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CHRONIC PAIN AND ARTHRITIS - STUDIES OF MECHANISMS IN THE REGULATION OF HYPERSENSITIVITY

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Stockholm 2016

Cover illustration: Immunohistochemical triple staining of human dorsal root ganglion showing α2δ1 (green), LPA1 (red) and DAPI (blue).

Published by Karolinska Institutet.
Printed by E-Print AB

ISBN 978-91-7676-291-2

Karolinska Institutet

Chronic pain and arthritis - studies of mechanisms in the regulation of hypersensitivity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

ACADEMIC DISSERTATION

for the degree of PhD at Karolinska Institutet

The thesis will be defended in public in the Hillarp Lecture Hall, Retzius väg 8, Karolinska Institutet, Stockholm, Sweden

Friday the 6th of May 2016, 09.00

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To my family

ABSTRACT

Chronic pain is one of the most devastating symptoms reported by rheumatoid arthritis (RA) patients. Current treatment strategies are focusing on dampening the inflammatory and immunological aspects of RA, but unfortunately subgroups of patients continue to suffer from pain, even when the disease is adequately controlled. In this thesis, the work is focused on investigating mechanisms underlying arthritis-induced pain using the collagen antibody-induced arthritis mouse (CAIA) model.

In Paper I, we describe 50 peptides that are only expressed, and 38 peptides that are predominantly expressed in the dorsal spinal cord using liquid chromatography mass spectrometry. Intrathecal injection of [des-Ser1]-cerebellin (desCER), one of the peptides predominantly expressed in the spinal dorsal horn, induces a dose-dependent mechanical hypersensitivity but has no effect on thermal sensitivity in mice. We also show that the precursor of desCER is expressed in excitatory interneurons of laminae II/III. This study suggests that desCER is a novel pain modulator, and provides a platform for continued exploration of novel neuropeptides involved in nociception.

In Paper II, we report that mice subjected to CAIA display transient inflammation and persistent mechanical hypersensitivity. We find an anti-nociceptive effect of a COX inhibitor, but only in the inflammatory phase of the CAIA model, and a time-dependent spinal glial activation.

In Paper III, we report neurochemical changes in dorsal root ganglia (DRGs) and spinal cord after induction of CAIA. In the CAIA DRGs, galanin, voltage-gated calcium channel subunit α2δ1 and the nerve injury markers ATF3 and GAP43 are up-regulated. Taken together, based on the pharmacological and neurochemical profile, our work suggests that antibody-driven joint inflammation lead to a partial neuropathic pain phenotype.

Finally, in Paper IV we focus on the role of lysophosphatidic acid (LPA) in arthritis-induced pain. The LPA synthesizing enzyme autotaxin is increased in DRGs from mice subjected to CAIA. Systemic treatment with a monoclonal antibody (Ab) against LPA completely reverses arthritis-induced mechanical and thermal pain-like behavior and also blocks the up-regulation of α2δ1 in peripheral sensory neurons of CAIA mice. We also find that the expression of α2δ1 remains at basal levels in *Lpa receptor-1* (*Lpar1*) deficient CAIA mice. These results provide new insights into the role of the LPA1/α2δ1 axis in arthritis-induced pain, suggesting new potential therapeutic targets for pain relief in RA.

In summary, we have described a battery of pain-related factors involved in CAIA-induced hypersensitivity. These results lead to a deeper understanding of pain mechanisms in RA and hopefully to new therapeutic approaches to treat the pain component in RA patients.

LIST OF PAPERS INCLUDED IN THE THESIS

I. **Su J***, Sandor K*, Sköld K, Hökfelt T, Svensson CI, Kultima K
Identification and quantification of neuropeptides in naïve mouse spinal cord using mass spectrometry reveals [des-Ser1]-cerebellin as a novel modulator of nociception
Journal of Neurochemistry 04/2014; 130(2): 199-214.

II. 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 & Rheumatology 12/2012; 64(12): 3886-96.

III. **Su J**, Gao T, Shi T, Xiang Q, Xu X, Wiesenfeld-Hallin Z, Hökfelt T, Svensson CI
Phenotypic changes in dorsal root ganglion and spinal cord in the collagen antibody-induced arthritis mouse model: Neuronal plasticity in arthritic mice
The Journal of Comparative Neurology 01/2015; 523(10): 1505-28.

IV. **Su J**, Barde S, Delaney A, Ribeiro J, Kato J, Agalave N, 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

* shared first authorship

RELATED PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Bas DB, **Su J**, Wigerblad G, Svensson CI
Pain in rheumatoid arthritis – models and mechanisms
Pain Management, in press.

II. Wigerblad G, Bas DB, Fernades-Cerqueira C, Krishnamurthy A,. Nandakumar KS, Rogoz K, Kato J, Sandor K, **Su J**, Jimenez – Andrade JM, Finn A, Farinotti AB, Amara K, Lundberg K, Holmdahl R, Jakobsson P, Malmström V, Catrina AI, Klareskog L, Svensson CI
Autoantibodies to citrullinated proteins induce joint pain independent of inflammation via a chemokine-dependent mechanism
Annals of the Rheumatic Diseases 04/2016; 75(4): 730-8.

III. 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 06/2014; 155(9): 1802-13.

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LIST OF ABBREVIATIONS

α2δ1	Alpha-2 delta-1, calcium ion channel subunit
Ab	Antibody
ACPA	Anti-citrullinated protein antibodies
AIA	Adjuvant-induced arthritis
ATF3	Activating transcription factor 3
ATP	Adenosine triphosphate
ATX	Autotaxin
CAIA	Collagen antibody-induced arthritis
CBLN1	Cerebellin precursor 1
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene-related peptide
CIA	Collagen-induced arthritis
CII	Collagen type II
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element binding protein
desCER	[des-Ser1]-cerebellin
DMARDs	Disease-modifying anti-rheumatic drugs
DRG	Dorsal root ganglion
GAP43	Growth associated protein 43
GFAP	Glial fibrillary acidic protein
IB4	Isolectin B4
Iba-1	Ionized calcium binding adaptor molecule 1
IL	Interleukin
i.p.	Intraperitoneal
IR	Immunoreactive
i.th.	Intrathecal
i.v.	Intravenous
JIP-1	JNK-interacting protein 1
JNK	c-Jun N-terminal kinase

LCMS	Liquid chromatography mass spectrometry
LI	Like immunoreactivity
LPA	Lysophosphatidic acid
LPA1-6	Lysophosphatidic acid receptor 1-6
LPS	Lipopolysaccharide
K/BxN	K/BxN serum transfer model
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
Nav	Sodium channel
NPs	Neuron profiles
NPY	Neuropeptide Y
OA	Osteoarthritis
PKCγ	Protein kinase C gamma
RA	Rheumatoid arthritis
RF	Rheumatoid factor
s.c.	Subcutaneous
TNF	Tumor necrosis factor

1 INTRODUCTION

Chronic pain is a prevalent and challenging problem affecting approximately 10-20 % of the population globally, resulting in marked reduced quality of life for the individual (Apkarian et al., 2009). Pain is also a substantial socio-economical problem due to costs of medication, loss of productivity and disability (Andersson et al., 2013). According to a survey in 15 European countries up to 40% of the patients with persistent pain reported having joint pain (Breivik et al., 2006). Osteoarthritis (OA), followed by rheumatoid arthritis (RA), is the most common cause of joint pain (Breivik et al., 2006, Lluch et al., 2014). Rheumatoid arthritis is an autoimmune and destructive joint disease occurring in around 0.5-1% of the population. It is important to note that despite improved disease control, many of the RA patients still suffer from persistent joint pain (Smolen and Steiner, 2003, McInnes and Schett, 2011). Drug development in the area of chronic pain has so far not been successful, and there are in fact currently only few available effective treatments for chronic pain conditions in general (Davila and Ranganathan, 2011, Walsh and McWilliams, 2014). Hence, it is critical to advance our understanding underlying chronic pain, in order to identify new targets for pain relief.

