From Department of Physiology and Pharmacology
Karolinska Institutet, Stockholm, Sweden

ROLE OF SPINAL AND PERIPHERAL HMGB1 IN ARTHRITIS-INDUCED PAIN

Nilesh Mohan Agalave

Karolinska Institutet

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Cover photo: Immunohistochemistry for HMGB1 colocalizing with neurons in the dorsal horn of the spinal cord

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Role of spinal and peripheral HMGB1 in arthritis-induced pain

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By

Nilesh Mohan Agalave

Principal Supervisor:
Camilla I Svensson
Karolinska Institutet
Department of Physiology and Pharmacology

Co-supervisor(s):
Helena Erlandsson Harris
Karolinska Institutet
Department of Medicine

Xiaojun Xu
Karolinska Institutet
Department of Physiology and Pharmacology

Opponent:
Pedro L Vera
University of Kentucky, LX,US
Department of Physiology

Examination Board:
Malin Ernberg
Karolinska Institutet
Department of Dental medicine

Bernd Fiebich
University medical centre, Freiberg, Germany
Department of Psychiatry

Maria Lindskog
Karolinska Institutet
Department of Neurobiology, care and society

To my parents,

Aai-Nana

ABSTRACT

Chronic pain is one of the most debilitating and repeatedly reported symptoms by rheumatoid arthritis (RA) patients. Despite good disease control achieved with disease modifying anti-rheumatic drugs (DMARDs), joint pain remains a major problem for a subgroup of patients. Therefore, it appears episodes of joint inflammation can have long-term effects on the peripheral sensory nervous system. Additionally, changes in the central nervous system may contribute to chronification of RA pain. High mobility group box-1 protein (HMGB1) is an important molecule in the pathogenesis of RA, but the role of HMGB1 in RA associated pain has not been studied. Thus, the involvement of spinal and peripheral HMGB1 in rheumatoid arthritis-induced pain is the focus of this thesis.

In Paper I, we characterized the collagen antibody-induced arthritis (CAIA) model from a pain perspective. As expected, injection of collagen type II antibodies induces transient joint inflammation and pain-like behavior. Surprisingly, pain-like behavior did not normalize when the inflammation resolved. We found that transient antibody-induced joint inflammation led to long-lasting mechanical hypersensitivity that outlasted the inflammation. Buprenorphine and gabapentin attenuated pain like behavior in both the inflammatory and late "post-inflammatory" phase of the model, whereas diclofenac was antinociceptive only during the inflammatory phase. This indicates that there is a temporal shift in the mechanisms that maintain arthritis-induced nociception. The CAIA model can thus be used to explore mechanisms of persistent pain induced by inflammation in the articular joint.

In Paper II and III, we investigated the spinal role of HMGB1 in arthritis-induced pain and sex-dependent microglial involvement in disulfide HMGB1 mediated nociception. Peripheral joint inflammation in the CAIA model increases expression and extranuclear levels of HMGB1 in the lumbar spinal cord. Blocking the endogenous action of HMGB1 with HMGB1 inhibitors attenuated CAIA-induced mechanical hypersensitivity in both male and female mice. A pronociceptive effect dependent on the redox state of HMGB1 was also revealed. The disulfide, but not the all-thiol or oxidized form, of HMGB1 induced nociception in male and female mice after intrathecal delivery. This effect was regulated via toll-like receptor 4 (TLR4) and associated with cytokine and chemokine production and elevated expression of factors related to increased glial cell reactivity. Intrathecal delivery of minocycline attenuated the disulfide HMGB1 induced hypersensitivity in male but not in female mice. Global protein analysis of lumbar spinal cords from male and female mice injected intrathecally with HMGB1 and vehicle or minocycline showed that 36 proteins were differentially expressed between male and female injected with HMGB1 and that 44 proteins in males and 8 in females were altered in mice receiving HMGB1 and minocycline. Interestingly, up-regulation of antinociceptive and anti-inflammatory molecules was found in male but not in female mice after intrathecal injection of HMGB1 and minocycline. This work points to a prominent and redox-dependent role of HMGB1 in spinal pain signal transmission.

In Paper IV, we demonstrated that a repetitive systemic injection of a HMGB1 neutralizing antibody attenuates CAIA-induced nociception in male but not in female mice. Intraarticular injection of disulfide but not all-thiol HMGB1 induced mechanical hypersensitivity in both male and female mice, but with a more pronounced induction of cytokine and chemokine mRNA expression in male compared to female mice. Moreover, nociception induced by disulfide HMGB1 is mediated by TLR4 expressed on nociceptors and myeloid cells in male and female mice, with a stronger contribution of TLR4 on myeloid cells in male mice.

In summary, we have described novel redox state and sex-dependent roles of HMGB1 in nociception at spinal and peripheral sites in a model of arthritis-induced pain. These results also reveal sex-dependent analgesic pharmacology and highlight the importance of taking sex into account in preclinical pain research. While further studies are warranted in order to further advance our knowledge on the role of HMGB1 in pain pathology, the work in this thesis highlights HMGB1 as an intriguing new target for pain relief.

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I Bas DB, Su J, Sandor K, **Agalave NM**, Lundberg J, Codeluppi S, Baharpoor A, Nandakumar KS, Holmdahl R, Svensson CI.

Collagen antibody-induced arthritis evokes persistent pain with spinal glial involvement and transient prostaglandin dependency

Arthritis Rheum. 2012 Dec;64(12):3886-96. doi: 10.1002/art.37686.

II **Agalave NM**, Larsson M, Abdelmoaty S, Su J, Baharpoor A, Lundbäck P, Palmblad K, Andersson U, Harris H, Svensson CI.

Spinal HMGB1 induces TLR4-mediated long-lasting hypersensitivity and glial activation and regulates pain-like behavior in experimental arthritis

Pain. 2014 Sep;155(9):1802-13. doi: 10.1016/j.pain.2014.06.007. Epub 2014 Jun 20.

III **Agalave NM**, Bersellini Farinotti A, Khoonsari PE, Krishnan S, Palada V, Umbria CM, Sandor K, Andersson U, Harris H**,** Kultima K, Svensson CI.

Sex-dependent role of microglia in disulphide HMGB1-mediated mechanical hypersensitivity

Manuscript

IV **Agalave NM**, Rudjito R, Bersellini Farinotti A, Lundäck P, Andersson U, Price T, Harris H, Burton M, Svensson CI.

Contribution of peripheral HMGB1 and TLR4 expressed on primary afferent sensory neurons and local immune cells in nociception evoked by collagen type II antibodies

Manuscript

Publications not included in the thesis

I Larsson M, **Agalave NM**, Watanabe M, Svensson CI.

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Pattern recognition receptors in chronic pain: Mechanisms and therapeutic implications

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Effect of intrathecal glucocorticoids on the central glucocorticoid receptor in a rat nerve ligation model

Scandinavian Journal of Pain, Volume 16, July 2017, Pages 1–9

V Pironti G, Bersellini Farinotti A, **Agalave NM**, Sandor K, Fernandez Zafra T, Jurczak A, Lund LH, Svensson CI, Andersson D.

Cardiac remodeling, oxidative stress and impaired cardiomyocyte Ca^{2+} handling in a mouse model of rheumatoid arthritis

European Heart Journal, Submitted

VI Su J, Barde S, Delaney A, Ribeiro J, Kato J, **Agalave NM**, Wigerblad G, Matteo R,Sabbadini R, Josephson A, Dolphin AC, Chun J, Kultima K, Peyruchaud O, Hökfelt T, Svensson CI.

Blockade of lysophosphatidic acid by monoclonal antibody reverses arthritis-induced pain via the LPA1/α2δ1 pathway

Manuscript*

VII Sandor K, Krishnan S, **Agalave NM**, Villarreal Salcido J, Fernandez Zafra T, Emami Khoonsari P, Svensson CI, Kultima K.

Spinal injection of newly identified cerebellin-1 and cerebellin-2 peptides induce mechanical hypersensitivity in mice

Submitted

CONTENTS

LIST OF ABBREVIATIONS

A1AT2	Alpha-1-anti-trypsin 1-2
A1AT4	Alpha-1-anti-trypsin 1-4
A1AT5	Alpha-1-anti-trypsin 1-5
atHMGB1	All-thiol HMGB1
CAIA	Collagen antibody-induced arthritis
CCI	Chronic constriction injury
CCL2	C-C motif ligand 2
CD11b	Cluster of differentiation molecule 11B
CIA	Collagen-induced arthritis
CXCL 1	Chemokine CXC motif ligand 1
CXCL2	Chemokine CXC motif ligand 2
DRG	Dorsal root ganglion
dsHMGB1	Disulfide HMGB1
GFAP	Glial fibrillary acidic protein
Hemo	Hemopexin
HMGB1	High mobility group box 1 protein
HPT	Haptoglobin
i.a.	Intraarticular
i.p	Intra peritoneal
i.pl.	Intraplantar
i.t.	Intrathecal
i.v.	Intravenous
Iba1	Ionized calcium binding adaptor molecule 1
IFN	Interferon
IL1-β	Interleukin 1 β
IL6	Interleukin 6
KYN	Kynurenic acid
LPS	Lipopolysaccharide

NeuN	Neuronal nuclear protein
NO	Nitric oxide
NY	Neuropeptide Y
oxHMGB1	Oxidised HMGB1
RA	Rheumatoid arthritis
RAGE	Receptor for advance glycation end product
s.c	Subcutaneus
SNI	Spinal nerve ligation
SPA3K	Serine protease inhibitor 3K
SPA3N	Serine protease inhibitor 3N
TBI	Tibial nerve incision
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TNF	Tumor necrosis factor
TrkA	Tyrosine kinase receptor A
VDBP	Vitamin D binding protein
VEGF	Vascular endothelial growth factor

INTRODUCTION

1.1 PAIN

International Association for the Study of Pain (IASP) states, 'Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (https://www.iasp-pain.org/Taxonomy). Chronic pain is one of the most under-recognized, under-treated medical problems of the twentieth century. Globally, approximately 10-20 % of the population suffers from pain, resulting in reduced quality of life for the individual (Apkarian et al., 2009; Macfarlane, 2016; van Hecke et al., 2013). Failure to recognize that chronic pain is a serious health problem, which results in substandard pain management, is a part of the problem. Also, drug development in the area of chronic pain has hitherto been insufficient, and there are currently few available effective treatments for chronic pain conditions. Without adequate pain relief, individuals with persistent pain often endure physical and psychosocial problems, such as an inability to work and perform daily chores, decreased activity and muscle wasting, fatigue, sleep disturbances, social isolation, anxiety and depression (Breivik et al., 2006; Hunt & Mantyh, 2001). As a consequence, chronic pain is not only devastating for the individual but also generates a huge socio-economical burden in the form of medical costs, sick leave, and loss of productivity (Breivik et al., 2006).

In a survey from 15 European countries 40% of the chronic pain patients reported having joint pain, mainly due to osteoarthritis and rheumatoid arthritis (M. L. Andersson et al., 2013; Lluch et al., 2014). Hence, it is critical to increase our understanding of how arthritis-induced chronic pain is regulated in order to identify new targets for pain relief.

1.2 PAIN BIOLOGY

Pain is a sensory experience that is basically unpleasant and associated with hurt and discomfort. It may vary with regard to intensity, quality, duration and referral. Pain can be adaptive or maladaptive. Adaptive pain provides a defense mechanism to protect the organism from injury and promotes healing in injured tissue (Julius & Basbaum, 2001; Woolf, 2004). In contrast, maladaptive pain is the type of pain that persists long after resolution of tissue damage and leads to problems rather than protecting the organism (Woolf, 2004). Painful stimuli are detected by the nociceptors (see below) and the information conveyed by relaying sensory neurons to different centers in the brain, ultimately giving us the sensation of pain. Furthermore, pain is differentially categorized based on how it is triggered, such as nociceptive (by chemical irritant, heat, cold, intense mechanical force), neuropathic (peripheral nerve injury) and inflammatory (tissue damage and activation of macrophages, mast cells, neutrophils and granulocytes) (Hunt & Mantyh, 2001; Julius & Basbaum, 2001; Woolf & Salter, 2000).

1.2.1 Nociceptors and transmission of pain

Nociceptors are the peripheral sensory nerves that detect painful noxious stimuli. Nociceptors are categorized based on their diameter, degree of myelination and conduction velocity; *C fibers,* smaller in diameter (0.2-1.5 µM) have a low conduction speed (0.5-2 m/s) and are activated by painful stimuli, *Aδ-fibers* are medium sized in diameter (1-5 µM) with thin myelination, a somewhat higher conduction speed (5-35 m/s) compared to C fibers, that mainly carry information related to the pain and temperature, *Aβ fibers* are large diameter (6-12 µM) with high myelination and high conduction speed (35-75 m/s) that carry information related to touch, and *Aα fibers* are large diameter fibers (13-20 µM) with high myelination and faster conduction speed (80-120 m/s) that carry information related to the proprioception. Nociceptors are also categorized based on the expression of peptides and ion channels. C-fibers that express substance-P and calcitonin gene-related peptide (CGRP) are referred to as peptidergic nociceptors (Basbaum et al., 2009). These neurons also express tyrosine kinase receptor (TrkA) for nerve growth factor and express transient receptor potential vanilloid 1 (TRPV1, also known as capsaicin receptor) that is activated by heat stimuli. Nociceptors that stain positive with Isolectin B4 and expressed P2X3 receptor for ATP, and typically do not release peptides such as substance-P and CGRP upon stimulation are referred to as non-peptidergic nociceptors (Basbaum et al., 2009; Zylka, 2005).

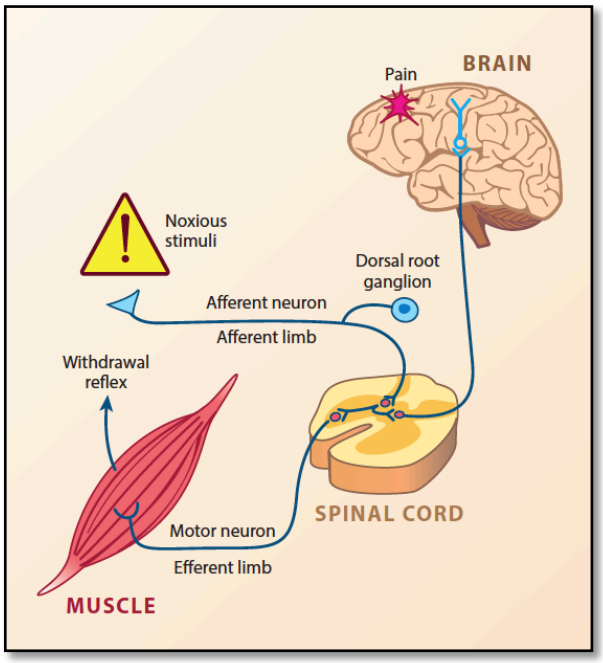

Figure 1. Schematic illustration for transmission of pain signal from periphery to the brain. Peripheral nociceptors (primary afferent neuron) detect the noxious stimuli and send signal to the spinal cord, where the signal transfer to the second order (projection neuron) which send signal to the brain (Reprinted with permission, Adapted from Talbot et al., 2016).

The cell bodies of nociceptors reside in dorsal root ganglia (DRG) with an exception for the sensory nerves innervating the face, which are located in the trigeminal ganglia (TG). Nociceptors are pseudo-unipolar in morphology with the cell body located in the DRG from which two axonal branches depart, one towards the peripheral tissue that it innervates and one towards the dorsal horn of the spinal cord where it makes synaptic contact with the second

order neuron (Basbaum et al., 2009). When nociceptors detect peripheral noxious stimuli, the peripheral nerve terminal is activated, and the stimulus is converted into electrical current (transduction) in the form of action potentials. The action potentials are conducted from the periphery to the central terminal. Nociceptors make synaptic contact with projection neurons and interneurons in the spinal cord, and the signal in the primary afferent nociceptor is transmitted to the second order neurons across the synapse (Basbaum et al., 2009; Woolf, 2004). The signal is conveyed to different centers of the brain giving us the perception of pain (Figure 1).

1.2.2 Innervation of nociceptors in joint

Different joint structures such as ligaments, fibrous capsules, periosteum, synovial layer, meniscus and surrounding bone structures are innervated by sensory nociceptive fibers. While Aβ fibers are mostly present in the ligament and fibrous capsule, Aδ and C fibers innervate in the ligament, fibrous capsule, meniscus, synovial layer, trabecular bone, periosteum and bone marrow (Mantyh, 2014). Some populations of nociceptive fibers in the joint structure are silent and become activated during the injury, damage or inflammation. Upon activation, the silent fibers can either be spontaneously active or display reduced threshold to the stimulus (Mantyh, 2014; Schaible et al., 2009). The noxious sensation from the joint structure can be evoked by different mechanical and chemical stimuli (Schaible et al., 2009).

