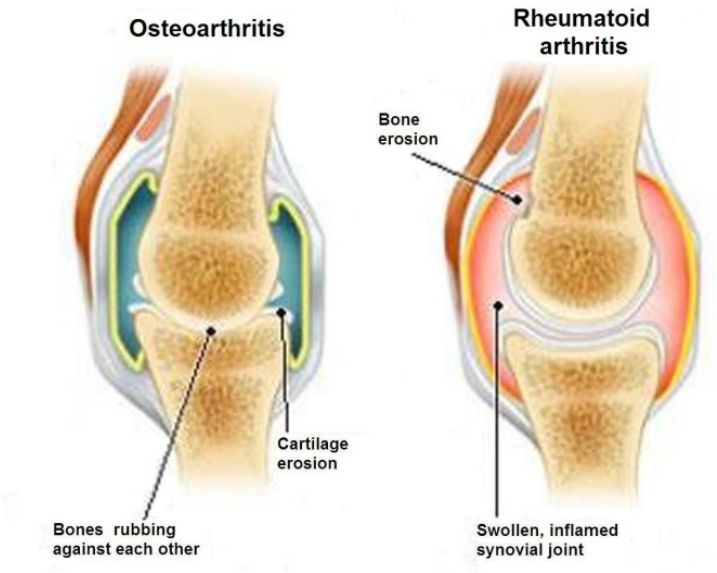


Figure 1: Schematic representation of joints affected by 'wear and tear' in Osteoarthritis (left), and the prototypical inflamed synovium in Rheumatoid arthritis (right). From Shiel, 2011.

1.2.1 Osteoarthritis

Osteoarthritis is often referred to as the oldest known disease. Being the most common joint disorder, it had surprisingly not been clinically characterized until the late 17th century, when the English physician Dr. William Heberden described bony swellings of the interphalangeal joints of the fingers and toes caused by osteophyte formation, later referred to

as "Heberden nodes" (Stecher, 1955). OA is primarily considered to be a degenerative disorder due to wear and tear on the joints resulting in a secondary inflammatory response arising within the synovium. However, we now know that the clinical and pathologic representation is far more complex. There is evidence for subchondral bone remodeling playing an important role in the pathogenesis of OA (Roman-Blas et al., 2009). Besides old age, genetics is another risk factor contributing significantly to its development. A study shows that monozygotic twins have a 70% higher concordance rate in developing OA compared to dizygotic twins (Storey, 2001).

1.2.2 Rheumatoid Arthritis

The earliest evidence of identification of RA as a disease does not appear until 1591, when the French physician Guillaume de Baillou coined the term "rheumatism" for the condition distinguished by joint soreness, inflammation, stiffness, and pain (Kaiser and Keitel, 2006). RA is an autoimmune disorder of unknown etiology affecting many tissues and organs, but which predominantly targets synovial joints. The disease hallmark consists of symmetric inflammatory polyarthritis of the joints. The pathological course begins with an inflammatory response of the synovium (the capsule around the joints), swelling of synovial cells and the formation of pannus, a fibrous membrane of granulation tissue, in the synovium. This ultimately leads to the destruction of articular cartilage and fusion of the joints. A genetic predisposition is also supported by studies reporting a RA clustering in families and this genetic influence is estimated at 50 to 60% (Winchester et al., 1992). Variants of two genes have been shown to cause a strong association with severe RA susceptibility, namely the inherited tissue type major histocompatibility complex (MHC) antigen HLA-DR4 and the T cell-associated gene PTPN22 (Shadick et al., 2007). The main

function of HLA class II molecules is to present antigenic peptides to CD4+ T cells, suggesting that RA is caused by an unidentified arthritogenic antigen.

1.3 Pain-Related Transmission

Because pain is the main symptom of arthritis, we will now describe briefly the mechanisms of pain-related transmission.

How the peripheral nervous system functioned was first theorized by René Descartes, a French scientist and philosopher in the 17th century. Contrarily to the thought that pain was a mystical experience, he brought to light the idea that it was in fact a physical disruption which traveled along nerve fibers until the message reached the processing center located in the brain. In his famous drawing of the kneeling boy by the fire (Figure 2), in the "Traité de l'homme" (Descartes, 1664), Descartes describes the "pain pathway" in a rather rudimentary form. In fact, pain is very complex, and its physical and mental dualist relationship is made clear by the International Association for the Study of Pain (IASP), which defines pain as both an "unpleasant *sensory* and *emotional* experience". In 1910, Charles Sherrington identified that pain is the "psychical adjunct of protective reflexes" and established the term

Figure 2: Illustration of the pain pathway from René Descartes' "Traité de l'homme", Descartes, 1664.

nociception, which comes from the Latin word *nocere* implying "to harm". Therefore, nociception provides alarming information about the external world to help protect us and maintain homeostasis by triggering reflexes and inducing learned averting behaviors to limit the potential or actual damaging consequences. The perception of acute pain begins suddenly and is usually sharp in quality. Accordingly, nociceptive fibers are terminations of primary afferents which respond to these pain-producing (noxious) stimuli. Depending on the stimulus type, different nociceptive fibers may become activated. On one hand, there are fibers that become activated by a selective type of noxious stimulation, that is, mechanical, thermal (heat or cold) or chemical stimulus. On the other hand, polymodal nociceptive fibers respond to a combination of stimuli, hence not stimulus-type specific (Bessou and Perl, 1969, Torebjork, 1974). The next sections will expand on nociceptive fibers in more detail.

Conversely, in the context of chronic pain lasting several months or years, the physiological protective effect of pain is no longer present and the pain becomes a pathological condition that is not only useless but also greatly distressing. There are two components of pain hypersensitivity. Allodynia is referred to when thresholds are lowered in such a way that a stimulus which is normally not painful can begin to provoke pain. For example, in the case of chronic pain, patients with RA report feeling pain while mechanically moving their joints in the normal working range. Another component is when the responsiveness is increased, termed hyperalgesia, defined as to when a noxious stimulus produces an exaggerated and prolonged pain. This unrelenting pain continues to be the single most common cause of disability that impairs quality of life. More specifically, patients afflicted with OA and RA together make up 42% of all chronic pain sufferers (Breivik et al., 2006). However, understanding the transition from acute to chronic pain is a crucial

challenge yet to be overcome in research to allow for the improvement of treating this long lasting debilitating condition.

1.3.1 Historical Perspective of the Organization of the Nervous System

In the 1830s, scientists were limited to the use of primitive microscopes to examine nerve cells. No one could determine the relationship between different shapes of nerve fibers until the two pioneers Camillo Golgi, from Italy, and Santiago Ramón y Cajal, from Spain, developed techniques to infuse microscopic amounts of stains into cells enabling them to highlight the intracellular portion of nervous tissue. While Golgi's invention comprised of using a reaction between silver nitrate and potassium dichromate to fill neurons, Ramón y Cajal, who is considered to be the founder of modern neurobiology, improved on Golgi's staining method by means of "double impregnation" while using different impregnation times depending on the type of tissue (Sotelo, 2003). In his influential work on the *Histology of the Nervous System of Man and Vertebrates,* Ramón y Cajal was also the first to describe a number of somatosensory structures, including several associated with pain (Ramón y Cajal, 1995).

1.3.2 Peripheral Nociceptive Innervation

Populations of cutaneous nociceptors can be characterised based on the primary afferent size and presence or absence of a myelin sheath as well as their responsiveness to diverse types of noxious stimuli. The nociceptive afferents are divided into the Aδ and C nociceptive fiber populations (Perl, 1968, Bessou and Perl, 1969, LaMotte and Thalhammer, 1982). Interestingly, more than 70% of the nociceptive fibers are C fibers.

1.3.2.1 Aδ fibers. Aδ fibers are thinly myelinated and are associated with transmitting thermal or mechanical fast pain sensation. In particular, this population of fibers is thought to be important in triggering the initial withdrawal reflex following noxious stimulation. Functional characteristics determined by recording discharges reveal that the Aδ nociceptors conduct in the range of 4 to 44 m/sec (Perl, 1968). However, a subset of Aδ afferents is non-nociceptive, as those fibers respond to low-threshold mechanical information, such as touch or brushing (Djouhri and Lawson, 2004). The central axons of the nociceptive Aδ fiber enter the spinal cord through the sensory root to terminate in the superficial dorsal horn (mainly lamina I), although some fibers may also terminate deeper in lamina V (Light and Perl, 1979).

Aδ fibers are further divided into 2 broad groups based on their physiological response properties: the high threshold mechanoreceptors (HTM) ant the mechanoheat (MH) sensitive nociceptors. The HTM Aδ fibers generally respond only to intense mechanical stimuli and are insensitive to noxious heat or the action of capsaicin (Perl, 1968, Georgopoulos, 1976). They make up over 73% of all Aδ fibers (Djouhri and Lawson, 2004). In the case where repeated heat is applied, HTM mechanoreceptors may become sensitized and begin to respond to heat stimuli (Fitzgerald and Lynn, 1977). The MH Aδ sensitive nociceptors respond both to noxious mechanical and heat stimuli. In the rat and monkey, MH nociceptive fibers were also found to display high chemical excitability to capsaicin (Szolcsanyi et al., 1988).

1.3.2.2 C fibers. C nociceptive afferents are unmyelinated axons, consequentially making them slowly-conducting with velocities ranging between 0.5 to 1 m/sec (Bessou and Perl, 1969). They are the most abundant nerve fiber in the skin, making up nearly 70% of all

primary sensory afferents, and have gathered much interest in their role in normal and pathological nociception (Nagy and Hunt, 1983). C nociceptive fibers can be activated by noxious mechanical, thermal, and chemical stimuli, although other C fibers have been described to have selective sensitivity to noxious mechanical and sometimes to cold stimuli. Lawson and colleagues characterized the distribution of subclasses of C nociceptive terminals by coupling immunohistochemistry and intracellular recordings. Similar to previous reports from different mammalian species, they found that 72% of C fibers were polymodal, indicating that they respond to moderate or strong mechanical stimulation as well as skin heating and chemical noxious stimuli (Lynn and Carpenter, 1982, Lawson et al., 2008). The C fibers responding only to mechanical or heat stimuli make up 11% and 9% of the population, respectively. It should be noted that, although few in number, not all C fibers are nociceptive, as some are low threshold mechanoreceptors (LTM), which is thought to encode pleasant touch sensation (Liu et al., 2007), or others may respond exclusively to innocuous thermal stimuli (Scholz and Woolf, 2002). Lastly, another population of C fibers has been found to be unresponsive to both mechanical and thermal stimulation at high thresholds, and are thus referred to as 'silent' nociceptors (Handwerker et al., 1991, Schmidt et al., 1995). These silent nociceptors may only become active following peripheral inflammation where they are sensitized to various stimuli; they may be considered relevant in pathophysiological conditions (Meyer et al., 1991, Kress et al., 1992).

1.3.2.2.1 Subdivision of nociceptive C fibers. The nociceptive C fibers are further subdivided into peptidergic and nonpeptidergic fiber populations, which differ in physiological, biochemical and anatomical properties. During prenatal development, sensory neurons form connections with their peripheral target tissues for neuronal survival. The

neuronal target cells are a source of neurotrophins, such as nerve growth factor (NGF), as well as other factors. During this developmental period, approximately 75% of dorsal root ganglion (DRG) neurons express the NGF receptor tyrosine kinase TrkA. These cells represent mostly small diameter neurons pertaining to the unmyelinated C fibers (Silos-Santiago et al., 1995). Therefore, the survival of the nociceptive small sensory neurons depends on NGF. However, as the organism develops into adulthood, the sensory neurons change their sensitivity to trophic factors. Evidence in rodents unveils that only about 50% of small diameter DRG neurons end up expressing the TrkA receptor by adulthood, particularly defined by the peptidergic nociceptive population (Molliver et al., 1997). The neurons that cease to express TrkA undergo a phenotypic switch with distinct histochemical composition compared to the peptidergic nociceptive population of C fibers. These 'non-peptidergic' C fibers begin to express Ret, another receptor tyrosine kinase, and downregulate TrkA production. Hence, following the postnatal period, they become sensitive to glial cell line–derived neurotrophic factor (GDNF), a characteristic that remains in adulthood (Molliver et al., 1997).

1.3.2.2.1.1 Peptidergic nociceptive C fibers. The NGF-dependent peptidergic C fibers contain neuropeptides, such as substance P (sP) and calcitonin gene-related peptide (CGRP), packaged into large dense core vesicles within the neuron. They project centrally and terminate in laminae I and outer II of the superficial dorsal horn, although some may also project deeper into lamina V (Ribeiro-da-Silva and Cuello, 1995). In the dorsal horn, these primary afferents form axo-dendritic or axosomatic synapses with projection neurons, which in turn relay pain-related information to supraspinal structures (Ribeiro da Silva, 2004). Peripherally, they innervate various structures such as the skin, joints, viscerae and muscle

(Perry and Lawson, 1998). In addition to their afferent function in mediating nociceptive sensation, they also play a key role in promoting neurogenic inflammation following a nociceptive stimulus or sensitization of the neuron, causing plasma extravasation and vasodilation via the release of sP and CGRP from their peripheral nerve endings, respectively (Beggs et al., 2010).

1.3.2.2.1.2 Non-peptidergic C fibers. On the other hand, as its name implies, non-peptidergic C fibers do not contain peptides in their terminals or cell bodies. This fiber population expresses the enzyme fluoride resistant acid phosphatase (FRAP) (Knyihar, 1971), now identified as the transmembrane isoform of prostatic acid phosphatase (Zylka et al., 2008), and binds the isolectin B4 (IB4) (Plenderleith and Snow, 1993). Interestingly, the non selective ligand gated ion channel $P2X_3$, which binds ATP, was found to be uniquely highly expressed in the IB4-binding, GDNF-sensitive group of nociceptive fibers unlike other purinergic receptors (Chen et al., 1995). Therefore, the non-peptidergic fiber population may display a different responsiveness to ATP. These neurons project centrally to the inner portion of lamina II in the superficial dorsal horn where they terminate in complex synaptic glomerular structures (Coimbra et al., 1974, Ribeiro-da-Silva and Coimbra, 1982) — for recent review see (Ribeiro da Silva and DeKoninck, 2009). Non-peptidergic fibers have recently been shown to innervate almost exclusively the skin, being entirely absent from deeper structures including joints and viscerae (Plenderleith and Snow, 1993, Taylor et al., 2009).

1.3.3 Innervation of the Skin

Ramón y Cajal was the first to illustrate the cutaneous "free nerve endings" in the dermis, some of them extending into the epidermis, but the primary afferents have since been

described in more detail. These free nerve endings belong to either finely myelinated or unmyelinated axons (Mense, 1996). In reality, ultrastructural evidence demonstrates that the plasma membrane of the terminal endings is enclosed by Schwann cells, where few restricted regions are freely exposed to the extracellular space. This region is thought to contain the receptors and channels that transduce sensory stimuli (Heppelmann et al., 1990). Peptidergic nociceptive C fibers terminate mostly in the dermis, in association with blood vessels, sebaceous glands and hair follicles, with some fibers penetrating the epidermis (Plenderleith and Snow, 1993, Ruocco et al., 2001, Grelik et al., 2005, Taylor et al., 2009). This close association with blood vessels supports their role in neurogenic inflammation (discussed later). The termination of non-peptidergic afferents expressing $P2X_3$ in skin was described in detail by our laboratory (Taylor et al., 2009). These afferents terminate mostly in the epidermis, where they represent the most abundant nerve fiber population, and in the upper part of the dermis (see below), but do not terminate around blood vessels (Taylor et al., 2009). The termination of myelinated mechanical nociceptive fibers in the rat skin has not been studied yet, due to a lack of a suitable marker.

Unlike sensory primary afferents, the sympathetic fibers do not innervate the upper dermis, but are located in the lower dermal and hypodermal regions where they are associated with blood vessels around which they branch in a net-like arrangement, and display numerous varicosities (Ruocco et al., 2000, Grelik et al., 2005).

1.3.3.1 Skin layer classification. The epidermis is the outermost layer of the skin characterized by the presence of stratified, keratinized epithelium, as well as melanocytes, Langerhans cells and Merkel cells (which are innervated by thick mechanoreceptors to form the Merkel disks) (Mescher, 2013). There are several types of mechanoreceptors in the

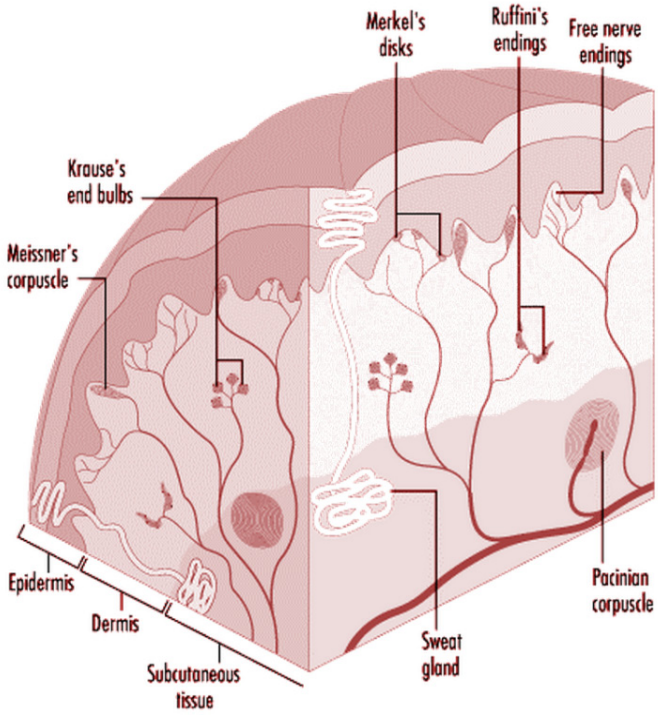


Figure 3: Glabrous skin layers, sensory free nerve endings and receptors (reproduced from Dykes and McBain, 2011).

dermis; the encapsulated Meisner corpuscles, located in the papillary layer of the dermis, respond to tactile stimuli as well as the Ruffini's and encapsulated Pacinian corpuscles, located in the reticular layer, which respond to pressure or vibration and tensile forces, respectively. In studies from our lab, the dermis has been subdivided into upper and lower dermis. The upper dermis begins at the dermal-epidermal junction and terminates at the level of the opening of the sebaceous glands, whereas as the lower dermis consists of the area below (Ruocco et al., 2000, Ramien et al., 2004). In the thick, glabrous skin of the rat hind paw, the upper dermis is defined as the portion of the dermis up to a distance of 150 µm from the dermal-epidermal junction (Yen et al., 2006b). For reference, the upper dermis comprises both the papillary and reticular layers, whereas the lower dermis is made up of the remainder reticular layer. This subdivision was established based on the observation that autonomic fibers (in rodents and primates) were never found above the layer that contains the sebaceous glands or the hairy skin or in the region close to the epidermis in glabrous skin (Ruocco et al., 2000, Grelik et al., 2005, Taylor et al., 2009).

1.3.4 Dorsal Root Ganglia

Primary afferent neurons are pseudounipolar neurons, meaning they have a smooth and rounded cell body that lies within the DRG and is contiguous to a single process splitting into two branches: a peripheral branch terminating in the skin and deeper structures, and a central branch terminating in the spinal dorsal horn (Kandel et al., 2000). These cells can be classified into large, medium and small-diameter sensory neurons (Lawson et al., 1993, Snider and McMahon, 1998).

As reported earlier, the C fibers are associated with small diameter cell bodies, whereas the myelinated Aδ and Aβ fibers have medium and large diameter cell bodies, respectively. Of particular interest for this thesis, DRG neurons projecting through the sciatic nerve are located in lumbar ganglia segments L3 through L6 in the rat (Swett et al., 1991). However, nearly 98-99% of all sciatic DRG perikarya reside in the L4 and L5 DRG segments. The L6 DRG contains merely 0.4% of its afferent neurons while the L3 ganglion contains 1.2% of the mid-thigh sciatic afferents.

1.3.5 Spinal Cord Neurons

The superficial layers of the dorsal horn are composed of lamina I, known as the marginal layer, and lamina II, known as the substantia gelatinosa. Contrasting to what is known about C fibers terminating almost exclusively in lamina I and II of the spinal cord, the Aβ fibers terminate in laminae III–V of the dorsal horn (Brown, 1983). Aδ fibers on the other hand terminate in laminae I, II, as well as in deeper layers V and VI (Nagy and Hunt, 1983, Swett and Woolf, 1985).

Among the superficial dorsal horn neurons are interneurons and projection neurons. Interneurons comprise more than 90% of the laminar neuronal population and are found to be presynaptic to projection neurons (Spike et al., 2003, Yu et al., 2005, Cordero-Erausquin et al., 2009). The projection neurons are excitatory and do not contain inhibitory neurotransmitters, such as gamma-aminobutyric acid (GABA) or glycine (Littlewood et al., 1995). Approximately 80% of projection neurons express the substance P receptor (NK-1r) (Todd et al., 2000). Their role in transmitting noxious stimuli has been demonstrated by several studies, where their activation causes NK-1r internalization or c-Fos expression (Mantyh et al., 1995, Doyle and Hunt, 1999).

1.3.6 Pain Hypersensitivity

'Sensitization' is referred to a heightened excitability of neurons, rendering them more sensitive to sensory stimuli. To better understand what produces pain hypersensitivity, it is best to subdivide it into the two mechanisms which are involved: peripheral and central sensitization. Because this thesis focuses on inflammatory and arthritic pain, our emphasis is on the mechanisms of pain hypersensitivity associated with inflammation.

1.3.6.1 Peripheral sensitization of primary sensory neurons. When peripheral terminals of nociceptors located in the skin, joints and viscera are sensitized, they become more responsive to previously innocuous stimuli and/or discharge more in response to a given noxious stimulus intensity, developing lowered thresholds to the sensation of pain (Bessou and Perl, 1969, Meyer and Campbell, 1981, LaMotte and Thalhammer, 1982). In other words, peripheral sensitization contributes to the hypersensitivity located at the site of tissue damage and inflammation by transferring more noxious sensory input to the spinal

cord. This is triggered by inflammatory chemicals or mediators, such as ATP, which can directly activate the primary sensory afferents. Two processes involved in the increase in peripheral sensitivity are post-translational changes to existing pro-nociceptive proteins and alterations in their gene expression (discussed in the next sections).

1.3.6.2 Central sensitization. Pain is not merely a reflection of peripheral inputs but also a dynamic reflection of central neuronal plasticity. Central sensitization arises from an increase in neuron excitability within the CNS, where normal inputs begin to generate abnormal responses. This immediate but transient phase is typically triggered by increased peripheral activity, altering the strength of synaptic connections between the primary afferents and the secondary sensory neurons of the spinal cord (Latremoliere and Woolf, 2009). The central terminals of the nociceptive primary afferents release signalling molecules. These include the excitatory amino acid synaptic transmitter glutamate, neuropeptides, namely sP and CGRP as well as the synaptic modulator brain-derived neurotrophic factor (BDNF), where they all act on their specific receptors expressed in dorsal horn neurons. This in turn results in the lowering of threshold and changes in the opening characteristics of these channels, thereby increasing the neuronal excitability.

Due to these activity-dependent synaptic changes, the low-threshold Aβ afferent fibers which are normally activated by light touch start activating spinal interneurons that generally only respond to noxious stimuli (Woolf and Salter, 2000), likely through a mechanism of disinhibition. Consequently, this otherwise low-intensity innocuous stimulation now begins to produce pain. Miraucourt and colleagues show that the selective blockade of glutamate NMDA receptors in the superficial dorsal horn prevents both

activation of the circuit and allodynia (Miraucourt et al., 2007). This demonstrates that a normally inactive circuit in the dorsal horn may convert touch stimulation into pain. They also provide evidence that glycine inhibitory dysfunction gates tactile input to nociceptive specific neurons. Additionally, there is some controversial evidence that myelinated fibers undergo a phenotypic switch in primary sensory neurons that comprises a change in their neurochemical properties due to alterations in transcription and translation, unlike in their naïve condition, to express and release sP and BDNF in the spinal cord when their peripheral terminals are exposed to inflammatory signals and NGF (Mannion et al., 1996, Neumann et al., 1996). However, this view has been challenged and no unequivocal morphological evidence of sP expression by these neurons has ever been shown (for review see (Todd and Ribeiro da Silva, 2007)). Research shows that the systemic NGF treatment causes the induction of central sensitization. TrkA-expressing nociceptive fibers begin to express higher levels of neuropeptides and other NGF-dependent proteins as a result of exposure to the increased NGF produced by inflammation (Woolf, 1996, Woolf et al., 1996). This topic will be further expanded below.

1.3.7 Nociceptive Transducers on C Fibers

Peripheral sensitization is the result of changes in key transduction proteins and ion channels that establish the excitability of the nociceptive end terminal. The transduction proteins are what essentially are responsible for converting a noxious stimulus into electrical activity. Following peripheral inflammation, though, the thresholds at which a stimulus can normally trigger a response fall considerably. Acidosis is a noxious condition associated with inflammation, ischemia or defective acid containment. As a consequence, acid sensing has evolved as an important property of afferent neurons with unmyelinated and thinly

myelinated nerve fibers. Protons evoke multiple currents in primary afferent neurons, which are carried by several acid-sensitive ion channels. Among these, acid-sensing ion channels (ASICs) and transient receptor potential (TRP) vanilloid-1 (TRPV1) ion channels have been most thoroughly studied.

1.3.7.1 TRPV1. Julius and colleagues first cloned the capsaicin receptor (Caterina et al., 1997). The receptor showed homology to *Drosophila* TRP proteins, identified to have a role in calcium homeostasis, and was classified under the subfamily VR1 due to the presence of a vanilloid moiety. TRPV1 was later observed to exhibit nonselective cation channel propertied with high Ca^{2+} permeability that is generated by rapid increases in temperature, whose heat activation threshold is lowered by the acidity caused by inflammation (Caterina and Julius, 2001, Immke and Gavva, 2006). When activated, TRPV1 facilitates the influx of cations, resulting in membrane depolarization and the production of action potentials (Szallasi and Blumberg, 1999). Inflammatory mediators, such as NGF and Bradykinin, increase TRPV1 activity via the phospholipase C (PLC)-mediated hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) (Prescott and Julius, 2003). This channel is also found to be up-regulated and sensitized during inflammation and injury (Di Marzo et al., 2002, Suh and Oh, 2005). Interestingly, prolonged stimulation of TRPV1 with capsaicin induces neurotoxicity in cultured dorsal root ganglion neurons (Chard et al., 1995) and selective degeneration of small diameter neurons in the adult rat (Sann et al., 1995). Hence, the TRPV1 channel confers responsiveness to both heat and chemical stimulation. Since TRPV1 is an important element that facilitates nociception by signaling specific types of sensory stimuli, it is obvious that its expression is confined to a subset of small diameter afferent neurons in the DRG. In rat, both peptidergic and nonpeptidergic C fibers respond to

capsaicin in the adult, although it remains uncertain whether TRPV1 is differentially expressed by the NGF- and GDNF-responsive nociceptors. It is important to note that there is a discrepancy between rodents regarding TRPV1 localization. In mice, TRPV1 is selectively expressed in the peptidergic C fiber population with virtually no overlap between IB4 staining (Dirajlal et al., 2003, Woodbury et al., 2004), whereas in rats, TRPV1 is present in both peptidergic and non-peptidergic C fiber populations (Guo et al., 1999).

1.3.7.2 TRPA1. Another member of the TRP family of ion channels is the transient receptor potential ankyrin-repeat 1 (TRPA1) (Venkatachalam and Montell, 2007). TRPA1 is expressed in a subset of C-fibers that express TrkA and the TRPV1 channel (Story et al., 2003). Activation of TRPA1 leads to depolarization that ultimately triggers action potentials to signal cold nociception or itch to the CNS. It is also shown to be a key regulator of neuropeptide release and the pharmacological inhibition of TRPA1 significantly reduces allodynia and hyperalgesia to thermal and mechanical stimuli induced by neurogenic inflammation (Bautista et al., 2006, Kwan et al., 2006, Eid et al., 2008). Likewise, TRPA1 mediates sensitization in a rat model of OA causing movement-evoked and spontaneous pain (Okun et al., 2012).

1.3.7.3 ASICs. ASICs are ion channels part of the H^+-gated subgroup of epithelial sodium channel (ENaC) family of proteins and become activated when the extracellular milieu becomes acidic (Waldmann, 2001, Krishtal, 2003). Acidification of the skin is known to produce pain, thus ASICs have the characteristics of functioning as a nociceptor since the lowering of pH level occurs with a variety of painful conditions, for instance, the onset of inflammation (Waldmann et al., 1997). Moreover, when administering an ASIC antagonist in

rats, a reduction in hyperalgesia is observed following CFA-induced inflammation (Dube et al., 2005).

1.3.7.4 Serotonin receptor 5-HT3. The 5-HT3 receptor is expressed by nociceptive primary afferents, specifically the Aδ HTM fibers. Its ligand serotonin, 5-hydroxytryptamine (5-HT), is a key neurotransmitter component of the inflammatory soup contributing to the pain through its action on multiple receptor subtypes (Dray, 1995). It is released by infiltrating cells or fragments at the site of injury, such as mast cells, basophils or platelets. When serotonin is administered peripherally, it is shown to evoke pronociceptive events that can be attenuated by selective 5-HT3 receptor antagonists (Sufka et al., 1992). In addition, central serotonergic circuits also modulate nociceptive transmission by facilitating spinal 5-HT3 receptors (Zeitz et al., 2002).

1.3.7.5 Voltage-gated sodium channels. Voltage-gated sodium channels (VGNC) are essential for the generation of action potentials. They consist of a family of nine structurally related subunits, all displaying electrophysiological and pharmacologically distinct properties. Specifically, the NaV 1.7, NaV 1.8. and NaV 1.9 channels are selectively expressed by small-diameter neurons and influence both acute and inflammatory pain (Wang et al., 2011). It is well known that local treatment with sodium channel blockers such as lidocaine can attenuate inflammatory and neuropathic pain. There is strong evidence supporting that altered sodium channel activity in peripheral neurons is associated with the development of inflammatory and neuropathic pain, such that NaV 1.8 channel knockouts have discriminating deficiencies in nociceptive processing (Akopian et al., 1999, Sleeper et al., 2000). More importantly, NGF regulates the expression of sodium channels through

mechanisms involving the control of trafficking from the DRG to the terminal of nociceptive fibers (Toledo-Aral et al., 1997, Wada et al., 2004).

1.3.8 Neuropeptides

In 1931, von Euler and Gaddum discovered sP, which is a member of the tachykinin family. It was later found that sP was the first pain-related peptide identified. sP serves as a sensory neurotransmitter/modulator at the spinal cord level and has axon-reflex vasodilatory actions when released by peripheral terminals (Euler and Gaddum, 1931, Hokfelt et al., 1975). sP binds to the NK-1r expressed on dorsal horn second-order projection neurons. Upon binding, sP elicits various physiological effects primarily via intracellular second messengers resulting in the depolarization of neurons. In blood vessels, sP acts on endothelial cells where its dilator action depends on the formation of nitric oxide (Bossaller et al., 1992).

CGRP was later found to colocalize with sP in the same small diameter afferent neurons and associate with smooth muscle of blood vessels and free nerve endings in the skin and joints (Skofitsch and Jacobowitz, 1985, Ribeiro-da-Silva and Hokfelt, 2000). The receptor for CGRP consists of a complex of a seven transmembrane-spanning protein, calcitonin receptor-like receptor (CLR), a single transmembrane-spanning protein designated receptor activity modifying protein 1, as well as an intracellular receptor component protein (RCP) (McLatchie et al., 1998, Evans et al., 2000). Like sP, CGRP is released from peptidergic nociceptive fibers to act both centrally as a neurotransmitter/modulator and peripherally. When CGRP is released in the peripheral terminals it causes a long-lasting vasodilation responsible for several of its proinflammatory actions. T lymphocytes and macrophages are found to express both the CGRP receptor and the NK-1r (McCormack et al., 1996, Simeonidis et al., 2003, Bracci-Laudiero et al., 2005). A study shows increased

number of CGRP-immunopositive DRG neurons during acute and chronic inflammatory lesions in the rat ankle joints (Hanesch et al., 1993), indicative of an upregulation, which parallels a similar upregulation of sP in DRG neurons (Minami et al., 1989).

1.3.8.1 Neurogenic inflammation. The phenomenon of neurogenic inflammation refers to the observation that stimulation of peptidergic nociceptive fibers mediates proinflammatory actions, such as vasodilation, plasma extravasation and recruitment of immune cells (Lembeck and Holzer, 1979). Bayliss was the first to describe the direction of the response by sensory afferent fibers to be "antidromic" in nature, denoting that these neurons not only conduct afferent information to the spinal cord, but also carry out an efferent function. Since then, abundant support has paved the way to validate the notion that activation of peripheral terminals of sensory neurons by local depolarization and axonal or dorsal root reflexes releases bioactive substances, namely sP and CGRP. The peripheral actions of sP and CGRP in the skin and joints lead to the first steps of neurogenic inflammation (Jancso et al., 1967, Ferrell and Russell, 1986). The inflammatory symptoms observed can be mimicked by administration of sP or CGRP agonists and reversed by antibodies or antagonists directed

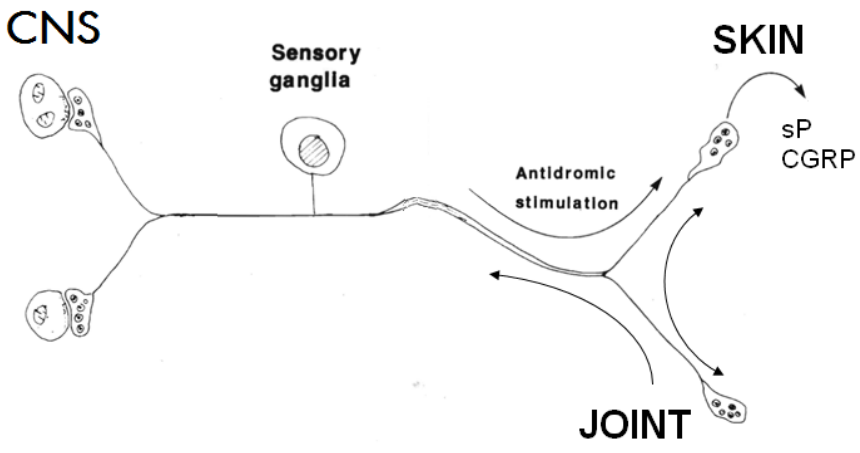


Figure 4: Neurogenic inflammation resulting from antidromic stimulation and neuropeptide release. Courtesy of Dr. Cuello and Dr. Ribeiro-da-Silva.

against these peptides or their receptors, respectively (Maggi, 1995). In fact, 90% of the sP synthesized by small diameter cell bodies in the DRG is transported to the peripheral terminals, where it is released by antidromic activation of the fiber (Levine et al., 1985b).

It is obvious that the peptidergic nociceptive C fibers are of major importance in the initiation of neurogenic inflammation, but particularly interesting is its subpopulation of fibers sensitive to capsaicin, the vanilloid found in hot peppers (Holzer, 1988). Intradermal injection of capsaicin produces hypersensitivity and an inflammatory flare,— symptoms that can be prevented by denervation or desensitization to capsaicin, resulting in the depletion of neuropeptide content in the terminals (Richardson and Vasko, 2002). Furthermore, destruction of TRPV1 expressing fibers attenuates neurogenic inflammation produced by antidromic stimulation of sensory fibers (Noble et al., 2006). Figure 4 shows a diagrammatic representation of neurogenic inflammation following nerve stimulation.

1.4 Normal Joint Anatomy

Articular joints are comprised of hyaline cartilage covering the joint surfaces, and is in turn covered externally by a fibrous membrane, called the perichondrium (Brewerton, 1992). The synovium is tightly sheathed by white fibrous ligaments connecting the two bones, thus limiting its movement. The synovial fluid lubricates the joint within the capsule. The periosteum is a fibrous connective tissue attached and surrounding the outer cortical surface of bone, except at joints where bone is lined by articular cartilage.

1.4.1 Innervation of the Joint

The overwhelming majority of synovial joint nerve afferents consist of unmyelinated axons, but some myelinated afferents of large diameter are also found (Langford and Schmidt, 1983). Specifically, the peptidergic C fiber population is found in all sections of normal joint tissue scattered throughout the fibrous capsule, ligaments, tendons and synovium (Mapp et al., 1990). At the articulating surface, there is little innervation detected. The periosteum is innervated and some reports suggest that bone pain originates predominantly, if not exclusively, from this structure (Mercadante, 1997). Autonomic fibers are also present in joint tissues, where most of these fibers are found to be adjacent to or within blood vessel walls (Ahmed et al., 1993). Some joint nociceptors are normally silent with respect to joint movements; however, they can be sensitized by inflammation, after which they become responsive even to the negligible movements of the joint (Schaible and Schmidt, 1985).

1.5 Evidence for the Development of Arthritis

1.5.1 Neurogenic Inflammation in Arthritis

Consistent with the view that sP and CGRP play an important role in mediating neurogenic inflammation, elevated concentrations of these sensory neuropeptides have been detected in synovial fluid of rheumatoid arthritis patients (Hernanz et al., 1993, Arnalich et al., 1994). They are released by the nerves innervating the joint into the synovium, bone marrow and periosteum. It has been long since proposed that the symmetric distribution of joint involvement observed in RA patients, as well as in adjuvant-induced arthritis models in rodents, may be determined by axon reflex mechanisms, a component of neurogenic

inflammation. In effect, a study shows that the electrical stimulation of the nerve fibers supplying the knee joint is found to evoke the release of sP from articular nerve fibers and produce neurogenically induced plasma extravasation into to synovial cavity (Yaksh et al., 1988).

A number of mechanisms induce joint inflammation in RA, where neuropeptides are found to contribute to the production of cytokines (Raap et al., 2000, O'Connor et al., 2004). Previous studies have identified that CGRP may augment production levels of interleukin (IL)-6 and IL-8 in synovial cells, specifically the fibroblast-like synoviocytes, of RA patients, while sP did not exhibit an effect on interleukin secretion by these cells. Guiducci's group show that neuropeptides alone are not able to directly increase interleukin synthesis in the synovial membrane of RA patients, while combining sP or CGRP with capsaicin strongly induces the expression of proinflammatory cytokines, demonstrating a cooperative role of TRPV1 and neuropeptides in inducing inflammatory stimuli (Terenzi et al., 2013). They also demonstrate that neuropeptides significantly increases TRPV1 expression in RA synoviocytes.

1.5.2 Pathological Bone Remodeling

Physiologic bone remodeling involves a continuous balance between resorption by osteoclasts and bone formation by osteoblasts. Under pathological conditions, this balance is altered, resulting in bone loss in diseases such as arthritis, causing decreases in bone density and disrupting skeletal architecture (Walsh and Gravallese, 2010, Almarestani et al., 2011). The alteration in bone remodeling within the RA bone microenvironment may also be influenced by the expression of pro-inflammatory cytokines and growth factors expressed by cells present within inflammed synovial tissues.