1.1 RHEUMATOID ARTHRITIS

Rheumatoid arthritis is a chronic, systemic autoimmune disease that affects multiple joints (Smolen and Steiner, 2003). The predominant symptoms are pain, stiffness, and swelling of peripheral joints. The principal pathophysiological feature of the disease includes inflamed synovia with a pronounced infiltration of inflammatory leucocytes, angiogenesis, cellular hyperplasia, and changes in the expression of cell-surface adhesion molecules, proteinases, and many cytokines. The presence of an inflammatory erosive synovitis (pannus) ultimately leads to destruction of cartilage, bone and soft tissues, resulting in long-term deformity and loss of joint function (Scott et al., 2010, Lillegraven et al., 2012). Another prominent reason for loss of joint mobility is chronic or episodic joint pain, which leads to psychological distress and impaired quality of life (Schaible et al., 2009). Rheumatoid arthritis is characterized by presence of various autoantibodies in serum and synovial fluid, for example antibodies against IgG and IgM (rheumatoid factor), anti-collagen type II antibodies (CII) and anti-citrullinated protein antibodies (ACPA) (Song and Kang, 2010, Willemze et al., 2012).

The main objectives of RA treatment are to decrease joint inflammation, maintain joint function, protect articular structures, control systemic involvement and relieve pain (Smolen and Aletaha, 2015). The identification of disease-modifying anti-rheumatic drugs (DMARDs) and biopharmaceuticals, in particular tumor necrosis factor (TNF) inhibitors, has led to great improvements in the treatment outcomes of RA. However, despite improved prognosis pain still is a big problem for the RA patients (Smolen and Steiner, 2003, Walsh and McWilliams, 2014). In fact, many patients continue to suffer from pain even though the disease is under good control (Lee et al., 2011, Taylor et al., 2011).

1.2 THE COLLAGEN ANTIBODY-INDUCED ARTHRITIS MODEL

Experimental animal models of arthritis are widely used to study disease pathways and to identify novel drug targets and therapeutic approaches (McNamee et al., 2015). In order to study chronic inflammatory pain in arthritis, selecting a relevant animal model is extremely important. Several models used in studies of arthritis-like pain involve intra-articular injection of agents such as Complete Freund's adjuvant (CFA) and carrageenan, which produce a pronounced inflammatory reaction and nociceptive behavior (Pearson, 1956, Sluka and Westlund, 1993, Inglis et al., 2005). These models are useful in studies of local short-lasting inflammation and the impact of such inflammation on the nervous system. However, they are not commonly used in the rheumatology field, as they do not reflect the main clinical picture, the chronicity and the pathology of human RA.

We are using an experimental arthritis mouse model, the CAIA model, in which arthritis is induced by an intravenous (i.v.) injection of CII antibody cocktails on day 0, and followed by an intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) on day 5, in order to synchronize the onset of inflammation and enhance severity and incidence of the disease (Nandakumar et al., 2003, Khachigian, 2006, Bas et al., 2012). One advantage, as compared to conventional inflammatory pain models, is that arthritis is induced with CII antibodies that are also found in serum from RA patients. This is a major strength from a translational research point of view. Collagen-induced arthritis (CIA) is the most commonly used RA animal model (Brand et al., 2007). Both CAIA and CIA mice have similarities with the pathogenesis of RA, and these models are characterized by joint swelling and redness in the front and hind paws, and occasionally also knee and vertebral joints are affected. Compared to CIA, CAIA has a more rapid and synchronized onset of joint inflammation. The CAIA model is commonly utilized in the RA field for disease mechanistic studies, but has so far not been employed to explore the pain aspects. Development of clinical signs of arthritis (paw and joint swelling and redness) is associated with a robust and highly reproducible behavioral hypersensitivity. Strikingly, while the inflammation resolves after 2-3 weeks, mechanical hypersensitivity outlasts the inflammation and remains pronounced until the termination of the experiment. Hence, it appears that the pain process in the CAIA model can be divided into inflammatory and post inflammatory phases. Thus, the CAIA model has the potential to provide new insights into how persistent inflammation in the joint affects pain-sensing neurons and pain processing in the peripheral as well as the central nervous system (CNS).

1.3 ARTHRITIS-INDUCED PAIN

Primary sensory neurons represent the interface between, on one hand, external stimuli at the peripheral sensory nerve endings in the skin, alternatively intrinsic organs, and on the other hand, the spinal cord and further to supraspinal levels (Melzack and Wall, 1965, Willis, 2007). To detect and transfer a particular nociceptive or non-noxious stimulus that impinges upon the body to the CNS, these sensory neurons have highly specialized adaptations that include a battery of neuropeptides, ion channels and receptors. They are also characterized by highly ordered patterns of innervations in peripheral targets and central circuits (Woolf and Ma, 2007). In the joint, the ligaments, fibrous capsule, meniscus, periosteum and synovial

layer are innervated by Aβ-, Aδ- and C-fibers. Noxious (pain) sensation can be evoked from these structures as shown in Figure 1 (Schaible et al., 2009, Yang et al., 2013). A different nomenclature, group I-IV fibers, can be used for afferent fibers from the joint. Group III and IV fibers are very similar to Aδ- and C- fibers, respectively. All primary afferent fibers are glutamatergic and many contain one or more neuropeptides, including substance P, calcitonin gene-related peptide (CGRP), somatostatin, and galanin.

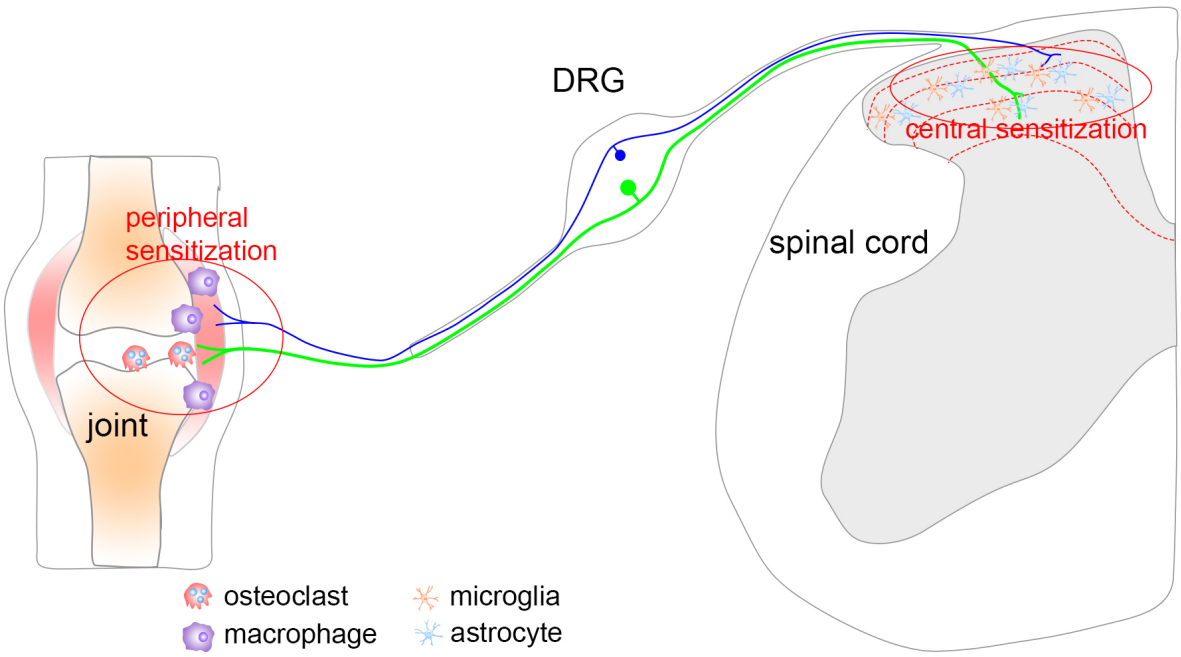

Figure 1. Pain pathways in RA. In the joint, many structures are innervated by pain fibers like Aδ- (green) and C-fibers (blue), also called nociceptive fibers. Their cell bodies are located in the dorsal root ganglia (DRGs). In RA, painful sensation can be evoked from the peripheral sites and then continuing to the spinal dorsal horn, where the primary afferents (peripheral sensitization) couple to interneurons or second order projection neurons (central sensitization), sending information towards the brain and giving rise to the sensation of pain (modified from Paper III).