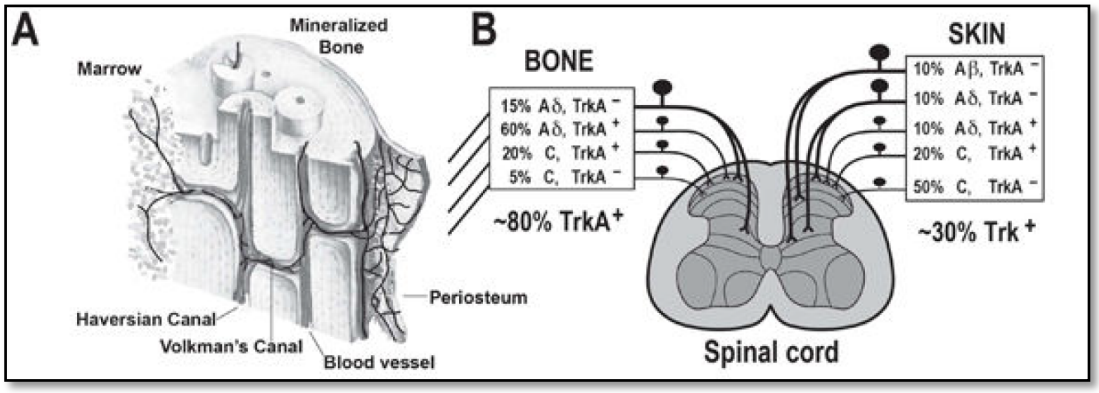

Figure 2. Schematic illustration of the termination of different sensory neuron populations in the dorsal horn of the spinal cord from joint and bone structure, and in the skin. Bone/joint structure innervated with TrkA + fiber, approximately 60% Aδ fiber and 20% of C fibers, and in the skin it is 10% Aδ-fiber and 20% C-fiber (Reprinted with permission, Adapted from Mantyh P. et al. 2014).

As compared to the skin, the majority of the sensory neurons innervating the bone and joint structure are thinly myelinated $TrkA^{+}$ and/or peptidergic fibers with minor innervation by Aβ and $TrkA^{-}$ peptide poor fibers (Figure 2) (Jimenez-Andrade et al., 2010; Zylka, 2005). Nerve fibers in the bone marrow and cortical bone are most often co-localizing with blood vessels, while the periosteum is densely innervated in a grid pattern (Martin et al., 2007). Intriguingly, the articular cartilage of the joint lack innervation of sensory nerve fibers, so nociception resulting from damage to the cartilage itself has likely originated in an adjacent structure, like subchondral bone and synovium (Mantyh, 2014). This literature provides the importance of

knowing the microenvironment of the joint structure, and subpopulations of nociceptors in the joint and skin.

1.2.3 Cytokines/chemokines as neuronal activators (peripheral sensitization)

Cytokines and chemokines are classical regulators of immune responses as well as major players in neuroinflammation. Cytokines are a diverse group of small proteins; chemokines represent one family of cytokines. They are involved in the interaction between immune and non-immune cells (Vilček, 2003). Under physiological and pathological conditions, cytokines and chemokines are released in response to stimuli from cells of both the immune and nervous systems. In the incidence of tissue injury, immune cells are attracted to the injury site (Figure 3) and secrete mediators, including cytokines and chemokines that can directly or indirectly sensitize the peripheral endings of primary afferents giving rise to peripheral sensitization, reviewed in (Taylor et al., 2011). Recent evidence shows that receptors for different cytokines/chemokines are expressed by nociceptors and glial cells (Miller et al., 2009). It has been reported that cytokines and chemokines activate nociceptors in different experimental models as well as in human (Alboni & Maggi, 2015; Khairova et al., 2009; Miller et al., 2009; K. Ren & Dubner, 2010; Schaible et al., 2010). Intraarticular injection of TNF, IL1-β IL6 and IL17 generates nociception in the normal knee joint in rodents (Schaible, 2014). Additionally, TNF, IL1-β and IL-6 have been shown to increase the synaptic plasticity in the dorsal horn of spinal cord (Lamina II) (Kawasaki et al., 2008; K. Ren & Dubner, 2010). A specific chemokine, CCL2, is expressed by peripheral neurons and released during the nerve injury (L. Zhang et al., 2017). In this thesis, cytokines (TNF, IL1-β, IL6), and chemokines (CCL2, CXCL1, CXCL2) are used as the readout for the tissue insult.

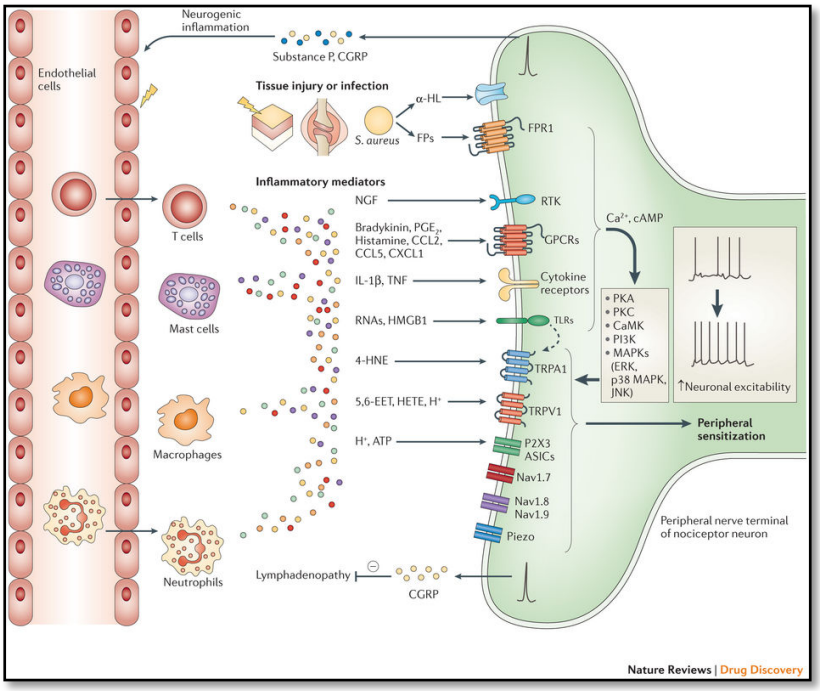

Figure 3. Schematic illustration of the molecules involved in the peripheral sensitization. Inflammatory mediators and other factors bind to their receptor present on the nerve terminal of the nociceptors to generate neuronal excitability in peripheral nociceptors. (Reprinted with permission, Adapted from Ji RR et al., 2014)

1.2.4 Spinal neuron-glial signaling in neuronal plasticity (central sensitization)

Historically the main function of glial cell (astrocytes and microglia) in central nervous system was thought to be providing structural and nutritional support for the neuronal network. However, emerging evidence suggests that central astrocytes and microglia perform a much wider range of functions. Rapidly increasing number of reports indicate that spinal glial cells plays an important roles in the maintenance of inflammatory and neuropathic pain in collaboration with neurons (Gosselin et al., 2010; McMahon & Malcangio, 2009; Milligan & Watkins, 2009; Tsuda, 2016). Peripheral injury and insult prolong and amplify nociceptive signal conduction, which promotes the release of neurotransmitters (glutamate, ATP), cytokines (CCL2) (L. Zhang et al., 2017) and neuropeptides (substance P, CGRP, BDNF) from the primary afferent neuron into the central synapse. Resident spinal glial cells express broad categories of receptors including purinergic, toll-like, cytokine, and neurotransmitter receptors (McMahon & Malcangio, 2009; Wolf et al., 2017). Thus factors released from nociceptors can activate receptors on glial cells, which activate intracellular signaling pathways like the p38/MAPK pathway in microglia and JNK/MAPK pathway in astrocytes. In turn, glia release factors that potentiate the activation of release of neurotransmitter from the presynaptic afferent and increase excitability of the second order (projection) neuron (McMahon & Malcangio, 2009). It has been shown previously that, proinflammatory cytokines such as TNF, IL1-β and IL6 regulate synaptic plasticity in the spinal cord (Alboni & Maggi, 2015; Kawasaki et al., 2008; Khairova et al., 2009). Studies have shown that spinal delivery of "glia inhibitors" or agents targeting glia-specific receptors or signaling pathways prevent or reverse pain-like behavior in a number of experimental models of pain, further supporting active role of glia cell in spinal pain signal transmission (Gosselin et al., 2010; McMahon & Malcangio, 2009; Milligan & Watkins, 2009; Tsuda, 2016). Beside is a cartoon illustrates the interaction between astrocytes, microglia and neurons (Figure 4).

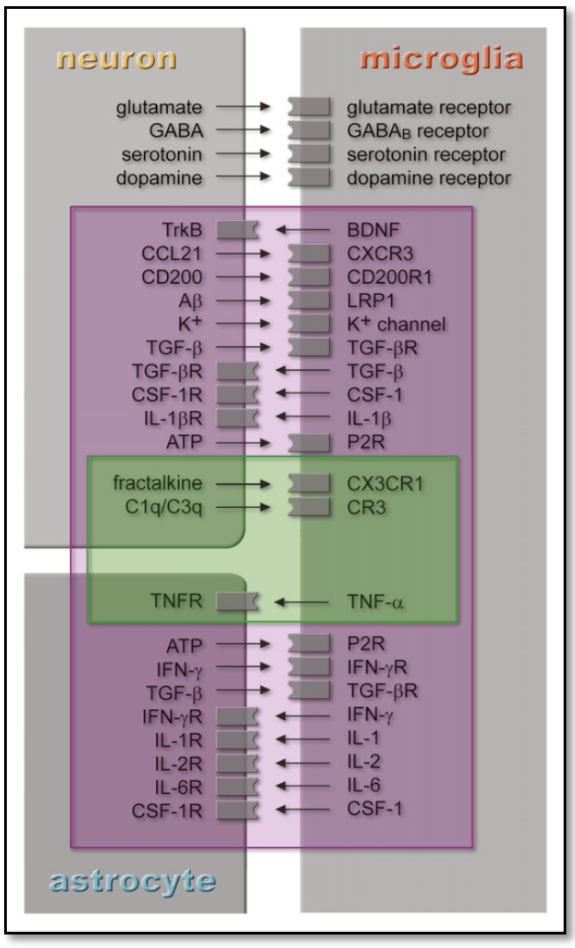

Figure 4. Schematic diagram represents the possible interaction between spinal neuron-.microglia and astrocytes, for maintenance of central sensitization (Reprinted with permission, Adapted from Kettenmann et al. 2012, Neuron)

1.2.5 TLRs in chronic pain

Toll-like receptors (TLRs) are transmembrane-signaling receptors that

play a critical role in innate and adaptive immune responses by recognizing factors of invading microbes. So far, more than 10 different TLRs with distinct ligand specificities have been identified. In the central nervous system, microglia and astrocytes express various TLRs, including TLR2 and TLR4. In particular, TLR4 has been associated with altered pain processing. TLR4 is activated by LPS (Miyake, 2004; Raetz et al., 2006) and intrathecal injection of LPS induces tactile allodynia (Christianson et al., 2011; Saito et al., 2010). Nociception observed following nerve injury and joint inflammation is attenuated in mice lacking functional TLR4 (Christianson et al., 2011; Tanga et al., 2005), in rats following spinal TLR4 knock-down (Tanga et al., 2005) and in mice receiving TLR4 antagonists (Bettoni et al., 2008; Christianson et al., 2011; Hutchinson et al., 2009). Importantly, preventing TLR4-mediated signaling suppresses spinal microglial activation and decreases nerve injury-induced spinal release of pro-inflammatory cytokines (Tanga et al., 2005). As TLR4 deficiency and TLR4 antagonists attenuate hypersensitivity in the absence of exogenous TLR4 ligands, TLR4 appears to be activated by endogenous ligands and play an important role in spinal nociceptive processing. Noteworthy, in earlier work, we found an increase in mRNA for the endogenous TLR4 ligand HMGB1 in spinal cords from mice subjected to experimental arthritis (Christianson et al., 2010). HMGB1 was originally considered to only have nuclear actions, but is now known to function also extra-cellularly as a pro-inflammatory molecule (U. Andersson & Tracey, 2011).

1.3 HIGH MOBILITY GROUP BOX 1 (HMGB1)

High mobility group (HMG) proteins are nuclear proteins, which were first extracted from calf thymus in 1973 (Goodwin & Johns, 1973; Kang et al., 2014). These proteins were characterized by their high solubility in 10% trichoric acid and fast migration on polyacrylamide gel electrophoresis without aggregation, hence explaining the attribution of the name (Goodwin & Johns, 1973). In 2001, the nomenclature committee organized in the National Cancer Institute USA categorized HMG into three different super families: HMGB (previously known as HMG-1/2), HMGA (previously known as HMG-14/17) and HMGN (previously known as HMG-I/Y) (Bustin, 2001).

High mobility group box 1 (HMGB1) protein, previously known as HMG-1, amphoterin or p30, is a 28 kDa non-histone protein that binds to nuclear DNA and therefore plays an important physiological role in DNA replication, transcription, recombination, repair and in genomic stability. It has been highly conserved in evolution and is ubiquitously expressed in most cell types. HMGB1 shuttles between nucleus and cytoplasm but in physiological conditions, it is found primarily in nucleus where it binds to chromatin structure (Isackson et al., 1980). More recently, extracellular HMGB1 was described as a danger signal mediating the activation of the immune system by binding to TLRs and receptor for advance glycation end product (RAGE). Thus, it plays a crucial pathological role in inflammation, cell growth, cell proliferation and cell death. The great potential of HMGB1 as danger/stress signal both in inflammatory, potentially painful conditions, motivated the studies developed during this

thesis. Thus, its more relevant properties and functions will be further explored in the following sections.

1.3.1 Structure and cellular localization

HMGB1 is a 215 amino acid protein consisting of two different HMG boxes and an acidic tail. HMG Box A resides from 9-79 amino acid (aa), HMG box B from 95-163 aa and the acidic tail from 186-215 aa (Bianchi et al., 1992). Box A and B are DNA binding domains which play a role in bending double-stranded DNA in helix strands to form DNA chaperone. Nuclear immigration signal (NES) is present in the DNA binding domain, which is mediated via nuclear exportin chromosome region maintenance 1 (CRM1). However, the steady state of HMGB1 in the nucleus is maintained by two NLS domains; nuclear localization domain 1 (NLS1), which resides from 28-44 aa, and nuclear localization domain 2 (NLS2), which resides from 179-185 aa (Bonaldi et al., 2003; Kang et al., 2014).

In physiological states, HMGB1 accumulates in the nucleus and binds to DNA acting as a chaperone and regulates various functions such as nucleosome stability and sliding, nucleosome release, genome chromatization, V(D)J recombination, as well as DNA replication and repair. Furthermore, it also acts as a transcriptional factor regulating the expression of certain genes (Kang et al., 2014). In general, the nuclear/cytoplasmic distribution ratio of HMGB1 is about 30:1 in many rat tissues (Kuehl et al., 1984).
During stress, injury and inflammation, the amino acid lysine in Box A and B undergoes acetylation and HMGB1 loses its binding affinity towards DNA. This results in its active/passive release into an extracellular milieu where it has different functions (H. Yang et al., 2013). HMGB1 interacts with RAGE through the amino acid sequence 150-183, which subsequently promotes cell migration and metastasis (Huttunen et al., 2002). The amino acid sequence 89-108 is responsible for TLR4 receptor binding that leads to cytokine production, whereas the amino acid sequence 7-74 is mainly responsible for the p53 transactivation binding domain for gene transcription. Extracellular Box A has been reported as the antagonist for HMGB1, and its antagonist activity is more potent when it is fused to the C-terminal acidic tail (Gong et al., 2010). Moreover, Box B acts as a pro-inflammatory mediator (J. Li et al., 2003) and C-terminal acidic tail displays antibacterial activity (Gong et al., 2009) (Figure 5).

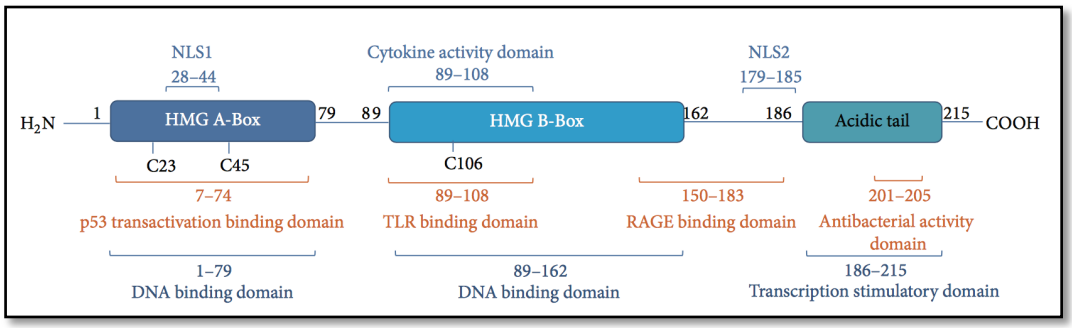

Figure 5. Detailed HMGB1 structure, with different receptor/functional binding domains and three cysteines (Open access, Adapted from Wan et al. 2016).