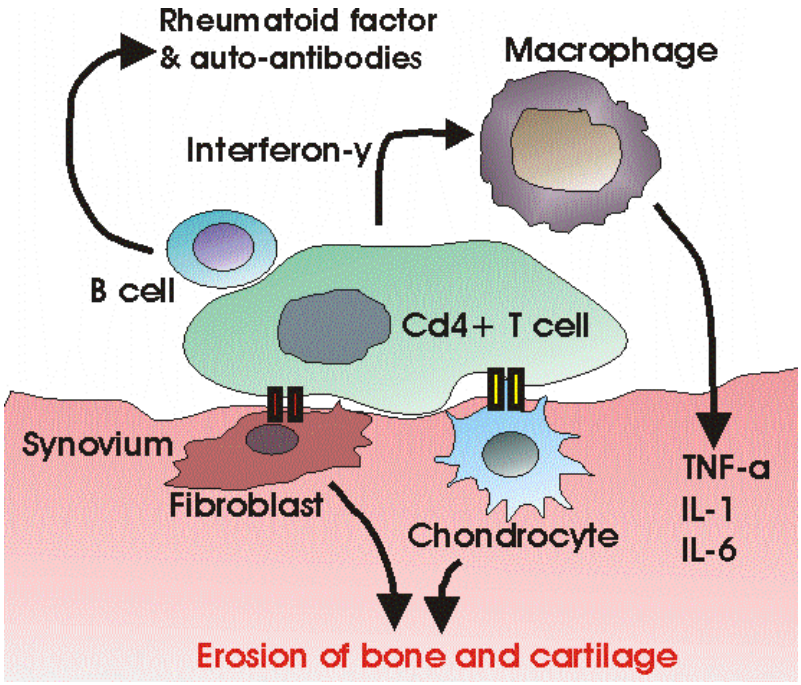
1.5.3 Development of Angiogenesis in Arthritis

Another pathological component observed in patients with RA and OA is the development of angiogenesis at the osteochondral margin (Walsh et al., 2010). Interestingly, the neurovascular invasion from the subchondral bone into articular cartilage is followed by a hyperinnervation of the area (Suri et al., 2007). NGF can stimulate angiogenesis (Nico et al., 2008), suggesting that the expression of NGF and sensory nerve growth may link osteochondral angiogenesis to the pain in arthritis.

1.5.4 Inflammatory Mediators Involved in Arthritis

Acute inflammation is still diagnosed from the presence of the five cardinal signs: rubor (redness), calor (increased heat), tumor (swelling), dolor (pain), and functio laesa (loss of function) (Tracy, 2006). These clinical symptoms result from an interconnected array of physiologic processes producing increased vascular permeability (plasma extravasation), vasodilation and sensitization of nociceptive afferents. A large variety of cells play a role in the perpetuation of synovitis and pathological progression of arthritis.

Of the infiltrating inflammatory cells, CD4+ T cells are responsible for triggering cell-mediated immune responses. Once they interact with MHC class II molecules, the antigen-activated CD4+ T cells further stimulate monocytes, macrophages, mast cells, and synovial fibroblasts to produce the cytokines IL-1, IL-6, and tumor necrosis factor (TNF)-α and to secrete matrix metalloproteinases (MMPs), through cell-surface signaling and release of soluble mediators such as interferon-γ and IL-17 (Choy and Panayi, 2001). TNF-α, IL-1 and IL-6 are the main cytokines driving inflammation in RA. Activated CD4+ T cells also stimulate B cells to produce immunoglobulins, such as the auto-antibodies against collagen type II. Additionally, the activated CD4+ T cells stimulate osteoclastogenesis, the cells



responsible for bone resorption. These activated macrophages, lymphocytes, and fibroblasts, as well as their products, can also stimulate angiogenesis. See Figure 5 for a diagrammatic representation.

Figure 5: Cytokine signaling pathway involved in inflammatory arthritis. The major cell types and cytokines involved in joint destruction mediated by TNF-α and IL-1 are shown. Adapted from Choy and Panayi, 2001.

1.5.5 Pathological Sensitization of Spinal Cord Neurons

In addition to the abovementioned triggers that initiate and maintain central sensitization, there are many other central mechanisms contributing to inflammatory pain. Some of them will be briefly described below.

Spinal cord neurons were found to display an enhanced excitability following inflammation (Hylden et al., 1989). Following the onset of inflammation, the hyperalgesia is maintained by the activation of N-methyl-D-aspartate (NMDA) receptors (Sluka and Westlund, 1993).

Another potential function of neuropeptides in nociceptive transmission is the sensitization of nociceptive dorsal horn neurons, contributing to symptoms of hyperalgesia

and allodynia which are characteristic of neuropathic and inflammatory pain. Sensitization of dorsal horn neurons in lamina I and in deeper laminae (III–VI) has been well documented following tissue injury and inflammation (Treede et al., 1992, Coderre et al., 1993).

Changes in the phenotype of dorsal horn lamina I neurons have been described under pathological pain conditions. In normal conditions, the NK-1r are mostly expressed by multipolar and fusiform spinal projection neurons, while most pyramidal neurons do not express the receptor. Interestingly, Almarestani and colleagues discovered in an adjuvant-induced arthritis (AIA) animal model a de novo expression of the NK-1r in pyramidal neurons (Almarestani et al., 2009) (Figure 6).

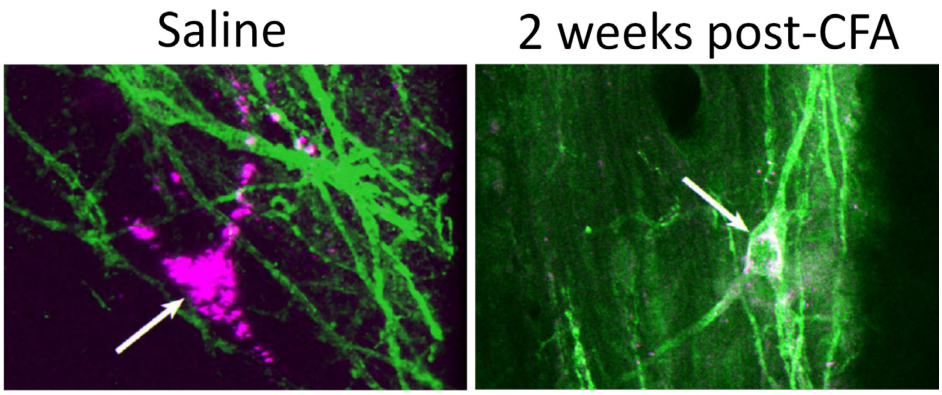


Figure 6: Confocal images illustrating the morphology in lamina I spinoparabrachial pyramidal neurons (magenta) in saline- and CFA-treated animals. NK-1 receptor (green) is expressed de novo in CFA-treated animals. From Almarestani et al., 2009.

Another important central pathway driving inflammatory pain hypersensitivity involves induction of cyclooxygenase-2 (Cox-2) in dorsal horn neurons, contributing to the production of prostaglandin E2 (PGE2) (Samad et al., 2001). PGE2 potentiates AMPA and NMDA receptor currents in these neurons, activates non-selective cation channels and

reduces inhibitory glycinergic neurotransmission by blocking glycinergic receptors with α3 subunits (Baba et al., 2001, Ahmadi et al., 2002).

Lastly, following peripheral inflammation, microglial cells are activated, changing their shape and chemical expression (Svensson et al., 2003, Raghavendra et al., 2004). Particularly, activation of p38 MAPK occurs leading to synthesis and release of pro-inflammatory cytokines, such as IL-1β and TNF-α, which give rise to the development of central sensitization by enhancing excitatory and reducing inhibitory currents as well as by activating the induction of COX-2 (Samad et al., 2001, Kawasaki et al., 2008b).

1.6 Pre-Clinical Animal Models of Arthritis

Animal models have been particularly important in the fast progression of health-related research. We owe much to the research carried out in animals in the last 60 years for allowing us to have a better understanding of arthritis and for testing potential therapeutic agents to treat both humans and animals afflicted with this disease.

1.6.1 Adjuvant-Induced Arthritis (AIA). The first animal model used for the study of arthritis was serendipitously designed in 1954 by Stoerk and colleagues while studying immune reactivity of homologous various organ tissues in the rat. A couple weeks following the injection of tissue extract emulsified in complete Freund's adjuvant (CFA), Stoerk observed redness and painful swelling in several joints. It was later discovered that the CFA alone was sufficient to cause the development of polyarthritis (Wooley, 2004). The arthritogenic component of CFA is made up of a suspension of heat killed mycobacteria administered in a mineral oil vehicle, prolonging its lifetime at the site of injection. The mechanism of action of CFA is carried out by stimulating the cell-mediated immunity.

Antigen-presenting cells, namely macrophages and dentritic cells, are recruited to the site of injection, where they phagocytose the slowly released emulsified mycobacterial antigens. This triggers antigen-specific cytotoxic T-lymphocyte responsiveness as well as the production of cytokines such as TNF-α and IL-12 (Billiau and Matthys, 2001). The AIA model produces rheumatoid arthritis-like symptoms and pathology, thus has proven to be a valid animal model. Previously, the administration was performed into the rat tail vein and the hallmark changes were joint inflammation, cartilage destruction and bone erosion, which persisted chronically for several weeks (Bendele et al., 1999).

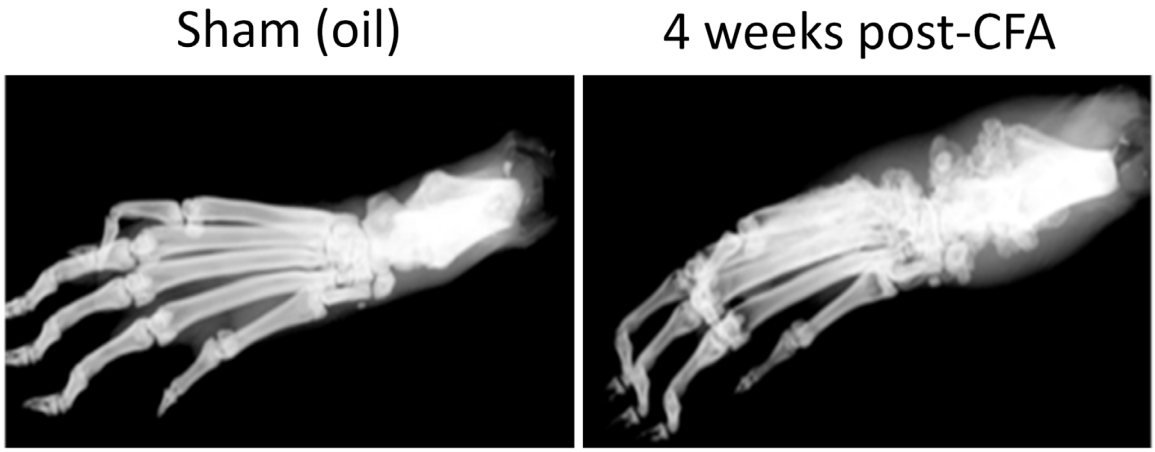


Figure 7: Radiographs of rat tibial-tarsal joints following intra-articular injection of oil (left) and CFA (right). Note in the CFA-treated joint marked signs of arthritis, edema and deformation of the digits.

Our laboratory has shown that the subcutaneous administration of a small dose of CFA in the plantar surface of the rat hindpaw causes a strictly unilateral arthritis of multiple joints of the paw and no systemic changes up to 4 weeks post-injection (Almarestani et al., 2011). In the mono-arthritis model (Figure 7), a low dose of CFA is administered intra-articularly, preventing the systemic spread of the disease. Changes are characterized by a

reliable onset of robust joint inflammation and marked bone resorption; all changes remain restricted to the injected joint (Bendele et al., 1999). Signs of cartilage destruction are observed but are mild when compared to the occurrence of inflammation and bone destruction. This AIA model has been used much more extensively for pharmaceutical testing than any other arthritis animal model, therefore more data is available for comparison with humans.

1.6.2 Collagen-Induced Arthritis (CIA)

It has been suggested that the powerful immune stimulant of the complete Freund's adjuvant may boost the reactivity of some constituent within the joint subsequently leading to the induction of arthritis. A potential candidate has been the type II collagen, a major structural component of connective tissue found specifically in cartilage (Trentham et al., 1977, Holmdahl et al., 1993). The cartilage antigens are emulsified with CFA and injected intra-dermally at the base of the tail. However, this model has a separate entity from the AIA model because it leads to the generation of homologous anti-type II collagen antibodies. This results in the destruction of cartilage, histologically mimicking RA. This model is only good while using specific rat and mouse strains, as they vary in susceptibility to autoimmune disease (Wooley, 1991, Moder et al., 1992). Although the CIA model closely resembles an autoimmune disorder, the damage caused by immunization against type II collagen is primarily associated with cartilage with only mild synovitis and periarticular inflammation, which is more likely a characteristic of OA.

1.6.3 Limitations to Using Animal Models of Rheumatoid Arthritis

As stated above, most of the RA animal models include pathological features that are in parallel to those transpiring in human disease, but there are important differences to keep

in mind. Firstly, animal models of RA develop much more quickly than the disease observed in human patients, thus these models are characterized principally by a strong inflammatory response. Additionally, since the epitope of the auto-antigen responsible for the human rheumatic disorder is not known, we are limited in accurately reproducing the RA pathology in animals.

1.6.4 Osteoarthritis-like Animal Models

The existing animal models of osteoarthritis may be induced chemically or surgically. The most commonly used chemically-induced OA model involves an intra-articular injection of monosodium iodoacetate (MIA). The MIA model is useful to investigate analgesic drug effects due to its reproducibility and mimics pathological changes developed in human OA patients. Following the intra-articular injection of MIA, joint degeneration is produced by inhibiting glycolysis, consequently leading to chondrocyte necrosis (Bendele et al., 1999). This results in a decrease in thickness of the articular cartilage, exposure of the subchondral bone and the reduction in bone mineral density (Fernihough et al., 2004, Pomonis et al., 2005).

Lastly, there are surgical models characterized by partial removal of the menisci combined with transection of the collateral or cruciate ligaments; these changes cause mechanical instability of the knee joint, thereby promoting the articulating surfaces to rub against one another (Bendele, 2001).

1.7 NGF and Arthritis

1.7.1 Discovery of NGF

In the 1950s, Rita Levi-Montalcini, a neuroembryologist, was the first to observe in her early experiments an abnormal network of nerve fibers engulfing the mouse sarcoma

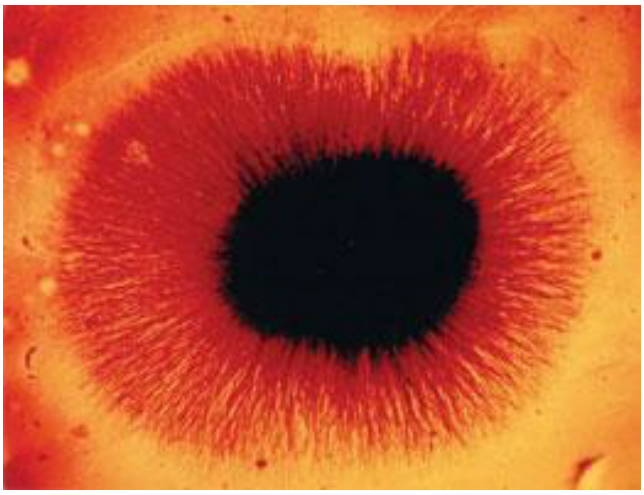


Figure 8: Levi-Montalcini's discovery of NGF causing nerves to sprout from chick sensory ganglia. From Cohen and Levi-Montalcini, 1956.

tissue she had grafted into the body wall of chick embryos. She speculated that some sort of released factor must have caused this vigorous nerve cell growth from the chick embryo toward the tumor. A few years later, Levi-Montalcini began to collaborate with Stanley Cohen, a biochemist in training. In 1956, they cracked the mystery behind this unidentified factor, and proposed that it be called "nerve growth factor". Cohen purified the NGF protein extract from salivary glands, where it occurs in surprisingly high concentrations, while Levi-Montalcini noticed a "fibrillar halo" shape grown around the chick embryo sensory ganglia when plated in a glass dish with this NGF extract (Weiss, 1988) (Figure 8).

1.7.2 Growth Factors and their Receptors

Target derived growth factors are essential for regulating neuronal survival during embryonic development, postnatal development and differentiation as well as mediating the functional properties of neurons in the adult. Two important growth factor subclasses consist of the neurotrophin and the glial cell line-derived neurotrophic factor (GDNF) families. Their role in development is well-known; however, their function in pain is raising an emerging interest among researchers. The neurotrophin family of structurally related growth factors includes NGF, BDNF, neurotrophin (NT)-3 and NT-4. Mature neurotrophins are homodimers derived by proteolytic cleavage from a precursor protein. Each neurotrophin

binds to two types of receptors. The first is $p75^{NTR}$, a member of the TNF receptor superfamily, which is a common receptor to all neurotrophins and binds them with a similar low affinity. The second type are members of the Trk family of receptor tyrosine kinases, TrkA, TrkB and TrkC, binding specific neurotrophins, for which tropomyosin-related kinase A (TrkA) is the high-affinity cognate receptor for NGF (Huang and Reichardt, 2003, Barker, 2004). The presence of $p75^{NTR}$ enhances the affinity of Trk receptors in responding to their preferred neurotrophin ligand. However, in the circumstance that Trk signalling is absent, $p75^{NTR}$ promotes apoptosis upon neurotrophin binding.

1.7.3 proNGF Signaling

The regulation of neuronal survival is generally attributed to mature neurotrophins, although a biological role for the precursor form of neurotrophins has recently been recognized (Lee et al., 2001, Fahnestock et al., 2004). A recent study describing that proNGF has apoptotic activity is contradictory with other results demonstrating that proNGF has neurotrophic activity. ProNGF has high affinity for $p75^{NTR}$ which can promote cell death at lower levels than mature NGF, suggesting that the balance between cell survival and apoptosis could depend on proNGF and mature NGF levels in tissues (Ibanez, 2002). Several other studies have reported that proNGF has neurotrophic activity similar to that of mature NGF (Chen et al., 1997, Rattenholl et al., 2001), although its activity is less potent than that of mature NGF (Edwards et al., 1988, Suter et al., 1991).

1.7.4 Mature NGF Signaling

Upon binding to TrkA, mature NGF plays an essential role in the development of the peripheral nervous system by promoting the growth and survival of neural crest-derived cells in developing embryos, particularly sensory and sympathetic neurons. However, sensory

afferents cease to rely on this factor for their survival in adulthood. This was found by early experiments administering an anti-NGF antibody in rats during early postnatal development, which revealed that DRG sensory neurons lose the NGF requirement for survival shortly after birth. Notwithstanding these results, NGF still has an influence on the phenotype of nociceptors in the adult CNS (Pezet and McMahon, 2006), as well as regulating the steady-state number of synapses (Sofroniew et al., 1990, Debeir et al., 1999). On the other hand, NGF remains critical for the survival of sympathetic fibers even in the adult. For instance, studies show that sympathetic ganglia are virtually absent in mice carrying mutations in the NGF or TrkA genes (Levi-Montalcini and Angeletti, 1968, Thoenen and Barde, 1980, Yankner and Shooter, 1982). Besides its obvious role in the maintenance of its peripheral targets, NGF also up-regulates the expression of several gene products, which will be discussed in the next section (Ruiz and Banos, 2005, Mousa et al., 2007).

1.7.5 Processing of NGF

In the CNS, members of the neurotrophin family are produced and secreted in an activity-dependent manner (Thoenen, 1995). Contrarily to what was originally believed, Claudio Cuello's group provide direct evidence that the precursor form of NGF (proNGF) is released in the extracellular space in an activity-dependent manner, along with the regulatory enzymes responsible for its conversion to the mature form (mNGF) and its degradation into inactive products (Bruno and Cuello, 2006). In more detail, tissue plasminogen activator, plasminogen, neuroserpin, precursor matrix metalloproteinase 9, and tissue inhibitor metalloproteinase 1 are what constitute the converting protease cascade and its endogenous regulators. Specifically, plasmin is found to be the key serine protease involved in the physiological maturation of proNGF. Moreover, matrix metalloproteinase 9 (MMP-9) is

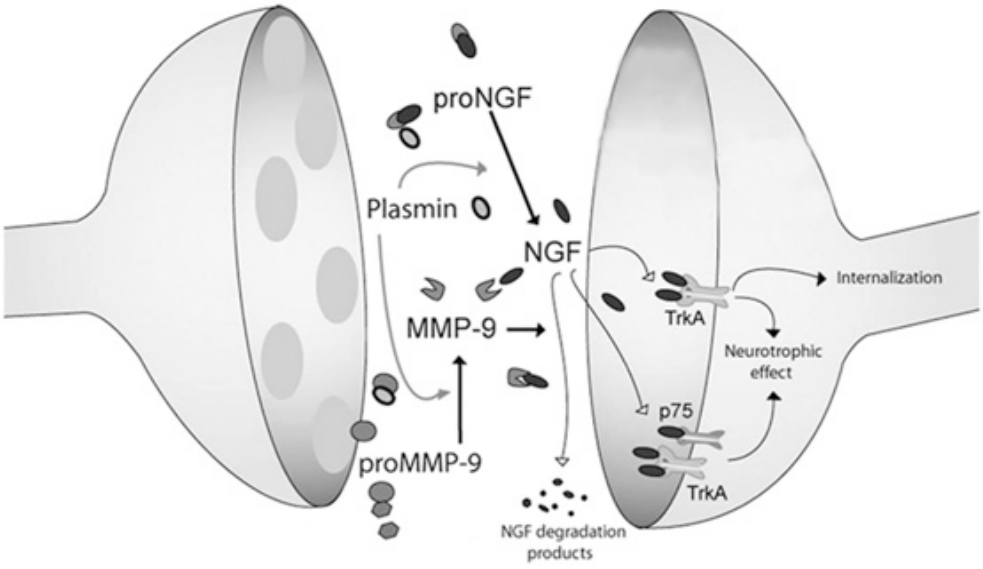


Figure 9: Schematic representation of the metabolic pathway leading to proNGF conversion into mNGF and its degradation. Adapted from Bruno and Cuello, 2006.

found to be the main protease degrading mNGF (Figure 9). This release of the precursor form follows the same proteolytic mechanism as for which BDNF is processed in the CNS (Pang et al., 2004). These proteolytic enzymes are found in neurons of the cerebral cortex and are released upon neuronal stimulation (Bruno and Cuello, 2006). However, conclusive evidence that NGF is also secreted in the precursor form in the periphery is still lacking.

1.7.6 NGF and Pain

NGF is well known to mediate algogenic effects. The significance of the role NGF plays in pain has been brought to light by recent observations made in different cases of patients afflicted with congenital insensitivity to pain, which is caused by different mutations in the TrkA receptor sequence. These mutations are induced by frame shifts, nonsense, splice

or mis-sense variations found in either the extracellular NGF-binding domain or the intracellular signal-transduction domain (Indo, 2001).

Local levels of NGF surrounding the peptidergic C fiber axon terminals are known to influence the function of nociceptors both acutely and chronically. Whilst depriving the periphery of NGF, there is an epidermal depletion of nociceptive end terminals resulting in reduced nociceptor sensitivity (Bennett et al., 1998a). Particularly, NGF levels are shown to be increased in peripheral tissues in a variety of inflammatory conditions in humans, which is also reflected in experimental animal models. Intra-dermal injection of NGF results in a rapid thermal hyperalgesia (Lewin and Mendell, 1994, Woolf et al., 1996). The contribution of NGF to inflammatory sensitivity may be generated directly by binding to TrkA receptors on peripheral terminals of primary sensory neurons (McMahon et al., 1994), but a significant contribution is also derived indirectly by acting on the peripheral cells which express the TrkA receptor, such as inflammatory cells and sympathetic neurons. NGF might produce its local sensitizing actions via the sympathetic nervous system by interacting with primary sensory neurons to produce the neurogenic component of inflammation (Levine et al., 1985b, Coderre et al., 1991).

1.7.7 NGF and Inflammatory Pain

The key role of NGF in inflammatory pain is illustrated by its expression and release from certain inflammatory cells, principally mast cells as well as eosinophils, lymphocytes and macrophages (Heumann et al., 1987, Leon et al., 1994). Furthermore, NGF is found to be up-regulated in experimental models of inflammation, including those induced by CFA (Donnerer et al., 1992, Woolf et al., 1994).

Interestingly, research shows that over expressing NGF levels causes small-diameter nociceptive fibers to sprout into inflamed joints and the skin tissue surrounding it (Mendell et al., 1999), thereby leading to abnormal nociceptive fiber presence. NGF also sensitizes these fibers to inflammatory mediators, leading to the enhanced nociception observed in chronic inflammation. Interestingly, the hyperalgesia resulting from inflammation is attenuated by administration of anti-NGF neutralising antibodies or a TrkA-IgG fusion molecule (McMahon et al., 1995).

1.7.8 NGF Action on Mast Cells

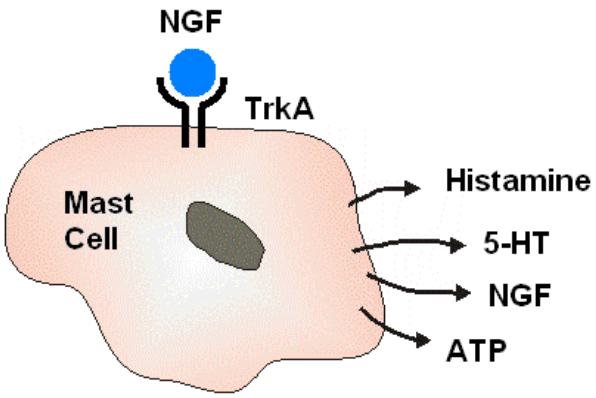


Figure 10: NGF binds to TrkA on mast cells causing the release of additional inflammatory mediators. Adapted from Mantyh et al., 2011.

NGF also indirectly sensitizes nociceptors by activating mast cells, as these cells also express the TrkA receptor. Exposing isolated mast cells to NGF induces them to degranulate and release their contents, such as of 5-hydroxytryptamine (5-HT) (Kawamoto et al., 2002). In addition to 5-HT, activated mast cells release other algogenic mediators such as histamine, amines, cytokines, bradykinin, prostaglandins, ATP and NGF itself (Harvima et al., 1994, Leon et al., 1994), creating a positive-feedback loop by further stimulating nociceptor terminals and potentiating the pain response associated with acute and chronic inflammatory processes (Figure 10).

1.7.9 NGF Alterations in Pronociceptive Gene Expression

It is known that the retrograde transport of the signaling endosomes following NGF-TrkA complex binding mediates the control of neuronal survival, growth, gene expression, and synaptogenic signaling events (Barker et al., 2002, Howe and Mobley, 2005, Pazyra-Murphy et al., 2009). The NGF-TrkA complex is internalized into signaling endosomes and are retrogradely transported from the peripheral terminals of nociceptive neurons to the DRG, enhancing the expression of various pronociceptive proteins, leading to further sensitization of primary afferents as well as activation of dorsal horn neurons (Campenot and MacInnis, 2004). Some of the proteins affected include sP, TRPV1, Nav 1.8, ASIC3, and lastly BDNF (Lindsay et al., 1990, Kerr et al., 2001, Winston et al., 2001).

1.7.10 NGF Modulation of TRPV1

The development of increased pain-related behavioral responses following inflammation is in part due to increased levels of TRPV1 on nociceptive C fiber terminals as well as the sensitization of existing TRPV1 channels. NGF upregulates TRPV1 expression and sensitizes TRPV1 channels to noxious stimuli by phosphorylating

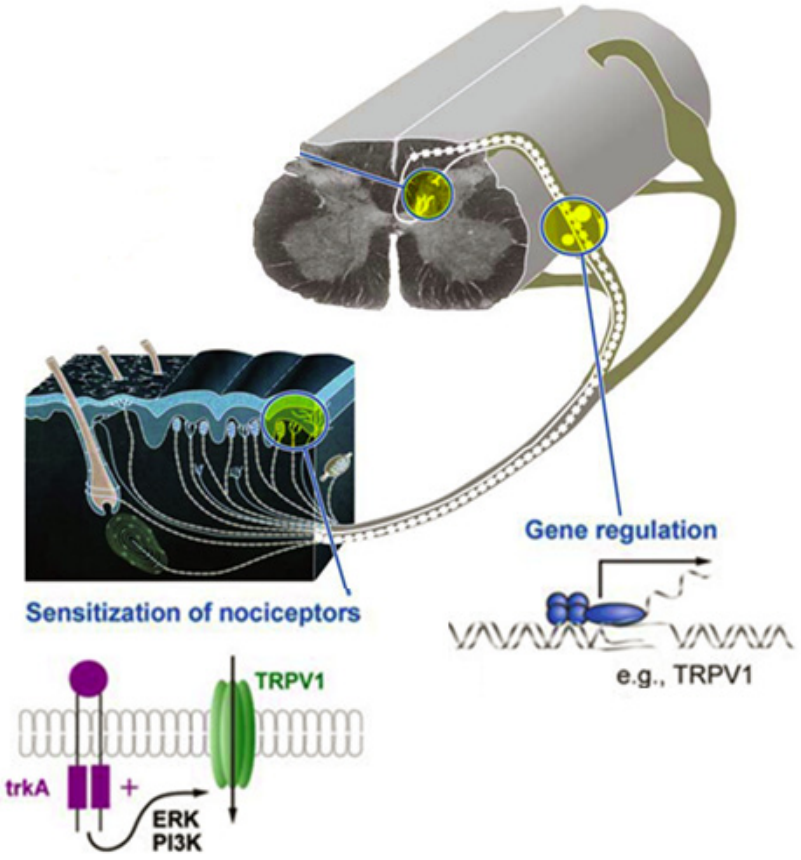


Figure 11: NGF modulation of TRPV1 expression at the peripheral end terminal and DRG. Modified from Pezet and McMahon, 2006.

and inserting TRPV1 into the plasma membrane (Amaya et al., 2004, Zhang et al., 2005b) (Figure 11). NGF can directly sensitize nociceptive neurons to pain-inducing stimuli by triggering rapid post-translational changes in the TRPV1 channel and modulating its gene expression. The sensitization caused by NGF is mediated via the PI3K pathway, with protein kinase C (PKC) acting as the downstream effector (Zhuang et al., 2004). PKC signaling induces TRPV1 channel activity by phosphorylation as well as its translocation to the plasma membrane surface (Premkumar and Ahern, 2000).

1.7.11 NGF Modulation of Neuropeptides

Moreover, endogenous NGF also plays an ongoing role in modulating nociceptor function and maintaining various characteristics of nociceptor phenotype in naïve animals. NGF is known to be an important regulator of neurokinin gene expression by increasing the transcription and translation of sP (Lindsay and Harmar, 1989). The opposite is also true, when a study shows that application of an NGF antiserum to the DRG of adult rodents in the absence of inflammation in turn reduces the expression sP and CGRP (Shadiack et al., 2001). Most of these NGF-induced changes in primary afferent fibers can influence the excitability in dorsal horn neurons, increasing the activation-induced release of SP, resulting in central sensitization which is associated with persistent pain states (Malcangio et al., 1997). Collectively, these findings illustrate the importance of NGF in promoting plastic changes associated with the generation of chronic pain states.

1.8 Autonomic Nervous System

The autonomic nervous system (ANS) functions as a largely involuntary sensory and motor control system of the periphery, including the viscera. The ANS affects many well known parameters such as heart rate, digestion, respiratory rate, salivation, perspiration and pupillary dilation in order to establish homeostasis greatly dependent on hypothalamic mechanisms. The three major divisions of the ANS are the sympathetic nervous system (SNS), the parasympathetic nervous system and the enteric nervous system. The physiologist Walter Cannon was the first to propose that the sympathetic and parasympathetic nervous system divisions had different and opposite functions (Kandel et al., 2000). He argued that the SNS is responsible for the emergency reaction, or "flight-or-fright" responses, in order for the system to respond to rapid changes in temperature, or blood loss. The parasympathetic nervous system, on the other hand, promotes "rest-and-digest" reactions, maintaining basal heart rate, respiration and metabolism under normal conditions. Unlike what Cannon suggested, the relationship between the SNS and parasympathetic nervous systems are not separate entities, where both divisions are tonically active and work in conjunction with one another. The rest of this section will focus on the SNS division.

1.8.1 Sympathetic Neurotransmitters

Sympathetic nerve fiber endings have several varicosities, which contain vesicles storing the catecholamine contents, namely noradrenaline (NA) as well as ATP and dopamine β-hydroxylase. The effects of NA and adrenaline are classically mediated by two major categories of catecholamine receptors: α- and β-adrenergic receptors. The α-adrenoceptors are further divided into several subtypes (α1A, α1B, α1D, α2A, α2B, and α2C)

and β-adrenoceptors into subtypes β1, β2, and β3 (Ruffolo and Hieble, 1994). Overall, the actions on adrenoceptors are mediated via guanine nucleotide-binding regulatory proteins (G proteins). The α2-adrenoceptor is G_i coupled and acts in decreasing intracellular adenyl cyclase activity, whereas α1-adrenoceptor is associated to G_q activating phospholipase C (PLC), which leads to intracellular Ca^{2+} influx (Summers and McMartin, 1993). Finally, the β-adrenoceptors increase adenyl cyclase activity through Gs. Peripherally, the main source of catecholamine comes from either local release of NA from postganglionic sympathetic nerve fibers or systemic release of adrenaline, predominantly, from the adrenal medulla. Interestingly, primary afferent neurons express several types of α-adrenoceptors that potentially arbitrate peripheral actions of noradrenaline. All three mRNA subtypes of the α1-adrenoceptor have been found in the DRG, for which subtype α1A is the most strongly expressed in naïve animals (Xie et al., 2001).

Specific transporter proteins carry monoamines in or out of the sympathetic neuron. Their transport into secretory vesicles is mediated by integral membrane proteins called vesicular monoamine transporters (VMATs). In mammals, two closely related monoamine transporters exist, namely VMAT-1 and VMAT-2, which are found within the membrane of intracellular vesicles (Erickson et al., 1992). These two subtypes differ in their substrate specificity, pharmacological properties and tissue distribution (Peter et al., 1994, Erickson et al., 1996). VMAT-1 is expressed exclusively in endocrine/paracrine cells associated with the intestine, stomach and SNS, whereas VMAT-2 is expressed in neurons of the SNS, and aminergic neurons in the enteric and CNS.

Another protein transporter important for proper sympathetic function is the noradrenaline transporter (NET), located in the outer neuronal cell membrane. NETs function

to transport synaptically released NA back into the presynaptic neuron. Furthermore, NETs are restricted to noradrenergic neurons and are not present on neurons that release dopamine or adrenaline (Torres et al., 2003). The transporters can be found along the cell body, axons, and dendrites of the neuron (Schroeter et al., 2000).

There are several pharmacological agents which use protein transporter systems as their mechanism of action. Of particular interest for this thesis is the anti-hypertensive drug Guanethidine, used to deplete cathecholamine stores. Being highly polar, Guanethidine does not cross the blood-brain barrier and is unlikely to exert any central effects (Freis, 1965). In addition, Guanethidine has no effect on the parasympathetic nervous system. Specifically, the drug is transported across the sympathetic nerve membrane by the same mechanism as NA itself, via NET (Joyce et al., 2002). Once Guanethidine has entered the sympathetic neuron, it is concentrated in transmitter vesicles, where it replaces NA. This leads to an irreversible depletion of NA stores in the nerve endings.

1.8.2 Sensory-Sympathetic Coupling

As early as 1904, Weber was the first to show that catecholamines can modulate pain-related withdrawal responses after applying adrenaline to the spinal cord of the cat (Kyles et al., 1993). Intra-dermal administration of NA in healthy subjects is not found to evoke pain, but it may induce hyperalgesia to thermal stimulation (Fuchs et al., 2001). However, in pathological conditions, peripheral administration of NA in inflamed or neuropathic human skin significantly influences sensory fiber terminals, aggravating pain and hyperalgesia (Drummond, 1995, Choi and Rowbotham, 1997). Additionally, it is the nociceptive C fibers, and to a lesser extent the nociceptive Aδ fibers, that become excited by sympathetic

stimulation and NA following inflammation or sensitization by heat or algogenic chemicals (Roberts and Elardo, 1985b, Hu and Zhu, 1989). A number of patients afflicted with neuropathic pain have been reported to have sympathetically maintained pain (SMP) and obtain relief using sympathetic blocking agents or after surgical sympathectomies (O'Halloran and Perl, 1997). Interestingly, sympathetic sprouting in neuropathic and inflammatory pain conditions occurs at the level of the DRG as well as in the upper dermis of the skin. Basket formations surrounding the DRG have been described in mice over-expressing NGF in the skin (Davis et al., 1994, Ramer and Bisby, 1999). More recently, at the level of the skin, sympathetic fibers were found to gradually migrate and branch into the upper dermis, an area where they are normally devoid, in both neuropathic and chronic inflammatory animal models (Ruocco et al., 2000, Grelik et al., 2005, Yen et al., 2006b, Almarestani et al., 2008). This sprouting may be associated with abnormal levels of NGF in the injured area; as mentioned earlier, sympathetic neurons not only express the TrkA receptor but depend on NGF for their maintenance and survival.

The sympathetic nervous system may also contribute to acute synovitis, as there is evidence that vascular permeability is elevated by sympathetic nerve stimulation and baseline plasma extravasation in the joint is significantly decreased following sympathectomy (Engel, 1941, Linde et al., 1974).

1.9 Current Treatments for Arthritis

Much progress in treatment of arthritis has been made in recent years but most therapeutic strategies have consisted of measures that are ameliorative rather than curative. Although the 'wonder drug' in preventing the development of arthritis has yet to be

discovered, scientific research has nonetheless come a long way. There are several disease modifying anti-rheumatic drugs currently on the market to treat RA. Unfortunately in terms of treatments for OA patients, they are limited in only providing symptomatic relief. As of yet, there are no existing disease modifying agents on the market to treat OA.

1.9.1 Corticosteroids

In 1930, Dr. Philip Hench observed in a patient with rheumatoid arthritis remission every time he developed jaundice. Interestingly, he also observed remission in female patients during their pregnancy (Hench, 1953). Therefore, Hench speculated that this "anti-rheumatic substance X" was a common hormone to both sexes and did not arise from liver disease exclusively. It was only years later that the steroidal hormone cortisone was isolated from the adrenal cortex and was shown to mediate strong effects in the relief of pain, swelling and stiffness. Unfortunately, although cortisone proved to have these beneficial anti-inflammatory effects, it was equally coupled with potentially serious and undesired effects. Therefore, although corticoids are indeed useful and valuable to RA and OA patients in the short-term, their long-term administration is not recommended, as they are insufficient in slowing down the progression of the disease (Dennison and Cooper, 1998).

1.9.2 NSAIDs

Nonsteroidal anti-inflammatory drugs (NSAIDs) help manage the chronic pain and inflammation associated with arthritis, not the underlying cause. The mechanism of action of NSAIDs is to block prostaglandins by inhibiting the cyclooxygenase (COX) enzymes responsible for its synthesis, specifically COX-1 and COX-2 (Vane, 2000). Side effects are yet ascribed when blocking the COX-1 enzyme, with tendencies to cause stomach ulcers and

promote gastrointestinal bleeding. Selective Cox-2 inhibitors are a relatively recent form of prescription NSAID and work similarly to older NSAIDs. Although Cox-2 inhibitors are less likely to cause stomach problems, they were found to be associated with risks in heart disease. In effect, the Cox-2 inhibitor Rofecoxib (Vioxx) was withdrawn from the market because of a strong link with myocardial infarctions and stroke. While Vioxx was removed, it has come to light that all prescription NSAIDs may have negative cardiovascular effects. Celecoxib (Celebrex), also a COX-2 inhibitor, is now prescribed as an alternative, while a large ongoing trial will help clarify whether Celebrex has a hazardous cardiovascular profile when compared to other standard NSAIDs, such as naproxen or ibuprofen.