During the development of RA, inflammation and other changes lead to sensitization of the primary afferent nociceptive neurons innervating the joint (Schaible, 2014, Walsh and McWilliams, 2014). This is mediated locally in the arthritic joint by release of various pro-inflammatory mediators such as TNF, interleukins (ILs), bradykinin, adenosine triphosphate (ATP), as well as neuropeptides. Increased input from such sensitized afferents can lead to an augmented release of glutamate and neuropeptides like CGRP and substance P on to spinal dorsal horn from central terminals. Together with the activation of microglia and astrocytes in the spinal dorsal horn, central sensitization will be induced as indicated in Figure 1.

1.3.1 Inflammatory and neuropathic pain

Traditionally, the pain experience is classified as three different types of pain according to initiation, causes and underlying mechanisms: acute pain, inflammatory (tissue injury) and neuropathic (nerve injury) pain (Scholz and Woolf, 2002). Obviously, the causes of inflammatory and neuropathic pain are different. Even though these two types of pain share certain mechanisms, expression patterns of pain-related factors like neuropeptides, ion

channels and nerve injury markers vary (Xu and Yaksh, 2011). Moreover, with regard to treatment there are several options. Inflammatory pain can often be attenuated by cyclooxygenase (COX) inhibitors (like diclofenac and ibuprofen) and opioids (like buprenorphine) and, further, pain improves as the inflammation resolves. In contrast, neuropathic pain is unresponsive to COX inhibitors and requires higher doses of opioids and may persist even after the healing of injury. Interestingly, clinical cases of inflammation-induced chronic pain exhibit both neuropathic and inflammatory components. For instance, in RA patients, inflammation and pain are not always parallel, in fact even not related to each other (van Laar et al., 2012). This is confirmed by the recent study showing that RA patients with well-controlled disease still have neuropathic pain symptoms (Koop et al., 2015).

1.3.2 Neuropeptides

Endogenous neuropeptides participate in intercellular signaling and thus can act as neurotransmitters or neuromodulators in the nervous system. They are involved in a variety of functions such as anxiety, depression, memory, sleep, inflammation and pain (Kilduff and Peyron, 2000, Taiwo and Taylor, 2002, Hokfelt et al., 2003). Neuropeptides comprise a diverse group of chemically distinct molecules, synthesized in, and released from, a range of sensory neurons, and involved in nociceptive signal transduction in different categories of experimental pain models (e.g. acute, neuropathic and inflammatory). Mounting evidence suggests that expression levels of certain neuropeptides in primary sensory neurons are altered in different directions in response to experimental manipulations. For example, after nerve injury the excitatory peptides substance P and CGRP are down-regulated, while a dramatic up-regulation of neuropeptide Y (NPY) and galanin is found in injured DRGs compared to their low basic expression (Hokfelt et al., 1984, Wakisaka et al., 1992, Nothias et al., 1993, Zhang et al., 1995, Solway et al., 2011, Zhang et al., 2011). In contrast, inflammatory pain, induced in CFA and carrageenan models, is associated with an increase of substance P and CGRP levels and release (Smith et al., 1991, Donnerer et al., 1993, Hanesch et al., 1993, Galeazza et al., 1995, Calza et al., 1998). However, no expression of NPY in DRGs was found in DRGs after tissue inflammation (Ji et al., 1994b). Although up-regulation of galanin in peripheral sensory neurons is induced after nerve injury (Hokfelt et al., 1987, Coronel et al., 2008), an increase of galanin expression has also been found in DRGs of CFA rats (Calza et al., 1998, Calza et al., 2000).

1.3.3 Ion channels

Neuronal excitability is regulated by ion channel activity. Open probability and expression levels of several ion channels located at the peripheral terminals of nociceptors are altered after injury, resulting in increased pain signaling (McCleskey and Gold, 1999, Waxman et al., 1999). In fact, voltage-gated Ca^{2+} and Na^{+} channels and ATP-gated P2X receptors are some of these ion channels implicated in the pathogenesis of arthritis-induced pain (Dube et al., 2009, Eid and Cortright, 2009, Micale et al., 2009, Burnstock et al., 2011).

1.3.3.1 Calcium channels

Voltage-gated calcium channels (VGCCs) are necessary for many critical functions like transmitter release and hormone secretion in the body. They are composed of five components including α1, α2, β, δ and γ subunits (Zamponi et al., 2015), which provide a battery of sites for selective pharmacological modification. According to their electrophysiological and pharmacological properties, calcium channels have been divided into low-threshold (T-types) and high-threshold (L-, N-, P/Q- and R-types) (Bourinet et al., 2016). Current therapeutic targets against VGCCs include L-type Cav1.2 calcium channels involved in hypertension treatment, T-type Cav3 channels involved in epilepsy, and auxiliary subunit α2δ1 for neuropathic pain (Zamponi et al., 2015). The auxiliary subunit α2δ1 expression is markedly up-regulated in DRG neurons and spinal dorsal horn after peripheral nerve injury (Luo et al., 2001, Newton et al., 2001, Li et al., 2006); and it is shown that the level of α2δ1 is also modestly elevated in cutaneous DRG neurons after subcutaneous injection of CFA (Lu et al., 2010). Moreover, α2δ1 is thought to be the target of the drugs gabapentin and pregabalin, which are widely used for treatment of, among others, neuropathic pain (Li et al., 2006). By binding to α2δ1 they block Ca^{2+}-flux into the cell and thereby attenuate hypersensitivity in both neuropathic and inflammatory pain models (Maneuf et al., 2006, Sills, 2006). Loss of function studies with α2δ1 knockout mice show dramatically reduced injury-induced hypersensitivity to mechanical and cold stimuli, which confirms that α2δ1 is a key component of the nociceptive pathways (Patel et al., 2013).

1.3.3.2 Sodium channels

Voltage-gated sodium channels play fundamental roles in the excitability of neurons, by initiating and propagating action potentials along axons (Mantegazza and Catterall, 2012). The distinct sodium channel subtypes like Nav1.3, Nav1.7, Nav1.8 and Nav1.9 are expressed on peripheral sensory neurons and have been implicated in persistent pain (Liu and Wood, 2011, Dib-Hajj et al., 2013). Nav1.3 is normally absent in the naïve DRGs, but dramatically up-regulated after sciatic nerve injury (Lindia et al., 2005). Nav1.7 expression is increased in DRGs neurons after peripheral inflammation, like CFA and formalin inflammation models, and blockage of Nav1.7 in nociceptors attenuates pain hypersensitivity in several inflammatory pain models (Gould et al., 2004, Nassar et al., 2004). But Nav1.7 has not yet been associated with neuropathic pain (Nassar et al., 2005). In contrast, increased expression of Nav1.8 is found in DRGs followed by both inflammation and nerve injury (Amir et al., 2006). Blocking Nav1.8 abolishes inflammatory pain (Yu et al., 2011), but only attenuates some types of neuropathic pain (Stirling et al., 2005, Kistner et al., 2010). Moreover, Nav1.9 is selectively expressed in small-sized DRG neurons (Fukuoka et al., 2008), and the expression level is significantly elevated after CFA injection (Tate et al., 1998) but reduced followed nerve injury (Berta et al., 2008). These findings suggest that sodium channels are differentially regulated in inflammatory and/or neuropathic pain conditions.

1.3.3.3 ATP-gated ion channel P2X3

Besides voltage-gated ion channels, ligand gated ion channels, including purinergic ionotropic receptors like P2X receptors activated by ATP, are considered to play significant roles in nociceptive signal transduction, as shown in different experimental pain models (Hamilton et al., 2001, Tsuzuki et al., 2001, Xu and Huang, 2002, Averill et al., 2004, Tsuda et al., 2010). A specific ATP-sensitive ligand-gated ion channel, the P2X3 receptor has generated considerable interest in view of its role in the initiation of nociceptive signaling (Xiang et al., 2008, Fukuoka et al., 2012). P2X3 is significantly increased in DRGs after nerve injury (Novakovic et al., 1999, Wang et al., 2010), and blockage of P2X3 by treatment with an antagonist can attenuate nerve injury-induced pain-like behavior (Hansen et al., 2012). However, a decrease in P2X3 receptor protein expression in primary sensory neurons has been shown after CFA inflammation, which is also significantly related to pain-like behavior in experimental rats (Xu and Huang, 2002).