A previous report has shown that cytoplasmic HMGB1 acts as a positive regulator of autophagy. During autophagic stimuli, HMGB1 translocates into the cytosol and cytoplasm, binds to beclin-1 and induces autophagy (D. Tang et al., 2010). Furthermore, the presence of HMGB1 in the cell membrane relates to its role in neurite growth, activation of platelets, cell differentiation, innate immunity as well as in cell adhesion and invasion (more details in Kang et al. 2012). In 1991, Merenmies et al. showed that HMGB1 distributed throughout filopodia (cytoplasmic projections) of neuroblastoma cells enhances neurite growth (Merenmies et al., 1991). HMGB1 also activates platelets to induce formation of neutrophil extracellular traps (NET) (Mitroulis et al., 2011). Lastly, extracellular HMGB1 plays an important role in different pathological conditions upon specific binding to its receptors. Actively and passively released HMGB1 triggers the innate and adaptive immune systems. For instance, binding of HMGB1 to TLRs activates immune cells such as macrophages, T cells, B cells and NK cells (G. Li et al., 2013).

1.3.2 Translocation and release

HMGB1 lacks the leader signal sequence to be released through the classical pathway; hence HMGB1 cannot be secreted via the classical endoplasmic reticulum secretory pathway. Several mechanisms have been reported concerning the translocation of HMGB1 from the nucleus to the cytoplasm and its subsequent extracellular release. These mechanisms include transcriptional modifications, CRM1-mediated nuclear export, reactive oxygen species (ROS), and calcium and nitric oxide (NO) signaling. These mechanistic cascades are also mediated by TNF-α, nuclear factor (NF)-kβ, Notch, mitogen-activated protein kinase (MAPK), signal transducer and activator of transcription (STAT), inflammasome, p53, PPAR and lysosomes in a dependent manner (Kang et al., 2014).

HMGB1 is actively released from the nucleus in response to exogenous microbial stimulus such as LPS (Wang et al., 1999), lysophosphatidylcholine (LPC) (Gardella et al., 2002), mycobacterial infection (Grover et al., 2008), CpG-DNA (Ivanov et al., 2007), as well as endogenous stimuli such as IFN-α (Jiang & Pisetsky, 2006), IFN-β (Lu et al., 2014), TNF (Wang et al., 1999), NO (Tamura et al., 2011), hydrogen peroxide (D. Tang et al., 2007), peroxynitrite hyperlipidemia (Haraba et al., 2011), kynurenic acid (KYN) (Tiszlavicz et al., 2011), ATP (Eun et al., 2014) neuropeptide Y (NY) (J. R. Zhou et al., 2013) and other stimuli (ethanol) (Whitman et al., 2013). Other than active secretion, HMGB1 can also be released passively after cellular death such as apoptosis, necrosis, autophagic or lysosomal cell death and tissue injury. The mechanisms underlying this passive release are dependent on several mediators such as PARP1, RIP3, cathepsin, anti-oxidant enzyme, DNase , caspase and ATG pathway

1.3.3 Redox state, receptors and signaling cascade

HMGB1 is passively and actively released from the nucleus into the extracellular milieu. It is predominantly present in the fully reduced form in the nucleus, and it can be oxidized in the cytoplasmic compartment after ROS production (Daolin Tang et al., 2011). Recently, it has

been shown that three cysteine amino acids (C), at positions 23, 45 and 106, play an important role in the HMGB1 redox state, receptor binding profile and the activation of the corresponding signaling pathway, which defines the physiological/pathological action of HMGB1 (H. Yang et al., 2012). When all three cysteines are reduced by an (SH) group, the protein is called All thiol (at) HMGB1 (all-thiol HMGB1 or HMGB1C23hC45hC106h). The all-thiol HMGB1 binds to RAGE) and acts as a chemo-attractant. It also forms a complex with the CXCL12 chemokine and potentiates chemotaxis by binding to the CXCR4 receptor. HMGB1 with a disulfide bond between C23 and C45, together with a thiol group at C106, is called disulfide (ds) HMGB1 (disulfide HMGB1 or HMGB1C23-C45C106h) (H. Yang et al., 2012). This form binds to TLR4 and displays cytokine-inducing properties via activation of intracellular MAP kinase signaling. When all cysteine positions have a sulphonyl group (SO_3H), HMGB1 is in its fully oxidized form (oxHMGB1 or HMGB1C23soC45soC106so), but the physiological function of this redox state of HMGB1 is still not clear. These redox forms of HMGB1 can be identified in different body fluids of patients with different pathological conditions (Antoine et al., 2014) (Figure 6).

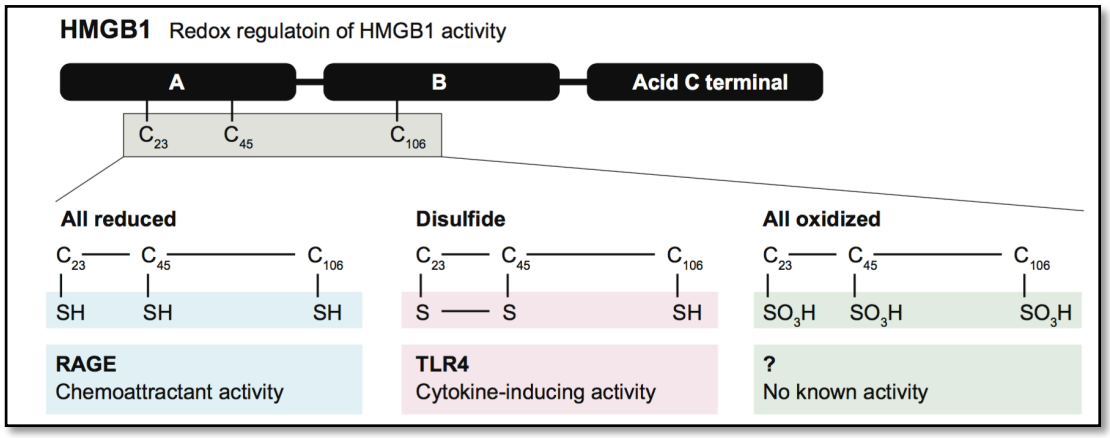

Figure 6. Three cysteines and their important roles in the redox regulation of HMGB1 (Reprinted with permission, Adapted from Jungo et al. 2015)

Besides the receptors mentioned above, HMGB1 may bind to partner molecules potentiating their effects. For instance, HMGB1 binds to IL-1β and LPS to potentiate their cytokine-inducing capacity via IL-1R and TLR4, respectively (Wahamaa et al., 2011). In addition, HMGB1-nucleosome complex have action through TLR2 receptors (Pisetsky, 2014) (Figure 7).

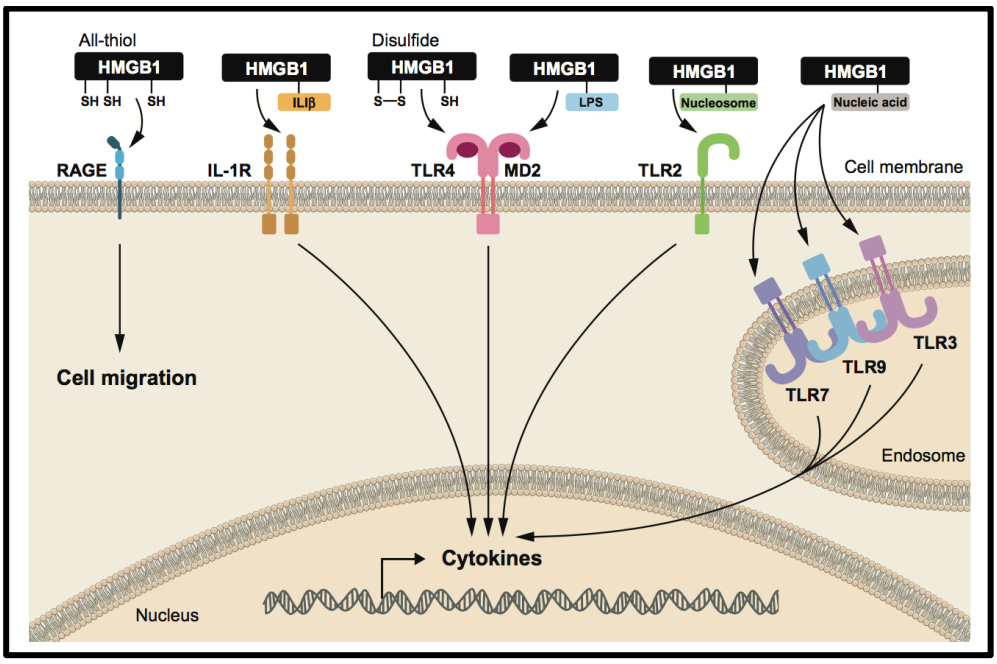

Figure 7. Different redox forms of HMGB1 and their action on different receptors (Reprinted with permission, Adapted from Kato et al.; 2015)

1.4 RHEUMATOID ARTHRITIS AND PAIN

Approximately 0.5-1% of the world's population suffers from rheumatoid arthritis (RA) (Cross et al., 2014). It is a chronic, systemic autoimmune disease characterized by synovial inflammation, cartilage destruction and bone erosion (Smolen & Aletaha, 2015). Prior to active disease, several antibodies can be detected in the systemic circulation such as rheumatoid factor (IgG and IgM), collagen type II and anti-citrullinated proteins (ACPA) (Nielen et al., 2004; Rantapaa-Dahlqvist et al., 2003). If circulating antibodies persist (thus becoming pathogenic), immune cells are recruited leading to the active state of RA disease.

Chronic pain is one of the most distressing symptoms in RA patients (Altawil et al., 2016). Recent studies have shown that pain symptoms start before the manifestation of RA disease. During the active phase, inflammatory mediators at the site of inflammation trigger the sensory neurons (Bas et al., 2016). These stimuli can become painful with persistent activation of peripheral neurons. Despite effective therapeutic results with disease modifying anti-rheumatic drugs (DMARDs), such as TNF neutralizing and IL-6 receptor binding antibodies, patients still report pain as their most disturbing problem (Altawil et al., 2016; Firestein, 2003; McInnes & Schett, 2007). Pain in RA has been associated mainly with tissue injury, and inflammatory processes in the small and big joints, but accumulating clinical and experimental data indicate that in addition to peripheral mechanisms, neurochemical and structural changes within the sensory system may affect central pain processing as well (Bas et al., 2016; Neumark et al., 1979). Even though the new DMARDs have improved the prognosis, RA-associated joint pain remains a big problem and the mechanisms that maintain pain in RA remain unclear. Thus it is crucial to identify new players in these processes.

1.4.1 HMGB1 and rheumatoid arthritis

Most of the evidence in the literature points towards the role of extra-nuclear HMGB1 in the pathogenesis of RA not only in human but also in experimental models. Levels of extra-nuclear HMGB1 are elevated in the serum, synovial tissue and synovial fluid of arthritis patients (Hamada et al., 2008; Kokkola et al., 2002; Taniguchi et al., 2003). The increased levels of HMGB1 in synovial fluid are much higher in RA than osteoarthritis (Taniguchi et al., 2003). Macrophages from the synovial fluid induce the release of cytokines (TNF, IL-1β and IL-6) after stimulation with HMGB1 since these cells have high expression of TLR2, TLR4 and RAGE receptor (Huang et al., 2007; Taniguchi et al., 2003).

In an experimental model of arthritis, HMGB1 expression is significantly elevated in the extracellular space and cytoplasm of fibroblasts, macrophages, synoviocytes and vascular endothelial cells (Palmblad et al., 2007). The presence of extranuclear HMGB1, TNF, IL-1β and VEGF (vascular endothelial growth factor) in proliferating synovial tissue may lead to the destruction of cartilage and bone (U. Andersson & Harris, 2010; Biscetti et al., 2016; Palmblad et al., 2007). Hypoxia is one of the prominent conditions in which HMGB1 leads to synovitis in the CIA (collagen-induced arthritis) experimental model. Immunohistological observations show that pimonidazole (hypoxic marker) co-localizes with HMGB1 in CIA model (Hamada et al., 2008). Elevated extranuclear HMGB1 enhances the function of tissue plasminogen activator and metalloproteinases, which leads to the destruction of cartilage and bone structure (Parkkinen & Rauvala, 1991). In addition to this, HMGB1 is also shown to play an important role in osteoclastogenesis by producing TNF via RAGE (Yamoah et al., 2008; J. Yang et al., 2008; Z. Zhou et al., 2008).

Injection of recombinant HMGB1 into murine knee joint leads to an inflammatory response with recruitment of immune cells and synovitis lasting for approximately 4 weeks, though the severity of the articular inflammation varies between mouse strains (Pullerits et al., 2003). It has been suggested that the inflammatory response of HMGB1 depends on IL-1 receptor activity since IL1-R knockout (KO) mice do not exhibit any signs of arthritis after HMGB1 injection in the knee (Pullerits et al., 2003). Others have shown that HMGB1 potentiates/synergizes the action of IL1-β by binding to its own receptor (IL-1R) (Wahamaa et al., 2011) with transactivation of the IL1-β promoter sites NF-IL6 (nuclear factor IL6) and PU.1 (myeloid and B cell-specific transcription factor) resulting in synergistic trans-activation of IL1-β (Mouri et al., 2008). Taken together these findings strengthen the importance of HMGB1 in the pathogenesis of rheumatoid arthritis.

1.4.2 HMGB1 and pain

Mounting data from the past decade indicate that HMGB1 has an important pathological role in different pain conditions. The first report showing this association was in 2010 by Chacur and colleagues. They demonstrated that application of HMGB1 on the sciatic nerve, using pre-implanted indwelling peri-sciatic catheters, induced mechanical hypersensitivity in rats in a dose-dependent manner (Chacur et al., 2001). Similar findings were reported after

application of HMGB1 from autologous pulposus to the sciatic nerve in rat (Shibasaki et al., 2010). Injection of HMGB1 to peripheral sites like intravesical, skin and joint, induce pain-like behavior. In the periphery, the redox state of HMGB1 has been proven to be important in the induction of hypersensitivity as intraplantar and intra-articular injections of disulfide HMGB1, but not all-thiol HMGB1, induce mechanical hypersensitivity in naïve mice (Agalave & Svensson, 2015; Tanaka et al., 2013; Yamasoba et al., 2016), which was reversed by rh-thrombudilin (Tanaka et al., 2013) and the selective TLR4 antagonist LPS-RS (Yamasoba et al., 2016). Furthermore, intravesical instillation of disulfide HMGB1 but not all-thiol HMGB1 elicited dose-dependent abdominal mechanical hypersensitivity (bladder pain), which was reversed by a TLR4 inhibitor (Ma et al., 2017). It should be noted, however, that a 10-fold higher dose of all-thiol HMGB1 (compared to the dose of disulfide HMGB1) also induces mechanical hyperalgesia after intraplantar injection (Yamasoba et al., 2016).

At the spinal level, injection of disulfide HMGB1 into the cerebrospinal fluid (but not all-thiol HMGB1 or oxHMGB1) induces mechanical hypersensitivity, activation of glial cells and cytokine mRNA expression in a TLR4 dependent manner (Agalave et al., 2014). In addition to evoking pain-like behavior upon injection, the involvement of HMGB1 at the periphery and spinal level in different experimental pain models has been reported. In the collagen antibody-induced arthritis (CAIA) model, inflammation in the paw leads to increased levels of extra-nuclear HMGB1 in the lumbar spinal cord (Agalave et al., 2014) and blocking spinal HMGB1 action by the HMGB1 inhibitor (HMGB1 neutralization antibody and Abox peptide) reverses CAIA-induced mechanical hypersensitivity (Agalave et al., 2014).

Moreover, elevated levels of extra-nuclear HMGB1 in DRGs, spinal cord and sciatic nerve have been reported in different neuropathic pain models with induction of mechanical hypersensitivity (Spinal nerve ligation (SNL), Partial sciatic nerve ligation (PSNL), Tibial nerve ligation (TNI)) (Feldman et al., 2012; Nakamura et al., 2013; Shibasaki et al., 2010). Consistently, mechanical hypersensitivity induced by the different nerve injuries was reversed by treatment with the HMGB1 inhibitor, glycyrrhizin, (Feldman et al., 2012) and HMGB1 neutralizing antibodies (Nakamura et al., 2013; Shibasaki et al., 2010). In addition, mechanical hypersensitivity in a bone cancer experimental model (Tong et al., 2010), type 2 diabetic-induce neuropathy model (P. C. Ren et al., 2012), cyclophosphamide induced bladder pain (Kouzoukas et al., 2016; Tanaka et al., 2014), Chemotherapy induced neuropathy (Nishida et al., 2016) and central stroke pain model (Harada et al., 2016b), is reversed by systemic treatment with a HMGB1 neutralizing antibody. Inhibiting endogenous HMGB1 with HMGB1 inhibitors shows reversal of pain-like behavior in different experimental pain model (Table 1).