1.9.3 DMARDs and Biologics for Rheumatoid Arthritis

In contrast to NSAIDs, disease modifying anti-rheumatic drugs (DMARDs) are therapeutic agents that are unrelated to each other but they commonly slow down the disease progression in RA (Donahue et al., 2012). Methotrexate, originally developed as a chemotherapy agent, acts by inhibiting the metabolism of folic acid. Lack of this cofactor interrupts the synthesis of purine nucleotides and the amino acids serine and methionine, thereby interfering with the formation of DNA, RNA, and proteins. Low doses of methotrexate are used for the treatment of certain autoimmune diseases. Due to its effectiveness, low-dose methotrexate is currently the first-line therapy for the treatment of RA. However, there are many side effects attributed to its use, such as a higher predisposition to infection, hair loss, nausea, headaches, abdominal pain, fatigue and pneumonitis; thus, there is a regrettable fine line between efficacy and tolerability.

Studies suggest antibiotic therapy, especially Minocycline, for its use as DMARD in milder RA disease (Langevitz et al., 2000). The anti-rheumatic effect of minocycline can be related to its immuno-modulatory and anti-inflammatory properties, rather than to its antibacterial. Although clinical trials have been contradictory, its use proves to be beneficial when given early in the disease course. Nevertheless, radiological progression has not been slowed by use of the drug.

Biologics target specific parts of the immune system to help turn down the inflammation process. These drugs can work quickly to reduce joint pain and swelling. In the longer term, biologics have been shown to slow the pace of joint damage and to improve joint use and movement. For example, Infliximab, an FDA approved monoclonal antibody binding with high affinity to the soluble and transmembrane forms of the cytokine TNF-α, is used to treat RA along with other autoimmune diseases (Maini et al., 1999). Similarly, Etanercept is a TNF-α inhibitor functioning as its decoy receptor, created by a fusion of TNF-receptor and the IgG1 antibody constant end region. However, Etanercept cannot neutralize receptor-bound TNF-α. Studies show that etanercept is better tolerated and more effective than methotrexate in patients with early RA (Weinblatt et al., 1999).

Lastly, Tafacitinib, a drug developed by Pfizer, is currently approved for the treatment of RA (Ghoreschi et al., 2011). Its mechanism of action is to inhibit the enzyme janus kinase 3 (JAK3), thereby interfering with the JAK-STAT signaling pathway and ultimately influencing DNA transcription. A study carried out in a mouse model shows rapid improvement of arthritis disease by inhibiting the production of inflammatory mediators.

Patients are however warned about possibly having a higher risk of opportunistic infections, tuberculosis, cancers and lymphoma.

1.9.4 DMARDs for Osteoarthritis

There is evidence from clinical trials using Glucosamine sulphate showing efficacy for pain relief in osteoarthritis as well as chondroprotection (Noack et al., 1994). Glucosamine sulphate is made from shrimp and crab shells and consists of glycosaminoglycans, the ground substance of cartilage. Follow-up patients treated for OA show a slight increase in joint space when compared to placebo groups.

Tanezumab is a humanized IgG2 monoclonal antibody directed against NGF that blocks its interaction with its receptors TrkA and p75 (Abdiche et al., 2008). A small phase 1 clinical trial revealed that a single intravenous injection of Tanezumab substantially decreased joint pain in patients with osteoarthritis (Hefti et al., 2006). Conversely, a proof-of-concept study showed that tanezumab was associated with mild and moderate adverse events, such as abnormal peripheral sensations, such as paresthesia and headaches, and radiographic evidence of bone necrosis, among some patients with moderate-to-severe osteoarthritis of the knee (Lane et al., 2010). Problematic radiographic issues emerged during phase 3 of the study which lead to the suspension of additional trials by the FDA as well as two other studies using Tanezumab were put on hold. It is postulated that the bone necrosis is most likely caused by excessive wear and tear in the absence of joint pain. Only recently, three years after Tanezumab has been put on hold, has the FDA agreed to eventually pursue these clinical investigations using lower doses and more restricted selection of patients.

1.9.5 Arthroplasty

Finally, surgical reconstruction of joints, or arthroplasty, is considered as the only remaining option when severe joint pain or dysfunction caused by arthritis is not alleviated by less-invasive therapies. A joint can be restored by resurfacing the bones or total joint replacement with a prosthesis may also be used. Total knee and hip arthroplasties are currently the most common joint replacement surgeries in patients with RA and OA.

1.10 General Objectives and Thesis Rationale

As described above, arthritic pain causes a significant clinical and societal burden and remains difficult to treat. Although there is extensive research being carried out in this field, we are still far from identifying the underlying mechanisms contributing to the pain associated with arthritis. It is clear that NGF is not only crucial for the maintenance of sympathetic and sensory nerve fibers, but also an important mediator of inflammatory pain. Recent advances have been made in understanding the processing of NGF in the CNS, though this still remains to be validated in peripheral tissues. This would be important for the development of novel therapeutic targets for the treatment of arthritis. Therefore, the general objective of the work described in this thesis is to investigate the peripheral changes of NGF in chronic inflammatory pain.

This thesis covers the three following experimental chapters:

1. In a rat model of inflammatory arthritis, the characterization of the behavior and innervation pattern of the skin and joint is performed using confocal imaging as well as the evaluation of alterations in the NGF processing cascade by use of western blotting.

2. A combination of behavioral, western blotting and confocal imaging is utilized to investigate the NGF processing mechanisms in the periphery. This is assessed by blocking MMP-9, the enzyme responsible for degrading mNGF, in naïve rats.

3. Pharmacologically modulation of NGF levels in inflammatory pain is carried out, using the same techniques as above. Specifically, the maturation of NGF is blocked by inhibiting plasmin, the enzyme responsible for the maturation process.

Chapter 2

Sympathetic fiber sprouting in inflamed joints and adjacent skin contributes to pain related-behavior in arthritis

Geraldine Longo, Maria Osikowicz, Alfredo Ribeiro-da-Silva

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2.1 Abstract

Although chronic pain is the most common symptom of arthritis, relatively little is known about the mechanisms driving it. Recently, a sprouting of autonomic sympathetic fibers into the upper dermis of the skin, an area that is normally devoid of them, was found in the skin following chronic inflammation of the rat hind paw. While this sprouting only occurred when signs of joint and bone damage were present, it remained to be clarified whether it was a consequence of the chronic inflammation of the skin or of the arthritis and whether it also occurred in the joint. In the present study, we used a model of arthritis in which complete Freund's adjuvant (CFA) was injected into the rat ankle joint. At 4 weeks following CFA treatment, there was an increase in sympathetic and peptidergic fiber density in the ankle joint synovium. We also observed a sympathetic, but not peptidergic, fiber sprouting in the skin over the joint, which may be a consequence of the increased levels of mature NGF levels in skin, as revealed by western blot analysis. The pharmacological suppression of sympathetic fiber function with systemic Guanethidine significantly decreased the pain-related behavior associated with arthritis. Guanethidine completely suppressed the heat hyperalgesia and attenuated mechanical and cold hypersensitivity. These results suggest that transmitters released from the sprouted sympathetic fibers in the synovial membrane and upper dermis contribute to the pain-related behavior associated with arthritis. Blocking the sympathetic fiber sprouting may provide a novel therapeutic approach to alleviate pain in arthritis.

2.2 Introduction

Arthritis is a debilitating disease affecting 21% of the US population (Lawrence et al., 2008). Patients suffering from osteoarthritis (OA) and rheumatoid arthritis (RA) make up 42% of all individuals afflicted with chronic pain (Breivik et al., 2006). The main symptom underlying arthritis is pain due to bone degeneration and inflammation affecting the joints. Although many symptomatic treatments do exist, there is no effective drug therapy to prevent or revert the underlying pathology.

Nociceptive fibers are responsible for relaying pain-related peripheral information from mechanical, thermal and chemical stimuli to the central nervous system. The glabrous skin of the rat hind paw is innervated by sensory and postganglionic sympathetic fibers. The peptidergic nociceptive fibers, recognized by means of immunoreactivity for the neuropeptides substance P and calcitonin gene-related peptide (CGRP), innervate mostly the dermis and to a minor extent the epidermis (Yen et al., 2006b, Taylor et al., 2009). Joints and the periosteum are also innervated by nociceptive primary afferents and sympathetic fibers (Hara-Irie et al., 1996, Mach et al., 2002).

Previous research has shown that a subcutaneous (SC) injection of complete Freund's adjuvant (CFA) into the rat hind paw is a model of polyarthritis suitable to study chronic pain and neuronal plasticity (Nagakura et al., 2003). In this model, there was persistent inflammation of soft tissues and joints; signs of joint damage developed at 2 weeks post-CFA (Vermeirsch et al., 2007, Almarestani et al., 2011). Interestingly, our group observed an ectopic presence of sympathetic fibers in the upper dermis of the skin (Almarestani et al., 2008). In normal conditions, postganglionic sympathetic fibers innervate blood vessels in the lower dermis (Yen et al., 2006b); however the ectopic sympathetic fibers were not associated

with the vasculature. Instead, they wrapped around peptidergic fibers (Almarestani et al., 2008). It was not known whether these changes in the innervation were a consequence of the chronic skin inflammation or resulted from the arthritis itself. This novel arrangement may favor sympathetic/sensory fiber interactions and may contribute to the hypersensitivity observed in arthritis.

The ectopic sprouting of sympathetic fibers is likely trophic factor mediated. One neurotrophic factor proposed to play a major role in this sprouting is nerve growth factor (NGF). During early post-natal development both sympathetic and primary afferent peptidergic fibers are dependent on NGF for neurotrophic support, whereas in adulthood NGF is required for survival of sympathetic neurons and proper phenotype maintenance in peptidergic afferents (Levi-Montalcini, 1987, Lewin and Barde, 1996). NGF plays an important role in mediating the inflammatory responses after tissue injury (McMahon et al., 2006). Here, we investigated the changes in innervation of the synovial membrane and skin over the ankle joint in an inflammatory arthritis model and correlated these changes with pain-related behavior. We investigated a possible role of the sprouted sympathetic fibers in pain by blocking their function pharmacologically with Guanethidine. Finally, we studied the changes in protein levels of both the precursor and mature forms of NGF in this animal model.

2.3 Methods

2.3.1 Animals

Experiments were performed on male Sprague Dawley rats (Charles River, Saint-Constant, Quebec, Canada) weighing 275-300g at the beginning of the study. The entire

experimental design was carried out following the guidelines contained in the Care and Use of Experimental Animals of the Canadian Council on Animal Care. Moreover, all studies were approved by the Faculty of Medicine Animal Care Committee of McGill University and were conducted in accordance with the Guidelines for Animal Research by the International Association for the Study of Pain (Zimmermann, 1983). All animals were kept on a 12h light 12 h dark cycle; food and water were available *ad libitum*. The animals were housed in cages with soft bedding. Animals were divided into three separate cohorts for morphological analyses, biochemical studies and pharmacological studies.

2.3.2 Model of Mono-Arthritis

Arthritis was induced by means of Complete Freund's Adjuvant (CFA). Animals were anesthetized with 5% Isoflurane in O_2 and injected with 40 μL of CFA, containing 150 micrograms of Mycobacterium butyricum, intra-articularly into the tibial-tarsal joint of the right hand paw (Butler et al., 1992). Control (sham) animals underwent the same procedure but were injected with the same volume of vehicle (mineral oil).

2.3.3 Tissue Edema of the Ankle Joint

One group of rats was used to assess the extent of tissue edema surrounding the inflamed joint in this model. Ankle joints were dissected out, weighed and dried in an oven at 60°C for 24 h. Edema in the ankle joint was determined as the loss in weight of each ankle joint after this dehydration procedure.

2.3.4 Behavioral Tests

Pain-related behavior was assessed using the von Frey, Hargreaves and acetone tests. Prior to any behavioral testing, animals were habituated to the testing environment. The baseline reaction values were measured one day before vehicle or CFA injection and were as follows: 21.6 ± 1.6 g for the von Frey test, 12.4 ± 1.1 s for the Hargreaves test and 0 for the acetone test.

2.3.4.1 Mechanical allodynia (von Frey test). Mechanical allodynia in rats was measured using a series of calibrated von Frey filaments (Stoelting, Wood Dale, IL, USA), ranging from 0.6 to 26 g. Animals were placed in plastic cages with a wire-mesh floor. The von Frey filaments were applied in ascending order to the midplantar surface of the ipsilateral hindpaw through the mesh floor. Each probe was applied to the foot until it bent. The time interval between consecutive filament administrations was at least 5 s. The calculations were performed as described previously (Osikowicz et al., 2008).

2.3.4.2 Cold allodynia (Acetone test). Cold allodynia was assessed using the acetone drop method (Choi et al., 1994). In the test environment described above, a 50µL droplet of acetone was applied to the midplantar region of the hind paw using a micropipette. Responses within the first 20 s were scored as follows: 0, no response; 1, one rapid hind paw flick/stamp; 2, two or more hind paw flicks/stamps; 3, periods of flicking/stamping with licking of plantar hind paw (Flatters and Bennett, 2004). Acetone application was repeated three times for each hind paw, with a 3 min interval between each application. For each rat, the sum of the three scores was then used for data analysis.

2.3.4.3 Thermal hyperalgesia (Hargreaves' test). The pain threshold to high temperature was tested using the plantar test (Hargreaves Apparatus, Ugo Basile, Type 7370, Comerio, Varese, Italy). Rats were placed into individual plastic cages with glass floors 5

min before the experiment. A noxious thermal stimulus was focused through the glass onto the plantar surface of a hind paw until the animal lifted the paw away from the heat source. The paw withdrawal latency was automatically displayed to the nearest 0.1 s. A cut-off latency of 20 s was used to avoid tissue damage. The latency of nociceptive reaction was measured in seconds under baseline conditions and weekly after CFA or vehicle injection.

2.3.5 Western Blot

Four weeks after CFA or vehicle injection, the rats were decapitated and the glabrous skin from the hind paws was collected, frozen in the liquid nitrogen and stored in the freezer (-80°C) for further processing. Tissue samples were homogenized in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 25 mL Tris–HCl, pH 7.6) containing protease inhibitors (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and cleared by centrifugation (13,000 rpm for 50 min at 4°C). Protein concentration of the supernatant was determined using the BCA Protein Assay Kit (Sigma). Homogenates (20 μL of 50 μg of total protein) were resolved in 4-12% polyacrylamide gels and transferred into a nitrocellulose membrane (Bio-Rad Laboratories, Inc). The blots were blocked in Tris Buffer Solution-Tween 20 (TBS-T) containing 5% non-fat dry milk at room temperature for 1 h on a rotomixer. Nitrocellulose membranes were probed with an primary antibody specific for nerve growth factor (NGF, 1:500, Santa Cruz Biotechnology). Blots were incubated overnight at 4°C with the primary antibody. After primary antibody incubation, membranes were washed 3 × 10 min in TBS-T. This was followed by incubation for 2 h at room temperature with a peroxidase-conjugated goat anti-rabbit Ig antibody (1:2500) (Jackson Immuno Research Laboratories, Inc.). The membranes were washed 3 × 10 min in TBS-T. The immunoreactive bands were visualized with the ECL enhanced

chemiluminescence kit (PerkinElmer Inc.) and using Kodak Biomax XAR imaging film. The immunoreactive bands were quantified by densitometry of the films using a MCID M4 image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). Membranes were rinsed and reprobed with a mouse anti-β-actin antibody (1:40,000; Sigma) diluted in 5% milk in TBS-T for 1 h at room temperature, washed with TBS-T, and incubated with a peroxidase conjugated donkey anti-mouse IgG (1:5000, Santa Cruz) in 5% dry milk in TBS-T for 1 h. The membranes were washed and the signal was detected and qualified as described above. The protein levels of the precursor (proNGF) and mature (mNGF) forms of NGF were normalized to the β-actin levels for each sample.

2.3.6 Immunohistochemistry

Animals were perfused with histological fixatives at 1, 2, 3 or 4 weeks after CFA or oil injections. For this, they were deeply anesthetized with Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 mL, i.p., per 100 g body weight) intraperitoneally and then perfused through the left cardiac ventricle with 100 mL of perfusion buffer, followed by 500 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PB), pH 7.4, at room temperature for 30 minutes. Subsequently, the glabrous skin and ankle joints were extracted and post-fixed in the same fixative for 1 hour at 4°C. A benefit of using this model of arthritis compared to the knee joint model is that we can use paw withdrawal thresholds to test for pain-related behavior induced by the arthritis. Ankle joints underwent a decalcification step in 10% ethylenediaminetetraacetic acid (EDTA) in dH_20 for 3 weeks. Subsequently, tissue was cryoprotected in 30% sucrose in PB overnight at 4°C for later immunohistochemical processing.

To study the glabrous skin, 50-µm thick sections were cut using a cryostat. All sections were collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS-T). Ankle joint sections were cut at 16 µm in thickness and attached directly onto gelatin-subbed slides for immunostaining. The tissue sections were incubated for 1 hour at room temperature in 10% normal goat serum (Gibco, Carlsbad, CA) in PBS to block unspecific labeling. To detect immunoreactivity of the peptidergic and sympathetic fiber populations, the sections were then incubated at 4°C for 24 hours using a rabbit anti-Calcitonin Gene Related Protein (CGRP) (Sigma-Aldrich, St. Louis, MO) antibody or rabbit anti-Vesicular Monoamine Transporter-2 (VMAT-2) antibody (Synaptic Systems, Göttingen, Germany) at a dilution of 1:2000 and 1:7500, respectively. After several rinses in PBS-T, the sections were incubated for 2 hours at room temperature with secondary antibodies, a goat anti-rabbit IgG conjugated to either Alexa Fluor 488 or Alexa Fluor 594 (Molecular Probes, Eugene, OR), at a dilution of 1:400 in PBS-T. For double-labeling of CGRP and VMAT-2, sections were processed as described above except that we used a mixture of a guinea pig anti-CGRP antibody at a 1:8000 dilution (Peninsula, San Carlos, CA) and of the VMAT-2 antibody; after washing, we used a mixture of an anti-guinea pig IgG antibody conjugated to Alexa Fluor 488 (1:800; Molecular Probes) with the anti-rabbit IgG conjugated to Alexa Fluor 594. Lastly, the sections were washed, mounted on gelatin-subbed slides, air-dried and cover slipped with an anti-fading mounting medium (Aqua PolyMount, Polysciences Inc., Warrington, Pa.). Some sections were processed omitting the primary antibody; no specific staining was observed. Slides were stored at 4°C until examined. Before quantitative analyses, all the slides were coded so that the person who performed the quantification was

completely blinded regarding the experimental groups. Codes were broken only after the quantification was completed.

2.3.7 CGRP-immunoreactive fiber quantification

We studied the innervation of the upper dermis of the glabrous skin of the hindpaw and the ankle joint synovial membrane on the ipsilateral side of the injection. As in previous studies from our laboratory, we considered as upper dermis as a band 150 μm in thickness residing immediately below the dermal-epidermal junction (Yen et al., 2006b). All the material used for quantification was single-labeled. For the measurement of the CGRP immunoreactive (IR) fiber innervation density, digital images were captured with a high resolution camera attached to a Zeiss Axioplan 2e imaging fluorescence microscope, using a PlanFluotar 40X objective. This microscope is connected to a computer equipped with the Zeiss Axiovision 4.8 software (Zeiss Canada). For each rat, three sections of the skin and three of the ankle joint were selected at random and, in each, 6 non-overlapping micrographs of the upper dermis or of and synovial membrane were taken , for a total of 18 images per region per animal. Images were exported in TIFF format for analysis using an MCID Elite image analysis system (Imaging Research, St. Catharines, Ontario, Canada). The region corresponding to the upper dermis was outlined with the tracing tool of the software. CGRP-IR fibers were detected and converted to 1 pixel in thickness to measure the total fiber length (μm) per scan area (μm^2). For statistical comparisons, we used a one-way analysis of variance (ANOVA) with Dunnett's post hoc test. Statistical significance was set at $p < 0.05$.

2.3.8 Sympathetic fiber quantification

VMAT-2-IR fibers in the ankle joint synovial membrane were quantified using the same approach as for the CGRP-IR fibers (described above). The quantification of the changes in autonomic innervation of the glabrous skin was performed using a different approach from that used for sensory fibers. Since the density of sympathetic fibers in the upper dermis is low, it is very difficult to measure directly. Therefore, we counted on each of 6 sections per animal all VMAT-2-IR fibers within the upper dermis. The mean number of fibers in the upper dermis per μm^2 was compared between groups using a one-way ANOVA and a Dunnett's post hoc test, with a statistical significance set at $p < 0.05$.

2.3.9 Drug Administration for Sympathetic Block

For the sympathetic block study, vehicle (saline) or 30 mg/kg Guanethidine Sulfate (Santa Cruz) were administered intra-peritoneally (i.p.) twice with a 24 hour interval, at the 2 and 4 weeks post-CFA time points (Xanthos et al., 2008). All three behavioral tests described above were carried out at baseline (day 0), 2 or 4 weeks post-injection (pre-drug in sham and CFA) and 4 hours after administration of the second dose of Guanethidine on either day 15 or 30 (post-drug). Animals were divided into different groups: Sham + vehicle, Sham + Guanethidine, CFA + vehicle and CFA + Guanethidine. Several studies show evidence that a single dose of systemic Guanethidine is sufficient for long-term sympathetic blockade (Maxwell et al., 1960, Kim et al., 1993). Data from the animals in the pre-drug group (CFA + vehicle and CFA + Guanethidine) were pooled together since no statistically significant difference was detected.

2.3.10 Statistical analyses

All statistical tests were carried out using GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego, CA, USA). All values are expressed as mean ± SEM. To compare for changes in the innervation of the skin, groups were compared by one-way ANOVA with Dunnett's post-hoc analysis. To analyze changes in the innervation of the synovial membrane, an unpaired t-test was performed to compare the control group to the CFA group. Group comparisons for the Guanethidine study were analyzed using a one-way ANOVA with Bonferroni's multiple comparison test to compare the CFA groups (vehicle vs. Guanethidine).

2.4 Results

2.4.1 Quantification of Edema around the Ankle Joint

To measure ankle edema, ankle joints were weighed before and after dehydration in an oven at 60^0C for 24 hours. Values represent the change in weight of the joint (+ surrounding tissues). The weight loss in sham animals was 0.94 ± 0.07g whereas in CFA-injected rats it was 2.81 ± 0.4g at the 4 week time-point ($p < 0.005$) . These values revealed that the CFA-treated rats had significantly more fluid in the tissues surrounding the ankle joints than the sham rats. In contrast, the values from the contraleral side in both sham and CFA-treated rats were not different from the ipsilateral side in sham animals.

2.4.2 Development of Allodynia and Hyperalgesia in rats following CFA administration

In the behavioral tests, all CFA-treated rats exhibited strong mechanical and cold allodynia as well as thermal hyperalgesia as measured on the ipsilateral paw using the von Frey, acetone and Hargreaves tests, respectively, at 1, 2, 3 and 4 weeks post-CFA (Figure **1**A, B, C). Behavioral tests were also performed on the contralateral hind paw; however, no

significant differences in sensitivity to mechanical and thermal stimulation were observed between experimental and control animals. The thresholds in the sham group were not changed during the observation period (Figure **1**), except with von Frey hairs at 1 week, when a mild hypersensitivity was observed (Figure **1**A).

2.4.3 Changes in the Pattern of Innervation of the Synovial Membrane

The innervation of the synovial membrane ipsilateral to the injection of CFA or oil (sham) was studied at the 4 week time point (Figure 2). In control rats, there was a sparse innervation by sympathetic (VMAT-2-IR) fibers (Figure 2A) and CGRP-IR fibers (Figure 2B). In CFA-injected animals, there was a significant increase in the density of both VMAT-2-IR fibers (**$p < 0.005$ (Figure 2D) and CGRP-IR fibers (**$p < 0.005$)(Figure 2E). In CFA-injected rats, the VMAT-2-IR fibers of the synovium were in close proximity to CGRP-IR fibers, wrapping around each other (Figure 2 F - white arrows).

2.4.4 Changes in the Pattern of Innervation of the Thick Skin Over the Ankle Joint

The CGRP-IR (peptidergic) fiber innervation density remained unchanged at all time points post-CFA injection studied (Figure 3). On the other hand, there was an ectopic presence of sympathetic fibers (as detected by VMAT-2 immunoreactivity) in the upper dermis beginning at 2 weeks post-CFA injection (Figure 4). The number of VMAT-2-IR fibers per unit area in the upper dermis was three fold higher in CFA-injected rats, compared to sham animals, at the 4 week time point (**$p<0.05$). Similarly to what was observed in the synovium, the VMAT-2-IR fibers in the upper dermis were in close proximity to CGRP-IR fibers and some appositions of the two fiber populations were seen (Figure 5 - white arrows).

2.4.5 Changes in NGF Protein Levels in the Glabrous Skin of Rats 4 Weeks Following CFA Administration

Western blot analyses of the glabrous skin samples were carried out at four weeks after CFA or oil injections. Both proNGF and mNGF were identified based on their molecular weights; proNGF migrated close to 40 kDa and mNGF migrated at 14 kDa (Figure 6). The localization of these bands is consistent with what is described in other publications using the same antibodies (Bruno and Cuello, 2006, Allard et al., 2012) and aligned with the corresponding bands from positive controls (for NGF, mouse submandibular gland extracts - data not shown). Our data revealed an increase in the protein levels of the mature form of NGF (mNGF) in the ipsilateral paw as compared to the contra-lateral paw; 0.49 ± 0.02 vs 0.4 ± 0.01; Figure 6A) and also when compared to ipsilateral paw samples from oil-treated rats (0.49 ± 0.02 vs. 0.39 ± 0.01). However, no significant changes in the protein levels of the precursor form of NGF (proNGF) were detected by Western blot analysis in the ipsilateral paw when compared to the results from the contra-lateral paw (0.74 ± 0.03 vs. 0.76 ± 0.13; Fig. 6B) and from the ipsilateral paw of oil-treated rats (0.74 ± 0.03 vs 0.75 ± 0.03).

2.4.6 Effect of Sympathetic Block on Pain Behavior Following CFA Administration

Figure 7 illustrates the pain-related behavioral data from rats before and after Guanethidine or vehicle treatment at 2 and 4 weeks post-CFA or oil injection. Guanethidine did not have an effect on the behavioral pattern of sham (oil-injected) animals (data not shown). In addition, no statistical difference was found when comparing the sham groups (baseline, and pre/post vehicle and Guanethidine treatment) to the baseline threshold levels of the CFA groups. Statistical assessment was performed using a one-way ANOVA with

Bonferroni post-hoc test comparing baseline values against all other treatments. At two weeks following CFA injection, Guanethidine injections did not have any significant effect on pain-related behavior (Figure 7A, B, C). Interestingly, at the 4 week time point, the CFA + Guanethidine group demonstrated a significant difference compared to the CFA + vehicle group in all 3 behavioral parameters tested. Indeed, mechanical (Figure 7A) and cold allodynia (Figure 7B) thresholds partially returned to baseline post-Guanethidine treatment (~50% reversal). Importantly, the CFA + Guanethidine group showed no significant difference compared to baseline levels in the Hargreaves test, indicating that the heat hyperalgesia was completely reversed (Figure 7C). There was a significant difference when comparing both CFA groups (vehicle vs. Guanethidine) in all 3 behavioral tests indicating that blocking sympathetic function increased the pain-related thresholds. These results correlate with the changes observed in the sympathetic fiber population in the glabrous skin surrounding the ankle joint at 4 weeks post-CFA injection.

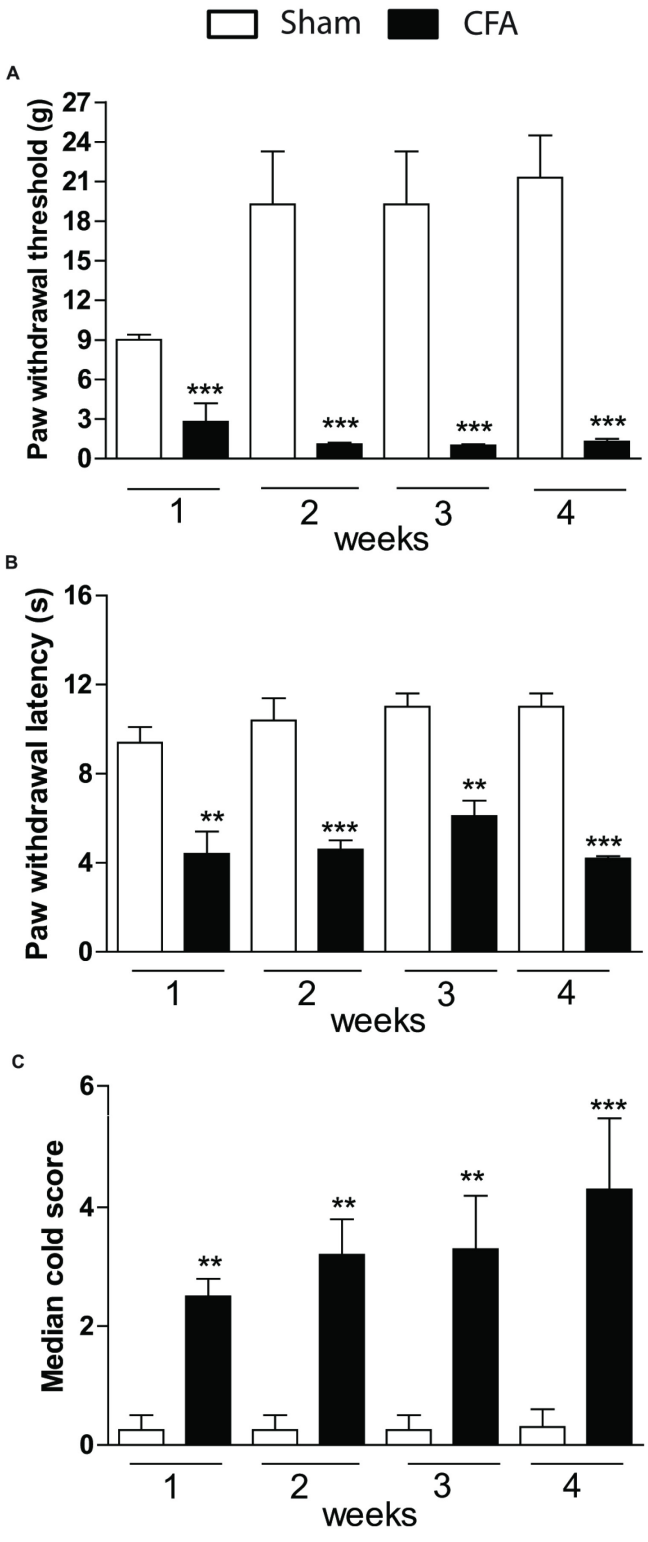
Sham
CFA
A
Paw withdrawal threshold (g)
27
24
21
18
15
12
9
6
3
0
***
***
***
***
1
2
3
4
weeks
B
Paw withdrawal latency (s)
16
12
8
4
0
**
***
**
***
1
2
3
4
weeks
C
Median cold score
6
4
2
0
**
**
**
***
1
2
3
4
weeks

Figure 1

Figure 1. Pain-related behavioral analysis of sham and CFA-treated animals. In the behavioral tests, all CFA-treated rats exhibited strong mechanical and cold allodynia as well as thermal hyperalgesia (Fig. 1A, B, C) as measured on the ipsilateral paw in the von Frey, acetone and Hargreaves tests respectively, at all experimental time points (1, 2, 3 and 4 weeks) . The pain thresholds in the sham group were not changed from baseline at any time point, except when applying von Frey hairs at the 1 week time point, where a significant lowering of the threshold was observed, possibly as a result of the solvent injection in the joint (Figure 1A). Statistical analysis was carried out using one-way ANOVA and Bonferroni's multiple comparison test. Error bars represent SEM (n = 6).

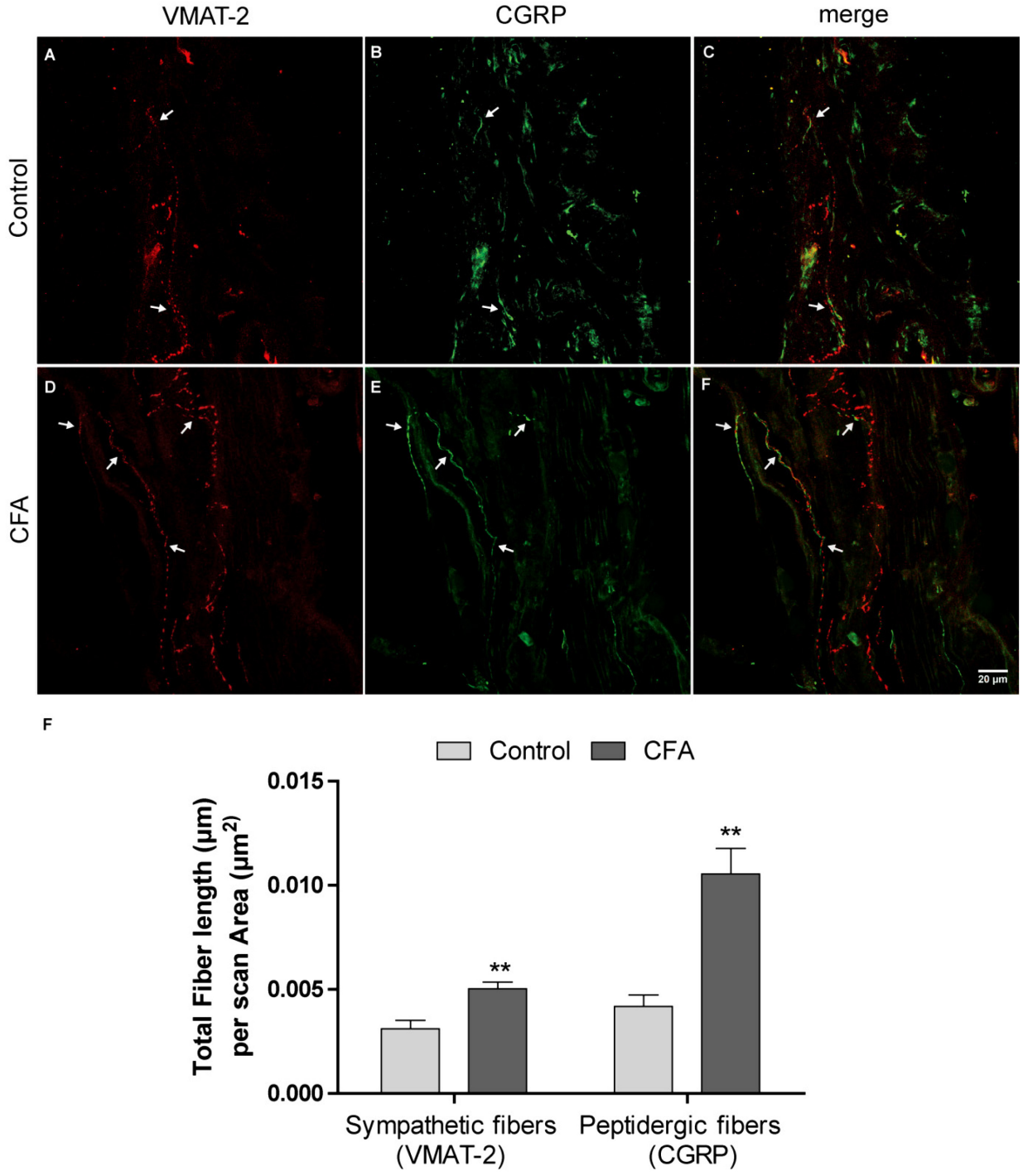
VMAT-2
CGRP
merge
Control
CFA
A
B
C
D
E
F
20 µm
F
Control
CFA
Total Fiber length (µm) per scan Area (µm²)
0.015
0.010
0.005
0.000
**
**
Sympathetic fibers (VMAT-2)
Peptidergic fibers (CGRP)

Figure 2

Figure 2. Changes in the nerve fiber innervation pattern of the synovial membrane of the ipsilateral ankle joint at 4 weeks after vehicle (A-C) or CFA (D-F) injection. CFA-injected animals displayed a net increase in both VMAT-2-IR (red) and CGRP-IR (green) fiber density when compared to the control group, as can be observed in the confocal images and in the graph (G). In panels C and F note that VMAT-2-IR and CGRP-IR fibers in control and CFA-injected animals sometimes are in close apposition to each other (white arrows) and that such appositions are more abundant in the CFA group (F) Images represent confocal microscope Z-stacks of optical sections Scale bar = 20 μm. See Materials and Methods for details of quantification. Comparisons between CFA and control groups were done by means of an unpaired t-test. Values represent means ± SEM (n = 4).

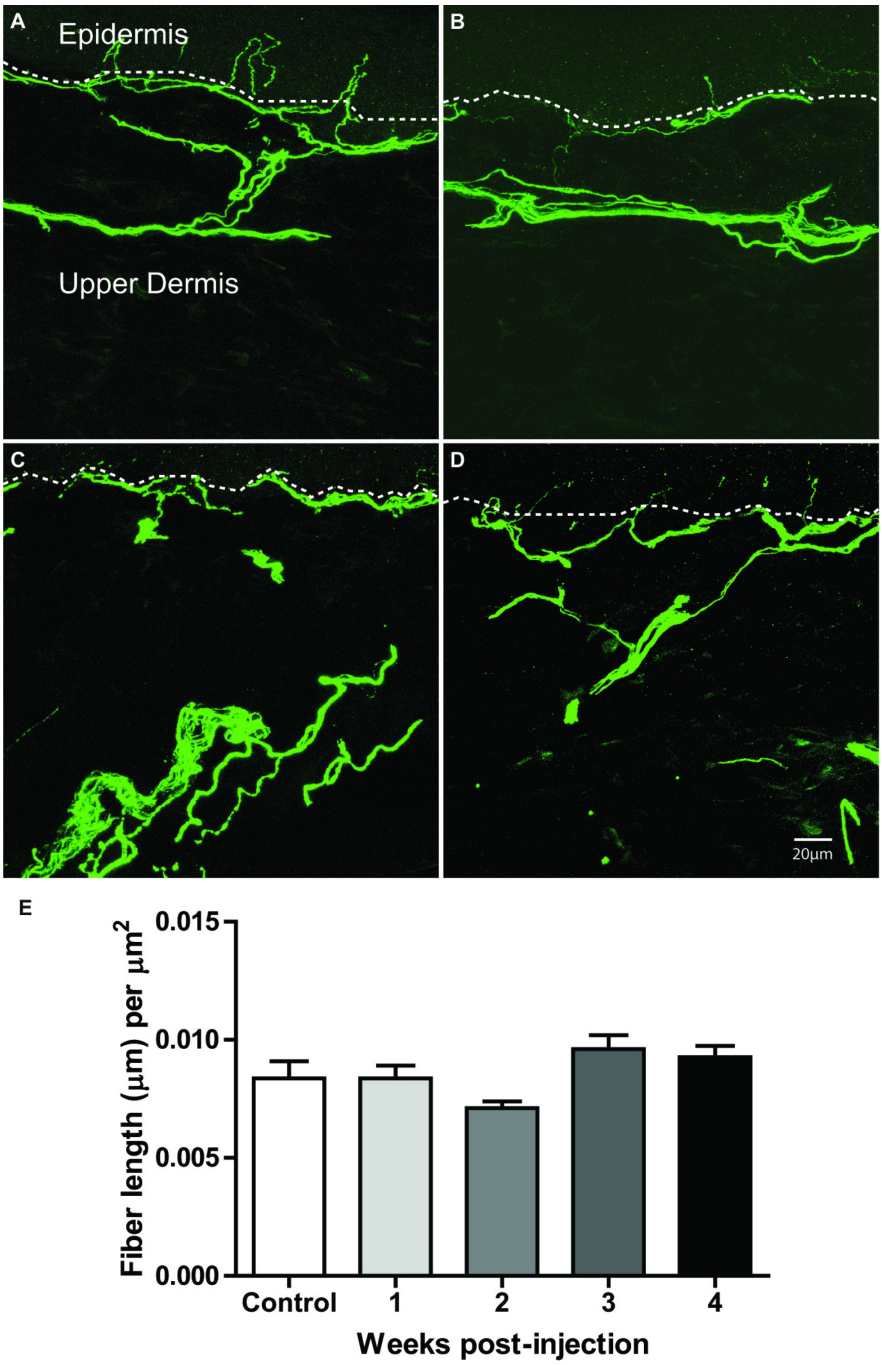
A
Epidermis
Upper Dermis
B
C
D
20µm
E
Fiber length (µm) per µm2
0.015
0.010
0.005
0.000
Control
1
2
3
4
Weeks post-injection

Figure 3

Figure 3. Changes in peptidergic (CGRP-IR) fiber innervation of the glabrous skin adjacent to the joint in CFA-treated rats. Photomicrographs show representative examples of CGRP-IR fiber innervation (green) in the hind paw skin of the rat in sham animals (A), as well as at 2 weeks (B), 3 weeks (C) and 4 weeks (D) post-CFA injection. Scale bar = 20 μm. (E) Bar graph showing average density of CGRP-IR fibers and fiber bundles in the upper dermis ($n = 6$, $p < 0.05$). No change in CGRP-IR fiber density was observed at any time point. See Materials and Methods for details of quantification. Comparisons were done by means of one-way ANOVA and Bonferroni's post-hoc correction. Error bars represent SEM.