1.3.4 Nerve injury markers

Activating transcription factor 3 (ATF3), a member of the ATF/CREB family of transcription factors originally identified in a variety of stressed tissues, is induced in DRG neurons upon nerve injury (Chen et al., 1996, Tsujino et al., 2000). ATF3, normally not or only very rarely expressed, is induced in DRG neurons as early as 12 hr, peaks after 3 days and decreases after 6 weeks of axotomy (Tsujino et al., 2000). Growth-associated protein 43 (GAP43), identical to growth associated protein B-50, is up-regulated in response to nerve lesion and involved in peripheral nerve regeneration (Jacobson et al., 1986, Van der Zee et al., 1989). Although ATF3 has also recently been shown to contribute to nerve regeneration by orchestrating gene expression responses in peripheral injured neurons (Seijffers et al., 2006, Seijffers et al., 2007, Chandran et al., 2016), it is still, together with GAP43, a useful nerve injury marker. The hypothesis that prolonged joint inflammation may have “nerve damaging” impact on sensory neurons has been examined using these factors. Interestingly, expression of ATF3 is elevated in DRGs in the late phase of the K/BxN-serum transfer arthritis (Christianson et al., 2010) and the CIA (Inglis et al., 2007), but not in the AIA model (Segond von Banchet et al., 2009). These markers appear to be specific to certain states of nerve injury-induced pain, as there is no increased expression of ATF3 or GAP43 in primary neurons following inflammation by CFA and carrageenan injection.

1.3.5 Glia activation

In the past two decades great progress has been made demonstrating that non-neuronal cells, that is glia, play crucial roles in the pathogenesis of pain, especially in the chronic pain state. Spinal glial activation has been observed in experimental studies on pain, including inflammatory, neuropathic and cancer pain models (Sweitzer et al., 2001, Zhang et al., 2003, Ji et al., 2013). Usually, glial activation displays morphological changes such as hypertrophy or process retraction/extension, including up-regulation of the glial markers such as ionized calcium-binding adapter molecule 1 (Iba1) in microglia, and glial fibrillary acidic protein

(GFAP) in astrocytes (Meller et al., 1994, Vale et al., 2004).

Historically glia has been considered to provide physical and nutritional support for the neuronal network. However, since the early 1990'ies increasing evidence suggests that glia plays a variety of roles in spinal sensitization. After painful stimulation or injuries (tissue or/and nerve), both microglia and astrocytes show morphological and functional signs of activation, including: i) increased activity in intracellular pathways, like the mitogen-activated protein kinases (MAPKs) pathways, as indicated by e.g. increased phosphorylation of p38 in microglia, and c-Jun N-terminal kinase (pJNK) in astrocytes and ii) production and release of glia mediators such as pro-inflammatory cytokines and growth factors; and these factors are thought to act on spinal neurons and contribute to spinal sensitization (McMahon and Malcangio, 2009). In addition, glia inhibitors can attenuate the initiation and reverse the maintenance stages of chronic pain (Watkins and Maier, 2003). It is an intriguing thought that difficulties in developing new pain therapeutics may in part be due to an overseen role of glia. Hence, it is critical to explore the role of these cells in pain transmission and to determine the feasibility of targeting astrocytes and/or microglia in conditions of chronic pain.

1.3.6 Lysophosphatidic acid

The bioactive glycerophospholipid lysophosphatidic acid (LPA) is an important signaling molecule, present in numerous tissues and fluids, notably within the developing and adult nervous system (Yung et al., 2015). While 18-carbon long unsaturated (C18:1) LPA is the most studied form (Figure 2), many other chemical forms of LPA with different acyl chain lengths, saturation, and position also exist (Yung et al., 2014). LPA is generated through several different enzymatic pathways with two major ones being actions of autotaxin (ATX) and phospholipases such as phospholipase A_2 enzymes (PLA_2). These pathways generate both intracellular and extracellular LPA (Pages et al., 2001). Extracellular LPA initiates various intracellular signaling cascades by binding and activating at least six 7-transmembrane G-protein coupled receptors denoted LPA1-LPA6 (Noguchi et al., 2009, Yung et al., 2014) (mouse gene and mRNA for these receptors are referred to as *Lpar1-Lpar6*) and therefore involved in both physiological and pathophysiological processes.

Accumulating data point to an important role of LPA and corresponding receptors in pain signaling. First, both i.p. and intrathecal (i.th.) injection of LPA (18:1) induce mechanical and thermal hypersensitivity in the paws lasting for at least 30 min and 7 days, respectively (Renback et al., 1999, Inoue et al., 2004), indicating that LPA is involved both in peripheral and spinal mechanisms of nociceptive signal transmission. Second, the use of transgenic mice and pharmacological tools demonstrates that several LPA receptors contribute to the development and/or maintenance of neuropathic, inflammatory and bone cancer pain (Inoue et al., 2004, Sevastou et al., 2013, Ueda et al., 2013). For instance, genetic removal of *Lpar1, Lpar3* or *Lpar5* prevents nerve injury-induced pain like behavior (Inoue et al., 2004, Lin et al., 2012, Ueda et al., 2013), and in addition, LPA3 and LPA5 have been implicated in nociceptive signal transmission in the spinal cord after nerve injury (Ma et al., 2009, Lin et al., 2012). Of note, both mechanical and thermal hypersensitivity induced by i.th. injection of LPA are abolished in either *Lpar1* or *Lpar3* deficient mice (Ma et al., 2010, Kashimoto et al., 2013).

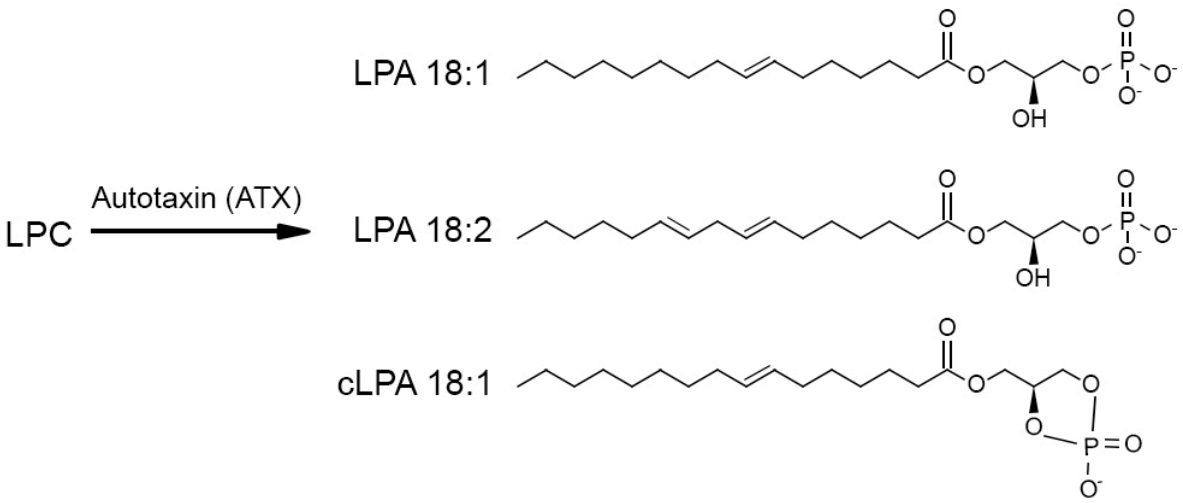

Figure 2. LPA synthesis by the enzyme autotaxin from LPC (modified from Paper IV).