Table 1. HMGB1 inhibition in different experimental pain model

Pain model	Species	Sex	Nociceptive assessment	HMGB1 inhibition	Route of administration	Reference
Nerve injury pain model						
SNL	Rat	Male	VF, hotplate	HMGB1 Ab	Peri-sciatic	(Shibasaki et al., 2010)
Nucleus pulposus	Rat	Female	VF	HMGB1 Ab	i.p.	(Otoshi et al., 2011)
TNI	Rat	Female	VF	Glycyrrhizin	i.p.	(Feldman et al., 2012)
Diabetes	Mice	-	VF	HMGB1 Ab	i.t.	(P. C. Ren et al., 2012)
PSNL	Rat	Male	VF	HMGB1 Ab	i.v.	(Nakamura et al., 2013)
PSNL	Mouse	Male	VF	HMGB1 Ab	Perineural	(F. F. Zhang et al., 2015)
Inflammatory pain model						
LPS paw	Rat		VF, thermal	HMGB1 Ab	i.pl.	(Tanaka et al., 2013)
Arthritis	Mouse	Male, female	VF	HMGB1 Ab, Abox	i.t.	(Agalave et al., 2014)
Other pain model						
Bone cancer	Rat	Female	VF	HMGB1 Ab	i.t.	(Tong et al., 2010)
Cyclophosphamide induced bladder pain	Mouse	Female	VF	HMGB1 Ab, thrombomodulin	i.p	(Tanaka et al., 2014)
Chemotherapy induced neuropathy	Rat	Male	VF, paw pressure	HMGB1 Ab	i.p.	(Nishida et al., 2016)
Central post stroke pain	Mouse	Male	VF	HMGB1 Ab	i.v., i.t	(Harada et al., 2016a)
Cyclophosphamide induced bladder pain	Mouse	Female	VF	Glycyrrhizin	i.p.	(Kouzoukas et al., 2016)

1.5 SEX DIFFERENCE IN PAIN PROCESSING

In the field of pain research, the importance of comparing mechanisms of nociception between males and females (both in rodents and patients) is currently gaining attention. From a preclinical perspective, Sorge et al. reported surprising data in 2011 suggesting that spinal TLR4 is important in inflammatory and neuropathic mediated mechanical hypersensitivity in male but not in female mice (Sorge et al., 2011). Later, it was shown that BDNF and purinergic receptor 4 (P2X4) in microglia are contributing to spared nerve injury (SNI) induced mechanical hypersensitivity in male but not in female mice. Moreover, it was indicated that in female mice, adaptive immune cells such as T-cells drive spinal sensitization, rather than microglia (Sorge et al., 2015). These findings have triggered many

researchers to explore sex-dependence of various pain-related mechanism in rodents, which have led to the insight that there is an underestimated complexity and discrepancy between studies associated with nociception and sex. For example, Sorge et al. reported that intrathecal injection of LPS induces mechanical hypersensitivity in male but not in female mice (Sorge et al., 2011). However, Woller et al. reported that intrathecal injection of LPS induces mechanical hypersensitivity equally in male and female mice and similarly, intrathecal injection of disulfide HMGB1 also induces mechanical hypersensitivity in male as well as in female mice (Agalave et al., 2014; Woller et al., 2016). However, sex differences were found after systemic treatment with the TLR4 antagonist (TAK-242) which reverses LPS-induced mechanical hypersensitivity in male but not in female mice (Woller et al., 2016). Blocking the effect of spinal HMGB1 did not show a sex-dependent dimorphism in the CAIA model (Agalave et al., 2014), which opens the possibility that endogenous HMGB1 may act on other receptors than TLR4 in some experimental models of pain. Moreover, formalin-induced allodynia (intraplantar injection) was delayed in both male and female mice after systemic treatment of TLR4 antagonist (TAK 242) and in TLR4 deficient mice (Woller et al., 2016), indicating that there may be differences in the sex-dependent engagement of TLR4 in nociception in the periphery compared to the spinal site.

Sex dimorphism reported in terms of production of pro-inflammatory cytokines after immune challenge show that females produce higher levels than males. Based on the literature, differences in cytokine production between males and females are dependent on the disease model in rodents (Aulock et al., 2006; Drew & Chavis, 2000; Engler et al., 2016; Loram et al., 2012). One example is that, following Complete Freund's Adjuvant (CFA) treatment, proinflammatory cytokines were highly elevated in trigeminal ganglia in male but not in female mice (Sorge & Totsch, 2017). Moreover, in the clinical field, LPS challenge in healthy humans elicits a higher cytokine production in the women compared to men (Karshikoff et al., 2015). Increases in the release of cytokines are correlated to higher hyperalgesia in females, as reviewed in Doyle and Murphy (2017). Cook, Nickerson and colleagues have shown that females develop more inflammation and hyperalgesia after immune challenges (CFA model) when compared to males (Cook & Nickerson, 2005). Thus, sex dimorphism mainly depends on the immune challenge and on the experimental disease model.

2 AIM OF THESIS

The overall aim of the study was to investigate the role of HMGB1 in an arthritis-induced pain (model) and specifically explore the central and peripheral role of HMGB1 in pain processing. Three specific aims of this thesis were;

1. Characterization of the collagen antibody-induced arthritis (CAIA) experimental model of arthritis as a model of arthritis-induced pain
2. Investigation of the spinal role of HMGB1 in the CAIA model with particular focus on the HMGB1-Microglia-TLR4 axis
3. Investigation of the peripheral role of HMGB1 in CAIA induced pain as a possible mechanism behind neuronal hypersensitivity

3 MATERIAL AND METHODS

3.1 ANIMAL MODEL

3.1.1 Animal

All animal experiments were approved by and conducted according to the regulations of the local ethics committee for animal experiments in Sweden and in the US (Institutional Animal Care and Use Committee of The University of Texas at Dallas). All animals were housed in standard cages (4-5 mice/cage) in an environment maintaining 12 h light/dark cycle with food and water ad libitum. Different WT strains, genetically compromised mice (both male and female) were used for this thesis. For **Paper I**; CBA, QB and BALB/c male mice were used. For **Paper II**, BALB/c male and female were used for induction of CAIA. Genetically modified mice were used which have a deletion of TLR2, TLR4 and RAGE with a C57BL/6 background. For **Paper III**, C57BL/6 male and female mice were used, and BALB/c and C57BL/6 male and female mice were used for **Paper IV**. Genetically modified and compromised mice, $TLR^{fl/fl}$ mice have been described previously (Jia et al., 2014). Mice with a TLR4 deletion in myeloid cells, or in peripheral nociceptors, were generated by crossing mice with the floxed TLR4 allele with mice expressing Cre under the control of the LysM or the Nav1.8 promoter, respectively. The resulting LysM-$TLR4^{fl/fl}$ and Nav 1.8-$TLR4^{fl/fl}$ and $TLR4^{fl/fl}$ (used as control mice) were backcrossed 8 generations to a C57BL/6 background at University of Texas at Dallas, US.

3.1.2 Collagen antibody-induced arthritis model

BALB/c male and female mice were injected intravenously with arthritogenic antibody cocktail (5 monoclonal CII antibodies), 1.25 mg/ mouse (Chondrex, Redmond, WA), on day 0; synchronized with an intraperitoneal injection of LPS, 25 µg/ mouse (Chondrex, Redmond, WA), on day 5 to developed joint inflammation (Described detail in **Paper I**). Two different groups of control mice were used for this study; saline control mice received saline on day 0 and on day 5 and LPS control mice received saline on day 0 with LPS on day 5. For this thesis work, the collagen antibody-induced arthritis model was used in **Paper I, II** and **IV.**

3.1.3 Arthritis score and joint histology

Clinical scores were measured by visual inspection of forelimbs and hind limbs. Clinical scores were allotted based on swelling and redness of toes, knuckles and ankle joints. An inflamed toe or knuckle gets 1 point, while an inflamed paw or ankle joint gets 5 points. Thus each limb gets a maximum of 15 points, and each mouse gets a maximum of 60 points. Clinical score data were plotted as arthritis score over time. Joint histology was performed on ankle joints, as we see the inflammation in the paw and ankle joint. Ankle joints were harvested during/after joint inflammation, post-fixed with 4% paraformaldehyde and decalcified for 3-4 weeks in decalcification solution (100 g EDTA, ethylenediaminetetraacetate, 75 g polyvinylpyrrolidone, 12.11 g Tris in 1 L Millique water adjusted to pH 7.0 with KOH (potassium hydroxide). After decalcification, ankle joints were

dehydrated using 70% ethanol and xylene followed by embedding in paraffin. Ankle joints were cut into 5 μm sections and mounted on glass slides and stained with hematoxylin and eosin staining.

3.2 DRUGS AND DRUG DELIVERY

3.2.1 HMGB1 inhibitor

HMGB1 neutralizing antibody was used to block the action of endogenous HMGB1, the mouse anti-HMGB1 antibody (2G7) was injected spinally at a dose of 7.25 μg and 15 μg, and systemically injected as 100 μg. HMGB1 box A peptide (Abox) (20μg) was injected i.t. and 300 μg injected systemically. The 2G7 anti-HMGB1 IgG2b noncommercial monoclonal antibody (mAb) (Chavan et al., 2012; Kokkola et al., 2003; Schierbeck et al., 2011) (developed at the former Critical Therapeutics, Boston, MA; now Cornerstone Therapeutics, Cary, NC) binds to an epitope within the amino acid region position 53 to 63 of the A box unit. Recombinant A box protein corresponds to 1 of the 2 highly conserved DNA binding domains of the HMGB1 protein (amino acid 1–89) and has been shown to block extracellular HMGB1 activities (Kokkola et al., 2003; Schierbeck et al., 2011).

3.2.2 Microglia inhibitor

The previously used microglial/glial inhibitor tetracycline antibiotic: minocycline was used for this study (Chen et al., 2017; Moller et al., 2016; Sorge et al., 2015). Minocycline was dissolved in saline and filter-sterilized with 0.22 mm millipore filter. The drug was freshly prepared and used within 3 days, because of its instability in solution. Minocycline was injected intrathecally (30 μg/mouse in 5 μl volume) whereas control mice were injected with 5 μl of saline.

3.2.3 Other drugs

3.2.3.1 HMGB1 (different redox forms)

Different redox forms of HMGB1 were used for the studies; the disulfide form of HMGB1 (TLR4 ligand, cytokine-inducing form), the all-thiol HMGB1 (RAGE ligand, chemoattractant form) and the oxidized form of HMGB1 (no known biological activity). In **Paper II** and **Paper III**, intrathecal delivery of the different forms (1 μg/mouse) was performed to investigate the central role of HMGB1 in pain processing. In **Paper IV**, intra-articular injection of disulfide HMGB1 and all-thiol HMGB1 was done to investigate the peripheral role of HMGB1 in pain processing.

3.2.3.2 Buprenorphine

Buprenorphine is a semisynthetic derivative of theabaine with mixed partial agonist opioid receptor modulator. Buprenorphine is well known to have high affinity towards E opioid receptors with antagonizing property. It has an inhibitory action on voltage-gated sodium channels with binding to the anesthetic binding site and has a local anesthetic property.

3.2.3.3 Pentoxifylline

Pentoxifylline is a xanthine derivative drug used to treat muscle pain in peripheral artery disease. It is a competitive phosphodiesterase inhibitor, which increases the intracellular levels of cAMP and activates PKA, which leads to inhibition of TNF and leukotriene synthesis and reduces inflammation. Systemic injection of pentoxifylline was performed in the inflammatory and late phase of CAIA model.

3.2.3.4 Gabapentin

Gabapentin is currently used in the clinic to treat neuropathic pain and seizures. It interacts with voltage-gated calcium channels, binding the α2δ subunit of the channel, which leads to a reduction in calcium current.

3.2.3.5 Diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug commonly used to treat inflammation and used as an analgesic drug. The mechanism of action of diclofenac is to inhibit cyclooxygenase II.

Table 2. List of the different categories of drugs, with route and dose

Drug	Study	Paper	Route	Dose
2G7	Central	Paper II	i.t.	15 µg / mouse
2G7	Peripheral	Paper III	s.c.	100 µg / mouse
Abox	Central	Paper II	i.t	20 µg / mouse
all-thiol HMGB1	Central/peripheral	Paper II/IV	i.t. /i.a.	1 µg /mouse
Buprenorphine	CAIA	Paper I	i.p.	0.1 mg/KG
Diclofenac	CAIA	Paper I	i.p.	30 mg/KG
disulfide HMGB1	Central/peripheral	Paper II/III/IV	i.t. /i.a.	1 µg /mouse
Gabapentin	CAIA	Paper I	i.p.	100 mg/KG
Minocycline	Central	Paper III	i.t	30 µg / mouse
oxHMGB1	Central	Paper II	i.t.	1 µg /mouse
Pentoxifylline	CAIA	Paper I	i.t.	30 µg / mouse

3.3 ASSESSMENT OF PAIN BEHAVIOR

3.3.1 Von Frey measurement

For measurement of mechanical hypersensitivity (pain-like behavior), the von Frey test was used. It is described in greater detail in **Paper I, II III** and **IV.** Briefly, animals were acclimatized to the Von Frey station on two different occasions. Three baseline measurements were performed on different days, followed by randomization of mice into the

different treatment groups. Mechanical hypersensitivity was measured by the assessment of paw withdrawal threshold in response to application of von Frey optihair filament, using the up-down method (Chaplan et al., 1994). A series of von Frey filaments with a logarithmically incremental stiffness of 0.5, 1, 2, 4, 8, 16, and 32 mN (converted to 0.051g, 0.102g, 0.204g, 0.408g, 0.815g, 1.63g, and 3.26g, respectively) were applied to the plantar surface of the hind paw and held for 2 to 3 seconds. Tissue damage was avoided by setting 4 g as a cutoff. Withdrawal of the hind paw was noted as a positive response. For the CAIA animal model and the intrathecal HMGB1 model withdrawal thresholds from both hind paws were averaged. However, in the peripheral project, only withdrawal thresholds of the injected (ipsilateral hind) paw were considered for the analysis. Data were plotted as 50% probability for withdrawal thresholds (which is the force of the filament to which an animal reacts to 50% of the presentations) expressed as the thresholds in gram.

3.4 PCR

3.4.1 On spinal cord

In **Paper II**, animals were deeply anesthetized with 4% isoflurane followed by decapitation. Lumbar spinal cord was dissected with laminectomy method with L3-L5 region collected. Tissues were flash frozen and stored in -70° C until use for the analysis. Spinal cord tissues were homogenized in Trizol with tissue lyser (25 Hz frequency for 2 min with 2 cycles; QAIGEN). mRNA was extracted according to the manufactures protocol, using TRIzol. Reverse transcription of RNA followed to make complementary DNA, which was further used in real time qualitative polymerase chain reaction (Step-one system, Applied biosystem, Foster city, CA). Below is the list of Taqman primer probes (Table no. 2) that were used.

Table 3. List of the Taqman primer probes

Analyte	Taqman primer	Name of analyte
Tnf	Mm00443258_m1	*Tumor necrosis factor*
IL1β	Mm00434228_m1	*Interleukin 1 beta*
Il6	Mm00446190_m1	*Interleukin 6*
MCP-1	Mm00441242_m1	*Monocyte chemoattractant protein 1*
Cxcl1	Mm04207460_m1	*Chemokine (C-X-C motif) ligand 1*
Cxcl2	Mm00436450_m1	*Chemokine (C-X-C motif) ligand 2*
Ngf	Mm00443039_m1	*Nerve growth factor*
Cox2	Mm00478374_m1	*Cyclooxygenase 2*
Cd11b	Mm00434455_m1	*Cluster of differentiation molecule 11B*
Gfap	Mm00546086_m1	*Glial fibrillary acidic protein*
Hmgb1	Mm00849805_gH	*High mobility group box 1 protein*

Hprt-1	Mm01545399_m1	*Hypoxanthine phosphoribosyltransfarase 1*
Pes-1	Mm00727566_s1	*Pescadillo homolog 1*

3.4.2 On ankle joint

In **Paper IV**, mice were injected intra-articularly with disulfide HMGB1 and all-thiol HMGB1, 4 hours later animals were deeply anesthetized with 4% isoflurane followed by decapitation. Ankle joints were dissected and trimmed as 6mm from ankle joint to tibia and paw, samples were flash frozen and stored in -70° C until further processing. Joint tissue was pulverized with biopulveriser followed with RNA extraction with TRIzol using tissue lyser (30 Hz frequency for 2min with 2 cycles, QAIGEN). Reverse transcription were performed to make cDNA followed with real time quantitative PCR (Step one System, Applied Biosystem, Foster city, CA).

3.5 IMMUNOHISTOCHEMISTRY

For immunohistochemistry, animals were anesthetized with isoflurane and intracardially perfused with saline/PBS solution followed by 4% paraformaldehyde (PFA). The Lumbar spinal cord tissue (L1-L6) and DRG tissue (L3-L5) was dissected, postfixed in 4% PFA for 12-20 h and cryoprotected in 20% sucrose. Lumbar spinal cord tissue (L3-L5) and DRG were immersed in OCT on filter paper, which were expose to CO2 stream. Spinal cord tissue was cut in 20 μM section and mounted on glass slide. Mounted sections were pre-incubated with 5% normal goat or donkey serum in 0.2% Triton X-100 in PBS solution to block nonspecific binding for antibodies. Consequently, sections were incubated with the primary antibodies overnight at 4 °C. After primary antibody treatment sections were incubated in respective secondary antibody then stained sections were coverslipped with prolong gold antifade medium with DAPI (Life technologies) and examined in Zeiss LSM710 confocal microscope.