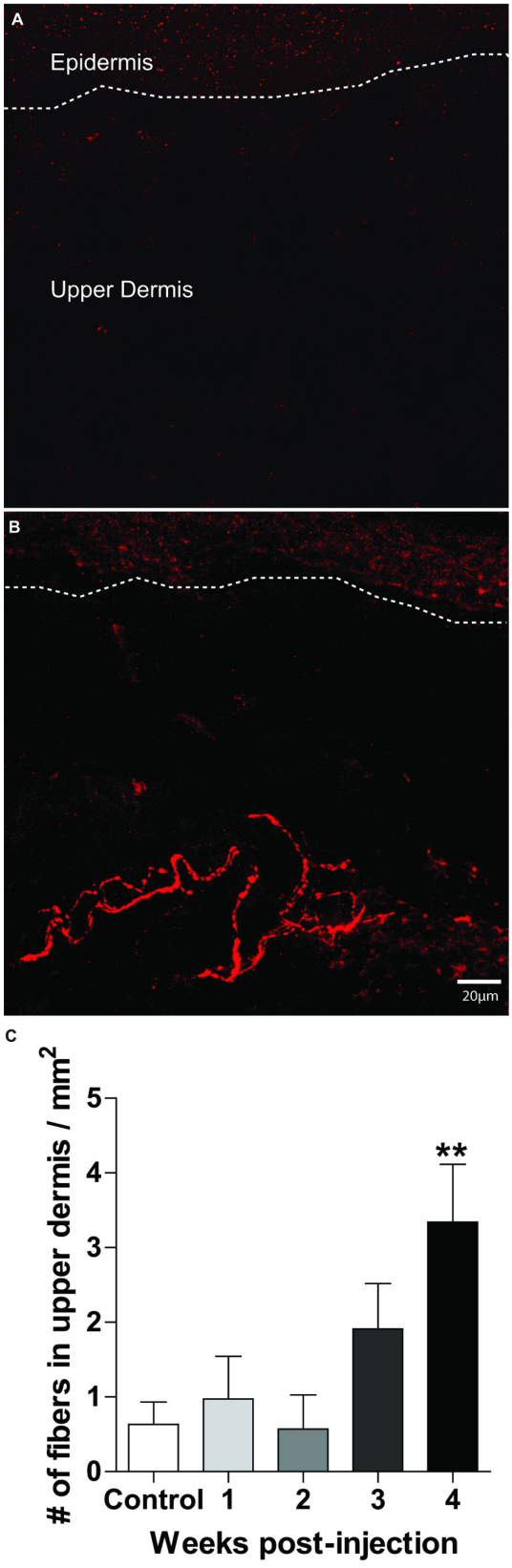
A
Epidermis
Upper Dermis
B
20µm
C
# of fibers in upper dermis / mm2
0
1
2
3
4
5
**
Control
1
2
3
4
Weeks post-injection

Figure 4

Figure 4. Changes in sympathetic (VMAT-2-IR) fiber innervation in the upper dermis of the skin adjacent to the joint after CFA injection. Representative photomicrographs showing changes in VMAT-2-IR fiber innervation (red) in sham animals (A) and CFA-injected animals (B) at the 4 weeks time-point. Scale bar = 20 μm. (C) Bar graph showing average number of sympathetic fibers in the upper dermis at several time points after CFA-injection. A significantly higher number of VMAT-2-IR fibers in the upper dermis per scanned area (mm^2), was detected in the upper dermis in animals at the 4 weeks post-CFA time point compared to sham animals ($n = 6$, ** $p < 0.005$). See Materials and Methods for details of quantification. Error bars represent SEM.

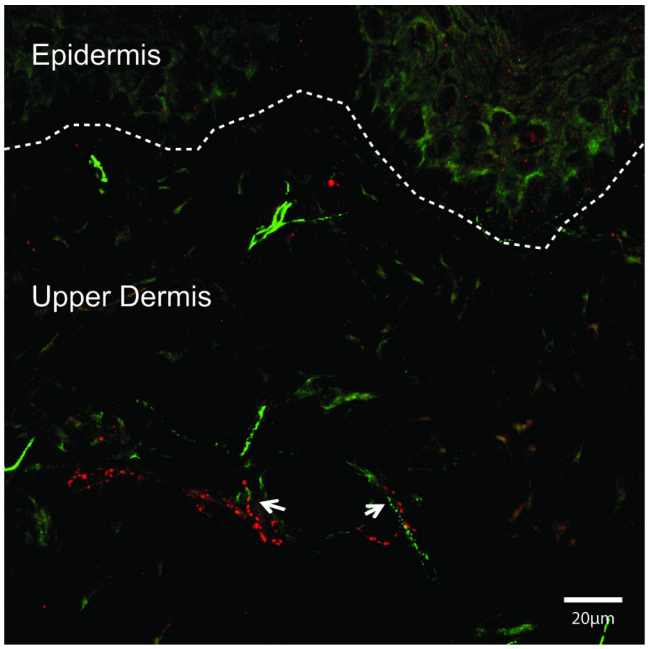

Figure 5. Confocal micrograph illustrating sympathetic and peptidergic fibers in close apposition in the upper dermis of CFA-treated animals. Note that the sympathetic fibers (VMAT-2-IR; in green) often run along the same trajectory as the peptidergic fibers (CGRP-IR; in red), suggesting an interaction between the sensory and autonomic fiber populations in the upper dermis.

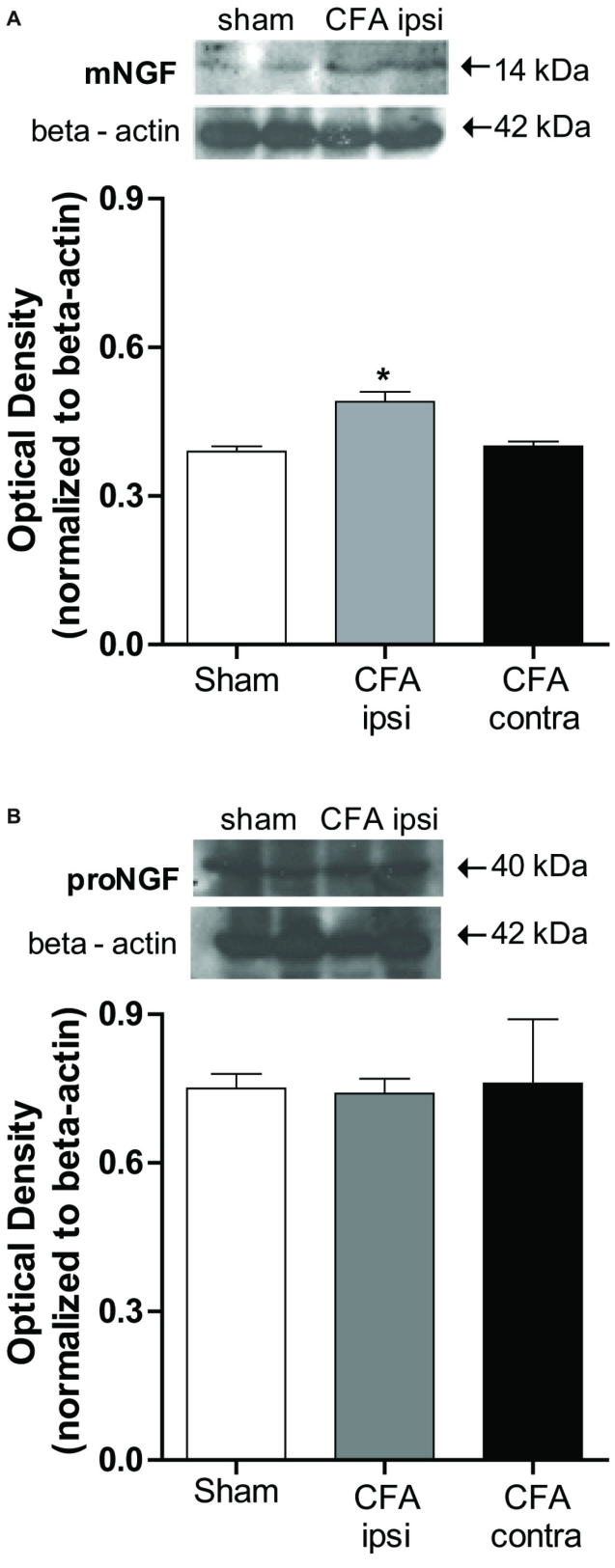
A
sham
CFA ipsi
mNGF
14 kDa
beta - actin
42 kDa
Optical Density
(normalized to beta-actin)
0.9
0.6
0.3
0.0
*
Sham
CFA ipsi
CFA contra
B
sham
CFA ipsi
proNGF
40 kDa
beta - actin
42 kDa
Optical Density
(normalized to beta-actin)
0.9
0.6
0.3
0.0
Sham
CFA ipsi
CFA contra

Figure 6

Figure 6. Changes in the protein levels of proNGF and mature NGF in the skin of CFA-treated rats. The Western blot analysis performed on the glabrous skin samples 4 weeks after CFA administration showed a significant increase in the protein levels of mature NGF (mNGF) (A), with no influence on the protein levels of the precursor proNGF (B). Examples of representative western blots are shown in the upper panels in A and B. The densitometry results are presented as the mean ± SEM for all samples (C and D). Inter-group differences were analyzed by a one-way ANOVA with a Bonferroni's multiple comparison test. ***$p<0.001$ indicates a significant difference as compared to glabrous skin sample of sham rats. The dashed lines on the graphs indicate protein analyses for mNGF and proNGF in the contralateral glabrous skin of CFA-treated rats.

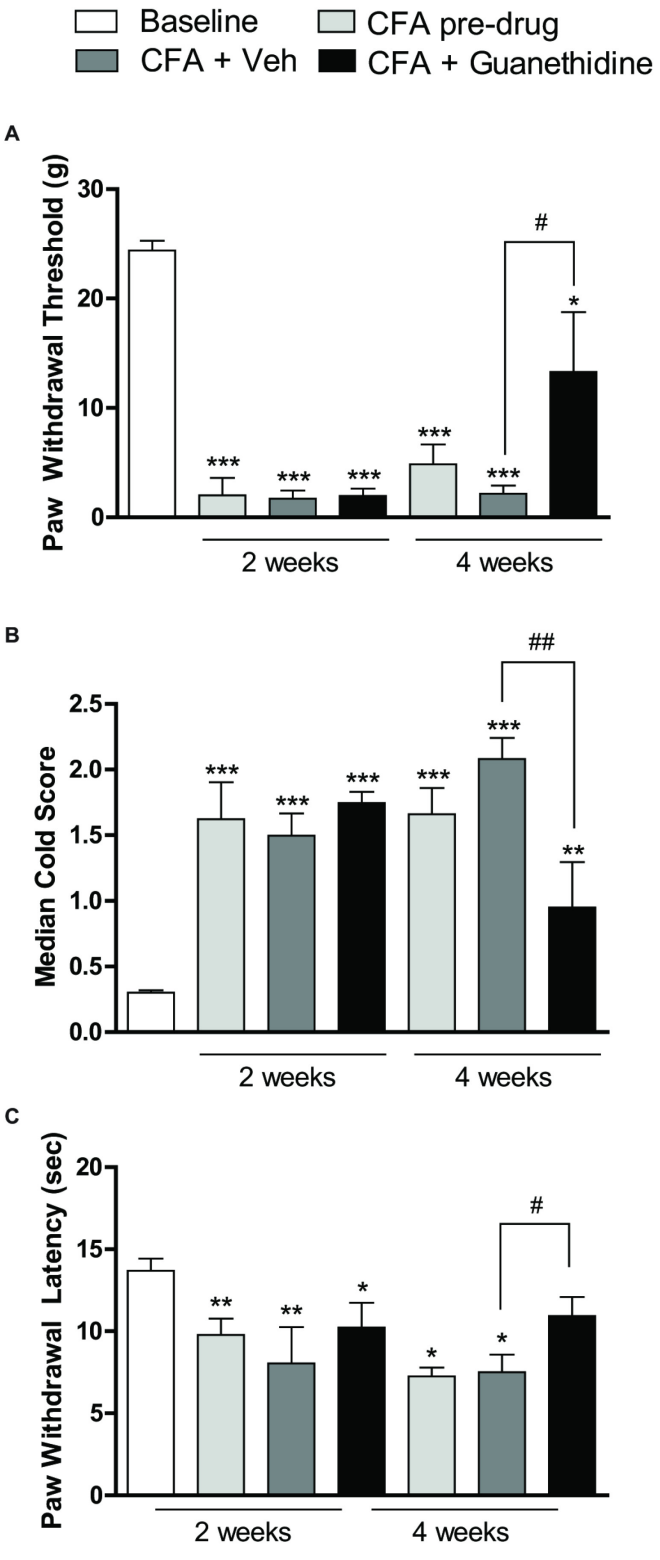
Baseline
CFA pre-drug
CFA + Veh
CFA + Guanethidine
A
Paw Withdrawal Threshold (g)
30
20
10
0
***
***
***
***
***
#
*
2 weeks
4 weeks
B
Median Cold Score
2.5
2.0
1.5
1.0
0.5
0.0
***
***
***
***
***
##
**
2 weeks
4 weeks
C
Paw Withdrawal Latency (sec)
20
15
10
5
0
**
**
*
*
*
#
2 weeks
4 weeks

Figure 7

Figure 7. Effect of sympathetic fiber activity suppression with guanethidine on mechanical allodynia, cold allodynia and heat hyperalgesia in animals at the 2 and 4 weeks time points post-CFA injection. At the 2 weeks time point, no statistical difference was found comparing CFA + vehicle and CFA + Guanethidine groups in all three behavioral tests (Fig. 7A, B, C). However, at the 4 weeks time point, animals from the CFA + Guanethidine group displayed a significant attenuation (~50% return to baseline) of mechanical and cold allodynia, respectively, compared to the CFA + vehicle group which displayed no change (as seen on panels A and B). On panel C, it can be observed that Guanethidine completely reversed the heat hyperalgesia in CFA treated rats, with thresholds returning to baseline levels ($n = 8$ for each group, $*p < 0.05$, $***p < 0.001$). Inter-group differences were analyzed by one-way ANOVA (significance represented as *) with a Bonferroni's multiple comparison test (CFA vehicle vs. Guanethidine group statistical difference symbolized by #). Error bars represent SEM.

2.5 Discussion

We used a well established mono-arthritis model consisting of the unilateral intra-articular injection of CFA in the ankle joint. We show that the CFA-injected animals displayed mechanical and cold allodynia as well as heat hyperalgesia, which was still detected at 4 weeks post-injection. Furthermore, we detected a sprouting of sympathetic fibers both in the ankle joint synovium and in adjacent skin. The density of nociceptive peptidergic nerve fibers was elevated in the synovial membrane but not in skin. The suppression of sympathetic fiber function with Guanethidine partially alleviated the pain-related behavior. Protein levels of mNGF, but not proNGF, were elevated in CFA-treated rats.

Although we observed similar ipsilateral pain-related hypersensitivity at 1, 2, 3 and 4 weeks post-CFA, mechanisms likely differ with the time points. Indeed, inflammatory mechanisms prevail at one week post-CFA. At this stage, inflammatory mediators, such as cytokines IL-1β and TNF-α, released into the synovium and surrounding tissues, maintain nociceptor hyperexcitability (Miller et al., 2009). In the polyarthritis CFA model, joint and bone damage were present as from 2 weeks post-CFA (Henry, 2004, Almarestani et al., 2011).Therefore, at later time points there is a component of the hyperexcitability associated with the destruction of cartilage and bone. Additionally, we observed plastic changes in the innervation pattern of the ankle joint synovium and glabrous skin surrounding the joint. At 4 weeks post-CFA, we found a significantly denser innervation of the synovial membrane by sympathetic fibers. This nerve fiber remodeling can be explained by the surge of proinflammatory mediators and growth factors attributed to synovitis and synovial pannus formation (Ishikawa et al., 1996). Also, there was a significant sprouting of sympathetic

fibers into the upper dermis of the skin surrounding the inflamed ankle joint. This sprouting was similar to the one we previously observed in the polyarthritis model (Almarestani et al., 2008) supporting the concept of a similar pathology underlying different models of arthritis.

NGF is likely involved both in sympathetic sprouting and in the pain-related behavior. Sympathetic neurons depend on NGF for survival (Levi-Montalcini, 1987) and express its high affinity receptor TrkA (McMahon et al., 2006). NGF levels detected by bioassay are increased in the synovial fluid of patients with rheumatoid arthritis compared to normal healthy controls which have an undetectable level of NGF (Aloe et al., 1992, Aloe and Tuveri, 1997). Importantly, in the current study, we detected that the levels of mNGF, but not proNGF, were significantly elevated in the skin at the time point in which we observed the alterations in innervation. We have still unpublished data in this arthritis model showing a significant increase in plasmin levels, enzyme which converts proNGF into mNGF, suggesting that the conversion turnover is increased, resulting in an absence of proNGF accumulation and increased levels of mNGF. Therefore, we suggest that chronic inflammation in the joint causes joint and bone destruction accompanied by elevated mNGF levels in surrounding tissues, triggering sympathetic fiber sprouting.

In contrast with the synovium, we found no change in CGRP-IR fiber density in skin of arthritic animals. This result differs from the polyarthritis model, where we observed significant CGRP-IR fiber sprouting beginning at 2 weeks post-injection, but this latter model has obvious skin inflammation besides the joint pathology (Almarestani et al., 2008), likely triggering higher NGF levels in the skin. This reasoning is in agreement with the concept that peptidergic primary afferents are less responsive to NGF changes. In fact, it is well known that sympathetic neurons remain dependent on NGF for survival as well as for

maintenance, whereas the post-natal peptidergic neurons cease to require NGF for survival (Gorin and Johnson, 1980, Lewin and Barde, 1996). Skin or mucosa inflammation in other pathological inflammatory diseases including psoriasis vulgaris, vulvodynia or interstitial cystitis induces sensory and/or sympathetic sprouting (Hohenfellner et al., 1992, Nakamura et al., 2003, Farmer et al., 2011) and involves different inflammatory components such as infiltration and degranulation of mast cells (Toyoda et al., 1994), neurogenic inflammation (Nakamura et al., 2003), plasma extravasation (Otten et al., 1984) and increased levels of proinflammatory cytokines (Darsow et al., 1997, Nakamura et al., 2003). The above changes are known to induce a substantial increase in NGF expression. Therefore, the moderate changes in mNGF in skin observed in this study may only be sufficient to cause sprouting of sympathetic and not sensory fibers in the skin.

Sympathetic fiber sprouting in the skin in arthritis is comparable to the sprouting previously observed by us in the skin in neuropathic pain models (Grelik et al., 2005, Yen et al., 2006b). This presence of ectopic sympathetic fibers in the upper dermis in both arthritis and neuropathic pain models led us to suggest a neuropathic pain component in arthritis (Almarestani et al., 2008), in agreement with the concept that arthritis and neuropathic pain are not two completely separate entities (Bennett, 2006). It is well known that sympathetic efferent activity can affect the firing rate of injured sensory fibers and evoke discharge in silent sensory afferents. In a neuropathic pain model, electrophysiological recordings from DRG neurons revealed a functional coupling of sympathetic and sensory fibers (Chen et al., 1996). In addition, it has been shown that the spontaneous discharge rate of C-fibers is greater when norepinephrine is applied (Xie et al., 1995). In some patients with peripheral neuropathies, sympathetic block results in pain reduction indicating a role of the sympathetic

fibers (Roberts, 1986). We observed that in inflammatory arthritis, like in neuropathic pain models, sympathetic fibers in the upper dermis wrapped around peptidergic fibers, instead of innervating blood vessels [(Almarestani et al., 2008) and current study]. Also, in the synovial membrane, sensory/sympathetic fiber appositions were more abundant in the CFA group. These abnormal fiber arrangements would favor an effect of transmitters/modulators released from the sympathetic fibers on primary afferent excitability. To confirm this, we blocked the function of sympathetic fibers using Guanethidine. We detected that at the 4 weeks time point, Guanethidine-treated arthritic rats had an amelioration of mechanical and cold allodynia thresholds, and a complete suppression of heat hypersensitivity. Conversely, in all 3 behavioral tests, no differences in thresholds were found between sham or CFA animals injected with vehicle or Guanethidine at the 2 week time point. These results are not surprising since ectopic sympathetic fibers only become significantly present at 4 weeks after CFA injection. Based on our data and that of others (Ghilardi et al., 2012), we suggest that Guanethidine suppresses the abnormal sympathetic activity in this arthritis model in both skin and joint. Our data following Guanethidine administration is comparable with that obtained in other chronic pain models (Malmberg and Basbaum, 1998, Xanthos and Coderre, 2008). Suppressing the sympathetic nervous system increases skin blood flow and temperature (Yanagiya et al., 1999) and alters bone architecture in adult growing rats (Pagani et al., 2008). Furthermore, clinical observations indicate that the sympathetic nervous system plays a role in the pathogenesis of inflammation (Kozin et al., 1976, Levine et al., 1986). These changes could explain in part the amelioration of pain-related behavior that we observed. However, no Guanethidine-induced behavioral changes were observed at the 2

weeks time point or in non-CFA treated rats, suggesting they are the result of the suppression of the pro-nociceptive effect of the ectopic sympathetic fibers.

However, Guanethidine treatment induced a partial amelioration of the pain-related behavior at 4 weeks, indicating other factors may contribute to the pain, such as persistent inflammation and bone and cartilage damage. Clinically, the persistence of synovitis and pain in patients with osteoarthritis are explained by the increased innervation by peptidergic free nerve endings releasing high amounts of substance P (Saito and Koshino, 2000). This increased peptidergic innervation in joints has also been found in animal models (Imai et al., 1997, Ghilardi et al., 2012). The increased activation of peptidergic primary afferents leads to neurogenic inflammation, which plays an important role in arthritis (Levine et al., 1985b). Importantly, through neurogenic inflammation, the skin surrounding the joints will also get inflamed (Ahmed et al., 1995). This phenomenon leads to activation of immune cells and mast cells in the surrounding tissues, amplifying the inflammatory cascade and resulting in increased release of NGF. This peripherally produced NGF is sufficient to maintain the sensitization of nociceptive sensory neurons and induce inflammatory hyperalgesia (McMahon et al., 1995, Rueff et al., 1996), besides inducing sympathetic sprouting, which plays a role in pain in itself as documented here.

In conclusion, we show in an animal model of monoarthritis a persistent pain hypersensitivity accompanied by an abnormal sprouting of sympathetic fibers in the skin adjacent to the inflamed joint. This abnormal growth of sympathetic fibers was accompanied by elevated levels of the mature NGF, but not of its precursor proNGF, making the modulation of mNGF levels an interesting target for the treatment of inflammatory arthritis. We also observed that the suppression of sympathetic fiber function led to an amelioration of

the pain-related behavior. These data reinforce the concept of a neuropathic component in arthritis and that sympathetic fibers play a role in the genesis of the pain associated with arthritis.

2.6 Acknowledgements

The authors would like to thank Manon St. Louis for her laboratory expertise, Magalie Millecamps for teaching us the intra-articular injection technique, Gary J. Bennett for allowing us to use his animal behavioral testing facility and Dr Patrick W. Mantyh and Magdalena Kaczmarska, from the University of Arizona, for assistance with the processing of hard tissues for immunohistochemistry. This research was funded by grants from the Canadian Institutes of Health Research (CIHR) (MOP 79411), the Louise and Alan Edwards Foundation and MITACS Accelerate Quebec.

Connecting Text: Chapter 2 to Chapter 3

In Chapter 2, we described the pattern of innervation in the synovial membrane and skin surrounding the inflamed joint in the CFA-induced arthritis model in the rat. We provide some evidence of a *de novo* interaction between sympathetic and peptidergic C fibers by assessing pain-related behavior following blockade of sympathetic function with Guanethidine. This correlated with increased levels of mNGF. These changes may partially explain the peripheral sensitization during chronic inflammation.

Therefore, in the next chapter, we investigated the hypothesis that altering the mNGF levels solely by targeting its degradation enzyme in naïve animals would produce similar changes in pain-related behavior and innervation pattern. These behavioral and morphological parameters were investigated following the inhibition of MMP-2/9 in naïve rats. The levels of proNGF and mNGF were also measured.This proof of principle study will help us get a better understanding of the peripheral processing mechanism for NGF.

Chapter 3

Inhibition of endogenous NGF degradation induces mechanical allodynia and thermal hyperalgesia in rats

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A. Claudio Cuello and Alfredo Ribeiro-da-Silva

Molecular Pain (2013) 9;37

3.1 Abstract

Background: We have previously shown a sprouting of sympathetic fibers into the upper dermis of the skin following subcutaneous injection of complete Freund's adjuvant (CFA) into the hindpaw. This sprouting correlated with an increase in pain-related sensitivity. We hypothesized that this sprouting and pain-related behavior were caused by an increase in nerve growth factor (NGF) levels. In this study, we investigated whether the inhibition of mature NGF degradation, using a matrix metalloproteinase 2 and 9 (MMP-2/9) inhibitor, was sufficient to reproduce a similar phenotype.

Results: Behavioral tests performed on male Sprague-Dawley rats at 1, 3, 7 and 14 days after intra-plantar MMP-2/9 inhibitor administration demonstrated that acute and chronic injections of the MMP-2/9 inhibitor induced sensitization, in a dose dependent manner, to mechanical, hot and cold stimuli as measured by von Frey filaments, Hargreaves and acetone tests, respectively. Moreover, the protein levels of mature NGF (mNGF) were increased, whereas the levels and enzymatic activity of matrix metalloproteinase 9 were reduced in the glabrous skin of the hind paw. MMP-2/9 inhibition also led to a robust sprouting of sympathetic fibers into the upper dermis but there were no changes in the density of peptidergic nociceptive afferents.

Conclusions: These findings indicate that localized MMP-2/9 inhibition provokes a pattern of sensitization and fiber sprouting comparable to that previously obtained following CFA injection. Accordingly, the modulation of endogenous NGF levels should be considered as a potential therapeutic target for the management of inflammatory pain associated with arthritis.

3.2 Background

Nerve growth factor (NGF) is the prototype of the neurotrophin family of growth factors (Lindsay, 1994, Cuello, 2010). As a trophic factor, NGF supports the development and survival of peptidergic primary sensory and sympathetic neurons (Patel et al., 2000). During early neuronal development, it has a critical role in the survival and growth of sympathetic and sensory neurons (Campenot, 1987, Crowley et al., 1994, Frade and Barde, 1998). Though NGF is no longer required for the survival of sensory neurons in adult animals, a body of evidence has shown that NGF continues to be involved in specifying the function of many sensory neurons and their interactions with sympathetic neurons (Chen et al., 1996). Within the mature organism, NGF plays a significant role in mediating inflammatory and immune responses after peripheral tissue injury (Herzberg et al., 1997). NGF levels are elevated in several conditions associated with pain in humans, including arthritis (Aloe et al., 1992, Halliday et al., 1998), cystitis (Lowe et al., 1997, Oddiah et al., 1998) and chronic headaches (Sarchielli et al., 2001). In animal studies, the concentration of NGF in the skin increases in response to inflammation produced by either injection of irritants (Oddiah et al., 1998) or ultraviolet-B irradiation (Woolf et al., 1994). NGF has been implicated in the sensitization of peripheral nerves to noxious stimuli (Dmitrieva and McMahon, 1996, Rueff and Mendell, 1996).

It has recently been shown by Bruno and Cuello (2006) that NGF is released as a precursor, proNGF, and not in the growth-promoting form, mature NGF (mNGF) (Bruno and Cuello, 2006). This work revealed a protease cascade responsible for proNGF maturation into mNGF and its degradation in the extracellular space. One of the key regulatory enzymes involved in proNGF processing within the CNS is plasmin, which is converted from

plasminogen by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). In the above report it was proposed that this newly reported metabolic pathway should have an impact on pain mechanisms. However, the validation of this pathway in the periphery and its impact on pain regulation remains unresolved. This biochemical pathway might be particularly relevant for pain because NGF over-expression studies have shown that NGF is an important signal for mechanical and thermal sensitization (Rueff and Mendell, 1996). Based on the above, we have advanced the hypothesis that interfering with the levels of endogenous mNGF by modulating its formation from proNGF should provide an attractive opportunity to develop a novel class of agents for the treatment of pain. In the present study we aim at exploring whether NGF processing within the periphery is analogous to that already shown in the CNS of naïve rats. As a proof of concept, we sought to verify whether the inhibition of endogenous NGF degradation in naïve rats — by the administration of a matrix metalloproteinase 2 and 9 (MMP-2/9) inhibitor — influenced: 1) the protein levels of molecules involved in NGF processing, 2) the innervation patterns of the skin by sensory and autonomic fibers as well as relationship between them, and 3) the pain thresholds.

3.3 Results

3.3.1 Effect of Repeated Administration of the MMP-2/9 Inhibitor on the Protein Levels of NGF and MMP-9

Western blot analyses of the glabrous skin samples were carried out on the 14th day after two weeks of chronic injections of the MMP-2/9 inhibitor - 20 μg, intraplantar (i.pl.). This time point and dose for biochemical analyses were selected on the basis of behavioral experiments (described below) where the maximum effect on pain-related behavior was

observed on the 14th day of repeated i.pl. injections of the MMP-2/9 inhibitor at a dose of 20 μg. Both proNGF, mNGF and MMP-9 were identified based on their molecular weights; proNGF migrated close to 40 kDa, mNGF migrated at 14 kDa, and MMP-9 migrated at 92 kDa. The localization of these bands is consistent with what is described in other publications using the same antibodies (Khan et al., 2002, Bruno and Cuello, 2006, Allard et al., 2012). Furthermore, they aligned with the corresponding bands from positive controls (mouse submandibular gland extracts for NGF and kidney homogenates for MMP-9) (data not shown). Our data revealed an increase in the protein levels of the mature form of NGF (mNGF) in the ipsilateral paw as compared to the contralateral paw (shown as an interrupted line on the graphs, 0.62 ± 0.06 vs 0.33 ± 0.02; Fig. 1A, n = 4) and also when compared to ipsilateral paw samples from vehicle-treated rats (0.62 ± 0.06 vs 0.36 ± 0.01). No significant changes in the protein levels of the precursor form of NGF (proNGF) were detected by Western blot analysis in the ipsilateral paw as compared to the results from the contralateral paw (0.71 ± 0.14 vs 0.59 ± 0.03; Fig. 1B, n = 4) and to the ipsilateral paw of vehicle-treated rats (0.71 ± 0.14 vs 0.62 ± 0.05). In addition, the treatment with the MMP-2/9 inhibitor resulted in the decrease of MMP-9 protein levels in the ipsilateral paw when compared to the contralateral side (0.38 ± 0.04 vs 0.61 ± 0.03; Fig. 1C) and when compared to the ipsilateral paw of vehicle-treated rats (0.38 ± 0.04 vs 0.62 ± 0.01). To validate this observation, we pre-treated the samples with urea, a strong denaturing agent that promotes protein unfolding (Bennion and Daggett, 2003). This treatment ensured that the primary antibody against MMP-9 was in fact recognizing its antigenic site and that the binding was not being masked by the MMP-2/9 inhibitor binding. Indeed, we did not find any difference in the data after

urea pre-treatment, an observation that provides sufficient evidence that the protein level is in fact decreased (data not shown).

3.3.2 Effect of Repeated Administration of the MMP-2/9 Inhibitor on the Enzymatic Activity of MMP-9

Zymography analyses performed on the glabrous skin samples from the hind paws revealed changes in the enzymatic activity of both MMP-9 (82-kDa) and MMP-2 (65-kDa) following the daily subcutaneous administration of MMP-2/9 inhibitor (20 μg, i.pl.; Fig. 2A, B). The treatment with the MMP-2/9 inhibitor reduced the enzymatic activity of MMP-9 and MMP-2 in the ipsilateral paw when compared to the contralateral paw (shown as an interrupted line on the graphs, 0.57 ± 0.03 vs 1.14 ± 0.05 and 0.72 ± 0.04 vs 0.94 ± 0.06, respectively) and when compared to ipsilateral skin samples from vehicle-treated rats (0.57 ± 0.03 vs 1.0 ± 0.06 and 0.72 ± 0.04 vs 1.0 ± 0.06, respectively; n = 4).

3.3.3 Effect of the Repeated Administration of MMP-2/9 Inhibitor on the Skin Innervation Pattern

In the current study, we hypothesized that inhibition of MMP-9 in naïve animals would prevent the degradation of endogenous mNGF and lead to sympathetic fiber sprouting in the skin. Indeed, there was an invasion of the upper dermis by sympathetic fibers, as detected by VMAT-2 immunoreactivity, in skin samples from animals treated for 2 weeks with daily injections of MMP-2/9 inhibitor (Fig. 3). The increased number of sympathetic fibers in the scanned area of the upper dermis was statistically significant at doses of 20 and 40μg of MMP-2/9 inhibitor. Interestingly, the density of peptidergic sensory fibers, as

detected by CGRP immunoreactivity, did not change compared to control levels (Fig. 4). One interesting observation in MMP-2/9 inhibitor treated animals was that, in the upper dermis, the VMAT-2-immunoreactive (IR)- fibers were often observed wrapping around CGRP-IR fibers (Fig. 5).

3.3.4 Effect of a Single Administration of the MMP-2/9 Inhibitor on Allodynia and Hyperalgesia

A single subcutaneous i.pl. administration of matrix MMP-2/9 inhibitor (10, 20, 40 μg) induced mechanical allodynia in naïve rats, as measured by the von Frey test. Pain-related behavior was evaluated at 30, 60, 180 minutes and 24 hours after single injection of this inhibitor (Fig. 6A). The MMP-2/9 inhibitor (20 μg; i.pl.) induced thermal hyperalgesia as measured by the Hargreaves test at 60 minutes following a single injection (Fig. 6B). In contrast, a single injection of the MMP-2/9 inhibitor (10, 20, 40 μg) did not influence cold allodynia as assessed by the acetone test at 30, 60, 180 minutes and 24 hours after the injection (Fig. 6C). Control animals were injected with the vehicle solution.

3.3.5 Effect of Repeated Administration of the MMP-2/9 Inhibitor on Allodynia and Hyperalgesia

The repeated administration of MMP-2/9 inhibitor (10, 20, 40 μg; i.pl.) to naïve rats once a day for two weeks resulted in the development of hypersensitivity to both mechanical and thermal stimuli as measured by the von Frey, Hargreaves and acetone tests, respectively (Fig. 7). The sensitivity to both mechanical and thermal stimuli increased over time. The analysis of the area under the curve (AUC), which allows for the assessment of the global effect of treatment, shows that the MMP-2/9 inhibitor treatment induced a significant allodynia and hyperalgesia in naïve rats ($p<0.05$) (Fig. 7A, B, C; right panels). We did not

observe any visible signs of inflammation (reddening or swelling of the skin) throughout the 14 days period at the MMP-2/9 inhibitor or vehicle injection site. Moreover, we found a statistically significant positive correlation between the number of sympathetic fibers in the upper dermis of the skin and mechanical allodynia, as measured by the von Frey test ($p=0.0029$), and with cold allodynia, as measured with the acetone test ($p=0.0086$). There was no positive correlation between the thermal hyperalgesia measured by the Hargreaves test and the sympathetic sprouting, although there was trend ($p=0.08$).

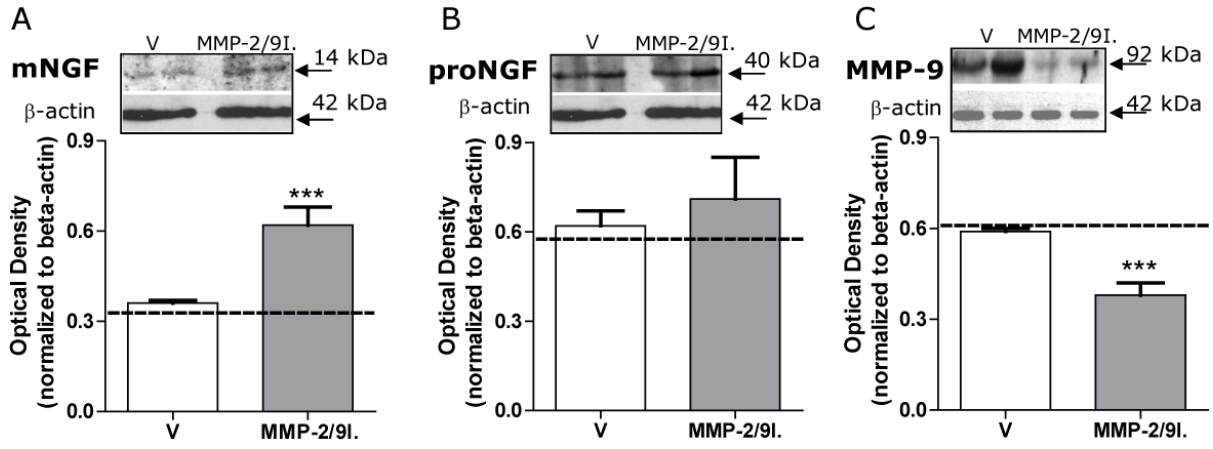


Figure 1: MMP-2/9 inhibitor administration altered the protein levels of NGF and MMP-9 in the skin of naïve rats. Western blot analysis performed on the glabrous skin samples following 14 days of repeated MMP-2/9 inhibitor (MMP-2/9I.; 20 µg, i.pl.) administration showed a significant increase in the protein levels of mature NGF (mNGF) (A), with no influence on the protein level of the precursor proNGF (B). Repeated administration of MMP-2/9I. (20 µg, i.pl.) significantly reduced the protein levels of MMP-9 (C) in the glabrous skin. Examples of representative western blots are presented in the upper panels A, B and C. The densitometry results are presented as the mean ± SEM from all samples. Inter-group differences were analyzed by ANOVA with a Bonferroni's multiple comparison test. ***$p<0.001$ ($n = 4$) indicates a significant difference as compared to glabrous skin sample of chronic vehicle-treated (V) naïve rats. The interrupted line on the graphs indicates protein analyses for mNGF, proNGF or MMP-9 in the contralateral glabrous skin of chronic MMP-2/9I.-treated naïve rats.