LPA and LPA receptors have gained attention as they have the potential to be crucial factors in multiple mechanisms leading to chronic pain, including the induction of peripheral sensitization, structural reorganization, demyelination and spinal glia activation. In experimental models of neuropathic pain, LPA1 activation has been coupled to up-regulation of the calcium ion channel subunit α2δ1 in DRG neurons and up-regulation of protein kinase C gamma (PKCγ) in the spinal dorsal horn (Inoue et al., 2004), contrasting LPA5 activation to the modulation of cAMP response element binding protein (CREB) in the spinal cord (Lin et al., 2012). Furthermore, increased expression of LPA3 has been observed in activated microglia after nerve injury (Ma et al., 2009), which is thought to serve as a positive feedback for amplifying LPA synthesis (Ma et al., 2013). This notion is supported by the observation that i.th. injection of LPA increases LPA production, suggesting that LPA itself plays important roles in the amplification of LPA biosynthesis. Interestingly, LPA-induced enhancement of LPA synthesis was abolished in *Lpar3*-deficient mice (Ma et al., 2013).

2 AIMS OF THESIS

The overall aim of this thesis is to explore, at the cellular and molecular level, mechanisms underlying pain processing at the lumbar spinal level in the CAIA mouse model of RA. These mechanisms are associated both with neurons and non-neuronal cells, that is glia. They encompass DRG neurons with peripheral branches in the skin and central projections in the dorsal horn, where the local interneurons and projection neurons participate. The thesis has three specific aims:

1. To identify peptides only expressed in the dorsal region of the mouse spinal cord using liquid chromatography mass spectrometry (LCMS). And, further, to analyse, as proof-of-concept, one identified, novel peptide with regard to involvement in pain processing.

2. To identify and quantify a number of molecules, including neuropeptides, channels and receptors in the mouse DRGs and spinal cord after collagen antibody administration with special focus on comparison between the two phases of the arthritis: the inflammatory phase and the chronic phase.

3. To investigate the role of LPA in arthritis-induced hypersensitivity, especially in the chronic phase.

3 MATERIALS AND METHODS

3.1 ANIMAL MODEL

3.1.1 Induction of CAIA

CBA male mice (Scanbur, Stockholm, Sweden) were kept in the animal facility at the Department of Physiology and Pharmacology, Karolinska Institutet according to established procedures. Arthritis was induced by i.v. injection of 150 mg 5 monoclonal collagen type II antibodies cocktail on day 0 (Chondrex, Redmond, WA), boosted with i.p. injection of 30 μg LPS on day 5 (Chondrex). After antibody injection, the inflammation started from day 6, reached a peak on day 15 and resolved by day 33. In contrast, the control mice injected either only with saline or with LPS did not show any signs of inflammation.

3.1.2 Pain-like behavior assessment

3.1.2.1 Mechanical hypersensitivity (von Frey test)

For mechanical measurement the mice were habituated to the test environment on two occasions before assessment of baseline. After three baseline recordings performed on different days, the animals were randomly assigned to control (saline and LPS) and CAIA groups. Mechanical sensitivity was determined by assessment of paw withdrawal using von Frey optiHair filament (Marstock OptiHair, Schriessheim, Germany) with the up-down method as previously describe (Chaplan et al., 1994). A series of filaments with a logarithmically incremental stiffness of 0.5, 1, 2, 4, 8, 16 and 32 mN (converted to 0.051, 0.102, 0.204, 0.408, 0.815, 1.63, 3.26 gram, respectively) was used and the top of the filament was applied to the plantar surface of the hind paw and held for 2-3 s. In order to avoid tissue damage a cut off of 4g was applied. A brisk withdrawal of the paw was noted as a positive response. The 50% probability withdrawal threshold (force of the von Frey hair to which an animal reacts to 50% of the presentations) was calculated (Chaplan et al., 1994).

3.1.2.2 Heat hypersensitivity

To assess heat sensitivity, a Hargreaves-type testing device (UARDG, Department of Anesthesiology, University of California, San Diego, 92103-0818) was used as previously described (Dirig et al., 1997, Svensson et al., 2005). Briefly, mice were placed in plexiglas cubicles on a glass surface maintained at 25 °C. The thermal nociceptive stimulus originates from a projection bulb below the glass surface, and the stimulus is delivered separately to one hind paw at a time. A timer was activated by the light source and automatically turned off by a motion sensor. Latency was defined as the time required for the paw to show a brisk withdrawal. Each hind paw was tested three times and the average withdrawal latency calculated.

3.1.2.3 Cold hypersensitivity

Cold hypersensitivity was measured by the acetone test. Acetone (one drop) was applied to the hind paw without touching the skin and the response of the mouse to acetone was

observed for 60 s and graded on a four-point scale, as modified from Flatters and Bennett (Flatters and Bennett, 2004). Based on the time spent expressing nocifensive behavior, the following scores were determined: 0 for no response, 1 for total reaction time less than 1 s, 2 for total reaction time between 1 and 3 s, 3 for total reaction time between 3 and 10 s, 4 for total reaction time longer than 10 s as previously described (Su et al., 2014). The test was repeated three times, and data from two hind paws were presented as median with interquartile range.

3.2 PRIMARY NEURON CULTURE

Around 30 DRGs were dissected (different levels of spinal ganglia) from adult CBA male mice and placed in 6 cm petri-dish with DPBS (Sigma, St. Louis, MO) on ice. The ganglia were digested in a 37 °C incubator with 3 ml papain (Sigma) for 30 min and followed by 1.5 ml collagenase and 1.5 ml dispases (Sigma) treatments. After digestion, ganglia were transferred to a 15 ml focal centrifuge tube with 6 ml of L15 medium containing 10% fetal bovine serum (FBS, Fisher Scientific, Waltham, MA) and 100 units/ml penicillin-streptomycin (Sigma). Ganglia were triturated with 1 ml pipette (Fisher Scientific) until the suspension was homogeneous. Single cell suspensions were filtered through a cell-strainer (100 μm, BD Biosciences, Stockholm, Sweden) and then incubated in 6 cm dishes to allow non-neuronal cells especially glia cells (satellite cells) to attach. After 90 min, the supernatant was collected and the cells (mixture of neurons and glia cells) were seeded 200 μl/well into 48-well plate pre-coated with L-type polylysine and laminin (Sigma).

3.3 MASS SPECTROMETRY

3.3.1 Sample preparation

Mice were deeply anesthetized with isoflurane and decapitated. Dissected spinal cord samples were immediately heat stabilized with the Stabilizor system (Skold et al., 2007). After stabilization, the spinal dorsal and ventral parts from cervical and lumbar enlargements were separated under a dissecting microscope. Peptides were extracted using Peptide extraction Kit (Denator AB, Uppsala, Sweden) according to the manufacturer's instructions with modifications (Svensson et al., 2003).

3.3.2 Peptide quantification and identification

Peptide separation was performed using a nano-flow liquid chromatography (LC) system and then directly electrosprayed into the mass spectrometer, a hybrid linear ion trap (LTQ) Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with a 7T ICR magnet. The mass-to-charge ratio of ions and their levels were recorded in the instrument. The acquired data was quantified using the software DeCyder MS2.0 (GE Healthcare, Uppsala, Sweden). Peptide identities were established using the software X! Tandem (Craig and Beavis, 2004) and MASCOT Daemon (version 2.3, Matrix Science, London, UK) to search the resulting data against an in-house made compilation of mouse precursors containing known neuropeptides and peptide hormones.

3.4 IMMUNOHISTOCHEMISTRY

3.4.1 Sample preparation

Mice were deeply anesthetized with sodium pentobarbital (60mg/kg) and transcardially perfused with 20 ml pre-warmed (37 °C) saline, followed by 20 ml pre-warmed 4% paraformaldehyde containing 0.2% picric acid in 0.16 M phosphate buffer (pH 7.2-7.4) and 50 ml cold fixative (Su et al., 2015). Lumbar 4 and 5 DRGs were dissected and post-fixed in the same fixative for 90 min at 4 °C. After cryoprotection with 10% sucrose in 0.1 M phosphate buffer containing 0.01% sodium azide (VWR International, Spanga, Sweden) and 0.02% bacitracin (Sigma) for 48 hr, DRGs were embedded with OCT (HistoLab, Vastra Frolunda, Sweden), frozen with liquid carbon dioxide and sectioned on a CryoStar NX70 cryostat (Thermo Scientific, Walldorf, Germany) at 12 μm thickness.