Table 4. List of the Primary antibody with its dilution

Primary Antibody	Vendor	Catalog number	Dilution
Rabbit anti-HMGB1	Abcam	Ab18256	1:500 dilution
Mouse anti-NeuN	Millipore	MAB377	1:1000 dilution
Anti-NeuN-Alexa 488	Millipore	MAB377x	1:100 dilution
Mouse anti-GFAP	Millipore	MAB 360	1:1000 dilution
Goat anti-Iba1	Abcam	Ab107159	1:2000 dilution
Rabbit anti-Iba1	Wako	01919741	1:2000 dilution

3.6 WESTERN BLOT

Western blot protocol details are described in **Paper II**. Briefly, mice were anesthetized, and spinal cord tissues was harvested by hydro-extrusion and immediately flash-frozen on dry ice and stored at -70°C until analysis. NE-PER (nuclear and cytoplasmic kit, Thermo Fisher Scientific, Lafayette, CO) was used to extract total protein from nucleus and cytoplasm (extranuclear). Total protein was separated by gel electrophoresis (NuPAGE 4–12% Bis Tris and MES running buffer, Invitrogen) and wet transferred to nitrocellulose membrane (Invitrogen). Membranes with separated protein were treated with 5% low-fat milk in Tris-based buffer (50 mmol/L Tris–HCl and 6 mmol/L NaCl with 0.1% Tween 20) to avoid nonspecific binding sites.

Blocked membranes were incubated with primary antibody overnight (48 hours for 2G7), followed by incubation in the secondary antibody conjugated to horseradish peroxidase (1:7500, Cell Signaling Technology, Danvers, MA). To visualize protein-antibody complex, chemiluminescent reagents (Supersignal, Pierce, Rockford, IL) were used, and signal intensity was measured using Quantity One software (Bio-Rad, Hercules, CA). Stripping solution (1X) were used to remove the antibodies (Millipore, Billerica, MA), in case the membranes were to be reprobed with different antibodies. Positive bands were normalized to their respective GAPDH band. Primary antibodies used for this study were 2G7 mouse anti-HMGB1 (1:1000; 1.2 μg), mouse anti-TATA binding protein (1:2000, catalog no. 051531, Millipore, Billerica, MA), rabbit anti-histone (1:5000, catalog no. ab1791, Abcam, Cambridge, UK), and mouse anti-GAPDH (1:10,000, catalog no. ab8245, Abcam). Data were analyzed and expressed as percentage change of control.

3.7 LIQUID CHROMATOGRAPHY- MASS SPECTROMETRY (LCMS/MS)

LCMS/MS analysis was performed to investigate different regulation of protein profile in male and female mice intrathecally injected with minocycline and saline; those previously injected with disulfide HMGB1. Total protein was extracted from the spinal cord collected at 6h after minocycline treatment with lysis buffer (1X PBS with 1% SDS + cocktail of protease inhibitor) with repetitive freeze/thaw process with sonication for 10-15 sec (0.3s on/0.7s pulse) using a tip sonicator. Total protein concentration was assessed using standard BCA method from Pierce. Alkylation and reduction of the protein were done by addition of dithiothreitol (DTT) and incubating at 56 °C for 30 min followed by addition of 25 mM iodoacetamide (IAA) and incubating for 30 min at 37 °C in dark. Acetone precipitation was done overnight to precipitate proteins. Digestion buffer (50 mM triethylammonium bicarbonate (TEAB) at pH 8.0 approx.) was used to resuspend the pellets. Combination of endoproteases Lys-C and trypsin to each sample vial at an E:S ratios of 1:50 and 1:100 respectively was done for enzymatic digestion. The samples were incubated overnight at 37 °C with shaking at 500 rpm. Prepared samples were run on TMT6 plex plate and labeling was performed according to standard protocol. Sample preparation and data analysis is described in detail in **Paper III.**

3.8 STATISTICAL ANALYSIS

For the statistical analysis, two-way analysis of variance (ANOVA) was used to compare changes over the time followed by the Bonferroni post hoc test. Comparisons of three or more groups were performed with one-way ANOVA followed by the Bonferroni post hoc test. Differences between two separate groups were performed using the student's t-test. Clinical scores (the arthritis scores) were compared using Kruskal-Wallis test, followed by Dunn's multiple comparisons post hoc tests. All data are presented as the mean ± standard error of the mean. Statistical significance was reported as *$p<0.05$, **$p<0.01$ and ***$p<0.001$. All statistical tests were performed using the Graph Pad prism-6 software (SanDiego, CA, US).

4 RESULTS

4.1 COLLAGEN ANTIBODY-INDUCED ARTHRITIS (CAIA)

For this thesis, We first characterized and then used the CAIA model as a model for arthritis-induced pain.

4.1.1 CAIA induces transient inflammation but persistent mechanical hypersensitivity in male and female mice

Three different mouse strains were used to investigate CAIA-induced joint inflammation and mechanical hypersensitivity. Intravenous injection of a cocktail of collagen type II antibodies followed by a low dose of intraperitoneal LPS induced transient joint inflammation and persistent mechanical hypersensitivity in QB, CBA, BALB/c mice. Though some variation in the temporal profile and degree of disease severity (**Paper I**, Fig.1) a transient inflammation with persistent mechanical hypersensitivity was observed in all three strains of mice. The model was divided into two different phases based on the resolution of visual signs of joint inflammation such that we refer to the inflammatory phase (flare of joint inflammation) and post-inflammatory phase or late phase (after resolution of inflammation with pain-behavior still detectable). For further studies in this thesis utilizing the CAIA model, BALB/c mice were used.

4.1.2 CAIA induces histopatholopathological changes in the ankle joint

To assess the degree of joint pathology subsequent to CAIA, joint histology was performed on ankle joints from three different mouse strains collected during the inflammatory and late phase. We found synovitis, bone erosion and cartilage destruction during the inflammatory phase and signs of residual bone erosion in the late phase in all three strain (Figure 8).

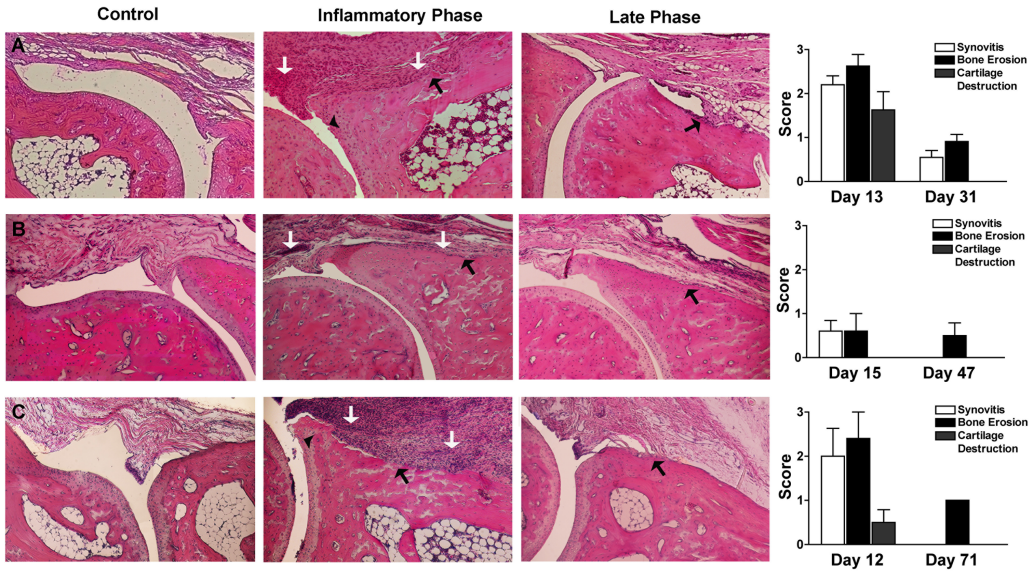

Figure 8. Joint histopathological changes in collagen antibody–induced arthritis (CAIA) male mice with hematoxylin and eosin staining for A) QB mouse strain, B) CBA mouse strain and C) BALB/c mouse strain. Bar graph represents synovitis, bone erosion and cartilage destruction for respective mouse strains (From **Paper I, Fig 2**)

4.1.3 Hypersensitivity in the two different phases of the CAIA model are maintained by different mechanisms

In order to investigate if the hypersensitivity in the two different phases in CAIA model are mediated by the same mechanisms we took a pharmacological approach and used different categories of conventional analgesic drugs. The outcome of these experiments is summarized in Table 5. Systemic injection of buprenorphine (weak partial μ-opioid receptor agonist) and gabapentin (voltage-gated calcium channel inhibitor) reverses CAIA induced mechanical hypersensitivity in inflammatory as well as in late phase. Interestingly, systemic injection of diclofenac (COX1/2 inhibitor) reverses CAIA induced hypersensitivity in inflammatory but not in late phase. Similarly, systemic injection of etanercept (soluble decoy TNF receptor) shows the same pattern as diclofenac (Bas et al., 2016).

Table 5. Pharmacological profiles of known analgesic and anti-inflammatory on CAIA induced inflammatory and late phase hypersensitivity in male mice. + Represents reversal of CAIA induced mechanical hypersensitivity and – represents, no effect on CAIA induced hypersensitivity (**Summarized from Paper I**).

Drug	Category	Inflammatory Phase	Late Phase
Diclofenac	NSAIDs	+	-
Buprenorphine	non-selective, mixed agonist–antagonist opioid receptor modulator	+	+
Gabapentin	voltage gated calcium ion channel inhibitor	+	+

4.1.4 CAIA induces spinal glial cell activation during and after joint inflammation

It is well established that, spinal glial cells play an important role in maintenance of pain in different experimental models of chronic pain. To investigate if a flare of joint inflammation has an effect on spinal glial cells in the inflammatory and late phase, we performed immunohistochemistry on lumbar spinal cord tissue using antibodies against Iba1 (microglia marker) and GFAP (astrocyte marker). In mice subjected to CAIA, we found a significant increase in the signal intensity for both the microglial and astrocytes markers, but with some strain differenece. In QB mice microglia as well as astrocyte were significantly elevated in both phases. However, in CBA mice microglia staining was significantly increased in both phases while astrocytes were activated only in the late phase (**Paper I**, Fig 4 and Fig 5).

4.2 SPINAL ROLE OF HMGB1 IN ARTHRTIS INDUCED PAIN

In **Paper II and Paper III,** the CAIA model was used to investigate the role of spinal HMGB1 in arthritis-induced mechanical hypersensitivity. Furthermore, the relationship between the redox state of HMGB1 and its pronociceptive properties was examined.

4.2.1 HMGB1 is constitutively expressed in spinal neuron, microglia and astrocytes

In order to examine if HMGB1 is present in the lumbar spinal cord, we performed immunohistochemistry using anit-HMGB1 antibodies and co-localized the immunoreactivity with different cell markers such as NeuN (neuronal marker), Iba1 (microglia marker) and GFAP (astrocyte marker). We found HMGB1 constitutively expressed in naive spinal cord colocalizing with neurons, microglia and astrocytes (Figure 9).

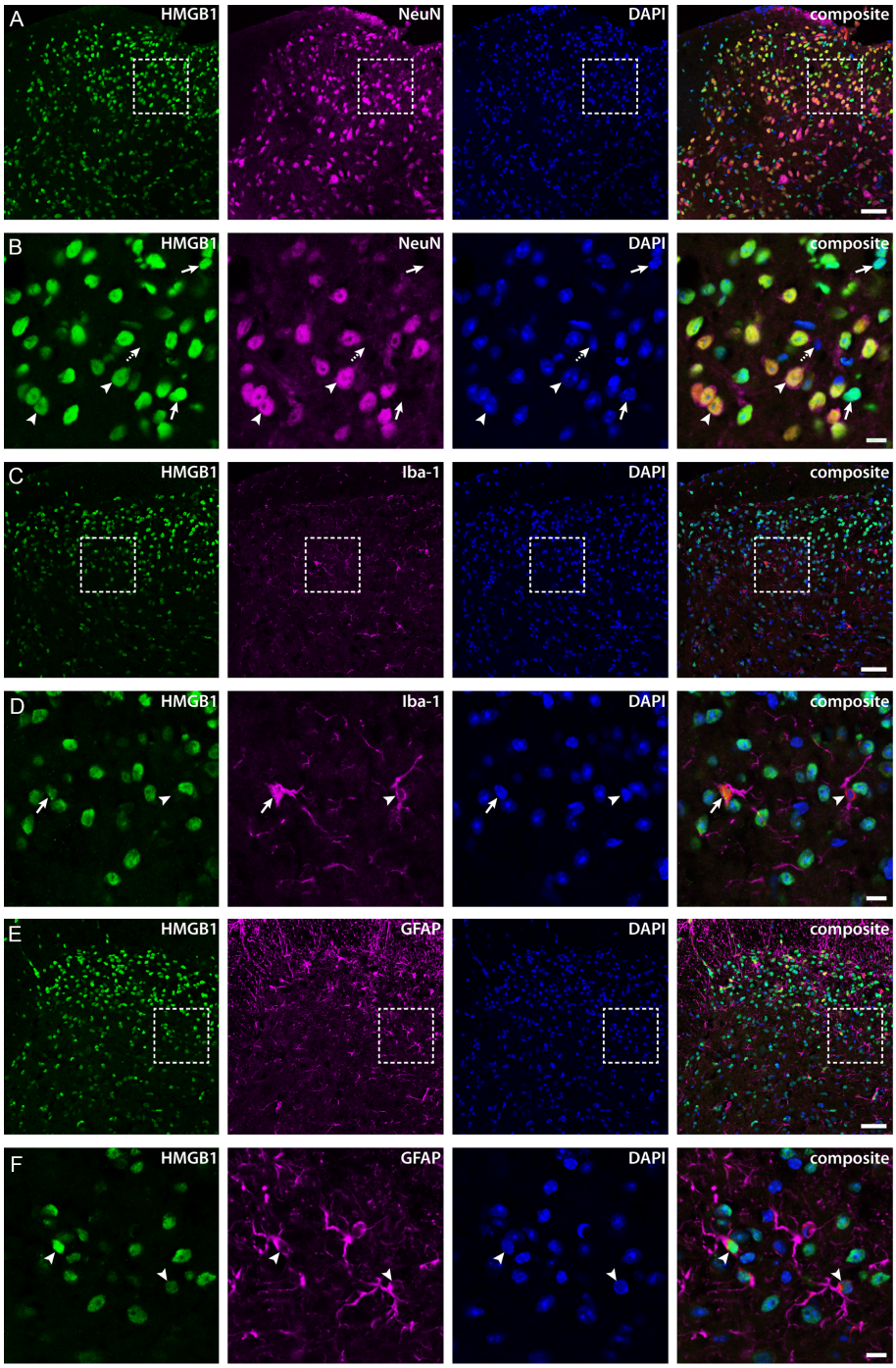

Figure 9. Immunohistochemistry for HMGB1 in neurons, microglia, and astrocytes in the dorsal horn of lumbar spinal cord. Panel A, B: HMGB1 co-localize with NeuN marker Panel C,D): HMGB1 cololize with IBA1 marker (microglia) Panel EF) HMGB1 cololize with GFAP marker (astrocytes). Scale bars are 50 μm (upper panels) and 10 μm (lower panels). Micrographs are single optical sections of the medial lumbar dorsal horn obtained with 20X(upper panels) or 40X (lower panels) objectives (From **Paper II, Fig 3**)

4.2.2 CAIA increases spinal HMGB1 mRNA levels in both phases and HMGB1 protein levels during joint inflammation

To investigate if CAIA-induced peripheral joint inflammation has an effect on HMGB1 expression in the spinal cord, we assessed HMGB1 mRNA levels by qPCR and protein levels by western blot. Spinal *Hmgb1* mRNA levels were significantly elevated in the CAIA group as compared to saline and LPS (control) group in both the inflammatory and the late phase (**Paper II**, Fig 4A, B). As extracellular HMGB1 has the capacity to function as a DAMP, we assessed the extranuclear levels of HMGB1 and found significantly increased levels of extranuclear HMGB1 in spinal cords from mice subjected to CAIA compared to saline and LPS control mice in inflammatory phase, but no difference between the groups in the late phase (Figure 10).

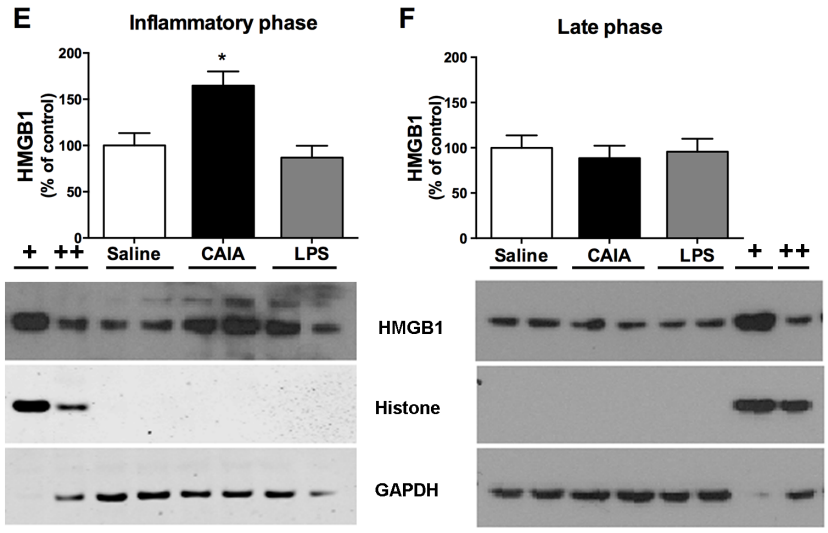

Figure 10. Spinal extranuclear HMGB1 elevated in CAIA inflammatory phase but not in late phase as compared to saline and LPS control. Density of HMGB1 signal was normalized against GAPDH and the data expressed as percentage of the saline control group. Histone was used as a negative control for extra nuclear fractionation (From **Paper II, Fig 4**).