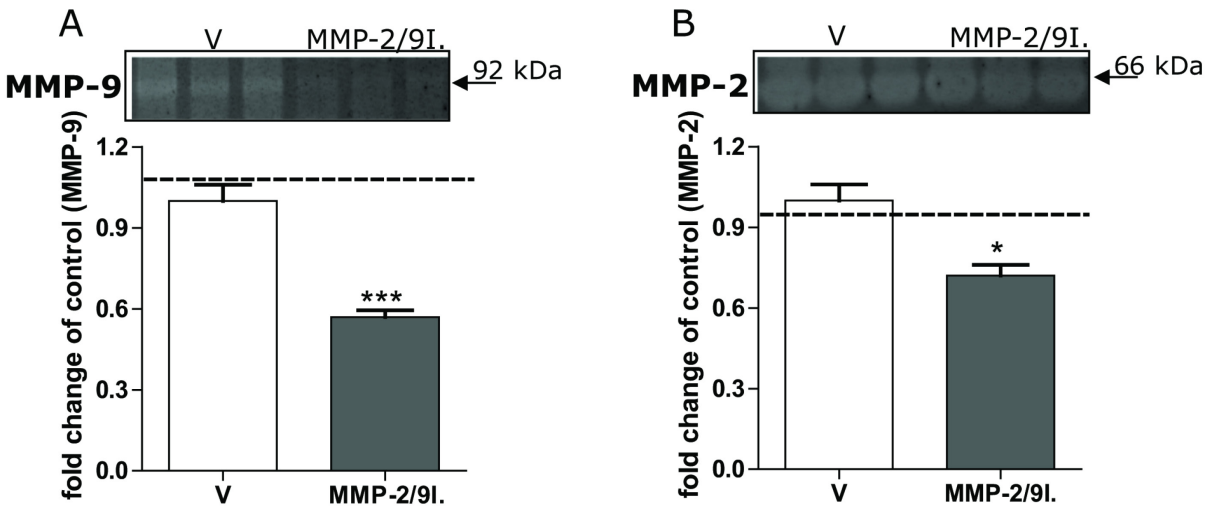
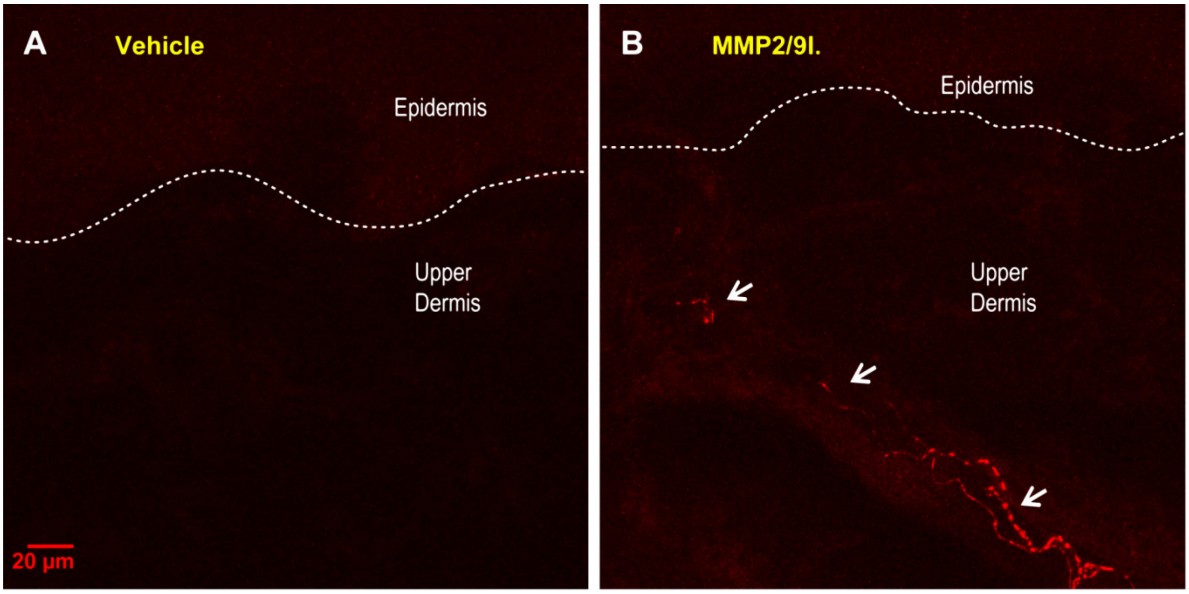


Figure 2: Effect of MMP-2/9 inhibition on the enzymatic activity of MMP-9 and MMP-2 in the skin of naïve rats. To evaluate MMP activity following repeated MMP-2/9 inhibition (MMP-2/9I.; 20 µg, i.pl.), zymography was performed on extracts from glabrous skin samples. The analysis revealed reduction in the enzymatic activity of both MMP-9 (A) and MMP-2 (B). Examples of representative zymograms are presented in the upper panels A and B. The densitometry results are presented as the mean ± SEM. Inter-group differences were analyzed by ANOVA with a Bonferroni's multiple comparison test. *$p<0.05$, ***$p<0.001$ ($n = 4$) indicate a significant difference as compared to glabrous skin sample of chronic vehicle-treated (V) naïve rats. The interrupted line on the graphs indicates zymogram analyses for MMP-9 and MMP-2 in the contralateral glabrous skin of MMP-2/9I.-treated rats.


A
Vehicle
Epidermis
Upper
Dermis
20 µm
B
MMP2/9I.
Epidermis
Upper
Dermis

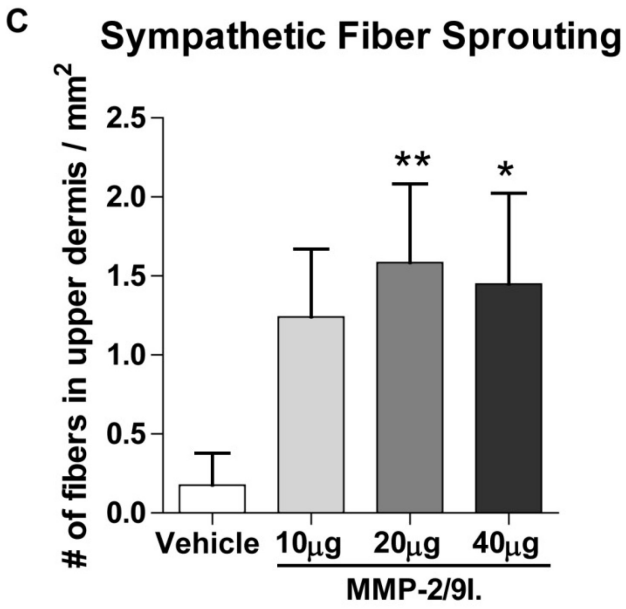

C
Sympathetic Fiber Sprouting
# of fibers in upper dermis / mm2
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Vehicle
10µg
20µg
40µg
MMP-2/9I.

Figure 3

Figure 3: Changes in sympathetic fiber innervation in the skin of naïve rats following MMP-2/9 inhibition. A and B represent confocal images of the rat's glabrous hindpaw skin in vehicle-treated and MMP-2/9 inhibitor (MMP-2/9I.; 20 µg, i.pl.) injected rats, respectively, at the 2 week end point. Note an invasion of sympathetic fibers into the upper dermis (B), mimicking what we had previously observed in a model of inflammatory arthritis (Almarestani et al., 2008). This fiber population was not normally found in control (vehicle-treated) animals as shown in A. Arrows in B indicate VMAT2-IR fibers in the upper dermis. The results in C are from all experimental groups and presented as the mean ± SEM. Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. *$p<0.05$, **$p<0.01$ ($n = 6$) indicates a significant difference as compared to glabrous skin sample of chronic vehicle-treated (V) naïve rats. The dashed lines represent the dermal-epidermal junction.

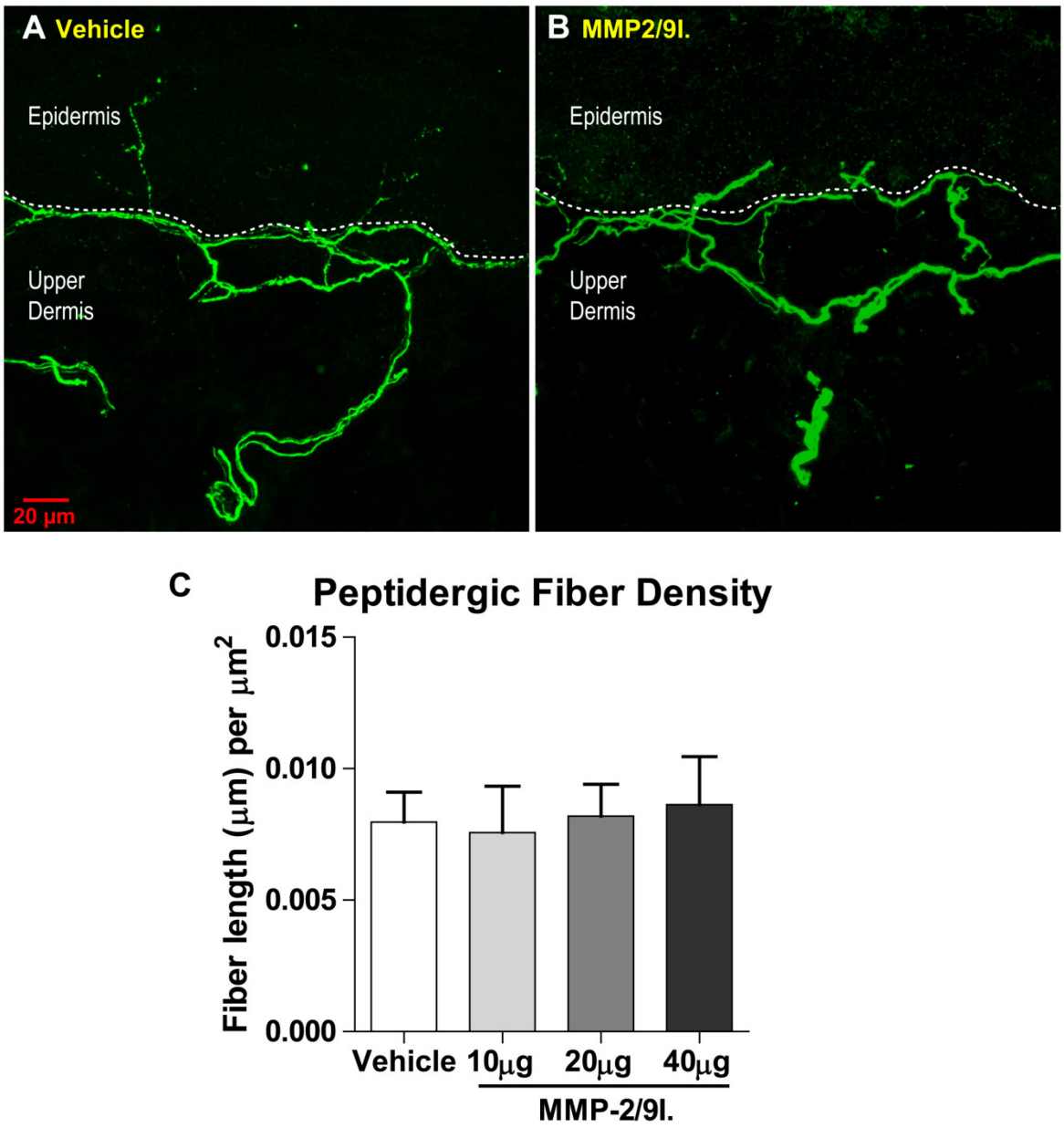


Figure 4: Density of sensory fibers in the skin of naïve rats following matrix MMP-2/9 inhibition. The density of CGRP-positive fibers following repeated matrix MMP-2/9 inhibitor (MMP-2/9I.; 10, 20, 40 µg, i.pl.) administration did not change significantly from control levels (C). A and B are representative confocal images of the rat's glabrous hindpaw skin in vehicle-treated and MMP-2/9 inhibitor (MMP-2/9I.; 20 µg, i.pl.) injected rats, respectively. The results are presented as the mean ± SEM (n = 6). Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. The dashed line represents the dermal-epidermal junction.

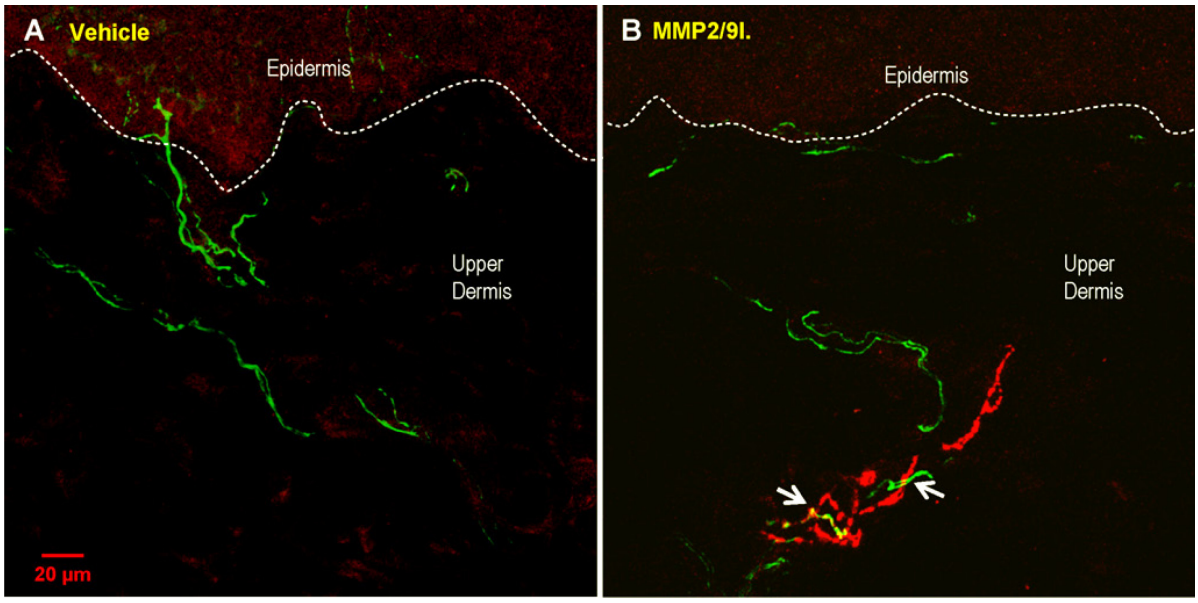


Figure 5: Sympathetic/sensory fiber interaction in the skin of naïve rats following MMP-2/9 inhibition. Note that close appositions of ectopic sympathetic fibers (VMAT2-IR as seen in red) with peptidergic nociceptive fibers (CGRP-IR as seen in green) in the upper dermis were observed at the 2 week end point following repeated injections of MMP-2/9 inhibitor (MMP-2/9I.; 20 µg, i.pl.). **A** and **B** represent confocal images of the rat's glabrous hindpaw skin in vehicle-treated and MMP-2/9 inhibitor (MMP-2/9I.; 20 µg, i.pl.) injected rats, respectively.

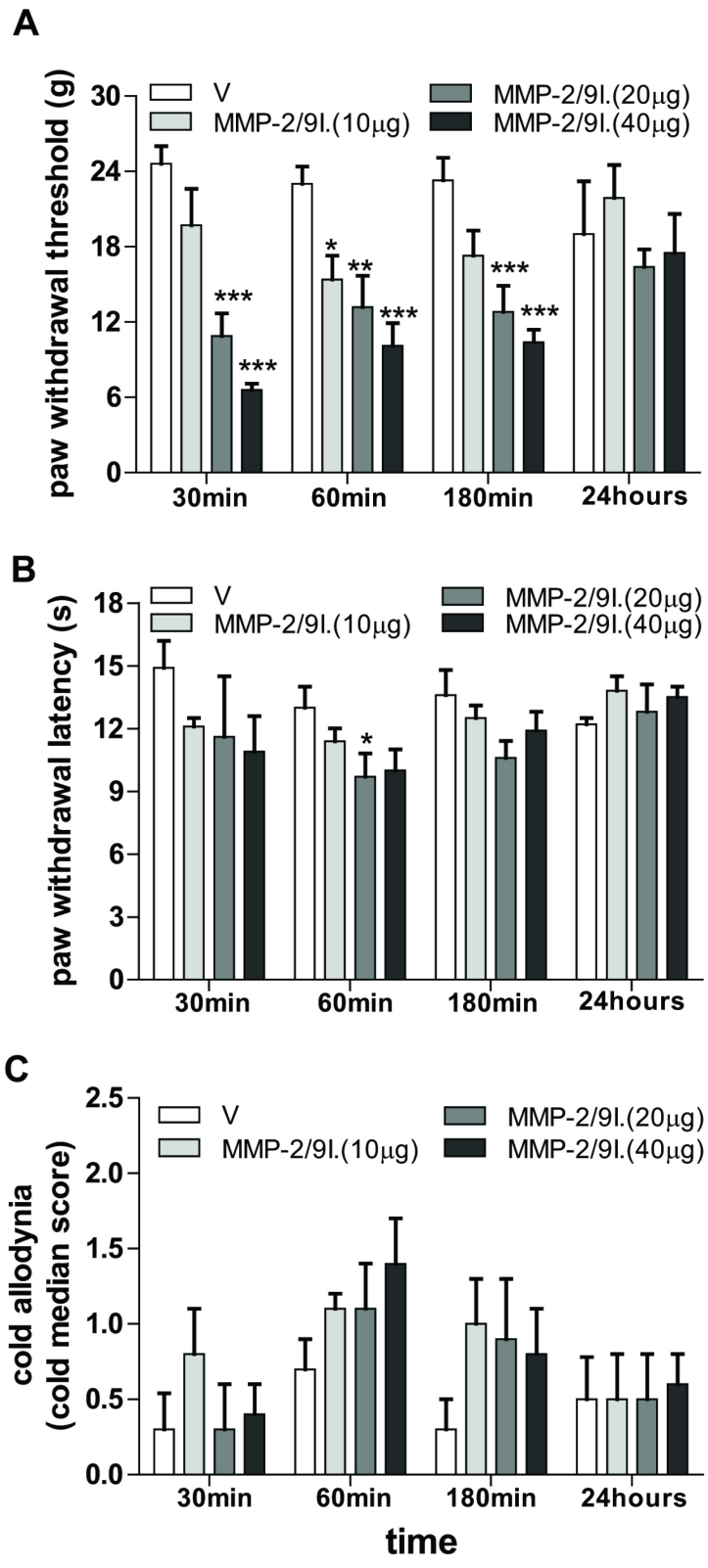

A
paw withdrawal threshold (g)
V
MMP-2/9I.(10μg)
MMP-2/9I.(20μg)
MMP-2/9I.(40μg)
30
24
18
12
6
0
*
**
***
30min
60min
180min
24hours
B
paw withdrawal latency (s)
18
15
12
9
6
3
0
C
cold allodynia
(cold median score)
2.5
2.0
1.5
1.0
0.5
0.0
time

Figure 6

Figure 6: Effect of a single MMP-2/9 inhibitor administration on allodynia and hyperalgesia in naïve rats. A single intra-plantar administration of MMP-2/9 inhibitor (MMP-2/9I.; 10, 20, 40 µg, i.pl.) induced mechanical allodynia and heat hyperalgesia as measured by von Frey and Hargreaves tests, with maximal effects using the 20 µg dose after 60 minutes. However, the treatment had no effect on cold allodynia as measured by acetone test. Behavior: the von Frey (A), Hargreaves (B) and acetone (C) tests were performed 30, 60, 180 min and 24 h after MMP-2/9 inhibitor administration. Results are presented as the mean ± SEM (n = 6-8 rats/group). Inter-group differences were analyzed by Bonferroni's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to vehicle-treated (V) naïve rats.

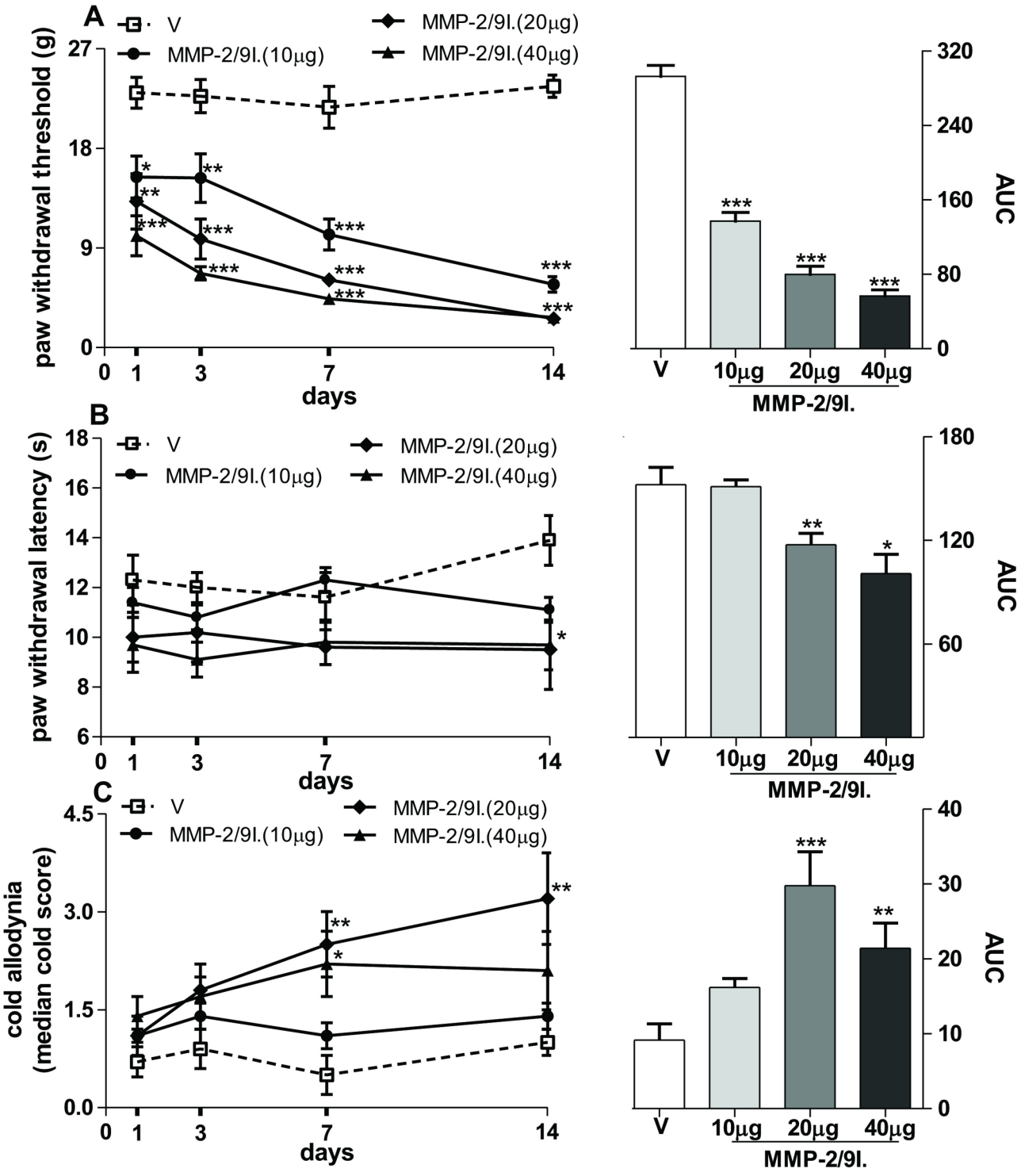

A
V
MMP-2/9I.(10μg)
MMP-2/9I.(20μg)
MMP-2/9I.(40μg)
paw withdrawal threshold (g)
27
18
9
0
0 1 3 7 14
days
320
240
160
80
0
AUC
V 10μg 20μg 40μg
MMP-2/9I.
B
paw withdrawal latency (s)
18
16
14
12
10
8
6
180
120
60
C
cold allodynia
(median cold score)
4.5
3.0
1.5
0.0
40
30
20
10

Figure 7

Figure 7: Effect of repeated MMP-2/9 inhibition on allodynia and hyperalgesia in naïve rats. Repeated intra-plantar administration of MMP-2/9 inhibitor (MMP-2/9I.; 10, 20, 40 µg, i.pl.) once daily for fourteen days induced allodynia and hyperalgesia. Behavioral tests: the von Frey test (A), Hargreaves test (B) and acetone test (C) were performed on the 1st, 3rd, 7th and 14th day of repeated MMP-2/9 inhibitor injections. Results are presented as the mean ± SEM (n = 6-8 rats/group). Inter-group differences were analyzed by Bonferroni's multiple comparison test. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to vehicle-treated (V) naïve rats. The area under the curve (AUC) was calculated by the Trapezoidal & Simpson's Rules, Pharmacologic Calculation System, version 4.0-03/11/86. Inter-group differences were analyzed by Bonferroni's Multiple Comparison Test. *$p<0.05$, **$p<0.01$, ***$p<0.001$ indicate a significant difference as compared to chronic vehicle-treated (V) naïve rats.

3.4 Discussion

The present study provides evidence that the intra-plantar administration of an MMP-2/9 inhibitor induced sensitization to mechanical, heat and cold stimuli in naïve rats. Our data also shows that the protein levels of mNGF and MMP-9 were affected in the hind paw skin following repeated MMP-2/9 inhibitor injections. Furthermore, we found that the MMP-2/9 inhibitor induced sprouting of sympathetic fibers into the upper dermis in the glabrous hind paw skin, but no change in the density of sensory peptidergic fibers. Our results support the hypothesis that sympathetic sprouting and pain related-behavior are due to an increase in mNGF levels.

3.4.1 NGF Metabolic Pathway

NGF is secreted from cells in an activity dependent manner as a precursor (proNGF), together with a full enzymatic cascade capable of leading to its breakdown into the mature (active) form and its degradation (Bruno and Cuello, 2006). The existence of the CNS protease cascade responsible for the conversion of proNGF into mNGF and for its degradation was demonstrated by a series of *ex vivo* as well as *in vivo* studies (Bruno and Cuello, 2006) that defined the relevance of tPA/plasminogen activation in the proNGF to mNGF conversion and of MMP-9 in the enzymatic degradation of mNGF. Although this NGF metabolic pathway is now well established in the CNS (Bruno et al., 2009, Allard et al., 2012), it has been much less studied in the periphery. The significance of such studies arise from the fact that up-regulation of NGF is a cause of many clinical conditions associated with pain (Aloe et al., 1992, Dicou et al., 1996, Lowe et al., 1997, Halliday et al., 1998, Oddiah et al., 1998, Miller et al., 2002, Sarchielli and Gallai, 2004, Watanabe et al., 2011) (Aloe et al., 1992, Dicou et al., 1996, Lowe et al., 1997, Halliday et al., 1998, Oddiah et al.,

1998, Miller et al., 2002, Sarchielli and Gallai, 2004, Watanabe et al., 2011). Therefore, understanding the mechanism underlying NGF processing and its eventual modulation provides attractive therapeutic opportunities. Our results provide for the first time an important validation in the periphery of the role of MMP-9 in mNGF degradation. Indeed, we were able to show that repeated intra-plantar administration of an MMP-2/9 inhibitor resulted in elevated levels of mNGF in the hind paw skin. Interfering with the degradation of endogenous mNGF was also sufficient to induce sprouting of sympathetic fibers in the skin and hypersensitivity to both mechanical and thermal stimulation. Our results confirm and expand the hypothesis that NGF plays an important role in pain mechanisms (see below).

3.4.2 NGF Induced Fiber Sprouting

NGF is a crucial neurotrophin for the definition of the innervation pattern of the skin during development (Albers et al., 1994). Adult sympathetic fibers are regulated by NGF in what regards their survival and phenotypic maintenance (Purves et al., 1988, Ritter et al., 1991) and can respond with fiber outgrowth to supplemental NGF (Ruit et al., 1990, Davis et al., 1994, Davis et al., 1996, Goodness et al., 1997). In the present study, we were able to show that inhibition of MMP-9 leads to an increase in mNGF levels and, likely as a consequence, to pain-related behaviors and a sprouting of sympathetic fibers into the upper dermis, a region of skin which is normally devoid of this fiber population. Interestingly, we did not observe any changes in the density of peptidergic sensory fibers following MMP-2/9 inhibitor injections. It has been demonstrated that sympathetic fibers are more responsive to NGF in the adult as they depend on it for survival, although NGF still plays a role in maintaining the phenotype of sensory peptidergic neurons (Gorin and Johnson, 1980, Lewin and Barde, 1996). Therefore, the lack of peptidergic sprouting in the current study would

suggest that the levels of mNGF in the skin resulted from MMP-2/9 inhibition were lower than in the chronic inflammation model (Almarestani et al., 2008). This is to be expected, because following a local subcutaneous injection of CFA there is extensive and long lasting skin inflammation which likely leads to sustained elevated local levels of mNGF. In the present study, we demonstrate that preventing the degradation of the NGF in the absence of any pathology is sufficient to increase the levels of endogenous mNGF. We have also observed that, in such conditions, the sympathetic fibers sprouted in a pattern comparable to what we previously observed in the arthritis model (Almarestani et al., 2008) and also in neuropathic pain models (Ruocco et al., 2000, Yen et al., 2006b). These studies demonstrate that the ectopic sympathetic fibers are not associated with blood vessels and often wrap around sensory peptidergic fibers suggesting enhanced sympathetic-sensory interaction. Under normal physiological conditions, the sympathetic nervous system is not associated with nociception and uninjured primary sensory nerve endings are not sensitive to catecholamines. However, noradrenaline injection following nerve lesions has been shown to excite small diameter nociceptors, but not in normal conditions (Sato and Perl, 1991, Bossut and Perl, 1995). This is in agreement with the sympathetic participation in some forms of neuropathic pain (Campbell et al., 1989). One possible mechanism for the role of the sympathetic system in pathological pain is the ectopic association of sympathetic fibers with sensory peptidergic fibers in upper dermis of the skin (Grelik et al., 2005, Yen et al., 2006b). Curiously, a similar ectopic association of sensory and sympathetic fibers was also observed in the thick skin of the rat hind paw following chronic inflammation (Almarestani et al., 2008). We observed here a similar fiber remodeling which paralleled the pain-like behavior following MMP-9 inhibition, in the absence of any lesion. Additionally, our results concur

with previous studies showing that sympathetic sprouting is related to increased levels of NGF. Thus, it has been shown that intraventricular infusion of nerve growth factor *per se* is able to induce sympathetic fiber sprouting in the sensory ganglia (Nauta et al., 1999), subpial region of the medulla, spinal cord (Winkler et al., 1997) and in the intracranial vasculature (Saffran et al., 1989, Isaacson et al., 1992) of naïve rats. Conversely, surgical or chemical sympathectomy has been shown to reduce the thermal and mechanical hyperalgesia evoked by NGF (Aloe et al., 1992, Andreev et al., 1995, Woolf et al., 1996).

3.4.3 NGF-Evoked Hyperalgesia

It has been shown that exogenous treatment with NGF evoked nociceptive behavior in animals (Lewin et al., 1993, Lewin and Mendell, 1994, Andreev et al., 1995, Pertens et al., 1999, Malin et al., 2006) and induced pain in humans (Petty et al., 1994, Dyck et al., 1997). A single NGF injection into the skin induced long-lasting mechanical and thermal hypersensitivity in humans (Rukwied et al., 2010, Weinkauf et al., 2011). Also, recently published data obtained in the rodent reported that the intra-plantar injection of NGF induced an immediate, long-lasting increase in mechanical and thermal sensitivity, as early as one hour post-injection (Mills et al., 2013). This study reported that the effect on thermal sensitivity was of shorter duration compared with that on mechanical sensitivity, what is in agreement with our results and indicates that mechanical hypersensitivity requires a considerable longer time to resolve than the heat hypersensitivity. This situation is similar to that in the human, where heat hypersensitivity resolved much faster than the long-lasting increase in mechanical sensitivity (Mills et al., 2013). In the current study, we observed changes in pain-related behavior when we inhibited NGF degradation in naïve rats, with a different time course for mechanical and thermal sensitivities. The temporal differences in

resolution between mechanical and thermal sensitivity suggest different mechanisms, however what drives these differences is still unclear. One of the possible mechanisms involved in NGF-induced hyperalgesia might be through sympathetic sprouting and abnormal sympathetic-sensory interactions (McLachlan et al., 1993, Xie et al., 2010). Indeed, sympathetic fibers express TrkA receptors and their activation results in noradrenaline release which would increase in excitability and spontaneous activity of petidergic sensory neurons (McLachlan et al., 1993, Xie et al., 2010). Also ATP, a co-transmitter released from sympathetic fibers and damaged tissues (Bertrand, 2003, Burnstock, 2011), has been shown to play a role in activating primary afferents via purinergic receptors (Mo et al., 2013) and may play a role in the hyperexcitability as well. Recent data from our laboratory provided some support to these sympathetic-sensory interactions in an inflammatory arthritis animal model, in which there is sympathetic fiber sprouting in skin over the joints as well as in the arthritic joint. Indeed, in that study we have shown that pharmacological suppression of sympathetic fiber function with systemic Guanethidine significantly decreased the pain-related behavior associated with inflammatory arthritis (Longo et al., 2013). Our data following Guanethidine administration was comparable with that obtained in other chronic pain models (Malmberg and Basbaum, 1998, Xanthos and Coderre, 2008). Moreover, there is also evidence from the literature that the sodium channel Nav 1.8, expressed on sensory neurons, may be a major player in NGF-induced thermal hyperalgesia (Kerr et al., 2001). Evidence shows that NGF modulates this sodium channel current density through the PKA pathway following TrkA binding (Brackenbury and Djamgoz, 2007). These changes in sodium channels might occur even in the absence of peptidergic fiber sprouting as consequence of mNGF increases induced by MMP-2/9 inhibition. Since many NGF-

responsive neurons contain the vanilloid receptor TRPV1, this receptor is also suspected to play a role in NGF-mediated hypersensitivity (Caterina et al., 1997, Tominaga et al., 1998, Michael and Priestley, 1999). Cultured DRG neurons treated with NGF display enhanced inward currents in response to the application of the TRPV1 agonist capsaicin (Shu and Mendell, 1999a, Caterina et al., 2000). NGF can increase TRPV1 expression (Donnerer et al., 2005, Xue et al., 2007) and promote TRPV1 insertion into the plasma membrane (Zhang et al., 2005a). NGF also acts indirectly by activating mast cells and neutrophils, which in turn release additional inflammatory mediators causing hypersensitivity (Lewin et al., 1994, Andreev et al., 1995, Amann et al., 1996, Woolf et al., 1996, Bennett et al., 1998b). There is a growing body of evidence indicating that NGF-evoked hyperalgesia is caused by a sensitization of primary afferent nociceptors that supply an area of damage or inflammation (Heppenstall and Lewin, 2000, Jankowski and Koerber, 2010).

Matrix metalloproteinases have multiple functions. Therefore, it is not surprising that there is evidence that MMP-9 inhibition at the spinal cord level alleviates the early phase of neuropathic pain, whereas intrathecal administration of MMP-9 itself is sufficient to produce neuropathic pain-like symptoms in normal animals (Kawasaki et al., 2008a). These results at the spinal cord level are very different from those we obtained through a peripheral inhibition of MMP-9, likely because, in the CNS, the MMP-9-induced pathophysiology involves interleukin-1β cleavage to its active form and microglial p38 activation (Kawasaki et al., 2008a), rather than effects through NGF. However, because MMP-2 is also responsible for the degradation of several pain-related mediators such as TNF-α, interleukin-1β, and substance P (Sternlicht and Werb, 2001), we cannot rule out that MMP-2 inhibition might

increase the levels of these mediators and subsequently contribute to the hyperalgesia through a peripheral mechanism.

3.4.4 Conclusions

Our data provides evidence that the metabolic processing of endogenous NGF can be modulated in the periphery in a manner similar to what was previously demonstrated in the central nervous system. We show that inhibition of NGF degradation in naïve rats induces sprouting of sympathetic fibers into the upper dermis of the skin, where they wrap around the peptidergic nociceptive afferents, suggesting an interaction between these two fiber populations. We propose that the observed sensitization to both mechanical and thermal stimuli is a consequence of elevated endogenous mNGF levels sensitizing peptidergic primary afferents and promoting abnormal sensory-autonomic fiber interactions in the skin. As NGF has been strongly implicated in different pain conditions, modulating its metabolic pathway provides an opportunity to influence its endogenous levels to alleviate pain.

3.5 Methods

3.5.1 Animals

Adult male Sprague-Dawley rats (275-300 g) were used in this study. Animals were housed in groups of four per cage with sawdust bedding under a standard 12 h/12 h light/dark cycle (lights on at 08.00 AM) with food and water available *ad libitum.* All experiments were carried out according to protocols approved by the McGill University Animal Care Committee and followed the guidelines for animal research from the International Association for the Study of Pain (IASP) (Zimmermann, 1983).

3.5.2 Drug Administration

An MMP-2/9 inhibitor (MMP-2/MMP-9 Inhibitor I, EMD Chemicals, Inc.), which selectively inhibits both MMP-2 (IC50 = 310 nM) and MMP-9 (IC50 = 240 nM), was administered subcutaneously into the plantar region of the right hind paw, once a day, for 14 days at doses of 10, 20 and 40 μg/20 μL. The control group of rats received vehicle (DMSO, 20mg/1ml) according to the same schedule. The doses of MMP-2/9 inhibitor were selected based on a previous study from our lab (Allard et al., 2012) and our preliminary experiments. The behavioral tests were conducted 30, 60, 180 minutes and 24 hours after the first MMP-2/9 inhibitor injection and then 60 min following the MMP-2/9 administration on days 3, 7 and 14.

3.5.3 Behavioral Tests

Signs of mechanical, heat and cold hypersensitivity were detected using the von Frey, Hargreaves and acetone tests, respectively. Prior to any behavioral testing, animals were habituated three times to the testing environment. The baseline reaction values were measured one day before the first MMP-2/9 injection and were as follows: 26 g for the von Frey test, 12.2 ± 1.9 s for the Hargreaves test and 0.5 ± 0.2 for acetone test.

3.5.3.1 Mechanical allodynia (von Frey test). Mechanical allodynia in rats was measured using a series of calibrated nylon von Frey filaments (Stoelting, Wood Dale, IL, USA), ranging from 0.6 to 26 g. Animals were placed in plastic cages with a wire-mesh floor. They were placed in this environment approximately 5 min before testing in order to allow behavioral accommodation. The von Frey filaments were applied in ascending order to the midplantar surface of the injured hindpaw through the mesh floor. Each probe was

applied to the foot until the filament bent. The time interval between consecutive filament administrations was at least 5 s. The calculations were performed as described previously (Osikowicz et al., 2008).