3.4.2 Normal method

For immunocyto-staining, the fixed cells were permeabilized with 0.3% Triton X-100/PBS for 10 min, and then non-specific antibody-binding sites were blocked with 5% normal goat serum in 0.3% Triton X-100/PBS for 30 min at room temperature (RT) and then incubated with rabbit anti-α2δ1 antibody and mouse anti-MAP2 monoclonal antibody. The corresponding Alexa-conjugated secondary antibodies (Invitrogen, Carlsbad, CA) and DAPI (Invitrogen) were used to visualize the cells. In each coverslip, 12 areas were selected randomly, and the α2δ1 positive neurons were counted, and its percentage in the MAP2 positive cells was calculated.

3.4.3 TSA plus method

Mounted sections were dried at RT for at least 30 min and then incubated with primary antibodies diluted in phosphate-buffered saline (PBS) containing 0.2% (wt/vol) BSA (Sigma) and 0.3% Triton X-100 (Sigma) in a humid chamber at 4 °C overnight. Immunoreactivities were visualized using the TSA Plus kit (PerkinElmer, Waltham, MA) as previously described (Su et al., 2015). For double labeling, sections of mouse and human ganglion already stained with LPA1 by using TSA plus kit were rinsed with PBS and incubated with α2δ1 antibody (1:400) in the humid chamber at 4 °C for 48 hr. After washing, the α2δ1 staining was visualized with secondary IgG (H+L) antibody conjugated with carbocyanin 3 (Cy3, 1:150, Jackson ImmunoResearch Laboratories, West Grove, PA) at RT (22 °C) for 90 min. Counterstaining was performed on single labeling sections with 0.001% propidium iodide (PI, Sigma) for 10 min at RT. Double labeled sections were counterstained with DAPI (Sigma) for 15 min at RT. After rinse in PBS, the sections were mounted with DABCO medium. Antibodies used in this thesis are listed in Table 1.

Table 1. Primary antibodies used for immunohistochemistry

Antigen	Description of Immunogen	Source, Host Species and Cat.	Concentration or Dilution
α2δ1	Synthetic peptide corresponding to amino acids 528-668 of human α2δ1	Sigma, rabbit polyclonal, HPA008213	0.05µg/ml (TSA)
ATF3	Recombinant protein corresponding to amino acids 194-212 of Human ATF3 (C-terminal)	Santa Cruz, rabbit polyclonal, sc-188	0.05µg/ml (TSA)
Calbindin D-28k	Recombinant rat calbindin D-28k	Swant, rabbit polyclonal, CB-38	1:4,000 (TSA)
CBLN1	KLH-conjugated synthetic peptide corresponding to amino acids 95-112 of mouse CBLN1	Morgan J.I. (Memphis, TN)	1:10,000 (TSA)
CGRP	Synthetic peptide corresponding to amino acids 23-37 of Tyr-α-r CGRP	Terenius L. (Stockholm, Sweden), rabbit polyclonal	1:20,000 (Normal)
galanin	Albumin-conjugated, synthetic peptide corresponding to amino acids 1-29 of rat galanin	Theodorsson E. (Linköping, Sweden), rabbit polyclonal	1:4,000 (TSA)
GAP43	Recombinant protein corresponding to full-length of rat GAP43	Chemicon, rabbit polyclonal, ab5220,	7.5µg/ml (TSA)
GFAP	GFAP isolated from cow spinal cord	DAKO, rabbit polyclonal, IS524	1:500 (Normal)
Iba1	Synthetic peptide corresponding to C-terminal amino acids of Iba1	WAKO, rabbit polyclonal, 019-19741	1:200 (Normal)
LPA1	Synthetic peptide corresponding to amino acids 342-359 of human EDG2	Abcam, rabbit polyclonal, Ab23698	1:1,000 (TSA)
MAP-2	Bovine brain microtubule protein	Millipore, mouse monoclonal, MAB3418	1:300 (Normal)
NPY	Synthetic peptide corresponding to full-length of porcine NPY	Wong H. and Walsh JH. (UCLA, CA), rabbit polyclonal	1:4,000 (TSA)
P2X3	Synthetic peptide corresponding to amino acids 383-397 of rat P2X3	Elde R. (Minneapolis, MN), rabbit polyclonal	1:2,000 (TSA)
substance P	Albumin-conjugated, synthetic peptide corresponding to full-length of substance P	Terenius L. (Stockholm, Sweden), rabbit polyclonal	1:2,000 (TSA)

3.4.4 Image analysis and quantification

Representative images were captured in an LSM710 confocal laser-scanning microscope (Carl Zeiss, Oberkochen, Germany) from one airy unit operated with ZEN2012 software (Zeiss). The proportion of neuron profiles (NPs) (number of immuno-positive NPs / total

number of NPs labeled by propidium iodide) in DRGs was calculated from three or four randomly selected sections.

3.5 QUANTITATIVE REAL-TIME PCR (TAQMAN)

Animals were deeply anesthetized (5% isoflurane) on day 15 and 45 after arthritis induction, and L4 and L5 DRGs were harvested after decapitation by dissection. The tissues were immediately flash frozen and stored at -70 ºC until analysis. Total RNA was extracted from DRGs using Trizol (Invitrogen) according to the manufacturer's protocol. Complementary DNA was prepared and quantitative real-time PCR (StepOne system) performed using hydrolysis probe following the manufacturer's protocol. Pre-developed validated primer/probe sets for *Lpar1-6* and as *Hprt1* were used for mRNA analysis (Table 2) (TaqMan Gene Expression Assays, Applied Biosystems, Foster City, CA). Threshold cycle values in each sample were used to calculate the number of cell equivalents in the test samples using the standard curve method (Boyle et al., 2003). The data were normalized to *Hprt1* mRNA levels, and the data expressed as percentage of control values. In some experiments the delta Ct method targets normalized to *Hprt1* expression was used to obtain relative concentration and presented as mean ± SD.

Table 2. Primers used for real-time qPCR

Genes	Taqman primer
Atf3	Mm00476033_m1
Cacna2d1	Mm00486607_m1
Enpp2 (Atx)	Mm00516572_m1
Galanin	Mm00439056_m1
Gap43	Mm00500404_m1
Hprt1	Mm01318743_m1
Lpar1	Mm01346925_m1
Lpar2	Mm00469562_m1
Lpar3	Mm00469694_m1
Lpar4	Mm01228532_m1
Lpar5	Mm01190818_m1
Lpar6	Mm00613058_s1
P2rx3	Mm00523699_m1
Scn9a (Nav1.7)	Mm00450762_s1
Scn10a (Nav1.8)	Mm00501467_m1

3.6 IN SITU HYBRIDIZATION

Antisense and sense RNA probes specific for mouse and human *Lpar1* were synthesized using sequence specific primers (mouse *Lpar1* forward primer 5'-ATGAGGACACTCCTGTACTTAACA-3'; reverse primer 5'-GAAAGGCATACTGAATACACACAC-3', human *Lpar1* forward primer 5'-

CCCCAGTTCACAGCCATGAATGAACC-3'; reverse primer 5'-CATGCGGAAAACCGTAATGTGCCTCT-3') and cDNA obtained from mouse brain and human hypothalamus. The PCR fragments were subcloned into pCRII-TOPO vector (Invitrogen) and transcribed by T7/Sp6 RNA polymerase (Life Technologies, Carlsbad, CA) in the presence of 35S-UTP (Perkin Elmer, Boston, MA). Sense probes were used as negative controls.