4.2.3 Intrathecal injection of HMGB1 induces pain like behavior in male and female mice in a redox state dependent manner

To investigate if elevated level of extranuclear HMGB1 contributes to nociception we injected different redox form of HMGB1 intrathecally and measured mechanical hypersensitivity. Significant reductions of the mechanical thresholds were found in male and female BALB/c and C57BL/6 mice after a single intrathecal delivery of disulfide HMGB1. No change in mechanical threshold was observed in mice injected intrathecally with all-thiol HMGB1 or oxidized HMGB1. The mechanical hypersensitivity induced by disulfide HMGB1 lasted for approximately 4 days in male mice, while female mice recovered somewhat faster. Hyperalgesic index data

were calculated for day 0 to day 3 for respective study (Figure 11).

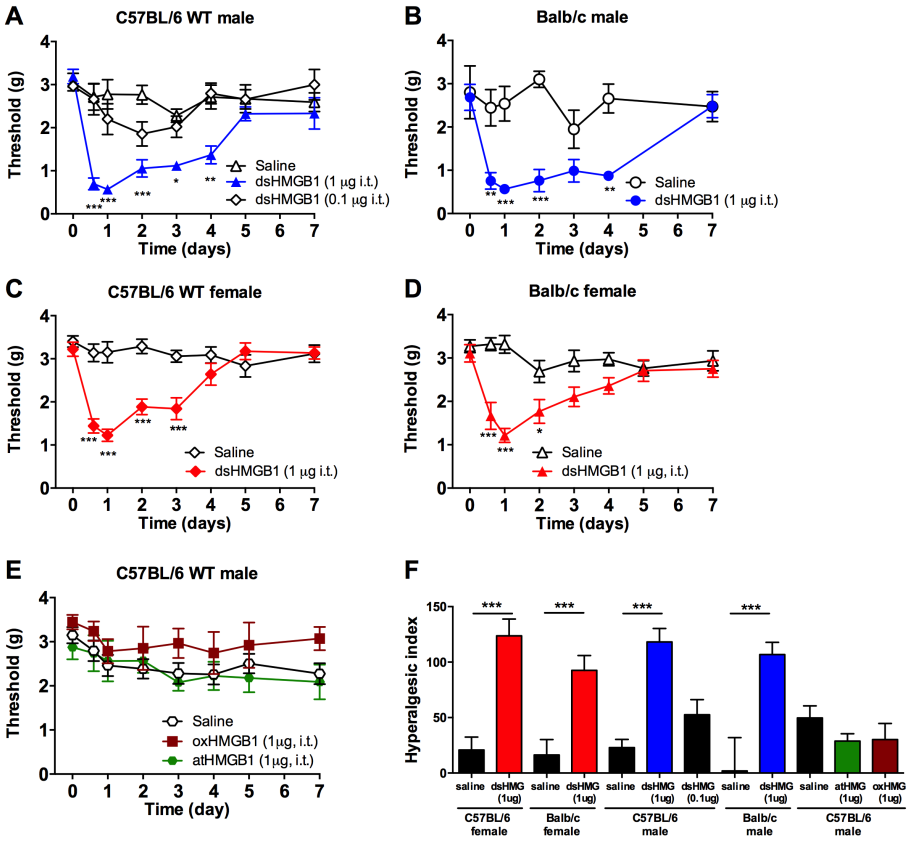

Figure 11. Single spinal injection of disulfide HMGB1 induces mechanical hypersensitivity in C57BL/6 and BALB/c male and female mice. Disulfide but not all thiol and oxidized HMGB1 induced mechanical hypersensitivity in C57BL/6 male mice. Hyperalgesic index calculated for 0-3 days. Data are presented as mean ± standard error of the mean, males: n = 6 mice per group; females n = 12 mice per group (From **Paper II,** Fig 5).

4.2.4 Disulfide HMGB1 mediate pain like behavior and induction of cytokine and glial cell associated mRNA via TLR4

To examine through which receptor disulfide HMGB1 initiate pro-nociceptive effect in naïve mice, we used genetically modified mice with deletion of some, but not all, receptors HMGB1 is thought to at through; TLR2, TLR4, and RAGE. Single intrathecal injection of disulfide HMGB1 led to a reduction in mechanical threshold in TLR2 and RAGE KO mice but not in TLR4 KO mice. Further, we investigated the expression level of cytokines, chemokine and glial markers after intrathecal injection of HMGB1. Disulfide HMGB1 but not all-thiol HMGB1 induced cytokine (*Tnf* and *Il1-β*) and chemokine (*Ccl2*, *Cxcl1* and *Cxcl2*) expression in WT male mice but not in TLR4 KO mice (Figure 12). Similar to cytokine and chemokine factors, glial factors such as (*Gfap*: astrocyte marker and *Cd11b*: microglia marker) were significantly elevated in after single intrathecal injection of disulfide HMGB1 in WT male mice but not in TLR4 KO mice (**Paper II, Fig 7**).

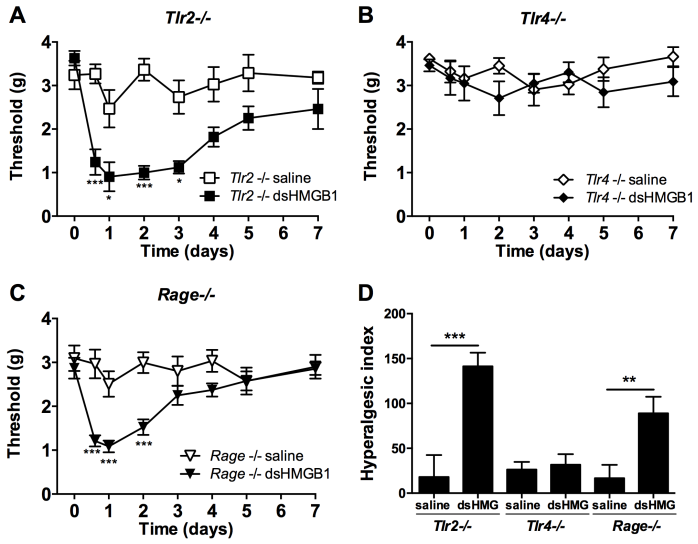

Figure 12. Single spinal injection of disulfide HMGB1 induces mechanical hypersensitivity in Tlr2$^{-/-}$ and Rage$^{-/-}$, but abrogated in Tlr4$^{-/-}$ mice. Hyperalgesic index calculated for 0-3 hours. Data are presented as mean ± standard error of the mean (From **Paper II, Fig 6**).

4.2.5 Blocking spinal HMGB1 reverses CAIA induced hypersensitivity during and after join inflammation in male and in female mice

To investigate if an elevated level of spinal HMGB1 contributes to the induction of mechanical hypersensitivity in the CAIA model we used HMGB1 inhibitors such as HMGB1 neutralization antibody (2G7) and Abox peptide (Abox). The HMGB1 inhibitors were injected intrathecally during the inflammatory and late phase, in male and female CAIA mice. We found that repetitive intrathecal delivery of the HMGB1 inhibitors reversed CAIA induced mechanical hypersensitivity during the inflammatory and late phase, in both male and female mice (Figure 13).

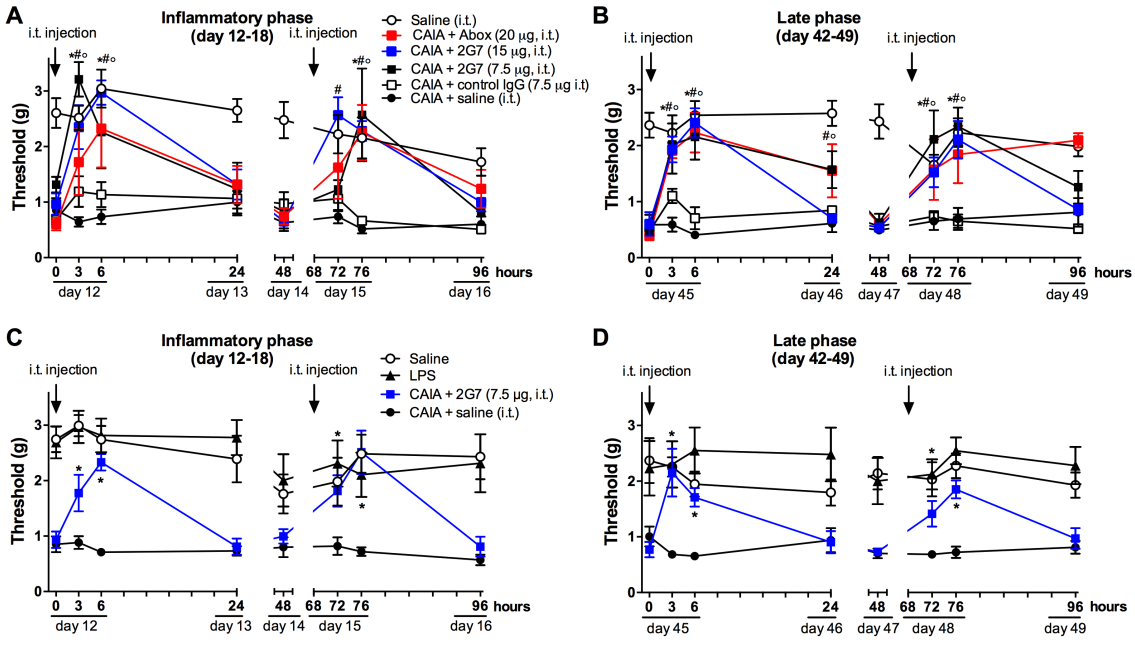

Figure 13. Repetitive intrathecal injection of 2G7 and Abox reverse collagen antibody-induced arthritis (CAIA)-induced mechanical hypersensitivity during the inflammatory and the late phase in male as well as in female

mice. Data are presented as mean ± standard error of the mean, males n = 6-8 mice/ group and females n= 6-8 mice/ group) (From **Paper II, Fig 2).**

4.3 SEX-DEPENDENT SPINAL HMGB1-TLR4-MICROGLIA INTERACTION FOR MAINTANANCE OF PAIN

Growing evidence shows sex dependence of spinal glial cells in maintenance of neuropathic and inflammatory pain (Chen et al., 2017; Sorge et al., 2015). This study (**Paper III**) was intended to examine if the pronociceptive property of disulfide HMGB1 display sex dimorphism.

4.3.1 Intrathecal injection of disulfide HMGB1 induces microglia activation in male and female mice

To investigate if disulfide HMGB1 shows a sex dependent effect on spinal microglia we assessed Iba1 signal intensity in the lumbar spinal 24 h after intrathecal delivery of disulfide HMGB1 in male and female mice. We found significantly increased Iba1 signal intensity of both male and female mice compared to respective control (Figure 14).

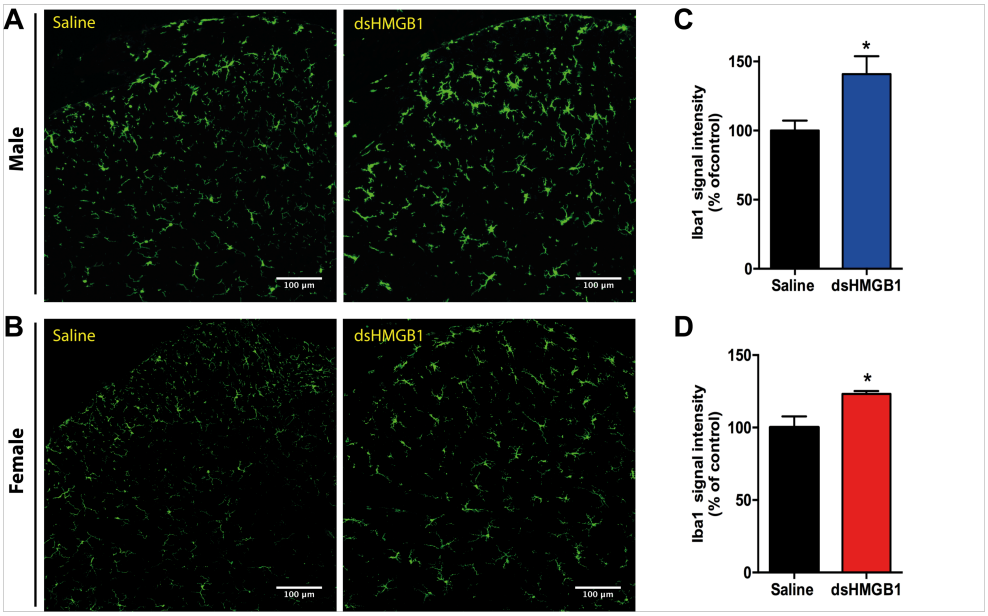

Figure 14. Spinal microglia reactivity in the lumbar level of spinal cord after intrathecal delivery of disulfide HMGB1. Immunoreactivity for IBA1 in A) male and B) female mice after 24 h post injection of disulfide HMGB1. Bar graph represents percentage signal intensity for IBA1 in C) male and D) female. Data are presented as mean ± standard error of the mean, males n = 5-6 mice/ group and females n= 5-6 mice/ group (From **Paper III, Fig 2**)

4.3.2 Blocking spinal microglia activity with minocycline reverses disulfide HMGB1 mediated hypersensitivity in male but not in female mice

As recent data point to a sex-associated contribution of microglia to spinal signal transmission (Chen et al., 2017; Sorge et al., 2015; Taves et al., 2016) we investigated if disulfide HMGB1 mediated hypersensitivity is microglia dependent in male and/or female. To assess this, we injected minocycline, commonly used as a microglia inhibitor, in male and

female mice together with and following intrathecal injection of disulfide HMGB1 and assessed mechanical hypersensitivity at 3 and 6h and after injections We found that minocycline significantly prevented and reversed disulfide HMGB1-mediated hypersensitivity at the 6 h time point in male but not in female mice (Figure 15). This data suggest that disulfide HMGB1-mediated hypersensitivity is dependent on microglia in male but not in female mice.

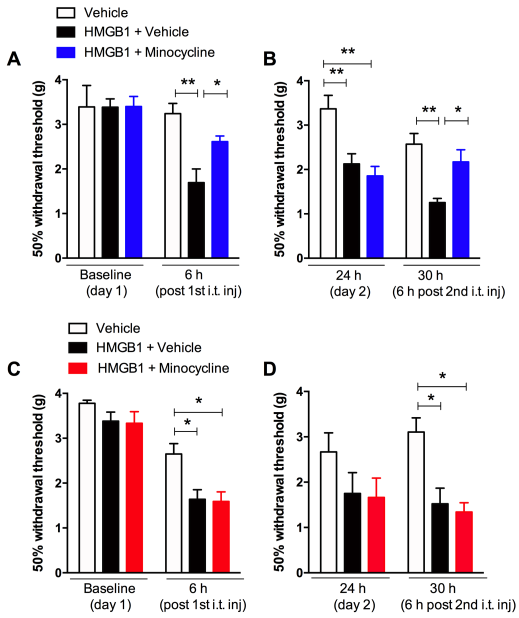

Figure 15. Intrathecal injection minocycline reverses disulfide HMGB1 induced mechanical hypersensitivity in male but not in female mice. Bar graph represents withdrawal threshold prior to and 6h after injection of HMGB1+minocycline or HMGB1+ vehicle on day 1 for A) male and C) female mice. On day 2, 24 hr after HMGB1+minocycline/vehicle injection withdrawal threshold were measured again, and minocycline and vehicle injected intrathecally a second time, and mechanical hypersensitivity assessed 6 hr later in B) male and D) female mice. Data presented as mean ± standard error of the mean. *p<0.05, **p<0.01 (From **Paper III, Fig 3**).

4.3.3 Sex dimorphism in response to minocycline using deep protein analysis

Mass spectrometry was used to investigate differences in the global protein expression in male and females injected with disulfide HMGB1 and vehicle (HMGB1 only) or minocycline treatment. We identified relatively quantified 2947 proteins. Using 2x2 factorial design, we found that male and female mice subjected to disulfide HMGB1 only, or in response to minocycline treatment showed differential regulation of 54 protein (q value < 0.05) (Supplementary table 1). Out of 54 proteins, 36 proteins showed the difference in regulation between male and females only injected with disulfide HMGB1. Surprisingly, 44 proteins showed differential regulation in males after treatment with minocycline, but only eight in females (7 protein of these showed differential regulation in males) (Supplementary Table 1). Intercept between 36 and 44 protein above was 26 proteins. In addition, and in line with male dependent differential regulations, 12 proteins showed the statistical significant interaction between gender difference and the addition of minocycline (Supplementary table 1).