3.5.3.2 Thermal hyperalgesia (Hargreaves' test). The pain threshold to high temperature was tested using the Plantar Test (Hargreaves Apparatus, Ugo Basile, Type 7370, Comerio, Varese, Italy). Rats were placed into individual plastic cages with glass floors 5 min before the experiment. A noxious thermal stimulus was focused through the glass onto the plantar surface of a hind paw until the animal lifted the paw away from the heat source. The paw withdrawal latency was automatically measured to the nearest 0.1 s. A cut-off latency of 20 s was used to avoid tissue damage. The latency to the nociceptive reaction was measured in seconds under basal condition and after drug treatment.

3.5.3.3 Thermal allodynia (Acetone test). Cold allodynia was assessed using the acetone drop method (Choi et al., 1994). In the test environment described above, a 50µl droplet of acetone was applied to the midplantar hind paw using a micropipette. Responses within the first 20 s were scored according to the following rating system: 0 - no response; 1- one rapid hind paw flick/stamp; 2 - two or more hind paw flicks/stamps; 3 - periods of flicking/stamping with licking of plantar hind paw (Flatters and Bennett, 2004). The acetone application was repeated three times for each hind paw, with a 3 min interval between each application. For each rat, the sum of the three scores was used for data analysis.

3.5.4 Biochemistry

3.5.4.1 Western blot. Following fourteen days of daily MMP-2/9 inhibitor administration, the rats were decapitated and the glabrous skin from the hind paws was

collected, frozen in the liquid nitrogen and kept for further processing. Tissue samples were homogenized in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 25 mL Tris–HCl, pH 7.6) containing protease inhibitors (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and cleared by centrifugation (13,000 rpm for 50 min at 4°C). Protein concentration of the supernatant was determined using the BCA Protein Assay Kit (Sigma). Homogenates (20 μL of 50 μg of total protein) were resolved in 4-12% polyacrylamide gels and transferred into a nitrocellulose membrane (Bio-Rad Laboratories, Inc). The blots were blocked in Tris Buffer Solution-Tween 20 (TBS-T) containing 5% non-fat powdered milk at room temperature for 1 h on a rotomixer. Nitrocellulose membranes were probed with primary antibodies specific for nerve growth factor (NGF, 1:500, E2610, Santa Cruz Biotechnology) and matrix metalloproteinase-9 (MMP-9, 1:500, AB19016, Millipore). Blots were incubated overnight at 4°C with the primary antibodies. After primary antibody incubation, membranes were washed 3 × 10 min in TBS-T. This was followed by incubation for 2 h at room temperature with a peroxidase-conjugated goat anti-rabbit IgG antibody (1:2500) (Jackson Immuno Research Laboratories, Inc.). The membranes were washed 3 × 10 min in TBS-T. The immunoreactive bands were visualized with the ECL enhanced chemiluminescence kit (PerkinElmer Inc.) and using Kodak Biomax XAR imaging film. The immunoreactive bands were quantified by densitometry of the films using a MCID M4 image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). Membranes were rinsed and reprobed with a mouse anti-β-actin antibody (1:40,000; Sigma) diluted in 5% milk in TBS-T for 1 h at room temperature, washed with TBS-T, and incubated with a peroxidase conjugated donkey anti-mouse IgG (1:5000, Santa Cruz) in 5% dry milk in TBS-T for 1 h. The membranes were washed and the

signal was detected and qualified as described above. The levels of NGF and MMP-9 were normalized to the β-actin levels for each sample.

3.5.4.2 Zymography. Gelatinolytic activity was determined by zymography using gelatin-containing gels following the protocol provided by supplier Millipore. The skin samples were collected at the same time point as those for western blot analysis (see above). The skin sample extracts were mixed with an equal volume of non-reducing sample buffer (0.5M Tris–HCl, pH 6.8, SDS, glycerol and bromophenol blue). The samples (50 µg of protein) were electrophoresed on an 8% SDS–PAGE containing 0.1% gelatin as the substrate. After electrophoretic separation, the gels were incubated for 30 min in renaturing buffer (2.5% Triton X-100 in distilled water) to remove the SDS, washed for 2X10 min with water and then incubated for 24 h at 37°C in a developing buffer containing 50mM Tris–HCl, pH 7.78; 5mM CaCl2 and 0.02% Brij 35. Following incubation, the gels were stained for 1 h with 0.5% Coomassie R-250 staining solution and then differentiated in a solution of methanol: acetic acid : water (50 : 10 : 40). Enzyme activity attributed to MMP-2 and MMP-9 was visualized (on the basis of molecular weight) in the gelatin-containing zymograms as clear bands against a blue background. The bands were quantified by densitometry using the MCID M4 image analysis system.

3.5.5 Immunohistochemistry

Two weeks after the daily injections of MMP-2/9 inhibitor, animals were deeply anesthetized with Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 mL, i.p., per 100 g body weight) and then perfused through the left cardiac ventricle with 100 mL of perfusion buffer, followed by 500 mL of 4% paraformaldehyde

(PFA) in 0.1 M phosphate-buffer (PB), pH 7.4, at room temperature for 30 minutes. Subsequently, the plantar glabrous skin was extracted and post-fixed in the same fixative for 1 hour at 4°C. The tissue was cryoprotected in 30% sucrose in PB overnight at 4°C for later immunohistochemical processing. Fifty-µm thick cross sections of skin were cut using a cryostat (Leica, Wetzlar, Germany). All sections were collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS-T). The tissue sections were incubated for 1 hour at room temperature in 10% normal goat serum (Gibco, Carlsbad, CA) in PBS to block unspecific labeling. To label the peptidergic and sympathetic fiber populations, the sections were then incubated at 4°C for 24 hours using either a rabbit anti-Calcitonin Gene Related Protein (CGRP) (Sigma-Aldrich, St. Louis, MO, C-8198, lot# 070M4835, diluted 1:2000) antibody or a rabbit anti-Vesicular Monoamine Transporter-2 (VMAT-2) (Phoenix Pharmaceuticals, Inc., CA, USA, H-V004, 01237-1, diluted 1:7500). As controls, some sections were processed omitting the primary antibody; no specific staining was observed. After three rinses in PBS-T, the sections were incubated for 2 hours at room temperature with goat anti-rabbit IgG conjugated to either Alexa Fluor 488 for CGRP or Alexa Fluor 594 for VMAT-2 (Molecular Probes, diluted 1:800). For double-labeling of CGRP and VMAT2, sections were processed as described above except that we used a guinea pig anti-CGRP antibody at a 1:4000 dilution (Peninsula, San Carlos, CA, T-5053) in a simultaneous incubation with the rabbit anti-VMAT2 antibody; after washing, we used a mixture of an anti-guinea pig IgG antibody conjugated to Alexa Fluor 488 (1:800; Molecular Probes) with the anti-rabbit IgG conjugated to Alexa Fluor 594. Finally, the sections were washed, mounted on gelatin-subbed slides, air-dried and cover slipped with an anti-fading mounting medium (Aqua PolyMount, Polysciences Inc., Warrington, Pa.). Slides were stored at 4°C

until examined. Since we aimed to localize and evaluate the morphological changes in innervation and to quantify the relative changes in the immunostaining intensity, the conditions of all procedures (dilutions of reagents and antibodies, washings, incubation time and temperature, blocking of nonspecific staining), were kept rigorously throughout the assays and were identical for the sections from all tested groups. Within each experiment, immunohistochemical processing of tissue sections sampled from all groups was carried out simultaneously. Before quantitative analyses, all the slides were coded so that the person who performed the quantification was completely blinded regarding the experimental groups. Codes were broken only after the quantification was completed.

3.5.6 CGRP-Immunoreactive Fiber Quantification

Quantitative analyses were performed on sections of glabrous skin from the hindpaw ipsilaterally to the injection of MMP-2/9 inhibitor or vehicle. In this study, as in previous work from our laboratory, we have divided the dermis into upper and lower dermis. We have defined the upper dermis as the area of the dermis spanning 150 μm below the dermal-epidermal junction (Yen et al., 2006b). For the measurement of the density of peptidergic fibers, as detected by CGRP immunoreactivity, we used a Zeiss Axioplan 2 imaging fluorescence microscope equipped with a PlanFluotar 40X oil-immersion objective. This microscope has a high resolution digital camera connected to a computer equipped with the Zeiss Axiovision 4.8 software (Carl Zeiss, Canada). Three sections per slide were randomly selected, and 6 microscopic fields including upper dermis were photographed at random, for a total of 18 images per animal. Images were exported in the TIFF format for analysis with an MCID Elite image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). The upper dermis was outlined with the use of a tracing tool in the software. CGRP-IR fibers

were automatically detected by the software using a brightness threshold and converted to 1 pixel in thickness to compute the total fiber length (μm) per scan area (μm^2).

3.5.7 Sympathetic Fiber Quantification

We used a different approach to quantify the changes in autonomic innervation. Since these fibers are much less abundant in the skin than the sensory, especially in the upper dermis, it was unpractical to measure fiber density. Therefore, we proceeded by photographing 6 entire skin sections per animal and counting all VMAT-2-IR fibers within the upper dermis, measured to be 150 μm from the dermal-epidermal junction. The mean number of fibers in the upper dermis per total area (μm^2) was then calculated.

3.5.8 Data Analyses

The Western blot and zymography results represent the densitometry analyses of all samples (4-6 samples per group). Inter-group differences were analyzed by one-way ANOVA and Bonferroni's multiple comparison tests (Figures 1, 2). The mean number of sympathetic fibers and density of sensory peptidergic fibers in the upper dermis was compared between groups by a one-way ANOVA and a Dunnett's post hoc test, with a statistical significance accepted at $p < 0.05$ (Figures 3, 4, 5). As no significant difference was detected among the control groups of all time points, they were pooled. The behavioral data are presented as the mean ± SEM (6-8 animals per group). The effect of single as well as repeated intra-plantar injections of MMP-2/9 inhibitor on mechanical and thermal sensitivity in rats (Figures 6, 7) was analyzed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. The effect of repeated injections of MMP-2/9 inhibitor on allodynia and hyperalgesia was also shown as the area under the curve (AUC,

Figure 7). To evaluate the AUC the Trapezoidal and Simpson's Rules, Pharmacologic Calculation System, version 4.0-03/11/86 was used (Tallarida and Murray, 1987). Using the GraphPad Prism software we performed a correlation analysis between the behavioral responses and sympathetic fiber sprouting, as this was the only fiber population which changed following administration of the MMP-2/9 inhibitor. The analysis was done on each behaviorally tested rat from which the skin tissue was collected for the immunohistochemical component.

3.6 Acknowledgements

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Connecting Text: Chapter 3 to Chapter 4

In the previous chapter, we described in a proof of principle study that the inhibition of the matrix metalloproteinase MMP-9 increased the protein level of mNGF and caused a pain-related hypersensitivity in naïve rats. These changes were correlated with a sprouting of sympathetic fibers into the upper dermis of the glabrous hind paw skin. Therefore, we provided support to the hypothesis that the peripheral NGF metabolic pathway is similar to that of the CNS and to the hypothesis that sympathetic sprouting and pain related-behavior are due to increased levels of mNGF.

In the next chapter, we were capable in manipulating mNGF levels by inhibiting plasmin, and thus preventing its conversion from proNGF in our model of inflammatory arthritis. Our objective was to prevent the abnormal changes in the pattern of innervation and alleviate the pain-related behaviour observed in the CFA model of arthritis, described in Chapter 2. Following the administering α2-antiplasmin, we assessed animal behavior as well as changes in the innervation of the skin adjacent to the inflamed joint. We also assessed the changes in TRPV1 and TrkA expression in the DRG following the induction of arthritis by immunocytochemistry in order to further confirm the peripheral effects the modulation of mNGF has at the level of the cell body.

Chapter 4

Local administration of α2-antiplasmin alleviates allodynia and hyperalgesia in a rat model of inflammatory arthritis

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(To be submitted)

4.1 Abstract

Recent evidence indicates that nerve growth factor (NGF) is a key mediator of inflammation and pain. Therefore, modulation of this neurotrophin should be considered as a potential therapeutic target for the management of inflammatory pain. NGF was shown to be secreted from cells in its precursor form (proNGF), together with a full enzymatic cascade capable of leading to its breakdown into the mature, active form (mNGF). We hypothesized that interfering with the levels of endogenous NGF by inhibition of plasmin (key enzyme that converts proNGF into mNGF) should reduce pain sensitization associated with chronic inflammation. To study this issue, we used a rat model of chronic inflammation in which complete Freund's adjuvant (CFA) was injected into the ankle joint of male Sprague-Dawley rats. To reduce the NGF levels, we used α2-antiplasmin in order to inhibit plasmin activity. Single or repeated administration of α2-antiplasmin significantly attenuated CFA-induced allodynia and hyperalgesia. In addition, western blot analyses revealed a reduction in CFA-induced mNGF level following plasmin inhibition. The previously demonstrated CFA-induced sympathetic fiber sprouting into the upper dermis, an area that is normally devoid of them, was blocked following repeated administration of α2-antiplasmin. This repeated α2-antiplasmin administration reduced CFA-induced increase in TRPV1 and TrkA expression in DRG cell bodies. We conclude that attenuation of NGF levels via α2-antiplasmin can be considered as a potential therapeutic option for the management of arthritis pain.

4.2 Introduction

Rheumatoid arthritis (RA) is a common chronic inflammatory disease, characterized by synovial inflammation and joint destruction. The main clinical sign of RA is chronic pain,

the management of which remains very difficult (Kean and Buchanan, 2005, Langford et al., 2006, Chen et al., 2008, Lang et al., 2010). The animal model consisting of the intra-articular injection of Complete Freund's adjuvant (CFA) into the rat tibio-tarsal joint space has been reported to produce a localized pathology that closely resembles some aspects of human RA (Butler et al., 1992, Laird et al., 1997, Kelly et al., 2007). Besides pain, swelling, limitation in the range of motion, CFA injection induces changes in the innervation of the ankle joint and surrounding tissues (Almarestani et al., 2008, Ghilardi et al., 2012, Longo et al., 2013). There are already several studies that describe an involvement of nerve growth factor (NGF) in the development of chronic inflammation and arthritis. Elevated levels of NGF were found in inflamed tissues from patients with conditions such as arthritis, psoriasis, prostatitis and pancreatitis (Fantini et al., 1995, Halliday et al., 1998, Friess et al., 1999, Miller et al., 2002, Nakamura et al., 2003, Raychaudhuri et al., 2003, Barthel et al., 2009) as well as in peripheral tissues in several animal models of chronic inflammatory pain conditions (Woolf et al., 1994, Sivilia et al., 2008, Longo et al., 2013). It has been shown that these elevated levels of NGF in the peripheral tissues induce sprouting of NGF-responsive sensory and sympathetic neurons into the affected region and further participate in sensitization of the nociceptors (Shu and Mendell, 1999b, a). Such NGF-induced hyperinnervation was observed in the ankle or knee joints in adjuvant-induced arthritis in rats (Wu et al., 2000) or mice (Ghilardi et al., 2012).

The obvious role of NGF in inducing sprouting of fibers, neuronal sensitization and hyperalgesia, provides evidence that anti-NGF treatments may constitute an effective means of treating pain in humans (Lowe et al., 1997, Saldanha et al., 1999, Sena et al., 2006, Jimenez-Andrade et al., 2007). The current treatment options for patients with painful

inflammatory conditions are inadequate. Commonly used non-steroidal anti-inflammatory drugs and opioid analgesics have significant side effects (Whelton et al., 2000, Kalso et al., 2004, Johnsen et al., 2005), which limit their long term use. Recent studies have shown that selective antagonism of NGF is highly effective in animal models of many pain states (Ugolini et al., 2007, Mantyh et al., 2010, Ghilardi et al., 2011, Zhu et al., 2012). Such therapeutic strategy could also be considered in the clinic. However, recent clinical investigations with the antibody that sequesters NGF showed some safety issues (Hill, 2011), what brings some concerns regarding this type of strategy in pain management. Therefore, we suggested an alternative way to reduce the elevated NGF levels in the inflammatory pain condition by modulating the conversion of proNGF into mNGF. In order to study this, we used α2-antiplasmin to inhibit plasmin, the enzyme that converts proNGF into mNGF, and we investigated its influence on 1) the protein levels of proNGF and mNGF, 2) the changes in innervation pattern of sensory and autonomic fibers in the skin, 3) the pain thresholds, and 4) TRPV1 and TrkA expression in DRG cell bodies from ankle nociceptive afferents of CFA-injected rats.

4.3 Materials and Methods

4.3.1 Animals

Adult male Sprague-Dawley rats (275-300 g) were used in this study. Animals were housed in groups of four per cage with sawdust bedding and were kept under a standard 12 h/12 h light/dark cycle (lights on at 08.00 AM) with food and water available *ad libitum.* All experiments were carried out according to protocols approved by McGill University Animal

Care Committee and followed the guidelines for animal research from the International Association for the Study of Pain (IASP) (Zimmermann, 1983).

4.3.2 Intra-Articular CFA Injection

Chronic joint inflammation was induced by an injection of CFA (150 μg of Mycobacterium butyricum) intra-articularly in the right tibio-tarsal joint (Butler et al., 1992), while the rat was briefly anesthetized with 2% isoflurane. The skin around the site of injection was sterilized with 75% alcohol. The right leg was held and the fossa of the lateral malleolus of the fibula was located. A 28-gauge needle was inserted vertically to penetrate the skin and turned distally to insert into the articular cavity at the gap between the tibiofibular and tarsus bones until a distinct loss of resistance was felt. A volume of 40 μl of CFA was then injected. Control (sham) animals underwent the same procedure but were injected with vehicle (mineral oil).

4.3.3 Drug Administration

A plasmin inhibitor (human α2-antiplasmin, Molecular Innovations, Inc.), was administered subcutaneously into the plantar (i.pl.) region of the right hind paw. A single i.pl. administration of α2-antiplasmin (8, 40, 80 μg) was performed two weeks after CFA intra-articular injection. Chronic i.pl. injections of α2-antiplasmin (8 μg) were given once a day, for 14 days, starting 1 hour or two weeks after CFA intra-articular injection. The control group of rats injected with CFA received vehicle [saline: 0.05 M NaC2H3O2 (EMD) and 0.1 M NaCl (EMD)] according to the same schedule. Sham animals were treated with vehicle or α2-antiplasmin. The doses of α2-antiplasmin were selected based on a previous study (Allard

et al., 2012) and our preliminary experiments. The behavioral tests were conducted 1 hour after the first α2-antiplasmin injection and then on days 3, 7 and 14 following repeated α2-antiplasmin administration.

4.3.4 Retrograde Tracer Administration

To identify the cell bodies of sensory neurons innervating the ankle joint, 5 μl of 2% fluorogold (FG; Fluorochrome) was injected into the right ankle joints of the rats, 7 days prior to the end of the experiments.

4.3.5 Behavioral Tests

Signs of mechanical, heat and cold hypersensitivity were detected using the von Frey, Hargreaves and acetone tests, respectively. Prior to any behavioral testing, animals were habituated three times to the testing environment. The baseline reaction values were measured one day before CFA injection and were as follows: 23.2 g ± 0.8 for the von Frey test, 14.1 ± 0.3 s for the Hargreaves test and 0.4 ± 0.1 for acetone test.

4.3.5.1 Mechanical allodynia (von Frey test). Mechanical allodynia in rats was measured using a series of calibrated nylon von Frey filaments (Stoelting, Wood Dale, IL, USA), ranging from 0.6 to 26 g. Animals were placed in plastic cages with a wire-mesh floor. They were placed in this environment approximately 5 min before testing in order to allow behavioral accommodation. The von Frey filaments were applied in ascending order to the midplantar surface of the injured hindpaw through the mesh floor. Each probe was applied to the foot until the filament bent. The time interval between consecutive filament

administrations was at least 5 s. The calculations were performed as described previously (Osikowicz et al., 2008).

4.3.5.2 Thermal allodynia (Acetone test). Cold allodynia was assessed using the acetone drop method (Choi et al., 1994). In the test environment described above, a 50µl droplet of acetone was applied to the midplantar hind paw using a micropipette. Responses within the first 20 s were scored according to the following rating system: 0 - no response; 1- one rapid hind paw flick/stamp; 2 - two or more hind paw flicks/stamps; 3 - periods of flicking/stamping with licking of plantar hind paw (Flatters and Bennett, 2004). The acetone application was repeated three times for each hind paw, with a 3 min interval between each application. For each rat, the sum of the three scores was used for data analysis.

4.3.5.3 Thermal hyperalgesia (Hargreaves' test). The pain threshold to high temperature was tested using Plantar Test (Ugo Basile). Rats were placed into individual plastic cages with glass floors 5 min before the experiment. A noxious thermal stimulus was focused through the glass onto the plantar surface of a hind paw until the animal lifted the paw away from the heat source. The paw withdrawal latency was automatically measured to the nearest 0.1 s. A cut-off latency of 20 s was used to avoid tissue damage. The latency to the nociceptive reaction was measured in seconds under basal condition and after drug treatment.

4.3.6 Western Blot

After fourteen days of daily α2-antiplasmin administration, the rats were decapitated and the glabrous skin from the hind paw region adjacent to the ankle joint was collected, frozen in the liquid nitrogen and kept for further processing. Tissue samples were

homogenized in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 150 mM NaCl, 25 mL Tris–HCl, pH 7.6) containing protease inhibitors (Complete, Roche Molecular Biochemicals, Indianapolis, IN) and cleared by centrifugation (13,000 rpm for 50 min at 4°C). Protein concentration of the supernatant was determined using the BCA Protein Assay Kit (Sigma). Homogenates (20 µL of 50 µg of total protein) were resolved in 12% polyacrylamide gels and transferred into a nitrocellulose membrane (Bio-Rad Laboratories, Inc). The blots were blocked in Tris Buffer Solution-Tween 20 (TBS-T) containing 5% non-fat dry milk at room temperature for 1 h on a rotomixer. Nitrocellulose membranes were probed with a primary antibody specific for nerve growth factor that recognizes both forms: precursor proNGF and mature mNGF (NGF, H-20: sc-548, 1:200, Santa Cruz Biotechnology). Blots were incubated overnight at 4°C with the primary antibodies and then washed 3 × 10 min in TBS-T. This was followed by an incubation of 2 h at room temperature with a peroxidase-conjugated goat anti-rabbit IgG antibody (1:2500) (Jackson Immuno Research Laboratories, Inc.) in TBS-T containing 5% non-fat milk. The membranes were washed 3 × 10 min in TBS-T. The immunoreactive bands were visualized with the ECL enhanced chemiluminescence kit (PerkinElmer Inc.) and using Kodak Biomax XAR imaging film. The immunoreactive bands were quantified by densitometry of the films using a MCID M4 image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). Membranes were rinsed and reprobed with a mouse anti-β-actin antibody (1:40,000; Sigma) diluted in 5% milk in TBS-T for 1 h at room temperature, washed with TBS-T, and incubated with a peroxidase conjugated donkey anti-mouse IgG (1:5000, Santa Cruz) in 5% dry milk in TBS-T for 1 h. The membranes were washed and the signal was

detected and quantified as described above. The levels of proNGF and mNGF were normalized to the β-actin levels for each sample.

4.3.7 Immunohistochemistry

Two weeks after the daily injections of α2-antiplasmin, animals were deeply anesthetized with Equithesin (6.5 mg chloral hydrate and 3 mg sodium pentobarbital in a volume of 0.3 mL, i.p., per 100 g body weight) and then perfused through the left cardiac ventricle with 100 mL of perfusion buffer, followed by 500 mL of 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffer (PB), pH 7.4, at room temperature for 30 minutes. Subsequently, the plantar glabrous skin adjacent to the ankle joint and the L4 dorsal root ganglion (DRG) were extracted in each animal and post-fixed in the same fixative for 1 hour at 4°C. The tissue was cryoprotected in 30% sucrose in PB overnight at 4°C for later immunohistochemical processing. Fifty-µm thick cross sections of skin were cut using a cryostat (Leica, Wetzlar, Germany). Sections were collected as free-floating in phosphate-buffered saline (PBS) with 0.2% Triton-X 100 (PBS-T). DRG sections were cut at a thickness of 16 µm and were attached to gelatin-subbed slides in an alternate manner so that every fourth section was collected on the same slide. Glass slides were numbered and stored at -20°C until processed for immunocytochemistry. A band of hydrophobic Barrier Paste (Invignome) was applied to slides around the sections to prevent solutions from spilling. The sections were incubated for 1 hour at room temperature in 10% normal goat serum (Gibco, Carlsbad, CA) in PBS to block unspecific labeling. To label the peptidergic and sympathetic fiber populations, the sections were then incubated at 4°C for 24 hours using either a rabbit anti-Calcitonin Gene Related Protein (CGRP) (Sigma-Aldrich, St. Louis, MO, C-8198, lot# 070M4835, diluted 1:2000) antibody or a rabbit anti-Vesicular Monoamine Transporter-2

(VMAT-2; Phoenix Pharmaceuticals, Inc., CA, USA, H-V004, 01237-1, diluted 1:7500). Sections of DRG were incubated with either a rabbit anti-TRPV1 antibody (Neuromics VR1 N-Terminus RA10110) or a rabbit anti-TrkA antibody (R&D Systems, ON, Canada, MAB1056), at a dilution of 1:8000 in PBS-T with 5% NGS. Some sections were processed omitting the primary antibody; no staining was observed. After three rinses in PBS-T, the sections were incubated for 2 hours at room temperature with a goat anti-rabbit IgG conjugated to either Alexa Fluor 488 for CGRP or Alexa Fluor 594 for VMAT-2 (Molecular Probes, diluted 1:800). For double-labeling of CGRP and VMAT2, sections were processed as described above except that we used a guinea pig anti-CGRP primary antibody at a 1:4000 dilution (Peninsula, LLC, San Carlos, CA, T-5053) in a simultaneous incubation with the rabbit anti-VMAT2 antibody; after washing, we used a mixture of a goat anti-guinea pig IgG antibody conjugated to Alexa Fluor 488 (1:800; Molecular Probes) with a goat anti-rabbit IgG conjugated to Alexa Fluor 594. DRG slides were incubated in goat anti-rabbit IgG conjugated to Alexa Fluor 594 (1:400; Invitrogen) for 2 hours in the dark at room temperature. Finally, the sections were washed, mounted on gelatin-subbed slides, air-dried and cover slipped with an anti-fading mounting medium (Aqua PolyMount, Polysciences Inc., Warrington, Pa.). Slides were stored at 4°C until examined. Within each experiment, immunohistochemical processing of tissue sections from all groups was carried out simultaneously. Before quantitative analyses, all the slides were coded so that the person who performed the quantification was completely blinded regarding the experimental groups. Codes were broken only after the quantification was completed.

4.3.8 CGRP-Immunoreactive Fiber Quantification

Quantitative analyses were performed on sections of glabrous skin from the hindpaw ipsilaterally to the injection of α2-antiplasmin or vehicle, immediately adjacent to the ankle joint. In this study, as in previous work from our laboratory, we have divided the dermis into upper and lower dermis. We have defined the upper dermis as the area of the dermis spanning 150 μm below the dermal-epidermal junction (Yen et al., 2006a). For the measurement of the density of peptidergic fibers, as detected by CGRP immunoreactivity, we used a Zeiss Axioplan 2 imaging fluorescence microscope equipped with a PlanFluotar 40X oil-immersion objective. This microscope has a high resolution digital camera connected to a computer equipped with the Zeiss Axiovision 4.8 software (Carl Zeiss, Canada). Three sections per slide were randomly selected, and 6 microscopic fields including upper dermis were photographed at random, for a total of 18 images per animal. Images were exported in the TIFF format for analysis with an MCID Elite image analysis system (Imaging Research Inc., St. Catharines, ON, Canada). The upper dermis was outlined with the use of a tracing tool in the software. CGRP-immunoreactive (IR) fibers were automatically detected by the software using a brightness threshold and converted to 1 pixel in thickness to compute the total fiber length (μm) per scan area (μm^2).

4.3.9 Sympathetic Fiber Quantification

We used a different approach to quantify the changes in autonomic innervation. Since these fibers are much less abundant in the skin than the sensory, especially in the upper dermis, it was unpractical to measure fiber density. Therefore, we proceeded by photographing 6 entire skin sections per animal and counting all VMAT-2 immunoreactive

fibers within the upper dermis. The mean number of fibers in the upper dermis per unit area (μm^2) was then calculated.

4.3.10 Quantification of TRPV1 and TrkA Expression

Fluorescent images of DRG sections were captured with a 10X objective, using the proper filters to detect Alexa 594 and FG retrograde tracer. We counted the number of FG-positive cells and the number of TRPV1 or TrkA positive cells that co-localized with FG-positive cells. As we were only interested in small diameter neurons (C-fiber population), we considered only those with diameters of less than 30 μm. We did not count faintly labeled neurons, as these were extremely difficult to distinguish from non-stained cells. For each DRG section, we obtained a [(TRPV1- or TrkA-positive cell) / (FG-positive cell)] ratio and calculated it as a percentage. This is the percentage of sensory neurons innervating the inflamed joint that also express TRPV1 or TrkA immunoreactivity. We calculated the mean TRPV1/FG and TrkA/FG co-localization percentages for each treatment group. Although we did not used stereology, only neurons with visible nuclei were counted.

4.3.11 Data Analysis

The behavioral data are presented as the mean ± SEM (6-10 animals per group). The effect of single as well as repeated intra-plantar injections of α2-antiplasminon mechanical and thermal sensitivity in rats (Figs. 1, 3, 4) was analyzed by one-way analysis of variance (ANOVA) and Bonferroni's multiple comparison test. The Western blot results represent the densitometry analysis of all samples. Inter-group differences were analyzed by one-way ANOVA and Bonferroni's multiple comparison tests (Fig. 2, 5). The mean number of sympathetic fibers and density of sensory peptidergic fibers in the upper dermis was

compared between groups by a one-way ANOVA and a Dunnett's post hoc test, with a statistical significance accepted at $p < 0.05$. As no significant difference was detected among the control groups of all time points, they were pooled. The mean co-localization percentages of TRPV1 or TrkA with FG were compared between groups by one-way ANOVA, followed by Dunnett's Multiple Comparison Test to detect differences between the groups. To see if the number of FG-positive cells varied among the four groups, one-way ANOVA was performed.

4.4 Results

4.4.1 Effect of Administration of α2-antiplasmin on CFA-Induced Allodynia and Hyperalgesia

All vehicle-treated CFA-injected rats exhibited strong mechanical allodynia, thermal hyperalgesia and cold allodynia, starting on the first day of behavior testing that lasted until the last day of testing (Figure 1). Treatment with α2-antiplasmin (8 μg; i.pl.), starting 1 hour after CFA injection and then once daily for 14 days, significantly attenuated mechanical allodynia, thermal hyperalgesia and cold allodynia in rats tested on the 14^{th} day after CFA (Figure 1A, B, C). Treatment with α2-antiplasmin (8 μg; i.pl.) had no effect on the pain-related behavior in sham animals injected with the mineral oil instead of CFA (Figure 1A, B, C).

4.4.2 Effect of Administration of α2-antiplasmin on CFA-Induced Changes in proNGF and mNGF Protein Levels

Western blot analyses of the glabrous skin samples were carried out on the 14th day after two weeks of daily injections of α2-antiplasmin (8 μg, i.pl.). Both forms of NGF

(proNGF and mNGF) were identified based on their molecular weights; proNGF migrated close to 40 kDa and mNGF migrated at 14 kDa. The localization of these bands is consistent with what is described in other publications using the same antibodies (Longo et al., 2013) and aligned with the corresponding bands from positive controls (mouse submandibular gland extracts for NGF) (data not shown). Our data revealed an increase in the protein levels of the mature form of NGF (mNGF) in the ipsilateral paw of vehicle-treated CFA rats as compared to the ipsilateral paw of sham rats (shown as an interrupted line on the graphs; Figure 2A) and also when compared to contralateral paw samples from vehicle-treated CFA rats (data not shown). The repeated treatment with α2-antiplasmin (8 µg, i.pl.) reduced the mNGF protein levels in the ipsilateral paw compared to that observed on the ipsilateral side of CFA-injected vehicle-treated rats. No significant changes in the protein levels of the precursor form of NGF (proNGF) were detected by Western blot analysis in the ipsilateral paw of vehicle-treated CFA rats when compared to the ipsilateral paw of sham rats (shown as an interrupted line on the graphs; Figure 2B) and to contralateral paw of vehicle-treated CFA rats (data not shown). Repeated α2-antiplasmin administration did not have a significant effect on proNGF levels.

4.4.3 Effect of a Single Administration of α2-antiplasmin on CFA-Induced Allodynia and Hyperalgesia

A single administration of α2-antiplasmin (at doses of 8, 40 or 80 µg, i.pl.) at two weeks after CFA injection resulted in a significant dose-dependent decrease in mechanical and cold allodynia and thermal hyperalgesia as measured by the von Frey, acetone and Hargreaves tests, respectively (Figure 3). The highest antiallodynic and antihyperalgesic

effects of α2-antiplasmin in all tests were observed after single administration of 80 μg of α2-antiplasmin. A single administration of the lowest dose of α2-antiplasmin (8 μg, i.pl.), which was the dose used for the chronic treatment experiments, had no effect on mechanical and cold stimulation data (Figure 3A and C) at all studied time points. However, it induced a significant reduction in the sensitivity to the hot stimuli at the one hour time point (Figure 3B). The pain thresholds in the vehicle-treated CFA-injected rats were not changed.

4.4.4 Effect of Delayed Administration of α2-antiplasmin on CFA-Induced Allodynia and Hyperalgesia

We have also studied the effect of chronic treatment with α2-antiplasmin on the already established inflammatory arthritis. In this experiment we started the repeated administration of α2-antiplasmin (8 μg, i.pl.) at two weeks after CFA injection. Similarly to what was observed when chronic treatment was started at the time of CFA injection, all vehicle-treated CFA rats exhibited strong allodynia and hyperalgesia that persisted for the entire observation period. Chronic treatment with α2-antiplasmin, starting two weeks after CFA injection and then daily for 14 days significantly attenuated mechanical allodynia and cold allodynia in rats tested on the 14th day after CFA injections (Figure 4A and C), but did not have any effect on the thermal hyperalgesia (Figure 4B). In short, we observed that α2-antiplasmin administered to an animal with established inflammatory arthritis had lower antiallodynic and antihyperalgesic effects than those observed in an animal treated with the drug from the time of the CFA injection.

4.4.5 Effect of Delayed Administration of α2-antiplasmin on CFA-Induced Changes in proNGF and mNGF Protein Levels

We studied changes in protein levels of both forms of NGF on the 14th day after two weeks of chronic injections of α2-antiplasmin (8 μg, i.pl.), at 4 weeks after CFA administration. We observed an increase in the protein levels of mNGF in the ipsilateral paw of vehicle-treated CFA rats compared to the ipsilateral paw of sham rats (shown as an interrupted line on the graphs; Figure 5A), and also when compared to contralateral paw samples from vehicle-treated CFA rats (data not shown). The repeated treatment with α2-antiplasmin (8 μg, i.pl.) reduced the mNGF protein level in the ipsilateral paw compared to the ipsilateral paw of CFA-injected rats which were not treated with α2-antiplasmin. No significant changes in the protein levels of proNGF were detected by Western blot analysis in the ipsilateral paw of vehicle-treated CFA rats compared to the ipsilateral paw of sham rats (shown as an interrupted line on the graphs; Figure 5B) and to the contralateral paw of vehicle-treated CFA rats (data not shown). Repeated administration of α2-antiplasmin had no effect on protein levels of proNGF.

4.4.6 Influence of Repeated α2-antiplasmin Administration on the Sympathetic Fibers and Sensory Fibers Density in the Glabrous Skin of CFA-Injected Rats

In the current study, we hypothesized that elevated levels of endogenous mNGF following CFA injection would lead to sympathetic fiber sprouting in the skin. Indeed, there was an invasion of the upper dermis by sympathetic fibers, as detected by VMAT-2 immunoreactivity, in skin samples of the vehicle-treated CFA rats at 4 weeks after CFA injection (Figure 6). The increased number of sympathetic fibers in the scanned area of the

upper dermis was statistically significant when compared to the same area of the upper dermis from sham animals. At 2 weeks after CFA injection there was only a trend to an increase in the number of sympathetic fibers. Repeated treatment with α2-antiplasmin blocked the CFA-induced sprouting of sympathetic fibers into the upper dermis (Figure 6).

Interestingly, the density of peptidergic sensory fibers, as detected by CGRP immunoreactivity, did not change following CFA injection compared to sham levels (Figure 7). Also, repeated treatment with α2-antiplasmin did not have any effect on the peptidergic sensory fiber density (Figure 7).

4.4.7 Influence of Repeated α2-antiplasmin Administration on TRPV1 and TrkA Expression in DRG Cell Bodies from Ankle Nociceptive Afferents of CFA-Injected Rats

We evaluated TRPV1 (Figure 8) and TrkA (Figure 9) expression in the cell bodies of small diameter primary afferents from the ankle joint as a proof of concept to assess the specificity of α2-antiplasmin action in inhibiting the conversion of NGF maturation in inflamed tissues. In animals with CFA injection, at the 2 weeks time point, there was a net increase in neurons co-localizing the tracer (FG) with TRPV1 and TrkA immunoreactivities (Figure 8D-E; Figure 9D-E). In contrast, the treatment with CFA followed by α2-antiplasmin neutralized this increase in co-localization (Figure 8G-H; Figure 9G-H). The quantitative analysis revealed the following TRPV1/FG co-localization percentages for the four groups: Sham+Vehicle (V), 19.4 ± 0.4 %; CFA+V, 48.7 ± 3.4%; CFA+ α2-antiplasmin, 15.7 ± 4.7% (Figure 8J). TrkA/FG co-localization percentages were: Sham+V, 17 ± 9.0%; CFA+V, 55.1 ± 9.9%; CFA+α2-antiplasmin, 20.6 ± 11.9% (Figure 9J). Statistical analysis revealed that CFA+V group differed significantly from all other groups when comparing co-localization

percentages in the L4 DRG ($p<0.001$). This indicates that a significantly greater number of FG-positive cells from the CFA+V group expressed TRPV1 or TrkA compared to the CFA+α2-antiplasmin and Sham (no CFA) groups. There was no difference in co-localization percentages between the treatment and Sham groups, indicating that treating CFA-injected animals with α2-antiplasmin kept TRPV1 and TrkA at baseline levels. No significant difference ($p>0.05$) in the number of FG-positive cells was found between the four groups.