In situ hybridization was performed as described previously with minor modifications (Le Maitre et al., 2013). Briefly, mouse and human DRG sections were post-fixed in 4% paraformaldehyde and treated with 0.25% acetic anhydride in 0.1M triethanolamine (pH 8.0). The sections were gradually dehydrated in a series of alcohol and stored at -20 °C until use. The sections were pre-hybridized with 50% (vol/vol) deionized formamide (pH 5.0), 50 mM Tris-HCl (pH 7.6), 25 mM EDTA (pH 8.0), 20 mM NaCl, 0.25 mg/ml, yeast tRNA and 2.5× Denhardt's solution for 4 hr at 55 °C and followed by hybridization with ^{35}S labeled *Lpar1* probe in a humidified chamber at 55 °C for 16- 20 hr. The ^{35}S-labeled *Lpar1* probes were diluted to a final concentration of $1.0x10^6$ cpm/200 µl in the hybridization solution containing 50% (vol/vol) deionized formamide (pH 5.0), 0.3 M NaCl, 20 mM DTT, 0.5 mg/ml yeast tRNA, 0.1 mg/ml poly-A-RNA, 10% (vol/vol) dextran sulfate, and 1× Denhardt's solution. After hybridization, sections were rinsed as follows: twice for 30 min in 1× SSC at 55 °C, 1 hr in 50% (vol/vol) formamide/0.5× SSC at 55 °C, 15 min in 1× SSC at 55 °C, 1 hr in RNase A buffer at 37 °C, twice for 15 min in 1× SSC at 55 °C, then dehydrated in ascending alcohol series (2 min each), and finally were air dried. The sections were exposed to Kodak BioMax MR film (VWR International) and then dipped in an autoradiographic emulsion. After a determined exposure time (6 weeks for mouse *Lpar1* and 8 weeks for human *Lpar1*), slides were developed using D19 developer (Kodak, Rochester, NY) for 3 min and AL4 fixative for 7 min, dried at room temperature. The sections were dehydrated with graded alcohol, dipped in xylene and mounted with Entellan.

4 RESULTS AND DISCUSSION

In this thesis, four projects were performed to study the molecular mechanisms underlying RA-induced pain.

4.1 NEUROPEPTIDES IN THE MOUSE SPINAL CORD AND DESCER AS A NOVEL PAIN MODULATOR

4.1.1 Identification and quantification of peptides in the mouse spinal cord

Neuropeptides were initially considered as transmitter-like substances and had emerged as the largest group of messenger molecules in the nervous system (Burbach, 2010). However, neuropeptides might in some cases be difficult to quantify using classical antibody-based techniques such as immunohistochemistry, western blotting and radioimmunoassay. We are using label-free liquid chromatography high-resolution tandem mass spectrometry (LCMS) to identify and semi-quantify neuropeptides, which is based on the amino acid-sequence, to explore potential new peptides that might be involved in pain. Using non-organic solvents we identified 204 peptides originating from protein precursors in naïve mouse spinal cord. Of these, 36 were previously well-characterized full-length neuropeptides, and 168 had not been previously characterized. Further, a majority of them had typical neuropeptide cleavage sites, thus they might represent potential functional peptides in the nervous system (27 already identified and 118 novel peptides).

From a pain perspective, neuropeptides released from central terminals of peripheral nociceptors and interneurons are more likely to play roles in pain transmission, if they are expressed in the dorsal horn than the ventral horn of the spinal cord. Totally 137 out of the 204 peptides were found in both dorsal and ventral parts, from cervical and lumbar spinal cord. We found 50 peptides that were only expressed in the dorsal region, while 38 peptides were predominantly expressed in the spinal dorsal part (Figure 3A). Some of the peptides that were exclusively or predominantly located in the dorsal horn had previously been proven important transmitters in nociception. For instance, protachykinin-1-drived peptides substance P, neurokinin A and C-terminal-flanking peptide were predominantly expressed in the spinal dorsal horn. Both substance P and neurokinin A participate in pain signal transmission (Ribeiro-da-Silva and Hokfelt, 2000). Thus, based on both the location and precursor protein, C-terminal-flanking peptide is a candidate for further study on pain.

4.1.2 desCER as a novel modulator in spinal nociception

In our first step in examining the roles of the novel peptides, we focused on one peptide called [des-Ser1]-cerebellin (desCER), originating from cerebellin precursor protein 1 (CBLN1), which was expressed 6 times more in dorsal region as compared to ventral part. The cerebellins (CBLNs) represent a family of secreted proteins, belonging to the C1q/TNF super family, which display wide but distinct expression patterns throughout the nervous system of many species, from chicken to man. The CBLN family comprises four members, CBLN1-4, which have distinct functions in synapses formation, maintenance and plasticity

(Joo et al., 2011, Martinelli and Sudhof, 2011, Matsuda and Yuzaki, 2011, Mishina et al., 2012) and are important during cerebellum development (Rucinski et al., 2009) and for long-term depression (Yuzaki, 2009). As there were no published studies describing the distribution of CBLNs in the nervous system outside the brain of adult mice, or a possible relationship to nociception, we decided to investigate the location of the precursor protein and the potential functional role of desCER in pain signal transmission in more detail. Immunohistochemistry showed expression of CBLN1 with a punctate staining in neuronal cytoplasma throughout the spinal gray matter. The immunofluorescence signal was much stronger in the dorsal compared to the ventral part, and predominantly present in lamina II and lamina III (Figure 3B). CBLN1-immunoreactive (IR) neurons co-localized with calbindin, a marker for excitatory interneurons, thus indicating that desCER could be released from excitatory neurons. Injection (i.th.) of desCER induced mechanical hypersensitivity in a dose-dependent manner, but did not alter the sensitivity to thermal stimuli in naïve mice, neither heat nor cold. This study provides evidence for the involvement of desCER in nociception through spinal pain circuits and, importantly, provides a platform for continued exploration of involvement of novel neuropeptides in the regulation of nociceptive transmission.

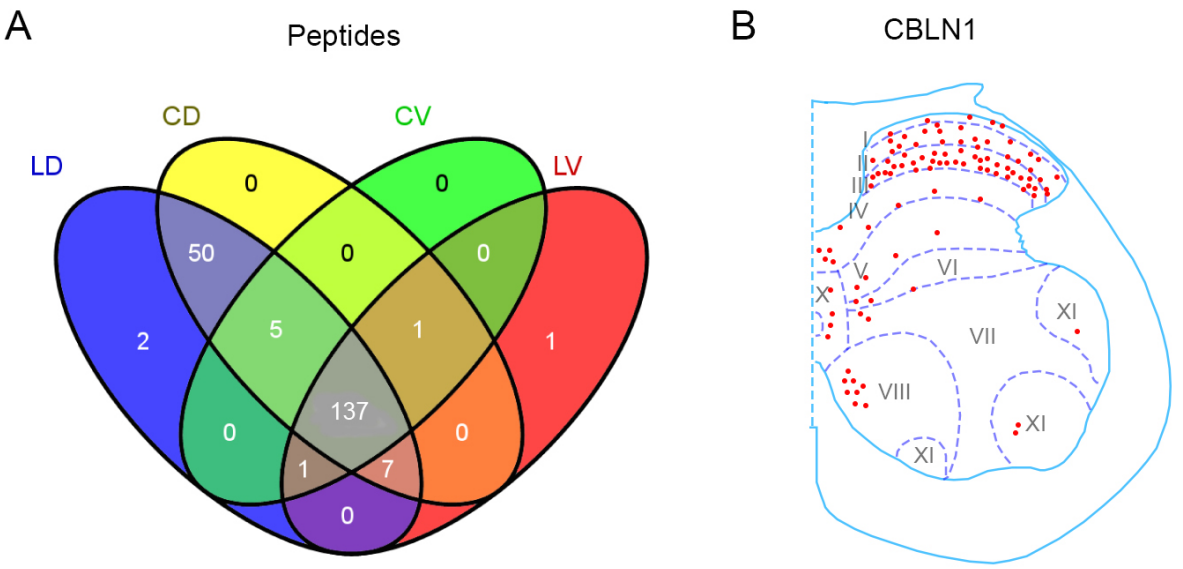

Figure 3. Neuropeptides in mouse spinal cord. (A) Using mass spectrometry, 50 neuropeptides were found exclusively expressed in dorsal spinal cord of naïve mouse, and 38 neuropeptides were predominantly expressed in the dorsal region. (B) Red docs showed the precursor protein CBLN1 expression in mouse spinal cord. CBLN1-drived peptide desDER is one of 38 predominantly dorsal expressed peptides found by mass spectrometry. CBLN1 expressed throughout the grey matter, predominantly in Laminae II_O/III. Abbreviations: LD = lumbar dorsal region; CD = cervical dorsal region; CV = cervical ventral region; LV = lumbar ventral region; CBLN1 = cerebellin 1 precursor. (Modified from Paper I)