All these protein results point at clear treatment effect of minocycline in males, with minor effect in females. Serine protease inhibitor 1-5 (A1AT5) was the protein with the largest difference between male and female after injection of HMGB1 only (Figure 16A). The proteins with the largest difference in response to minocycline treatment in male mice were alpha 1 antitrypsin 1-5 (A1AT5) (Figure 16A), serine protease inhibitor A3K (SPA3K) (Figure 16D) and haptoglobin (HPT) (Figure 16F), of which none of them showed a significant treatment effect in female.

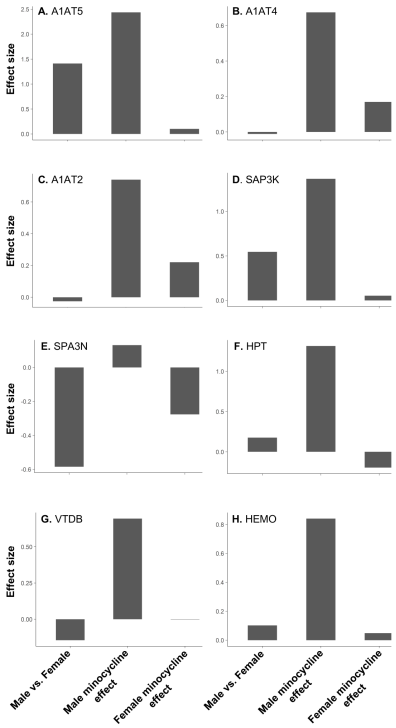

Figure 16. Depth protein analysis in male and female mice after treatment of minocycline (From **Paper III, Fig 5**).

Other serine family members alpha-1-antitrypsin 1-2 (A1AT2), Alpha-1-antitrypsin 1-4 (A1AT4) and serine protease inhibitor A3N (SPA3N) (Figure 16B, C, E) showed statistically significant increased in expression in males treated with minocycline but did not find treatment effect in females. Addition to other proteins, Vitamin D binding protein (VDBP) (Figure 16G) and hemopexin (Figure 16H) are other examples of proteins which showed statistically significant increased in expression in response to minocycline treatment in males, but not females.

4.4 PERIPHERAL ROLE OF HMGB1 IN ARTHRTIS INDUCED PAIN

The role of of HMGB1 the joint during arthritis-induced pain and the importance of the redox state of HMGB1 and interaction with TLR4 have been investigated in detail in **Paper IV.**

4.4.1 Systemic injection HMGB1 inhibitor reverses CAIA induced pain like behavior in male but not in female mice

To investigate if blocking peripheral HMGB1 shows sex dimorphism in CAIA-induced mechanical hypersensitivity, we injected the HMGB1 neutralizing antibody 2G7 systemically from day 12 to day 17 after injection of collagen type II antibodies. Interestingly, while blocking the action of HMGB1 did not alter the degree of arthritis, we found that 2G7 reversed CAIA-induced mechanical hypersensitivity in male mice but not in female mice (Figure 17).

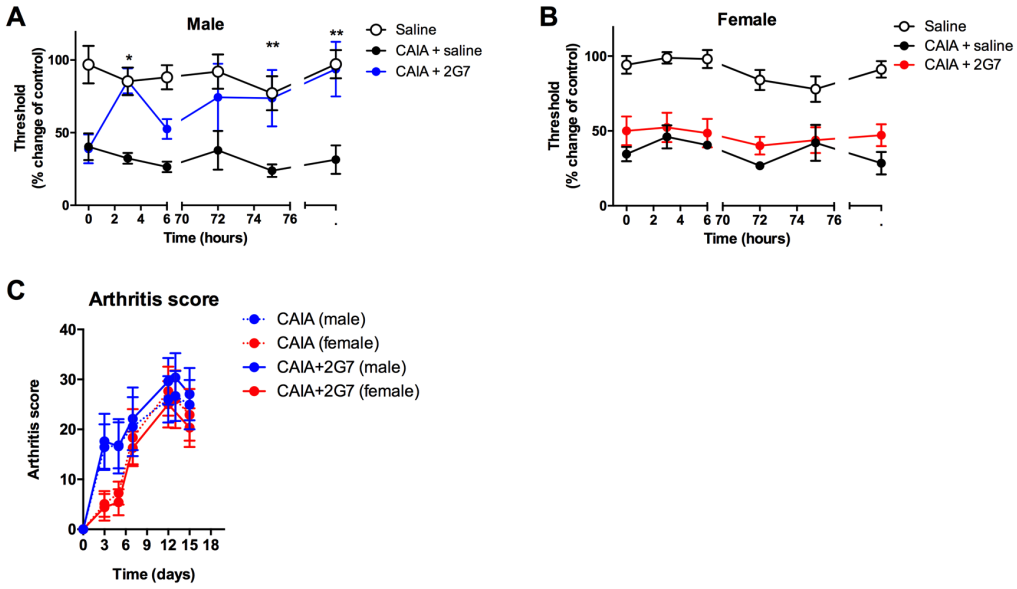

Figure 17. Repetitive systemic injection of HMGB1 inhibitor reverses CAIA induced hypersensitivity in A) male but not in B) female mice in inflammatory phase (From **Paper IV**, Fig 2).

4.4.2 Intraarticular injection of disulfide HMGB1, but all-thiol HMGB1 induces hypersensitivity in male as well as female mice with induction of cytokine and chemokine expression

To investigate if presence of HMGB1 in the joint induces hypersensitivity in a sex and redox state dependent fashion, we inject different redox form of HMGB1 in male and female naïve mice. Intra-articular injection of disulfide HMGB1 and all-thiol HMGB1 were performed in male and female mice C57/BL6 and BALB/c mice. We found that disulfide HMGB1 but not all-thiol HMGB1 induced hypersensitivity in BALB/c and C57BL/6 male and female mice 3 and 6 hours after injection (Figure 18). We found a significant increase in mRNA levels of cytokines (*Tnf, Il1-β* and *Il6*), chemokines (*Ccl2, Cxcl1* and *Cxcl2*) and other pain-associated factors factors (*Ngf* and *Cox2*) in disulfide HMGB1-injected ankle joint but not in all-thiol HMGB1 injected mice. Comparing male to female mice showed that disulfide HMGB1-

induced cytokine-chemokine expression was more prominent in male mice compared to female mice (Figure 19).

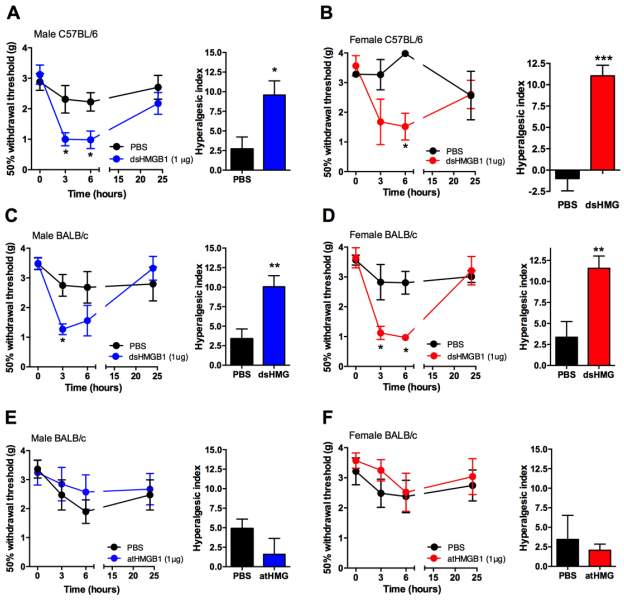

Figure 18. Single intraarticuar injection of disulfide HMGB1 but not all-thiol HMGB1 induces mechanical hypersensitivity in male and female mice in two different strains (A-F). Hyperalgesic index calculated for 0-6 h for C57BL/6 A) male and B) female and BALB/c C,E) male and D,F) female mice (From **Paper IV, Fig 3**).

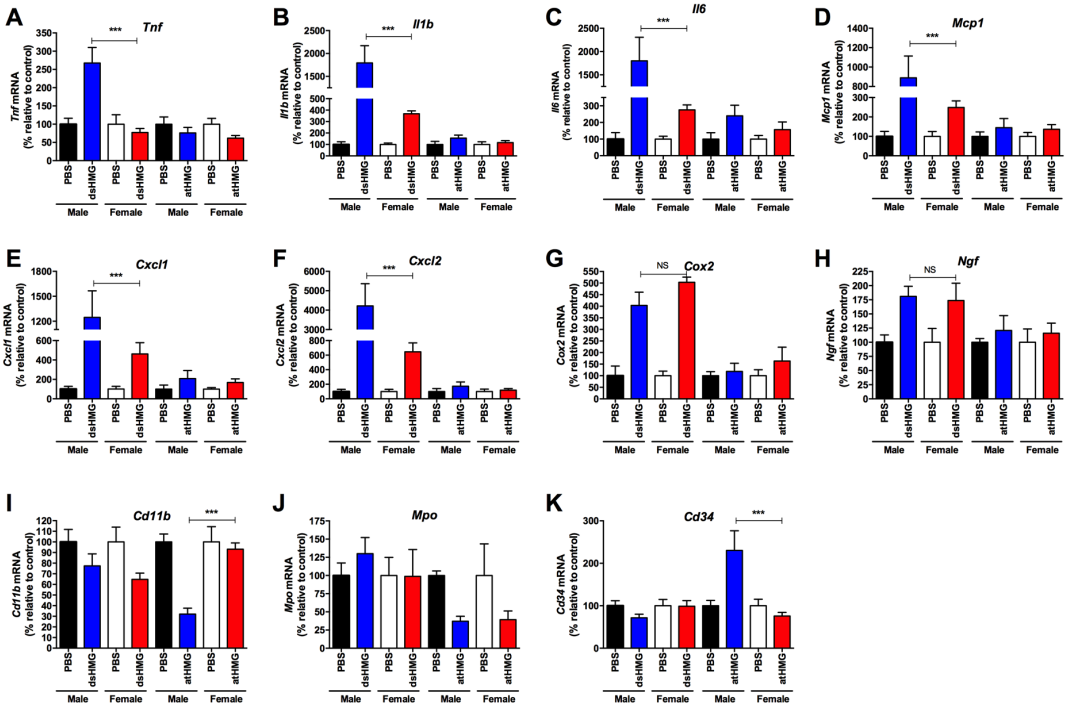

Figure 19 Expression of cytokine (A-C), chemokine (D-F), Other factors (G-H) and immune cells (I-K) in ankle joint injected with disulfide HMGB1 in C57BL/6 male and female mice (From **Paper IV, Fig 4**).

4.4.3 Disulfide HMGB1 mediated hypersensitivity is induced by TLR4 expressed both on nociceptors and myeloid cells in male and female mice, but with a more pronounced immune cell contribution in male mice

To examine if disulfide HMGB1 induces mechanical hypersensitivity via TLR4 on peripheral neurons or local myeloid cells genetically modified mice were used. These mice had a deletion of TLR4 in Nav1.8 positive peripheral neurons (Nav1.8-TLR4$^{fl/fl}$) or myeloid cells (LysM-TLR4$^{fl/fl}$). TLR4$^{fl/fl}$ mice were used as a WT control. At the 6-hour time-point female Nav1.8-TLR4$^{fl/fl}$ mice were completely, whereas Nav1.8-TLR4$^{fl/fl}$ male mice were partially protected from the development of hypersensitivity after intra-articular injection of disulfide HMGB1 (Figure 20A, B). Both male and female LysM-TLR4$^{fl/fl}$ mice were protected from disulfide HMGB1-induced hypersensitivity at the 6-hour time point. However, female LysM-TLR4fl/fl mice were not protected against disulfide HMGB1 induced mechanical hypersensitivity at the 3-hour time point, though it should be noted that TLR4$^{fl/fl}$ (WT) mice injected with disulfide HMGB1 did not show reduction in withdrawal thresholds at the 3-hour time point in female mice. Moreovr, LysM-TLR4$^{fl/fl}$ male mice were protected from disulfide HMGB1-induced hypersensitivity at all time points. This data suggest that disulfide HMGB1 mediated mechanical hypersensitivity induced by TLR4 expressed both on nociceptors and myeloid cells in male and female mice, but with a more pronounced immune cell contribution in male and neuronal contribution in female mice (Figure 20C, D).

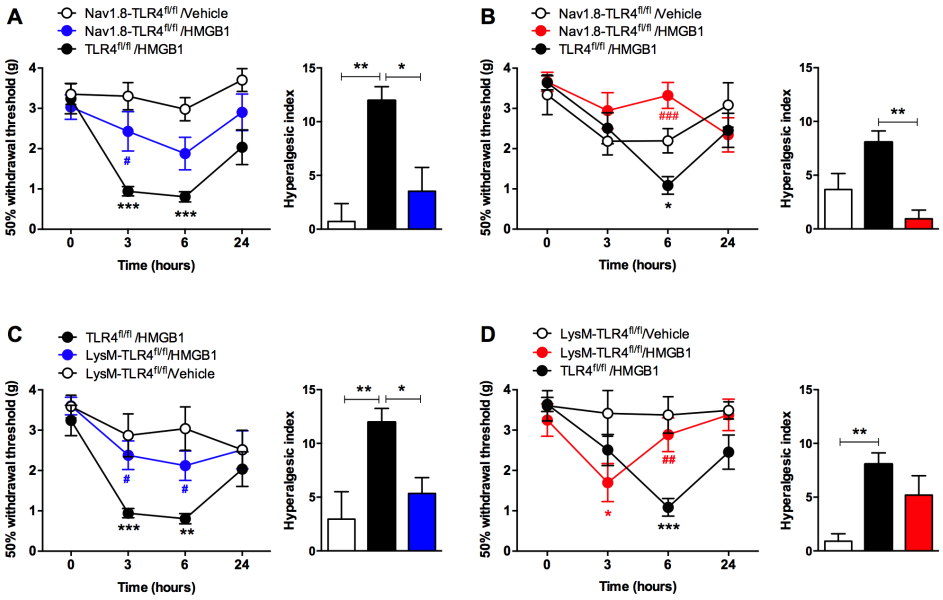

Figure 20 Disulfide HMGB1 induces hypersensitivity in male and female WT mice, whereas Nav1.8 TLR4$^{fl/fl}$ female completely protected and Nav.1.8 TLR4$^{fl/fl}$ male partially protected after injection into ankle joint (A, B). LysM TLR4fl/fl male but not LysM TLR4fl/fl and WT were protected from disulfide HMGB1 induced mechanical hypersensitivity (C,D) (From **Paper IV, Fig 5, 6**).

5 DISCUSSION

The aim of this thesis was to advance our understanding of the DAMPs molecule HMGB1 and if and how it is involved in spinal and peripheral mechanisms of pain signal transduction and transmission, with a specific focus on the arthritis-induced pain. This study points to an intriguing role of disulfide HMGB1 both in the joint and the spinal cord in arthritis-induced pain and that the redox state of HMGB1 is critical in this process. To our surprise, we found a sex- and cell type-dependent coupling between the pronociceptive properties of disulfide HMGB1 and activation of TLR4.

In **Paper I**, the CAIA model was characterized from a pain perspective. Collagen antibodies were injected intravenously against a collagen type II epitope that is shared between rodents and humans, synchronizing with an intraperitoneal injection of LPS to increase the incidence and severity of disease (Nandakumar et al., 2003). Previous work has shown that both systemic and intrathecal injection of LPS induces pain like behavior (Christianson et al., 2011; Maier et al., 1993; Meller et al., 1994) and glial cell activation in the spinal cord in rodents (Guo & Schluesener, 2006). However, the dose used in our study was a subthreshold dose of LPS, which does not have effect on pain-like behavior and on glial activation at spinal level. Mice subjected to CAIA displayed transient inflammation but a long-lasting hypersensitivity in three different mouse strains with alterations in joint histopathology. Similarly, also in another passive model of arthritis, the K/BxN serum transfer model (naïve mice injected i.p. with polyclonal serum containing anti-glucose-6-phosphate isomerase antibody) transient joint inflammation with long-lasting hypersensitivity was observed (Christianson et al., 2010) indicating that an episode of antibody-driven joint inflammation has long-term consequences on neuronal excitability. Interestingly, the pharmacological profile achieved using antinociceptive and anti-inflammatory drugs reveals that CAIA-induced hypersensitivity is driven via different mechanisms at different stages of the disease. Blocking cyclooxygenase 1/2 activity and the action of TNF (Bas et al., 2016) during joint inflammation, but not after resolution of joint inflammation, reverses CAIA-induced mechanical hypersensitivity; which indicated that prostaglandins and TNF play an important nociceptive role during joint inflammation, but there is a temporal shift in the mechanism maintaining the hypersensitivity. Moreover, gabapentin (approved for the treatment of neuropathic pain) reverses CAIA and K/BxN-induced hypersensitivity during both phases. Also, factors such as activating transcription factor 3, alpha-2-delta and galanin, associated with nerve injury, are elevated in DRG neurons in the late phase of the CAIA and/or K/BxN model (Su et al., 2015, Christianson et al., 2010). Thus it is possible that changes that have some aspects in common with neuropathic pain are activated after antibody-induced joint inflammation. Clearly, the CAIA model is an interesting model for investigation of the spinal and peripheral role of HMGB1 in arthritis-associated pain.