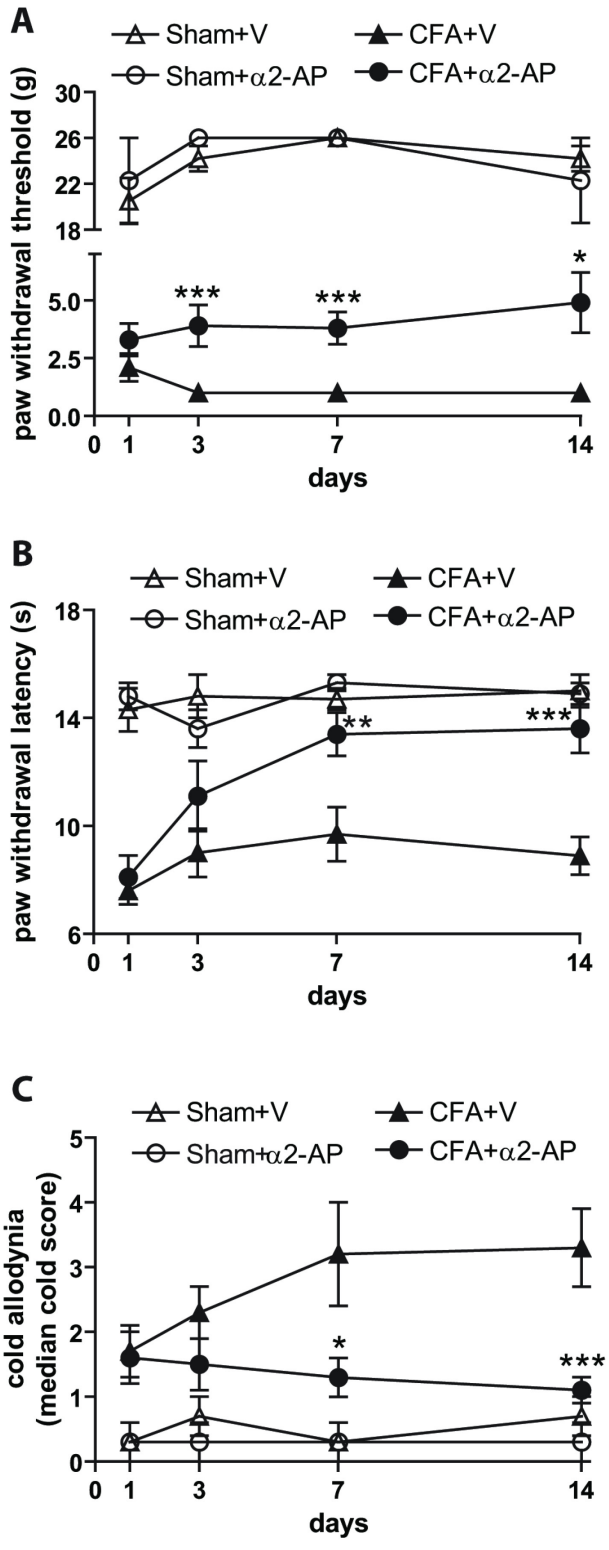

A
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
paw withdrawal threshold (g)
30
26
22
18
5.0
2.5
0.0
***
***
*
0
1
3
7
14
days
B
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
paw withdrawal latency (s)
18
14
10
6
**
***
0
1
3
7
14
days
C
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
cold allodynia
(median cold score)
5
4
3
2
1
0
*
***
0
1
3
7
14
days

Figure 1

Figure 1: Chronic treatment with α2-antiplasmin decreased the CFA-induced allodynia and hyperalgesia. Repeated treatment with α2-antiplasmin (α2-AP; 8 µg, i.pl.), starting at one hour after CFA injection, and then once daily for 14 days, significantly attenuated CFA-induced mechanical and cold allodynia and thermal hyperalgesia as measured by von Frey, acetone and Hargreaves tests, respectively. Behavior: the von Frey (A), Hargreaves (B) and acetone (C) tests were performed 60 min after α2-antiplasmin administration on the 1, 3, 7 and 14^{th} days after CFA injection. Results are presented as means ± SEM (n = 6-10 rats/group). Inter-group differences were analyzed by Bonferroni's multiple comparison tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to vehicle-treated (V) CFA rats.

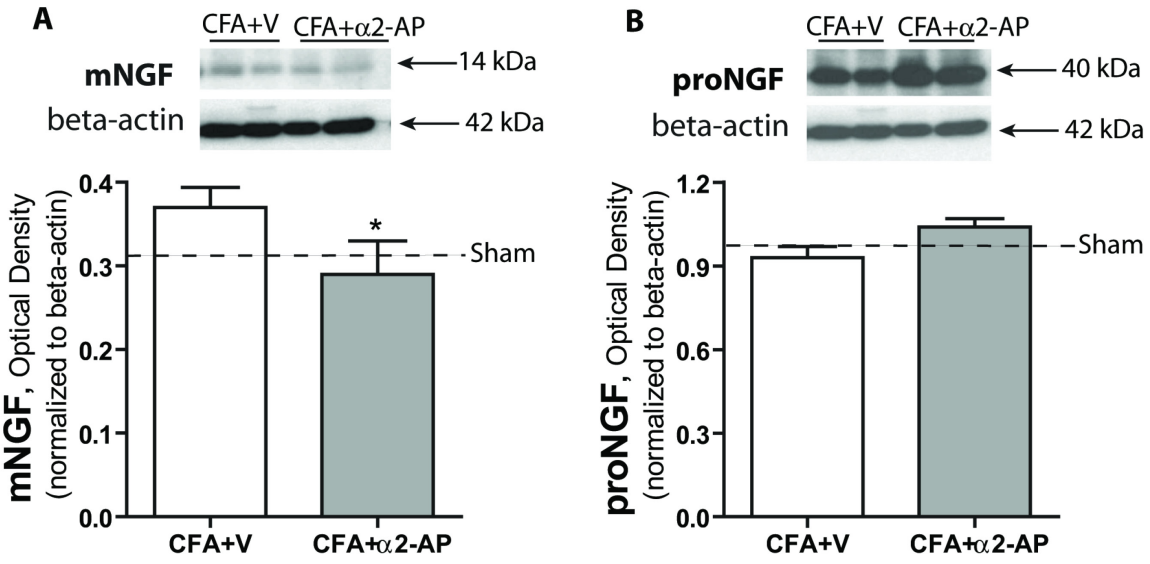
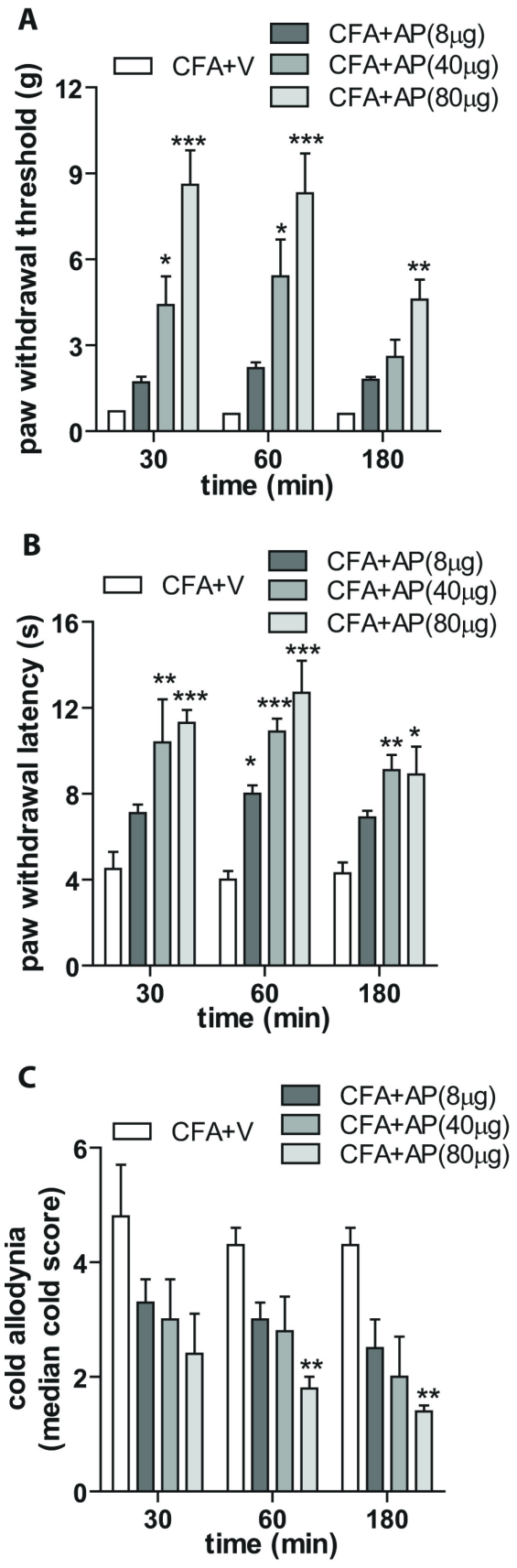


Figure 2: Chronic treatment with α2-antiplasmin influenced CFA-induced changes in the protein levels of NGF in the rat skin. Western blot analyses performed on glabrous skin samples at two weeks after CFA injection showed a significant increase in the protein levels of mature NGF (mNGF) (A), with no influence on the protein level of the precursor proNGF (n = 4) (B). Repeated treatment with α2-antiplasmin (α2-AP; 8 µg, i.pl.), starting at one hour after CFA injection and then once daily for 14 days, reduced the CFA-induced increase in mNGF protein levels; however, it had no effect on proNGF levels. Representative western blots are presented in the upper panels A and B. The densitometry results are presented as the means ± SEM from all samples. Inter-group differences were analyzed by ANOVA with a Bonferroni's multiple comparison test. *$p<0.05$ indicates a significant difference compared to glabrous skin sample from vehicle-treated (V) CFA rats. The interrupted line on the graphs indicates protein analyses for mNGF and proNGF in the ipsilateral glabrous skin of vehicle-treated sham rats.


A
CFA+V
CFA+AP(8μg)
CFA+AP(40μg)
CFA+AP(80μg)
paw withdrawal threshold (g)
12
9
6
3
0
*
***
*
***
**
30
60
180
time (min)
B
CFA+V
CFA+AP(8μg)
CFA+AP(40μg)
CFA+AP(80μg)
paw withdrawal latency (s)
16
12
8
4
0
**
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*
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*
30
60
180
time (min)
C
CFA+V
CFA+AP(8μg)
CFA+AP(40μg)
CFA+AP(80μg)
cold allodynia
(median cold score)
6
4
2
0
**
**
30
60
180
time (min)

Figure 3

Figure 3: A single injection of α2-antiplasmin decreased CFA-induced allodynia and hyperalgesia. A single administration of α2-antiplasmin (α2-AP; 8, 40, 80 μg, i.pl.) at two weeks after CFA injection, attenuated CFA-induced mechanical and cold allodynia and thermal hyperalgesia in a dose-dependent manner as measured by von Frey, acetone and Hargreaves tests, respectively. Behavior: the von Frey (A), Hargreaves (B) and acetone (C) tests were performed at 30, 60 and 180 minutes after α2-antiplasmin administration on the 14^{th} day after CFA injection. Results are presented as the means ± SEM (n = 6-8 rats/group). Inter-group differences were analyzed by Bonferroni's multiple comparison tests. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared to vehicle-treated (V) CFA rats.

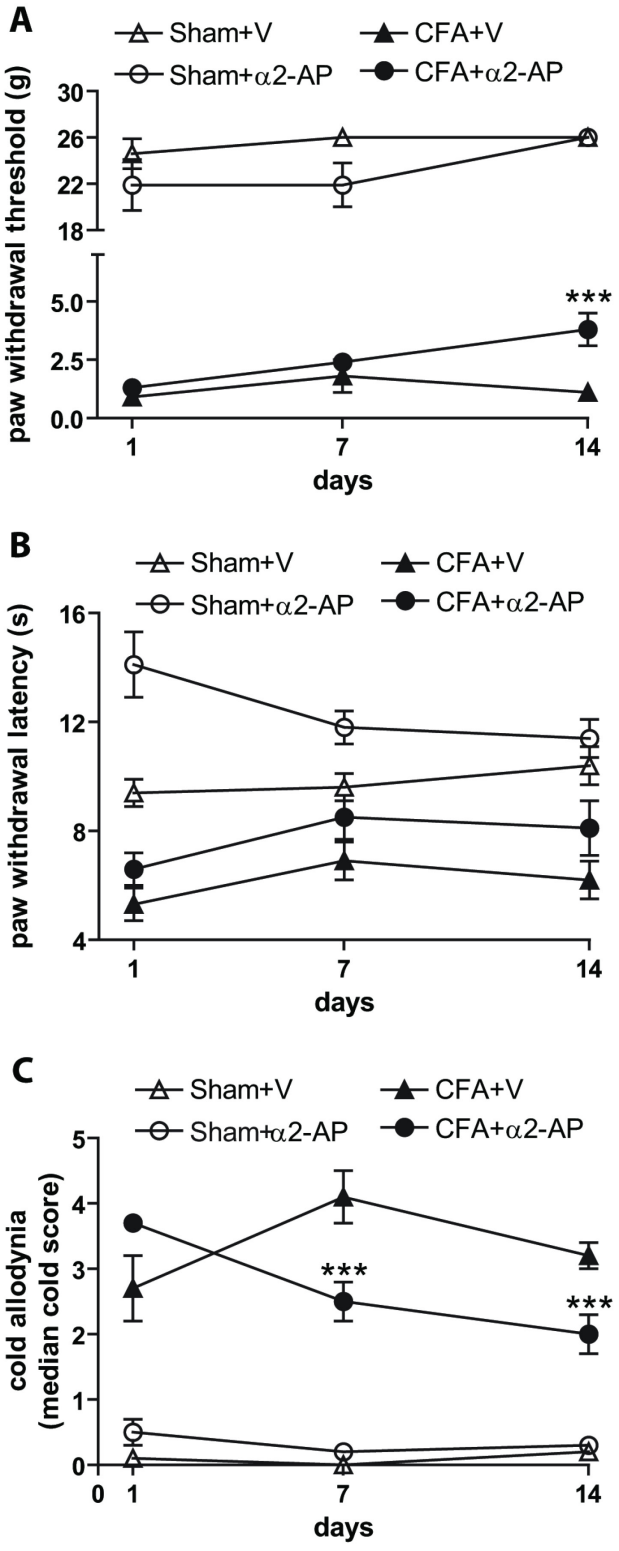

A
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
paw withdrawal threshold (g)
30
26
22
18
5.0
2.5
0.0
***
1
7
14
days
B
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
paw withdrawal latency (s)
16
12
8
4
1
7
14
days
C
Sham+V
CFA+V
Sham+α2-AP
CFA+α2-AP
cold allodynia
(median cold score)
5
4
3
2
1
0
***
***
0
1
7
14
days

Figure 4

Figure 4: Delayed onset chronic treatment with α2-antiplasmin decreased CFA-induced allodynia. Repeated injections of α2-antiplasmin (α2-AP; 8 μg, i.pl.), starting at two weeks after CFA injection and then once daily for 14 consecutive days, significantly attenuated CFA-induced mechanical and cold allodynia but not thermal hyperalgesia as measured by von Frey, acetone and Hargreaves tests, respectively. Behavior: the von Frey (A), Hargreaves (B) and acetone (C) tests were performed 60 min after α2-antiplasmin administration on the 1, 7 and 14^{th} days after CFA injection. Results are presented as the mean ± SEM (n = 6-10 rats/group). Inter-group differences were analyzed by Bonferroni's multiple comparison test. ***$p<0.001$, compared to vehicle-treated (V) CFA rats.

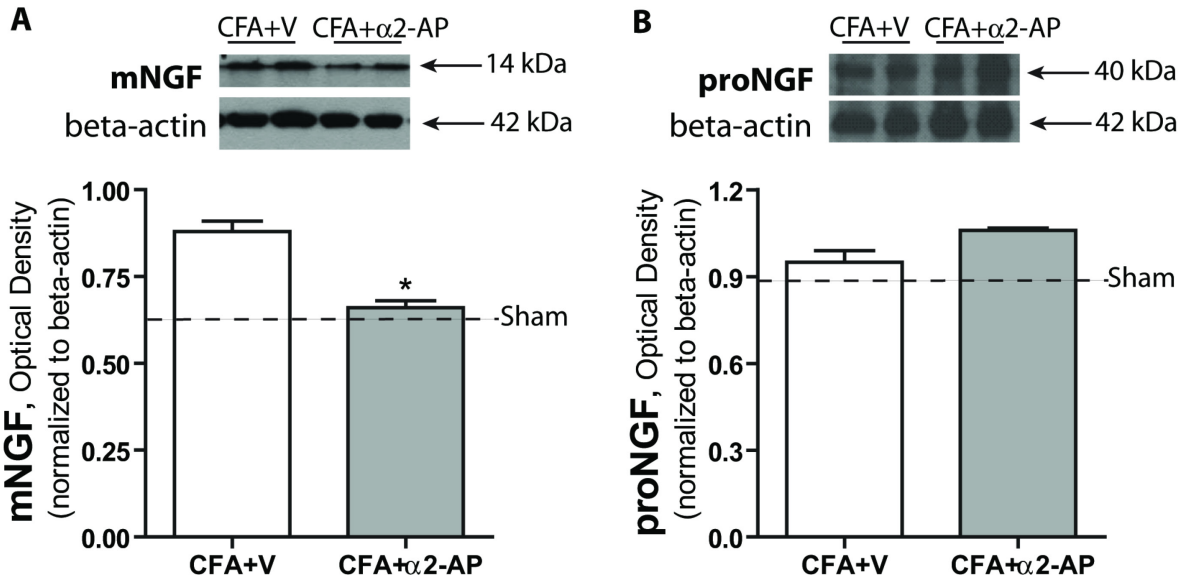
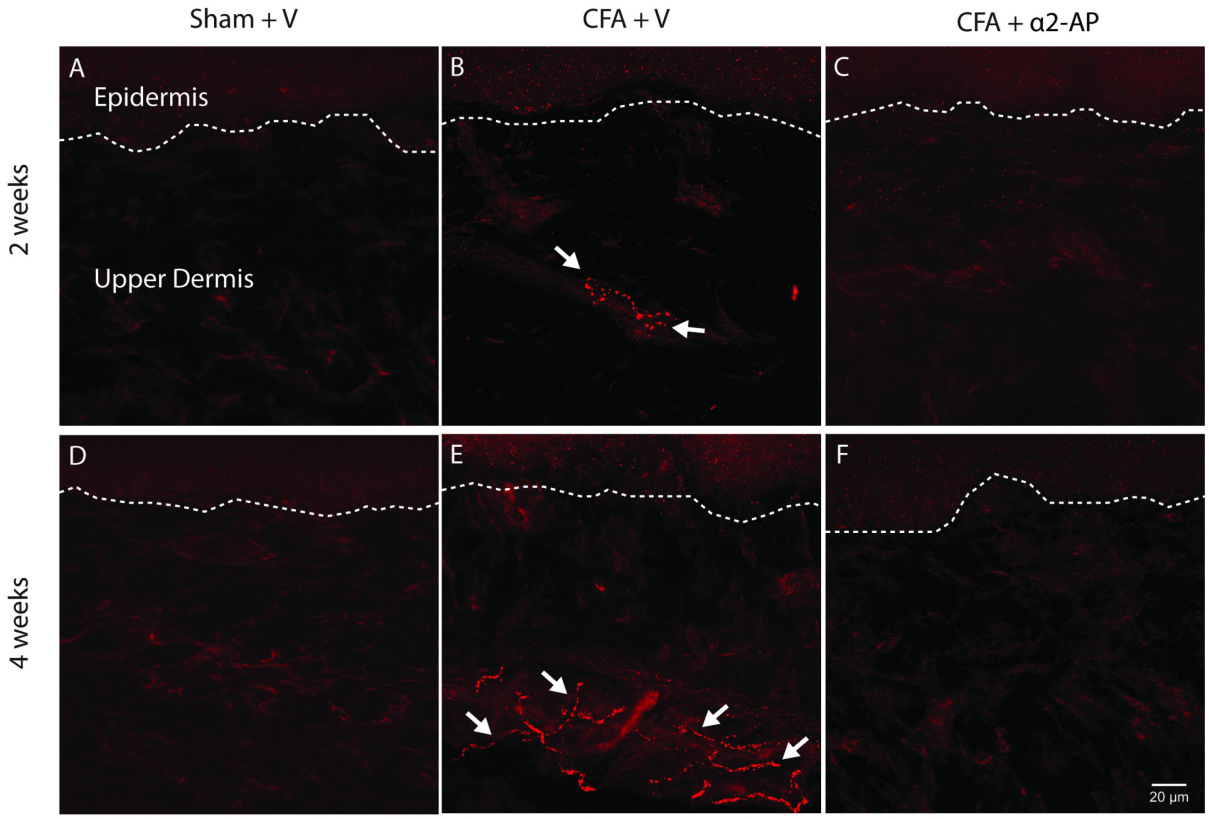


Figure 5: Delayed onset chronic treatment with α2-antiplasmin influenced the CFA-induced changes in NGF protein levels in the rat skin. Western blot analysis performed on samples of glabrous skin adjacent to the joints at 4 weeks after CFA injection showed a significant increase in the protein levels of mature NGF (mNGF) (A), with no influence on protein levels of the precursor proNGF (n = 4) (B). Repeated treatment with α2-antiplasmin (α2-AP; 8 µg, i.pl.), starting at 2 weeks after CFA injection and then once daily for 14 days, reduced the CFA-increased mNGF protein levels, without any effect on proNGF levels. Representative western blots are shown in the upper panels A and B. The densitometry results are presented as the means ± SEM from all samples. Inter-group differences were analyzed by ANOVA with a Bonferroni's multiple comparison test. *$p<0.05$ indicates a significant difference compared to glabrous skin sample from vehicle-treated (V) CFA rats. The interrupted line on the graphs indicates protein analyses for mNGF and proNGF in the ipsilateral glabrous skin of vehicle-treated sham rats.


Sham + V
CFA + V
CFA + α2-AP
2 weeks
4 weeks
A
Epidermis
Upper Dermis
B
C
D
E
F
20 μm

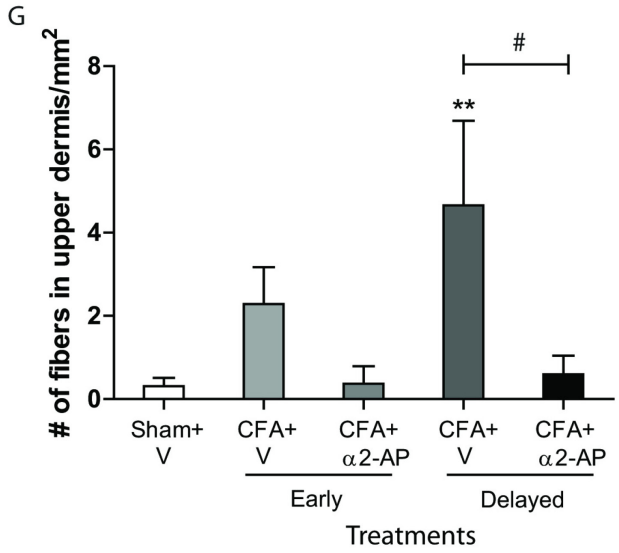
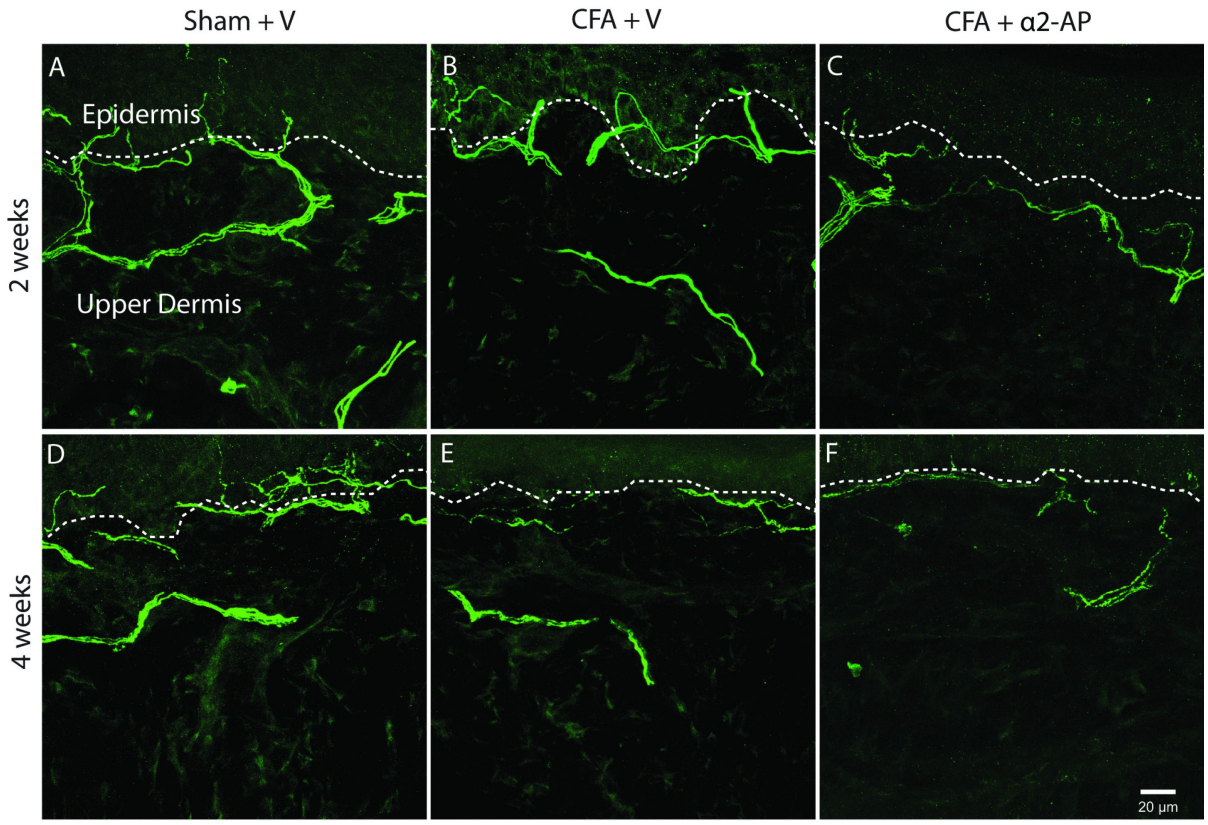

G
# of fibers in upper dermis/mm2
0
2
4
6
8
#
**
Sham+ V
CFA+ V
CFA+ α2-AP
CFA+ V
CFA+ α2-AP
Early
Delayed
Treatments

Figure 6

Figure 6: Changes in the sympathetic fiber innervation following chronic treatment with α2-antiplasmin in the skin of CFA-injected rats. At the two week time point after CFA injection, there was trend to an increase in the number of sympathetic fibers in the upper dermis (B, G). The number of sympathetic fibers is the upper dermis was significantly increased only at four weeks following CFA administration (E, G). This fiber population was virtually absent from the upper dermis of vehicle-treated sham animals as shown in A and D. Repeated administration of α2-antiplasmin starting either at one hour after CFA injection (C) or two weeks after CFA injection (F) reversed the CFA-induced sprouting of sympathetic fibers into the upper dermis. Arrows in B and E indicate VMAT2-IR (sympathetic) fibers in the upper dermis. The results in G are presented as means ± SEM (n = 6). Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. **$p<0.01$ indicates a significant difference compared to glabrous skin samples from vehicle-treated sham animals. #$p<0.05$ indicates a significant difference compared to glabrous skin samples of vehicle-treated CFA rats. The dashed line represents the dermal-epidermal junction. White arrows indicate sympathetic fibers.


Sham + V
CFA + V
CFA + α2-AP
A
B
C
Epidermis
Upper Dermis
2 weeks
D
E
F
4 weeks
20 µm

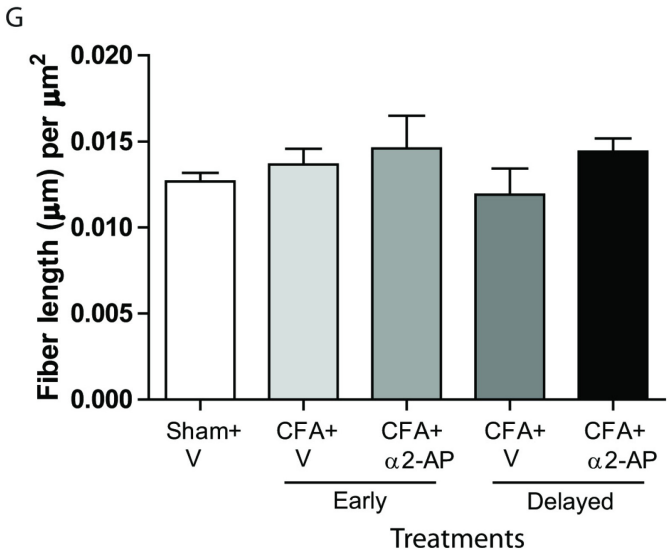

G
Fiber length (µm) per µm2
0.020
0.015
0.010
0.005
0.000
Sham+ V
CFA+ V
CFA+ α2-AP
CFA+ V
CFA+ α2-AP
Early
Delayed
Treatments

Figure 7

Figure 7: Changes in sensory peptidergic fiber innervation following chronic treatment with α2-antiplasmin in the skin of CFA-injected rats. The density of CGRP-positive fibers at two (B) and four (E) weeks after CFA injection did not change significantly from sham levels (A, D). Also, chronic administration of α2-antiplasmin starting either at one hour after CFA injection (C) or at two weeks after CFA injection (F) did not influence the density of CGPR-positive fibers (G). The values in G represent means ± SEM. Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. The dashed line represents the dermal-epidermal junction.

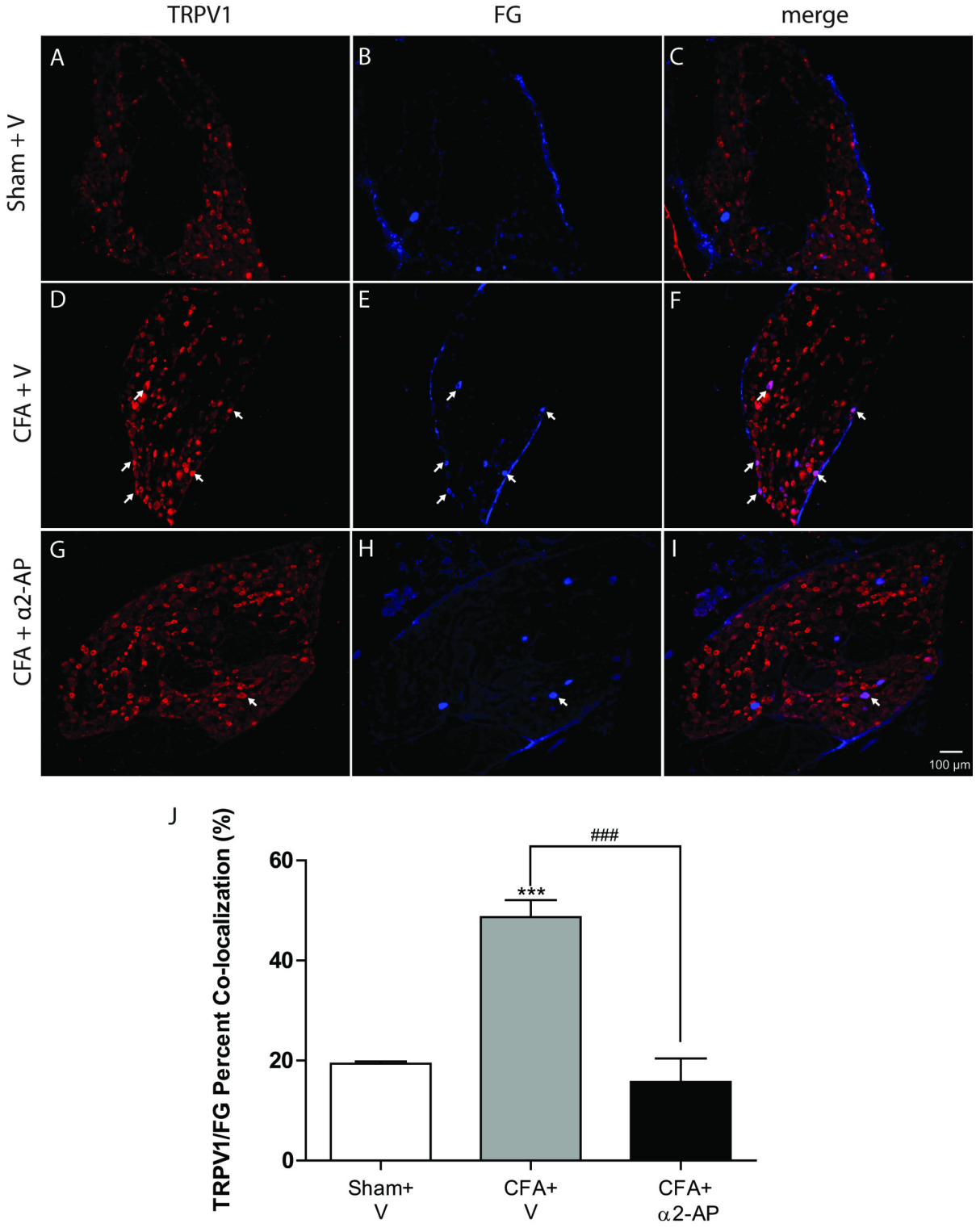

TRPV1
FG
merge
Sham + V
CFA + V
CFA + α2-AP
A
B
C
D
E
F
G
H
I
100 µm
J
TRPV1/FG Percent Co-localization (%)
60
40
20
0
###
***
Sham+ V
CFA+ V
CFA+ α2-AP

Figure 8

Figure 8: Changes in TRPV1 expression in the DRG neurons following chronic treatment with α2-antiplasmin in CFA-injected rats. A, D, and G show TRPV1 immunoreactivity. B, E, and H show signal from the fluorogold tracer that labeled retrogradely neurons innervating the ankle joint. Following CFA administration there was a significant increase in the number of TRPV1- and FG-positive neurons in the L4 DRG ($p<0.001$). Chronic administration of α2-antiplasmin starting at 1 hour after CFA injection, effectively prevented the increase in the number of TRPV1- and FG-positive neurons observed in vehicle-treated CFA rats. The results in J represent the means ± SEM. Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. ***$p<0.001$ indicates a significant difference compared to vehicle-treated (V) Sham rats; ###$p<0.001$ indicates a significant difference compared to vehicle-treated (V) CFA rats. White arrows point to FG-positive cell bodies that colocalize with TRPV1 immunoreactivity (n = 4-6).

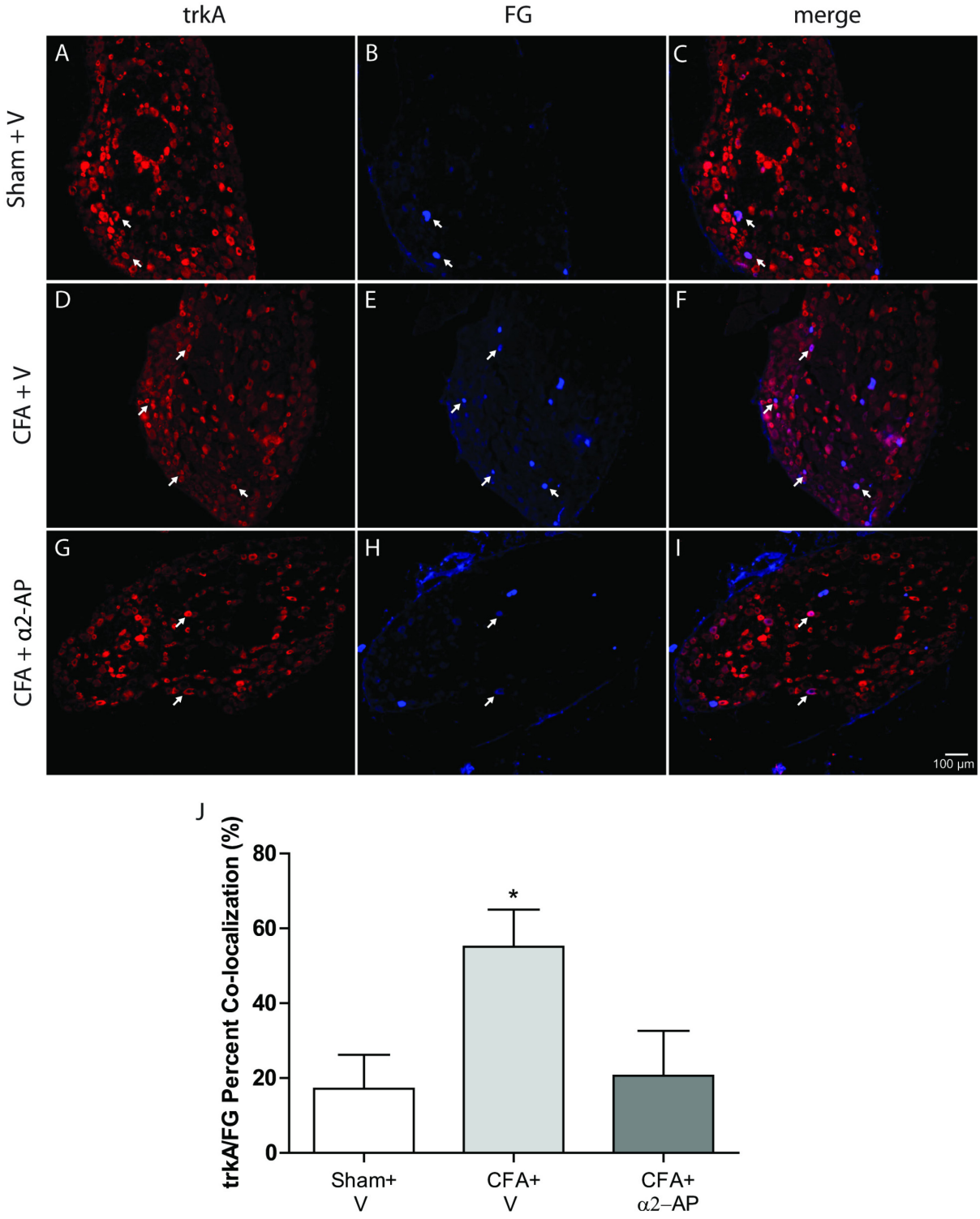

trkA
FG
merge
Sham + V
CFA + V
CFA + α2-AP
A
B
C
D
E
F
G
H
I
100 µm
J
trkA/FG Percent Co-localization (%)
80
60
40
20
0
*
Sham+ V
CFA+ V
CFA+ α2–AP

Figure 9

Figure 9: Changes in the TrkA expression in the DRG neurons following chronic treatment with α2-antiplasmin in CFA-injected rats. A, D, and G show TrkA immunoreactivity. B, E, and H show signal from the fluorogold tracer that labeled retrogradely neurons innervating the ankle joint. In the L4 DRG, the vehicle-treated CFA group had a significant increase in the number of TrkA- and FG-positive neurons ($p<0.05$). Repeated α2-antiplasmin administration effectively prevented the increase in the number of TrkA- and FG-positive neurons observed in vehicle-treated CFA rats. The values in J represent the means ± SEM. Inter-group differences were analyzed by ANOVA with a Dunnett's post hoc test. *$p<0.05$ indicates a significant difference compared to vehicle-treated (V) Sham rats. White arrows point to FG-positive cell bodies that colocalize TrkA immunoreactivity (n = 4-6).

4.5 Discussion

The present study provides novel evidence suggesting that inflammatory pain associated with arthritis may be managed by reducing the abnormally elevated mNGF levels in the region of the lesion. We have shown for the first time that intra-plantar administration of a plasmin inhibitor (α2-antiplasmin) produced some alleviation of CFA-induced hypersensitivity to mechanical and thermal stimuli in rats. Our data also demonstrate that CFA-induced changes in the protein levels of mNGF were normalized in skin following repeated α2-antiplasmin injections. Furthermore, we found that α2-antiplasmin blocked the CFA-induced sprouting of sympathetic fibers into the upper dermis in the glabrous hind paw skin adjacent to the joint, but had no effect on the density of sensory peptidergic fibers. Moreover, α2-antiplasmin blocked the upregulation of TRPV1 and TrkA in DRG cell bodies corresponding to ankle joint afferents.