In **Paper II and III**, we explored the role of extranuclear HMGB1 in the spinal cord and how the different redox states of HMGB1 contribute to pain signal processing. In **Paper II,** we examined the presence of HMGB1 in different cell types at the spinal level. In agreement with previously reported findings in rats (Feldman et al., 2012; Nakamura et al., 2013; Shibasaki et al., 2010), we found constitutive expression of HMGB1 in spinal neurons, astrocytes and microglia in mice. Importantly, the extranuclear levels of HMGB1 were found to be elevated at the lumbar level of spinal cord subsequent to induction of CAIA indicating that peripheral joint inflammation leads to the spinal release of HMGB1 (Figure 21). This notion is further supported by the analgesic effect of spinal injection of an HMGB1 neutralizing antibody, which most likely is exerting its action by binding extracellular HMGB1 and thereby preventing the actions of HMGB1. Since HMGB1 is expressed in both neurons and glial cells, we cannot draw any conclusions about which cell type is the source of the extranuclear HMGB1. However, mounting data suggest that HMGB1 translocate from the nucleus to the cytoplasm in the dorsal root ganglions (DRGs) and dorsal horn neuron subsequent to the nerve ligation (Feldman et al., 2012; Nakamura et al., 2013). Other studies have shown a dose-dependent release of HMGB1 from brain slices cultured after exposure to ethanol (Zou & Crews, 2014).

HMGB1 activates cells through multiple membrane receptors such as TLR2, TLR4 and RAGE receptors (Harris et al., 2012). Several TLRs have been suggested to play a role in the regulation of inflammatory and neuropathic pain (Christianson et al., 2011; Kim et al., 2013; Liu et al., 2012). Moreover TLR2, TLR4 and RAGE are expressed on glial cells and inflammatory cells, and TLR4 and RAGE are expressed on sensory neurons (Vincent et al., 2007; Wadachi & Hargreaves, 2006). Thus pattern recognition receptor those are critical for mounting an innate immunoreaction are potentially also vital in pain processing through their expression on both neurons and glial cells. In our study, we found that disulfide HMGB1 induces pain-like behavior in male and female mice, which is mediated mainly via TLR4 (Figure 21) and to some extent through RAGE, as RAGE KO mice recovered faster than WT mice. Under normal conditions, HMGB1 is in its reduced all-thiol state in the intracellular compartment (Hoppe et al., 2006). The outcome of our study suggests that all-thiol HMGB1 is reduced to the disulfide form after release to become a TLR4 ligand that induces nociception and activates glial cells (Figure 21).

Previous reports have shown that blocking the actions of endogenous HMGB1 activity attenuates pain-like behavior in experimental models of diabetic, bladder, cancer and nerve-injury induced pain, indicating that HMGB1 is an important factor in pain pathology in multiple conditions (Feldman et al., 2012; Ma et al., 2017; Nakamura et al., 2013; Otoshi et al., 2011; P. C. Ren et al., 2012; Shibasaki et al., 2010; Tanaka et al., 2013; Tong et al., 2010; Yamasoba et al., 2016). Our work further expands our insights on the role of HMGB1 as we demonstrate that blocking the actions of both peripheral and spinal HMGB1 reverses CAIA-induced hypersensitivity (Figure 21). However, the sex dimorphism that we observed after systemic injection of the HMGB1 neutralizing antibody was unexpected. Even though it has been reported that blocking TLR4 in inflammatory and neuropathic-induced pain is

associated with sex dimorphism, this effect has been coupled to spinal TLR4. Interestingly, we found that blocking the action of spinal HMGB1 reduced hypersensitivity in both male and female mice and without signs of sex-dependence (Figure 21).

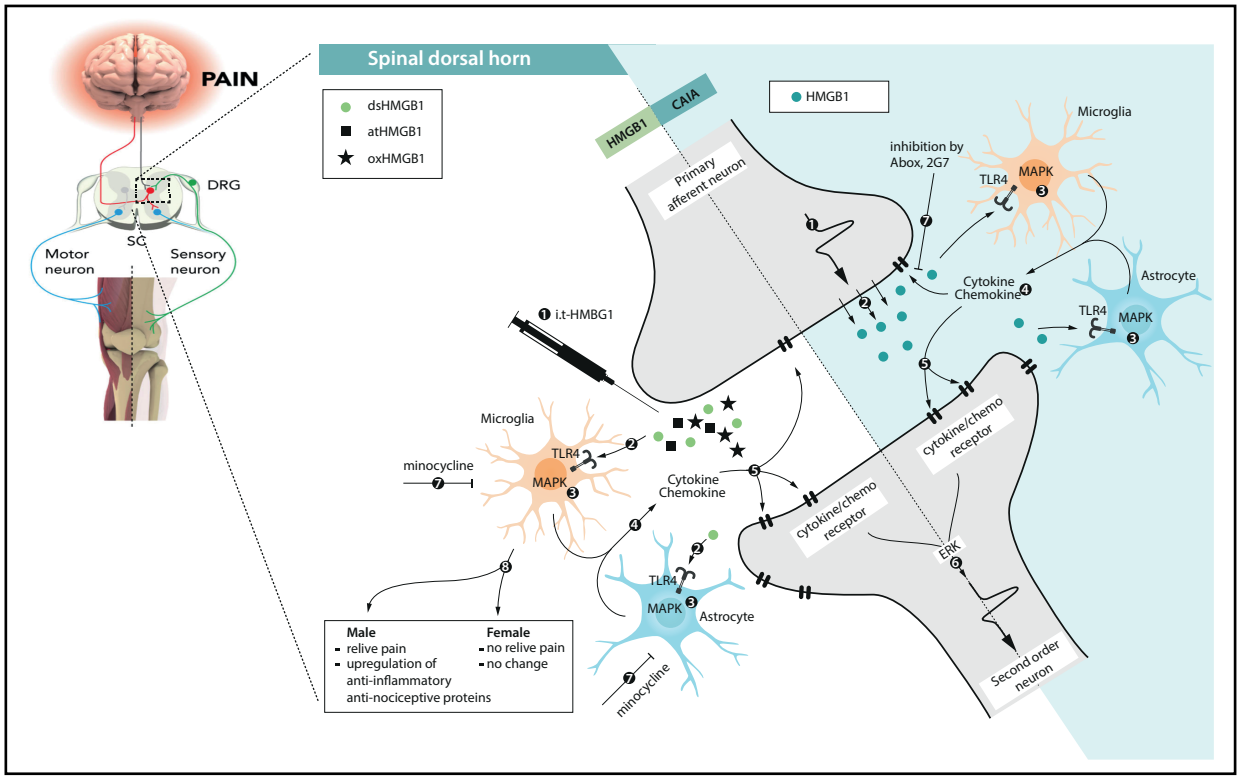

Figure 21. Proposed HMGB1 mechanism in spinal cord. **In CAIA**, Persistent action potential from periphery (1) triggers to release HMGB1 into the synaptic cleft (2) which could bind to its receptor on glial cells (3), that activates the intracellular MAPK pathway resulting in activation of transcriptional factors to produce cytokine and chemokine (4). Released cytokine and chemokine bind to their respective receptor on pro-pre synaptic neuron (5) to activate transduction phenomenon and increase hyperexcitability in second order neuron (6). Blocking spinal endogenous HMGB1 with HMGB1 inhibitors (2G7 and Abox), dampen the hyperexcitability in the second order neuron (7) in male and female mice. **i.t. injection of different redox form of HMGB1**, Intrathecal delivery of disulfide HMGB1 (dsHMGB1) but not all-thiol HMGB1 (atHMGB1) and oxidized HMGB1 (oxHMGB1)(1) binds to TLR4 receptor present on the glial cell (microglia and astrocytes) (2), which triggers intracellular MAPK pathway (3) with subsequent production of cytokine and chemokine (4). Released cytokine and chemokine bind to their receptor on pro-presynaptic neuron (5) and stimulates transduction phenomenon to increased hyperexcitability in second order neuron (6). Blocking microglial activity with minocycline (7) resulted into inhibition of action potential in male but not in female mice with up regulation of anti-inflammatory and anti-nociceptive proteins (8).

In **Paper III,** we investigated the effect of disulfide HMGB1 on microglia and studied sex-dependent mechanisms behind the induction of mechanical hypersensitivity using minocycline, a drug that in addition to its antibiotic properties has been associated with microglia inhibition. Accumulating animal and human data reveal sex differences in pain, both in terms of sensitivity to painful stimuli and effects of pain treatment. The overall aim of this study was to determine if there is a sex difference in disulfide HMGB1-induced microglial activation and mechanism by which it drives nociception. We found the intrathecal delivery of disulfide HMGB1 drives nociceptive signal transmission in a similar fashion in male and female mice. Similarly, LPS drives nociception in male and female mice after intrathecal delivery (Woller et al., 2016), in contrast other found, intrathecal delivery of LPS induces nociception only in male but not in female mice (Sorge et al., 2011).

Mounting data support a role of spinal microglia in the development of hypersensitivity in different experimental models of pain, which is frequently visualized as an increase in Iba1 immunoreactivity associated with a change in microglial morphology (larger cell body and more ramified processes). We found that disulfide HMGB1-induced morphological signs of enhanced microglial reactivity to a similar degree in male and female mice (Figure 21). Interestingly, inhibiting microglial activity with minocycline attenuated disulfide HMGB1-induced hypersensitivity in male but not in the female mice. This finding is in agreement with other studies showing sex-dependent effects of inhibiting microglial activity in different experimental models of pain (Chen et al., 2017; Sorge et al., 2015; Sorge & Totsch, 2017). LCMS analysis of spinal cords from mice treated with minocycline in conjunction with intrathecal injection of disulfide HMGB1 revealed a striking difference in regulation of protein expression in male and female mice (Figure 21). As minocycline most likely is acting on other cells in addition to microglia, (Moller et al., 2016) these data have to be interpreted very carefully from a microglia perspective. Anyhow, it is noteworthy that the minocycline treatment up-regulates a number of anti-inflammatory and anti-nociceptive proteins, rather than blocking protein synthesis, in male but not in female mice. Proteins of particular interests are members of the serpin family. Serpins exert their action by binding and inhibiting specific serine proteases, SPA3K was identified as a specific inhibitor of tissue kallikrein and alterations of the kallikrein-kinin system lead to anti-inflammatory, antinociceptive and anti-allergic effect (Bhoola et al., 1992; Clements, 1989; Murray et al., 1990). In addition, SPA3N has been shown to attenuate neuropathic pain by inhibiting leukocyte elastase activity (Vicuna et al., 2015). Haptoglobin, has been reported to induce an anti-oxidative, anti-inflammatory and immunoregulatory effect and suggested to suppress cellular immune response by activating macrophages and inhibiting TNF production (Theilgaard-Monch et al., 2006). Hemopexin has been shown to be anti-inflammatory and to downregulate LPS-induced TNF and IL-6 secretion from murine macrophages (Liang et al., 2009). Lastly, immunomodulatory function of the vitamin D binding protein (VDBP) has also been reported (Ghoreishi et al., 2009) together with musculoskeletal pain and migraine attacks with vitamin D inadequacy (Abbasi et al., 2012; Nagata et al., 2014). Overall, our findings in paper II support, at least to some extent, that there is a sex dimorphism associated with microglia inhibition. However, further studies are warranted in order to understand the specific mechanism, and the cellular target(s) of minocycline, which differentiates the effects observed in male and female mice.

In **Paper IV**, we investigated the peripheral role of HMGB1 in CAIA-associated pain in male and female mice. Interestingly, while injection of disulfide HMGB1 into the ankle joint induced pain-like behavior in male and female mice, systemic inhibition of HMGB1 attenuates pain-like behavior in male but not in female mice. It is surprising that there is a sex dimorphism in blocking peripheral HMGB1 activity and but no difference when injecting HMGB1 into the articular joint, especially as systemic delivery of TLR4 antagonists has not been associated with differential effects in male and female mice in formalin models (Woller et al., 2016). This suggests that HMGB1 may act through receptors other than TLR4 in the

periphery, something that we are currently exploring in our laboratory. However, while focusing on TLR4, we investigated if there are cell-specific sex-dependent actions of disulfide HMGB1 in the ankle joint.

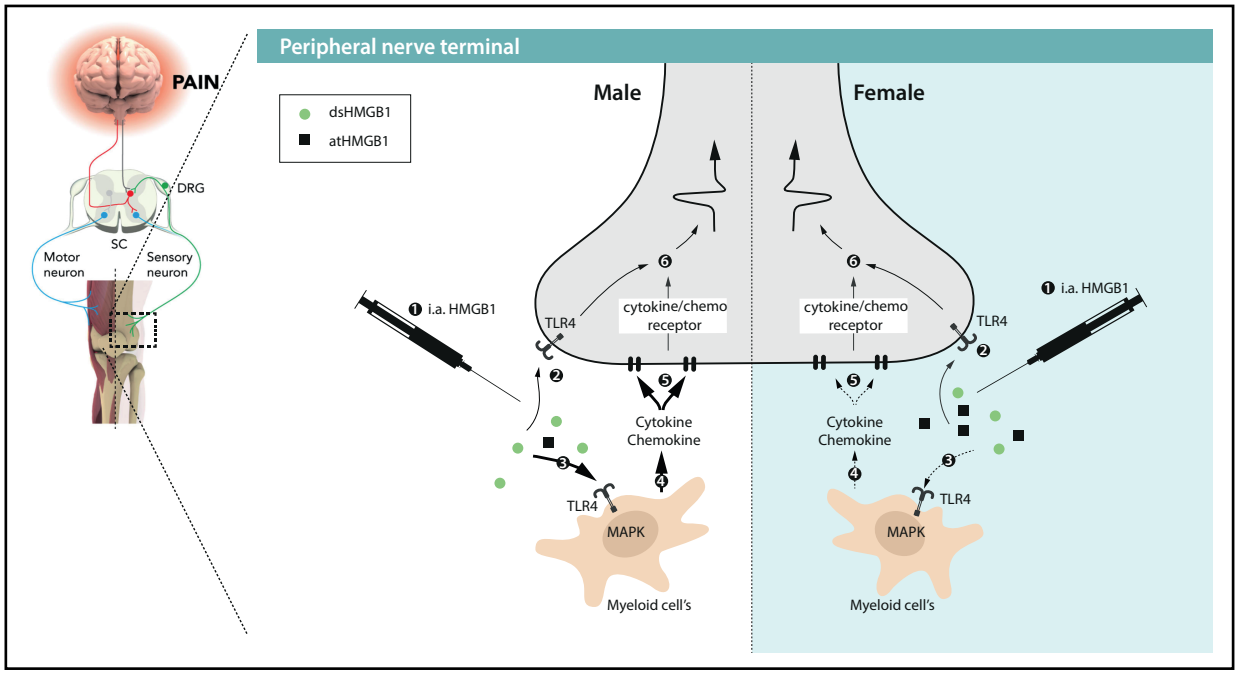

Figure 22. Proposed HMGB1 mechanism at periphery. Intraarticular injection of disulfide HMGB1 (dsHMGB1) but not all-thiol HMGB1 (atHMGB1) (1) develops pain like behavior (action potential) in male and female mice. In male and female mice, disulfide HMGB1 binds to TLR4 on nociceptors (2) which generates action potential (6) and/or it binds to TLR4 on myeloid cells (3) initiating MAPK pathway to produce chemokine and cytokines (4), that bind to their receptor on the nerve terminal (5) to generate action potential (6). Triggering pain like behavior in male and female mice is different to some extends i.e. Nerve terminal activation by cytokine and chemokine from myeloid cells is more prominent in male mice (thick arrow) than female mice.

Our findings do not demonstrate an exclusive cell specificity of HMGB1 in the joint, but, though not consistent at all time points, our findings indicate that TLR4 on nociceptors and myeloid cells have differential involvement in nociception in male and female mice (Figure 22). Furthermore, analyses of cytokines and chemokines revealed that male mice responded with a more pronounced induction of cytokines and chemokines in response to disulfide HMGB1 compared to the female mice. Similar to this, other studies have reported that intraperitoneal injection of LPS increases blood levels of IL-6 to a greater extent in males (Marriott et al., 2006) and LPS stimulation of macrophages induces a larger cytokine release when the cells come from males compared to females (Kahlke et al., 2000; Marriott et al., 2006). The reason for this difference deserves careful investigation, as it is potentially very important for deciphering the role of the innate immune system in inflammation and pain processes.

Concluding remarks

Altogether, these studies have contributed to advance our understanding of mechanisms underlying the pain in arthritis. For the first time, spinal and peripheral HMGB1 was shown to be involved in arthritis pain processing. Moreover, we show that the redox state of this molecule is critical for its pronociceptive properties, bringing more light on the biological

functions of HMGB1 and its action on receptors like TLR4. Finally, I hope that our data will be of importance from a translational point of view as it points to HMGB1 as a novel target for pain relief, but with the notion that such approach would require advancement of our understanding of the sex dimorphism associated with TLR4. Our work indeed brings attention to the need of exploring pain mechanisms in both males and females

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