Several studies have reported increased expression and release of NGF, as well as of its receptors, in different peripheral tissues including skin, synovial fluid and sciatic nerve in inflammatory conditions associated with pain both in animal models and in humans (Aloe et al., 1992, Woolf et al., 1994, Laird et al., 1997, Halliday et al., 1998, Sivilia et al., 2008, Barthel et al., 2009). Furthermore, exogenous NGF administration evoked nociceptive behavior in animals (Lewin et al., 1993, Andreev et al., 1995, Malin et al., 2006) and induced pain in humans (Petty et al., 1994, Dyck et al., 1997). Therefore, antagonizing NGF should provide therapeutic opportunities in pain management, specifically in pain associated with arthritis. One of the strategies that aimed at alleviating pain via NGF antagonism is through the use of anti-NGF antibodies (Eibl et al., 2012). Antiallodynic and antihyperalgesic effects of anti-NGF antibodies have been shown in many pre-clinical studies in which different

models of inflammation or neuropathy were used, including rodent models of CFA-induced inflammation (Ghilardi et al., 2012), chronic pancreatitis (Zhu et al., 2012), bone cancer pain (Mantyh et al., 2010), fracture pain (Koewler et al., 2007), and neuropathic pain (Ramer and Bisby, 1999). Clinical trials with humanized monoclonal anti-NGF antibodies alleviated pain in patients with chronic pain conditions, such as osteoarthritis (Lane et al., 2010, Nagashima et al., 2011, Schnitzer et al., 2011), low back pain (Katz et al., 2011) and interstitial cystitis (Evans et al., 2011). However, significant side effects were reported during the trials, including, in some cases, bone necrosis requiring joint replacement (Lane et al., 2010, Wood, 2010), which raised some serious questions regarding the use in the clinic of anti-NGF antibodies. It is possible that NGF may have significant functions in normal bone and that its levels should not be lowered excessively, what may be very well the case with anti-NGF antibodies.

Previous studies indicated the existence in the CNS of a protease cascade responsible for the conversion of proNGF into mNGF and also the possibility of its modulation *in vivo* (Bruno and Cuello, 2006, Allard et al., 2012). Bruno and Cuello (2006) suggested that lowering the levels of mNGF in pathological conditions associated with pain such as chronic arthritis might be of clinical interest. In the current study, we show that by inhibiting plasmin, we were able to reduce abnormally elevated mNGF levels and alleviate allodynia and hyperalgesia in rats with inflammatory arthritis. Our results confirmed that the repeated intraplantar administration of a small dose of α2-antiplasmin attenuated the development of behavioral hypersensitivity after CFA injection in rats, but had smaller effects on the already established allodynia and hyperalgesia when the onset of treatment was delayed. Moreover, a single administration of α2-antiplasmin, at a ten times higher dose, significantly reduced the

CFA-induced hyperalgesia. The less effectiveness when the α2-antiplasmin treatment was started only at two weeks post-CFA compared to immediately after CFA may be a consequence of the fact that, in the later phase of inflammation, a broader spectrum of different peripheral and central components is involved in the hyperalgesia, such as infiltration of mononuclear cells, neurogenic inflammation, synovial hyperplasia, cartilage and bone destruction, as well as central neuroimmune activation (Gough et al., 1994, DeLeo et al., 2004, Almarestani et al., 2011). Therefore, inhibiting only one of the elements contributing to the inflammation is not sufficient to completely reverse the progression of pain, particularly because at the late stages it also arises from changes in the joint and bone. A longer period of treatment with α2-antiplasmin might be required to achieve more effective therapeutic outcomes. Further studies may clarify these issues.

A surprising result from this study was the lack of a proNGF accumulation following chronic α2-antiplasmin administration, contrary to what was recently shown in the medial prefrontal cortex of normal adult rats (Allard et al., 2012). Different mechanisms might be involved in proNGF levels regulation in periphery compared to the CNS, what could explain such difference in effects.

As it has been previously shown, NGF plays a role in inflammatory hyperalgesia as a result of its indirect action on sympathetic neurons (Andreev et al., 1995, Woolf et al., 1996). In the current study we observed an increase in mNGF levels following CFA injection. These elevated mNGF levels likely triggered sympathetic fiber sprouting into the upper dermis, a region of skin normally devoid of them. Interestingly, we did not observe any changes in the density of peptidergic sensory fibers following CFA injections. Our results revealed that the repeated α2-antiplasmin administration, likely through a normalization of elevated mNGF

levels, blocked the CFA-induced sympathetic fiber sprouting. It has been previously reported that sympathetic fibers are more responsive to NGF in the adult as they depend on it for survival, although NGF still plays a role in maintaining the normal phenotype of sensory peptidergic neurons (Gorin and Johnson, 1980, Lewin and Barde, 1996). Therefore, the lack of peptidergic sprouting in the present study would suggest that the levels of mNGF in the adjacent skin, following CFA injection in the joint, were not sufficient to induce sensory fiber sprouting. These observations are in agreement with a recent study from our laboratory using the same arthritis model in which we observed an identical sprouting of sympathetic, but not peptidergic, fibers in skin (Longo et al., 2013). We also observed that the sprouted sympathetic fibers were not associated with blood vessels as they normally are, but often wrapped around the sensory peptidergic fibers suggesting enhanced sympathetic-sensory interactions. This observation is in agreement with what we previously observed in the same model (Longo et al., 2013) and is comparable to what we observed in an inflammatory polyarthritis model (Almarestani et al., 2008) and in neuropathic pain models (Yen et al., 2006a).

The above observations support the concept that under physiological conditions sympathetic fibers do not play a significant role in nociception, but following plastic changes induced by inflammation or nerve injury they do play a role in the triggering or maintenance of the pain (Campbell et al., 1989). Interestingly, it was observed in this model that sympathetic fiber function suppression with Guanethidine led to an alleviation of pain-related behavior at 4 weeks post-CFA, when sympathetic sprouting was present, but not at 2 weeks, a time point in which there was no significant sprouting (Longo et al., 2013). The most likely mechanism of this pro-nociceptive effect of the sprouted fibers is through the release of

noradrenaline acting on adrenergic receptors localized on sensory neurons, causing increase in their excitability and spontaneous activity (McLachlan et al., 1993, von Kugelgen et al., 1994, Xie et al., 2010). A role for noradrenaline in pain sensitization has been postulated many years ago (Nathan, 1947).

There is evidence from the literature that the elevated levels of NGF have influence on other inflammatory mediators, receptors or channels, by regulating the transcriptional factors locally, at the site of inflammation, in the sensory signaling pathway, or in the DRG (Mantyh et al., 2011). Along this line, we also studied changes in TRPV1 and TrkA in the DRG neurons innervating the ankle joint, and observed an upregulation of both TRPV1 and TrkA. Interestingly, such upregulation was blocked by the α2-antiplasmin administration, providing evidence that NGF is involved, at least in part, in the inflammation-induced over-expression of TRPV1 and TrkA. This concurs with previous studies showing that exogenous NGF administration can increase the TRPV1 expression and that anti-NGF treatment can suppress TRPV1 upregulation induced by inflammation (Ji et al., 2002). This effect might be a consequence of a direct or indirect influence of NGF on hypersensitivity in inflammation. Our data is also in agreement with a previous immunohistochemical study which showed that after inflammation the number of TRPV1-positive neurons increased and that TRPV1 was detectable in neurons corresponding to A-delta fibers (Amaya et al., 2003). As in other comparable studies in the DRG, the changes in the number of neurons expressing TRPV1 or TrkA just represent changes in the detection of the marker by immunocytochemistry and are a reflection of changes in protein expression levels. They do not mean that populations of neurons express *de novo* TRPV1 or TrkA after CFA.

In conclusion, in an inflammatory arthritis model, a pharmacologically induced chronic downregulation of the maturation of proNGF to mNGF led to a normalization of abnormally elevated levels of mNGF. This was accompanied by a blockade of sympathetic fiber sprouting and alleviation of hypersensitivity to mechanical and thermal stimuli. Our data provide evidence that the metabolic processing of endogenous NGF can be modulated in the periphery in a manner similar to what was previously demonstrated in the central nervous system. This raises the interesting possibility that elevated levels of mNGF can be reduced to alleviate pain in arthritis.

4.6 Acknowledgements

The authors would like to thank Dr. Gary Bennett for allowing us to use his animal behavior testing facility. This work was supported by grants from CIHR, Louise and Alan Edwards Foundation, and the Tri-Council MITACS Accelerate Program in partnership with Pfizer Canada.

Chapter 5

General Discussion

5.1 Overview

The work presented in this thesis expands on our current understanding about the role of NGF in inflammatory arthritis and provides evidence suggesting the occurrence of new mechanisms driving the chronic pain associated with this condition.

In our second chapter, we described a reorganization of nerve fibers in the joint and adjacent skin following inflammation of the joint. The observations made in this study suggest a significant involvement of the sympathetic fiber population, as evidenced by the hyperinnervation of the synovial membrane by sympathetic efferents and the ectopic presence of these fibers in the upper dermis of the skin at 4 weeks after the induction of arthritis. This correlates with a significant increase in mNGF levels. Blocking sympathetic function alleviated the pain sensitivity only when these sympathetic fibers were already present in significant numbers in the upper dermis and associated with the peptidergic C fibers.

In the third chapter, a proof of principle study was carried out to validate if the metabolic processing of endogenous NGF is similar in the periphery to that of the CNS. We observed that inhibiting MMP-9 in naïve rats induced sprouting of sympathetic fibers into the upper dermis of the skin. Moreover, these sympathetic fibers were found to wrap around the peptidergic nociceptive afferents, similar to what was observed in the skin following chronic inflammation as described in Chapter 2. Thus, we propose that the observed hypersensitivity to pain stimuli is, at least in part, a consequence of elevated endogenous mNGF levels, leading to sensitization of peptidergic primary afferents and abnormal sensory-autonomic fiber coupling in the skin.

Lastly, our fourth chapter extends upon the results presented in both chapters 2 and 3, which indicate that sympathetic sprouting is likely NGF-related. We demonstrated that the NGF processing cascade can be modulated by interfering with plasmin, the enzyme responsible for converting proNGF to mNGF. Indeed, following the administration of the plasmin inhibitor α2-antiplasmin, mNGF levels in skin adjacent to the inflammed joint were reduced back to the endogenous baseline levels. Furthermore, we found a direct correlation between this decrease in mNGF and the reduction in the hypersensitivity to stimuli in our α2-antiplasmin-treated CFA-injected animals compared to the vehicle-treated CFA-injected animals.

The significance of our results will be discussed in the next sections. We will suggest future experiments in order to continue this line of research. Finally, we will also point out the potential caveats attributed to our studies, being that we were limited in using research techniques and expertise available to us at the time the experiments were performed.

5.2 Sympathetic Fiber Involvement in Pain

In this body of work, we have demonstrated that sympathetic fiber efferents display a dynamic innervation pattern. The VMAT-2 antibody was used to visualize the sprouting of sympathetic efferents into the upper dermis of the skin in a time-dependent manner post-CFA injection. As reported in other studies from our group, our results are similar to those observed in neuropathic pain models, in which an ectopic presence of sympathetic fibers in the upper dermis following nerve injury occurs (Ruocco et al., 2000, Grelik et al., 2005, Yen et al., 2006b). Likewise, we rarely found any sympathetic fibers in this region in sham animals. The striking similarity of this sympathetic invasion into the upper dermis and

association with peptidergic fibers between arthritis and neuropathic pain models may be related to a presence of a neuropathic component in arthritis, as suggested in a previous study from our lab (Almarestani et al., 2008). However, if we consider only the monoarthritis model used in this thesis, we do not observe any change in the peptidergic C fiber population post-CFA injection, contrary to what is observed in chronic constriction injury (CCI) or other nerve ligation models. This may be explained by the fact that there is no physical nerve damage in skin in our model and that the observed plastic changes are likely triggered by a surge of inflammatory mediators, ultimately leading to an abnormal source of NGF. The issue that in the localized polyarthritis model, in which CFA was injected in skin adjacent to the joints, there was also peptidergic fiber sprouting lends support to this hypothesis. Indeed, in this latter model there is strong skin inflammation (Almarestani et al., 2008). These differences raise interesting questions regarding the possible mechanisms underlying the development of the pathologies in each chronic pain model, as well as the relative contribution of inflammation and nerve injury to the morphological changes affecting NGF-responsive nerves.

5.2.1 Alpha Adrenergic Receptors

Previous studies have shown that cutaneous administration of noradrenaline in healthy subjects does not evoke pain, although it may induce selective hyperalgesia to thermal stimulation (Fuchs et al., 2001). On the other hand, in pathological conditions, peripheral noradrenaline might play a role on the nerve endings mediating pain. This was shown by the finding that administration of noradrenaline or adrenaline in inflamed or neuropathic skin in humans exacerbated the associated hyperalgesia (Chabal et al., 1992, Choi and Rowbotham, 1997, Ali et al., 2000). Also, inflammation or sensitization of the

receptive field by algogenic chemicals may lead to conditions in which noradrenaline and/or sympathetic stimulation activate nociceptors (Roberts and Elardo, 1985a, Banik et al., 2004, Ren et al., 2005). We postulate that the noradrenaline released from the ectopic sympathetic fibers acts on novel or upregulated adrenoceptors on sensory fibers. Moreover, based on our abovementioned findings illustrating a close relationship between the ectopic sympathetic fibers and the peptidergic fibers, we believe that this phenomenon may contribute to the maintenance of unrelenting pain. However, the peripheral neurons are very complex since they co-express a variety of adrenoceptor subtypes (Gold et al., 1997). As a result, activation of these peripheral sensory fibers by noradrenaline is likely to contribute to a large range of noradrenaline-induced effects.

Following nerve injury, C fibers can develop new adrenergic receptors and sensitivity, which may help explain the mechanism of sympathetically maintained pain. Therefore, this atypical sympathetic fiber-mediated pain phenomenon may be explained by changes in the noradrenergic receptors expressed on sensory fibers. In agreement with this, some animal studies have provided evidence for the development of adrenergic sensitivity in nociceptive fibers contributing to the pronociceptive responses (Ouseph and Levine, 1995). The receptors involved in the hyperalgesia seem to be α1 adrenergic receptors (AR). It is known that noradrenaline released by the sympathetic efferents is capable of binding to α1- (Lee et al., 1999, Nam et al., 2000). Conversely, pain modulation is thought to be mediated via the α2-AR, where activation by agonists might induce a potent analgesic effect (Fairbanks et al., 2002). In addition, it has been shown that α1-AR agonist activity can counteract α2-AR agonist-induced analgesia (Gil et al., 2009). Furthermore, treatment with α1-AR antagonists was found to completely block the enhanced responses of C-fibers after

capsaicin injection in sympathetically intact animals (Ren et al., 2005). On the other hand, antagonizing the α2-AR only slightly decreased the capsaicin-evoked responses (Ren et al., 2005). Thus, since Guanethidine depletes noradrenaline stores, its release from the ectopic sympathetic fibers will be suppressed, resulting in the elimination of the potential interaction between the ectopic sympathetic and sensory fibers in the upper dermis of the skin. This may explain the partial return of pain thresholds to baseline levels in mono-arthritic animals treated with Guanethidine.

Furthermore, the administration of an α2-AR agonist significantly attenuated postoperative pain caused by surgery of the knee joint in humans (Gentili et al., 1996). In a mouse model of arthritis, both an α2A-AR knockout or intra-articular, but not intrathecal, administration of an α2-AR antagonist reversed the antihyperalgesic effect caused by transcutaneous electrical stimulation (King et al., 2005). These finding suggest that the α2A adrenoceptors are involved in mediating the suppression of peripheral sensitization.

5.2.2 Future Experiments

5.2.2.1 Alpha adrenergic receptor density. In human patients with reflex sympathetic dystrophy, which is a form of neuropathic pain, the density of cutaneous α1-AR, measured using the selective radio-ligand 125I-hydroxyphenyl-ethyl-aminomethyl-tetralone (HEAT) and autoradiography to label α1-adrenoceptors, is significantly greater than in healthy control subjects suggesting that α1-AR may contribute to the hyperalgesia associated with this disorder (Drummond et al., 1996). A quantitative analysis of the density of these receptors on sensory neurons using immunohistochemistry coupled with confocal microscopy in our inflammatory pain model would help explain their functional role in pain. It would be interesting to look at the ratio between α1 to α2 adrenergic receptor density on C

fibers by double-labelling each α-AR receptor with an anti-CGRP antibody. Therefore, a shift in the balance towards a greater α1-AR concentration would indicate a greater pronociceptive output relayed by the peptidergic fibers. The major obstacle to perform such an experiment using modern approaches is that there is presently no satisfactory antibody against the α1-AR available. However, previous work by Stone's group, in which our lab was involved, shows by using confocal microscopy the presence of $\alpha 2_A$-adrenergic receptors in peptidergic afferents in skin of normal rats (Riedl et al., 2009).

To follow up on the issue of sympathetic and peptidergic fiber coupling, another experiment that should be carried out would be to observe this relationship at the ultrastructural level. Hopefully, the use of an electron microscope can reveal more information, such as the detection of physical close appositions or the possibility of synaptic contacts between the two fiber populations. This would help us understand the mechanisms at which sympathetically mediated pain may function by and determine whether it is merely a question of receptor expression density or also involves a physical interaction whereby sympathetic fibers synaptically activate the sensory nerve fibers.

5.2.2.2 Physiological function of sprouted sympathetic fibers. The systemic administration of Guanethidine is used as a pharmacological tool to block sympathetic function. This compound is a valuable tool to measure changes in sensitivity associated with the sympathetic system and was chosen because it does not cross the blood-brain-barrier (BBB) due to its high polarity (Freis, 1965), thereby avoiding any central effects on the behavior phenotype. For an alternative more direct assessment of the sprouted sympathetic fiber function, it would be attractive to carry out electrophysiological recordings from sensory fibers. For instance, isolated skin nerve preparations can be used to record electrical

discharges in peripheral nerve fibers in the upper dermis and assess whether the electrophysiological profile of these nerve fibers changes at different time points post-CFA injection and after administration of alpha adrenergic agonists and/or antagonists. However, our lab is not equipped with the technology to perform these measurements, although they could be done in collaboration with an electrophysiology laboratory.

5.3 Bone Hyperinnervation in Arthritis

The degenerative joint process accompanying synovitis results in joint pain in several patients (Myers et al., 1990). Unfortunately, the clinical severity of arthritis does not necessarily correspond to the radiological signs of bone damage making it a poor predictor of pain intensity (Silman, 2000). The idea that the nervous system may contribute to the pathophysiology of rheumatoid arthritis has already been proposed by Levine and colleagues in 1985 (Levine et al., 1985a). In Chapter 2, we provide morphological evidence suggesting a sympathetic and peptidergic C fiber coupling in the skin surrounding the joint, as well as hyperinnervation of the synovial membrane, which occur at a time the joint pathology is fully developed. In inflammatory arthritis, many neurons begin to fire spontaneously and others fire at much higher rates in response to stimulation (Salo, 1999). Although many of the key inflammation-associated mediators have been proposed to contribute to these changes in neuronal response properties, it was shown that almost all of the observed changes may be induced by locally increased NGF levels (Lewin and Mendell, 1994). Moreover, the increased density of peptidergic fibers in the joint suggests a greater role for neuropeptides whereby their release from the peripheral terminals of these fibers may trigger neurogenic

inflammation. However, this mechanism has not yet been fully investigated and its role is often underplayed in reviews about arthritis.

5.4 Peripheral NGF Processing Pathway

Although in agreement with what has been revealed in the CNS, the studies presented in this thesis are only scratching the surface and we are only beginning to understand the processing of NGF in the periphery.

5.4.1 Plasminogen/Plasmin Complex

The production of the serine proteinase plasmin from the extracellular zymogen plasminogen can be catalyzed by specific plasminogen activators. Besides the well-known role of plasminogen/plasmin in the fibrinolytic system and how it is critical for clot lysis, this complex system plays other biological roles (Lijnen, 2001). For example, plasmin is known to be important for ovulation, tumor invasion and metastasis (Ny et al., 1999). Plasmin also plays a direct or indirect role in cartilage and bone matrix degradation, contributing to inflammatory joint disease in RA (Del Rosso et al., 1999, van der Laan et al., 2000). It has also been shown to directly degrade extracellular matrix components including the glycoproteins fibronectin, laminin, and elastin, as well as proteoglycans (Kwaan, 1992, Andreasen et al., 2000). Additionally, plasmin indirectly contributes to matrix degradation by activating matrix metalloproteinases and proteoglycanases (Herren et al., 2003). Plasmin is also implicated in the recruitment of inflammatory mediators, and induces cytokine expression in human monocytes (Plow et al., 1999, Syrovets et al., 2001). Moreover, the zymogen plasminogen is present at micromolar concentrations in human plasma and in the extracellular fluid (Kwaan, 1992). In contrast, there are no known functions for plasminogen

itself, relegating it to the role of an inactive precursor. Since plasmin carries out many functions, its generation and activity are tightly regulated by specific activators and inhibitors. The two types of plasminogen activators (PA) are tissue-type PA (tPA) and urokinase-type PA (uPA) (Lijnen, 2001). On the other hand, as reported in this thesis in Chapter 4, α2-antiplasmin is the major endogenous inhibitor of plasmin. Therefore, besides our emphasis on using α2-antiplasmin to block the cleavage of proNGF into its mature form, the inhibition of plasmin itself may also provide potential therapeutic effects by attenuating plasmin's pro-inflammatory actions and its associated effects in the degradation of cartilage and bone. Interestingly, Li and colleagues have implicated plasmin as an essential component of the early phase of Rheumatoid arthritis (Li et al., 2005). They revealed that uPA-deficient mice have a lower severity and incidence of arthritis when compared to wild-type mice in the CIA model. This strengthens the argument that plasmin is essential for the induction of pathological inflammatory joint destruction in arthritis. An alternative approach to what was presented herein would be to target this enzyme upstream by inhibiting the conversion of plasminogen to plasmin, as opposed to inhibiting the plasmin itself with α2-antiplasmin. That is, to target the plasminogen activators, uPA or tPA. This treatment approach should provide comparable benefits to targeting plasmin itself with the inhibitor.

As mentioned above, plasmin is the key mediator of fibrinolysis. Thus, a valid concern about targeting plasmin is that this strategy may impact and favor the formation of fibrin clots. However, studies have shown that when α2-antiplasmin is added to human or rabbit plasma *in vitro*, tPA-induced fibrinogenolysis is inhibited but the lysis of fibrin clots is insignificantly affected (Weitz et al., 1993). In accordance, α2-antiplasmin supplementation also blocks tPA-induced fibrinogenolysis and is devoid of any major effects on clot lysis *in*

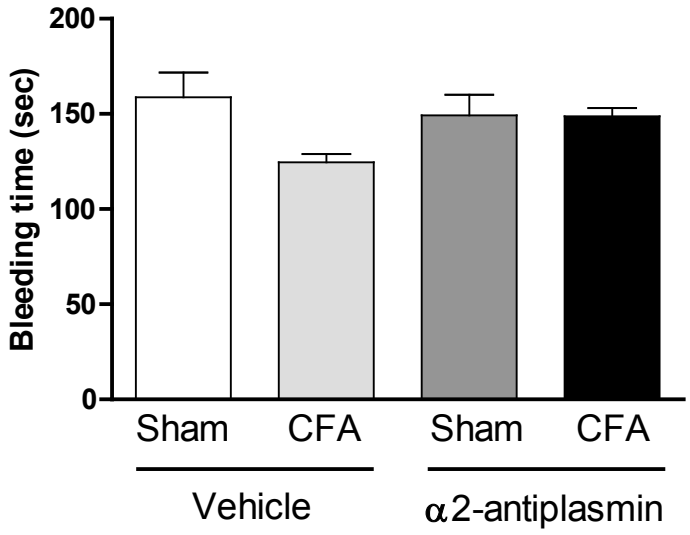


Figure 1: No change in bleeding time (sec) was found across sham or CFA-treated groups following the daily administration of either a vehicle or α2-antiplasmin for 2 weeks (one-way ANOVA)

vivo. When compared to the study by Weitz et al., which used a 1mg/kg dose of α2-antiplasmin, our use of doses 40 times smaller suggests that the effects on blood coagulation should be minimal. Indeed, we did not observe any changes in bleeding times measured at the end point of 2 weeks of daily s.c. injections of α2-antiplasmin (refer to the unpublished data shown in Figure 1). Indeed, as believed by extrapolation, the locally administered doses of α2-antiplasmin that we used are not sufficient to cause systemic effects on coagulation. In the future, to fully ascertain the safety of α2-antiplasmin as a therapeutic option, a more accurate approach to determine blood clotting time should be used. Furthermore, in the instance of hypoplasminogenemia, a disorder with severe plasminogen deficiency, no significant increased risk of deep venous thrombosis is observed after α2-antiplasmin treatment (Schuster et al., 2007). This indicates that, up to certain levels, the fibrinolytic imbalance could be well-compensated (Schuster et al., 2007).

Lastly, inflammation can generate local thrombosis (Fox and Kahn, 2005). Thus, treatment with α2-antiplasmin should, in theory, be counteracting the possibility of thrombosis and concomitantly decreasing inflammation. Although we did not assess the level

of inflammation or edema remaining in the ankle joint following the α2-antiplasmin treatment, we believe that it will be reduced due to the inhibition of mNGF, a key pro-inflammatory mediator. This should be confirmed by quantitative assessment like we did in the study in Chapter 2 of this thesis.

5.4.2 MMP-9

Joint destruction in RA is attributed to matrix-degrading enzymes, namely matrix metalloproteinases and serine proteases, because of their capacity to degrade a variety of extracellular matrix proteins and to activate latent forms of matrix metalloproteinases (MMP) (Werb et al., 1977, Cunnane et al., 1998). In particular, the gelatinases MMP-2 and MMP-9 are overproduced in the joints of patients with RA and may play an important role in cartilage degradation through digestion of denatured collagen. Studies have shown that MMP-9 levels are substantially elevated in the sera and synovial fluid from patients with RA (Gruber et al., 1996, Yoshihara et al., 2000). The tissue distribution of MMP-9 within the synovium of RA patients is localized to sites of inflammation including: surface synovial lining cells, endothelium and leukocytes. These observations suggest that connective tissue turnover arises from excessive MMP activity in the invading pannus or synovial fluid (Ahrens et al., 1996). Within the NGF processing cascade, the enzymatic actions of plasmin are twofold. Indeed, plasmin not only cleaves proNGF into mNGF but also cleaves the proMMP-9 form into active MMP-9. Therefore, using α2-antiplasmin as a novel therapeutic approach may provide additional benefits while treating RA. As described in chapter 2, the resulting increase in mNGF outweighs the likely benefits of inhibiting MMP-2/9 in naïve animals since basal levels of MMP-9 are normally low in physiological conditions.

5.4.3 Future Experiments

Bruno and Cuello (2006) have shown direct *in vitro* evidence of an activity-dependent release of the components of the proteolytic cascade responsible for the extracellular conversion of proNGF to mNGF using superfused slices of cerebral cortex. Therefore, a prospective study to be considered is to investigate all the enzymes that influence the processing of NGF in the periphery. There is yet to be a detailed study showing direct changes to every component of the enzymatic cascade, namely tPA, uPA, plasminogen and plasmin, in the synovium in physiological conditions and upon the development of inflammatory arthritis. It would be important to correlate the abovementioned alterations, if observed, in a time-dependant manner, with the levels of proNGF or mNGF and the progression of the arthritis pathology.

Furthermore, it would be interesting to investigate the proteases involved in the NGF processing cascade in a more simple system, such as cultured keratinocytes or mast cells, in order to observe their dynamics *in vitro*. Mast cells are present in limited numbers in normal human synovium, but in RA this population can expand to constitute 5% or more of all synovial cells (Nigrovic and Lee, 2005). Therefore, the quantification of infiltrating mast cells in

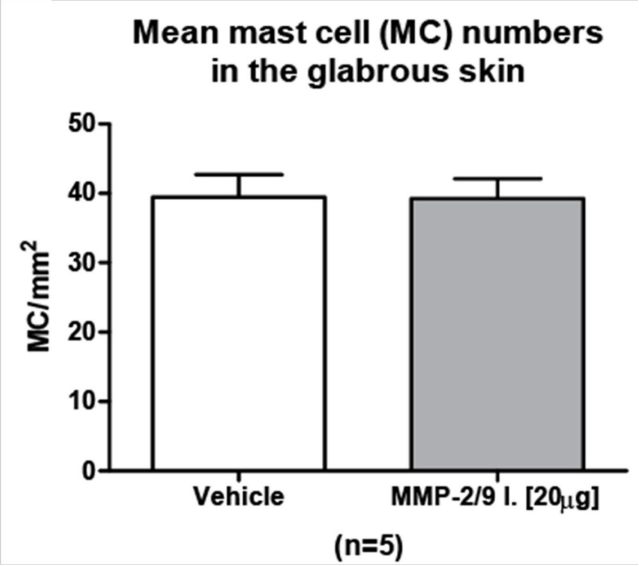


Figure 2: The mast cell (MC) count per scanned area of skin section was unchanged following the administration of either vehicle or MMP-2/9 inhibitor to naïve rats.

the upper dermis and synovium following chronic inflammatory arthritis should be assessed. In our preliminary results, we detected no change in the number of mast cells following MMP-2/9 inhibition in skin of naïve animals when compared to the vehicle-injected animals (Figure 2). This indicates that although we see changes in the mNGF protein levels, the MMP-2/9 inhibitor was not sufficient to trigger an inflammatory response as in the case of RA. However, the situation may be very different in CFA-induced arthritis and the number of mast cells both in skin and synovial membrane should be specifically assessed.

5.5 Neuroprotective Role of NGF

Throughout this thesis, a strong emphasis was made on establishing the negative role of NGF in the context of pain under pathological conditions. However, NGF also has neuroprotective functions. For instance, NGF stimulates the growth of TrkA-expressing sensory neurons following dorsal rhizotomy, resulting in the regrowth of axons into the CNS and the formation of functional connections to restore some of the lost sensory functions caused by the lesion (Ramer et al., 2000). NGF additionally plays a crucial role in maintaining synaptic connections of cholinergic neurons in the brain and its expression is affected early during the pathogenesis of Alzheimer's disease (Allen et al., 2011). Thus, the complete sequestration of NGF via antibodies such as Tanezumab may have detrimental effects if it implicates the CNS. It is well established that the anti-NGF antibodies do not cross the blood brain barrier (BBB). A study, which needs confirmation, has shown that, in the CIA model, the influence of a chronic inflammatory state increased the cerebrovascular permeability, thereby disrupting the BBB (Nishioku et al., 2010). In case it would be confirmed that the BBB becomes leaky with the development of arthritis, anti-NGF

antibodies could potentially cross the BBB and jeopardize the maintenance of synaptic connections within the CNS. Conversely, targeting locally the enzymatic cascade with s.c. injections of α2-antiplasmin, or other compounds interfering with the maturation of mNGF, around the synovial joint, is a more subtle way to modulate NGF and reduces the chances of having the compound reach the systemic circulation.

5.6 Other Future Experiments

5.6.1 VEGF

Vascular endothelial growth factor (VEGF) promotes the migration and proliferation of vascular endothelial cells upon binding to VEGF receptors on the cell surface (Lamalice et al., 2007). VEGF gene expression is up-regulated by a variety of growth factors and cytokines, including TNF-α and IL-1 (Ramanathan et al., 2007). In addition, VEGF activates nuclear factor-κB and stimulates the synthesis of a broad variety of pro-inflammatory cytokines and chemokines (Marumo et al., 1999). Moreover, it has been shown that angiogenesis can be induced following continuous infusion of VEGF into adult rat brain tissue (Croll et al., 2004). Interestingly, elevated VEGF levels in the plasma or tissue have been associated with autoimmune diseases such as RA (Hetland et al., 2008). Vascular endothelial dysfunction is involved in the induction of negative cardiovascular events and this process is also observed in patients with RA. Importantly, endothelial dysfunction also impairs the integrity of the BBB (Granger and Senchenkova, 2010). Recently, NGF has been identified as a novel angiogenic molecule exerting a variety of effects on endothelial cells (Jadhao et al., 2012). For example, a study by Kim et al. has demonstrated that TrkA activation in the retina by NGF leads to VEGF elevation via the PI3K-Akt pathway,

suggesting that TrkA could be a stimulator of vascular development (Kim et al., 2013). As reported in the introduction, a pathological observation found in RA and OA patients is the development of neovascularization of non-calcified articular cartilage, which precedes the hyperinnervation of sensory and sympathetic fibers (Suri et al., 2007). This phenomenon may be linked to the involvement of both VEGF and NGF. To complement the studies presented in this thesis, it would be interesting to look at alterations in VEGF levels. Thus, investigating the possibility of using a combined approach in which both NGF and VEGF levels are modulated might be important as it is possible that there are synergistic effects in reducing the pathological changes that arise in arthritis, including inflammation, angiogenesis and hyperinnervation of joint tissues.

5.6.2 Sequestration of NGF Using Monoclonal Antibodies

The work presented in this thesis was carried out using an animal model of inflammatory arthritis, mimicking to a certain extent the RA pathology observed in humans. Targeting NGF as a treatment for arthritis is not a novel idea. For example, we have discussed herein studies that have used sequestering monoclonal antibodies against NGF in humans (Abdiche et al., 2008, Lane et al., 2010) and others that have employed TrkA–IgG soluble fusion proteins to target its receptor (Hefti et al., 2006). Given that bone pain may be a major target for NGF-TrkA therapies, understanding how these therapeutic strategies affect individuals that have advanced bone degeneration is essential. However, the clinical trials for NGF sequestration are only examining its effects in patients afflicted with OA, whereas the preclinical studies are predominantly carried out in inflammatory models (e.g. AIA or CIA) in rodents. OA causes bone destruction and subsequently triggers mild inflammation, whereas in RA, synovitis is the first pathological symptom. Additionally, not only is the

proportion of RA patients compared to OA patients much more limited, due to its autoimmune nature, but many RA patients have, in general, already undergone treatments with other agents, including sometimes antibodies, in the form of DMARDs. Nonetheless, more emphasis in targeting NGF as a therapeutic option for RA patients should be considered since our work demonstrates that the neuropathological changes stemming from chronic inflammation involve maladaptive alterations to the innervation pattern of the joint and surrounding tissues. Moreover, an attractive study to complement the work in this thesis would be to perform similar experiments in a model of osteoarthritis, such as the MIA model, and determine if the phenomenon of sympathetic-sensory coupling also occurs in other arthritic pathologies.

5.7 Caveats

There are a number of limitations regarding the techniques used in this thesis. Some important issues are addressed below.

5.7.1 Type of Drug Administration

Ideally, rather than repeatedly injecting subcutaneously into the skin surrounding the joint, an intra-articular drug delivery system would be more effective and its development should therefore be considered. In recent years, extensive research has been undertaken to develop sustained-release systems, such as polymeric micro- and nanoparticles, liposomes and hydrogels, for intra-articular drug delivery purposes. This new technology has the advantage of prolonging the drug's effect at its site of action and provides increased intra-articular drug concentrations to the joint space. More importantly, it provides the recipient with a decreased systemic exposure to the drug. Many factors can influence the efficacy of a

drug delivery vehicle for promoting sustained intra-articular residence time, including particle size, vehicle safety and hydrophobicity (Zhang and Huang, 2012). Although we did not detect any side effects following our subcutaneous intraplantar administration, preventing the potential negative effects of inhibiting plasmin would be achieved with these novel methods of intra-articular drug administration since the compounds would be slowly released.

5.7.2 Antibodies Against pro/mNGF for IHC

We have successfully detected both proNGF and mNGF by Western blotting. However, a limitation we are faced with is that we presently lack an anti-NGF antibody designed for immunocytochemistry which would recognize both mNGF and proNGF. The only available antibody that recognizes satisfactory any form of NGF was directed specifically against proNGF, as the sequence it recognizes is not part of the mNGF molecule. This antibody was used in a study from our lab that assessed the distribution of proNGF immunoreactivity in the glabrous skin of naïve and neuropathic animals (Peleshok and Ribeiro-da-Silva, 2012). In that study it was observed that with the development of neuropathic pain, mast cells followed by keratinocytes and peri-vascular cells significantly up-regulated proNGF. As expected, in our study looking at MMP-2/9 inhibition in naïve rats, the source of proNGF immunoreactivity appeared to be mast cells in the upper dermis as well as the keratinized epithelial layer in the epidermis. Unfortunately, no quantification of changes in proNGF levels in the inflammatory model was assessed in this body of work.

5.8 Contributions to Original Work

In recent times, studies into the mechanisms of inflammatory pain and the role of NGF are beginning to emerge. In this thesis, we describe some novel observations that, in our view, make original contributions to our understanding of the underlying pathologies of arthritic pain. These contributions are summarized below;

1. Sympathetic fiber sprouting in inflamed joints and adjacent skin contributes to pain related-behavior in arthritis. Geraldine Longo, Maria Osikowicz and Alfredo Ribeiro-da-Silva. Journal of Neuroscience 33 (24): 10066-10074, 2013

Using a pharmacological tool to block sympathetic function, we determined a role for the sympathetic fibers that ectopically sprout in the skin around the joint at 4 weeks following intra-articular CFA injections. We found that blocking sympathetic function causes a reduction in the pain-related behavior only at the time point where these fibers significantly invade the upper dermis and adjoin the peptidergic C fibers, suggesting an interaction between these fiber populations. Moreover, we observe that the protein levels of mature NGF are significantly elevated in the inflamed skin surrounding the joint compared to sham animals.

2. Inhibition of endogenous NGF degradation induces mechanical allodynia and thermal hyperalgesia in rats. Maria Osikowicz, Geraldine Longo, Simon Allard, A. Claudio Cuello and Alfredo Ribeiro-da-Silva, Molecular Pain, 9:37, 2013.

This study details, for the first time, the ability to modulate the NGF enzymatic cascade in the periphery. As a result, we were the first to show, in this proof of concept study that blocking the inactivation of mNGF is sufficient to cause: 1) hypersensitivity to pain

stimuli in naïve rats, 2) increases in mNGF levels at the site of MMP-2/9 inhibitor injection, and 3) sprouting of fibers into the upper dermis of skin.

3. Local administration of α2-antiplasmin alleviates allodynia and hyperalgesia in a rat model of inflammatory arthritis. Geraldine Longo, Maria Osikowicz, A. Claudio Cuello and Alfredo Ribeiro-da-Silva. *To be Submitted*

The experiments described in chapter 4 offer the first demonstration of the benefits of the indirect reduction of abnormally elevated endogenous NGF levels in arthritis. This was achieved by inhibiting plasmin, therefore blocking its ability to cleave proNGF into mNGF. Our data may provide the basis for a potential novel therapeutic strategy for arthritis. We showed that mNGF levels significantly decrease following daily administration of α2-antiplasmin. Furthermore, as a result of these daily injections, we observed alleviation in the pain-related behavior, as well as an effective suppression of CFA-induced sprouting of sympathetic fibers in our chronic inflammatory animal model.

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