4.2 COLLAGEN ANTIBODY–INDUCED ARTHRITIS EVOKES PERSISTENT PAIN WITH SPINAL GLIAL INVOLVEMENT

4.2.1 CAIA induces transient inflammation and persistent hypersensitivity

We successfully induced CAIA in three different mouse strains: QB, BALB/c and CBA. All three stains developed transient inflammation and persistent hypersensitivity after injection of

collagen type II antibodies and LPS. Interestingly, even though there was a strain-dependent distribution of the inflammation (fore versus hind paw) and time to resolution, they developed similar mechanical pain-like behavior, and importantly, in all three strains mechanical hypersensitivity persisted for weeks after the visual signs of inflammation disappeared. I focused on the CBA strain in this thesis, and induction of CAIA in CBA mice led to an increase in arthritis scores 6 days after injection of the collagen antibody cocktail, with the disease severity peaking around day 15. The signs of visual inflammation (arthritis) were transient, and were no longer detectable by day 36. Interestingly, CBA mice subjected to CAIA displayed more severe signs of arthritis in the fore paws compared to the hind paws, which was not the case for BALB/c or QB mice, where the distribution of inflammation was more even between fore and hind paws. The CBA CAIA mice showed reduced tactile paw withdrawal thresholds from day 3, even before arthritis was observed, and lasted till the end of the experiment. No signs of inflammation or hypersensitivity were found in saline or LPS control groups. We refer to the days with visible inflammation as the "inflammatory phase", and the period that starts 10 days after the visible signs of inflammation has disappeared as the "post-inflammatory phase" or late phase. In CBA mice this typically meant that the post-inflammatory phase started around day 45.

During the inflammatory phase (day 16), the CAIA mice displayed higher acetone scores compared to the saline and LPS groups. However, no differences of acetone score were found between CAIA and control groups on day 42, indicating that CAIA induced transient cold allodynia only in the inflammatory phase. Moreover, using Hargreaves box for assessment of heat sensitivity long-lasting heat hypersensitivity in the CAIA mice was recorded. After induction of CAIA, mice showed a significantly decreased latency to heat stimulation compared to the control groups, both on day 17 and on day 43. These results showed that CAIA evokes transient joint inflammation and cold allodynia but persistent mechanical and heat hypersensitivity. In the RA patients, thermal (cold and heat) and pressure sensitivity are increased in a variety of anatomical sites, including both inflamed joints and non-inflamed tissues (Jahanshahi et al., 1989, Edwards et al., 2009). In this case, the CAIA model displays similar pain hypersensitivity as RA patients and could be relevant for studies of underlying mechanisms of the disease.

4.2.2 COX inhibition attenuates CAIA-induced mechanical hypersensitivity only in the inflammatory phase

We assessed the effect of analgesic drugs, such as gabapentin (anticonvulsant used for treatment of neuropathic pain), buprenorphine (opioid) and diclofenac (COX inhibitor), on mechanical hypersensitivity in the CAIA mice during both phases. During the inflammatory phase, all three drugs attenuated the mechanical hypersensitivity 2 and/or 4 hours after drug administration. In the postinflammatory phase, gabapentin and buprenorphine increased mechanical threshold in the CAIA mice, but no analgesic effect was observed after injection of diclofenac. Thus, COX inhibition was anti-nociceptive only during the inflammatory phase, indicating a time-dependent prostaglandin dependence in arthritis-induced pain. In contrast, gabapentin and buprenorphine attenuated hypersensitivity to mechanical stimulation in the CAIA mice in both phases. These results further underline the view that joint

inflammation can cause a drift towards mechanisms that partly resemble neuropathic pain in the chronic pain state.

4.2.3 CAIA induces time-dependent glia activation in the spinal cord

Spinal glia activation has been detected and involved in central sensation following nerve injury. In the spinal cord of CAIA mice, signs of increased microglia and astrocyte activity were detected by immunohistochemical assessment of Iba-1 and GFAP, respectively. Spinal Iba-1-like immunoreactivity (LI) was increased and microglia morphology altered in the dorsal horn of mice subjected to CAIA, most prominent during the inflammatory phase but still detectable in the late phase. In contrast, an increased immunofluorescence signal of spinal GFAP and altered astrocyte morphology were only detectable in the late phase of the CAIA model. By western blotting, we found that the levels of phosphorylated JNK in the spinal cord were pronouncedly increased in the late phase of arthritis in the CAIA model. In addition, i.th. injection of the JNK inhibitor JIP-1 partially reversed mechanical hypersensitivity in the late phase, indicating that spinal JNK participated in the modulation of arthritis-induced pain transmission. We were, however, not able to demonstrate in which cell type JNK phosphorylation was elevated due to technical problems (none of the antibodies bought from Promega, Santa Cruz Biotechnology or Cell Signaling Technology worked for immunohistochemistry). Others have demonstrated that JNK is activated in spinal astrocytes in models of chronic pain, and one may thus speculate that this is the case also in our study. However, i.th. injection of the non-specific "glia inhibitor" pentoxifylline reversed CAIA-induced mechanical hypersensitivity. Thus taken together, these data point to an involvement of spinal glia in regulation of spinal nociceptive signal transmission initiated by a RA-like joint pathology. Our finding shows that CAIA represents a multifaceted model for studies exploring the mechanisms of pain induced by inflammation in the joint, while a time-dependent spinal glial activation contributes to antibody-induced pain.

4.3 PHENOTYPIC CHANGES IN DORSAL ROOT GANGLIA AND SPINAL CORD IN THE CAIA MICE

4.3.1 CAIA alters expression of neuropeptides and ion channels in DRGs

The focus of this study was to investigate the molecular basis for long-term alterations in nociceptive pathways induced by polyarthritis using the CAIA mouse model. In this model, mechanical hypersensitivity is persistent even after the joint inflammation is resolved. We have examined expression levels of pain-related markers including neuropeptides, ion channels and nerve injury markers in DRGs and spinal cord, both during the peak of visual joint inflammation (day 15) and when the hypersensitivity persists without sign of inflammation (days 45-47).

In lumbar DRGs, based on immunohistochemistry staining results with antisera to substance P, CGRP, NPY and galanin, the latter was the only neuropeptide that was significantly increased in the CAIA group. While the number of galanin-IR NP was increased both 15 and 47 days after antibody injection, no difference in galanin signal intensity or size distribution

was found between CAIA and control groups. In spinal cord, galanin-LI in L4/L5 spinal dorsal horn was analyzed but showed similar expression levels in the three groups. Galanin is a well-kwon neuropeptide involved in nociceptive signaling transmission (Ji et al., 1994a, Liu and Hokfelt, 2002). After nerve injury, there is an increase of galanin in the small sized DRG neurons, with a typical shift to expression also in medium-to-large sized neurons (Villar et al., 1988, Zhang et al., 1998, Zhang et al., 2011). This increase in galanin expression is thought to be a part of recruitment of a program associated with neuropathic pain. Of note, while no changes in galanin expression in DRGs during peripheral inflammation induced by carrageenan (Ji et al., 1995), an increase in galanin expression was found in rat DRGs after CFA injection at the base of the tail (Calza et al., 2000). Thus, an increase in the percentage of galanin-IR DRG neurons was found in the CAIA mice, which suggests that antibody-driven joint inflammation may cause injury-like changes in the peripheral sensory neurons, or/and that galanin up-regulation can be induced by a certain type(s) of inflammatory processes, including arthritis.

Besides neuropeptides, we also examined, if induction of CAIA alters the expression of ion channels such as calcium channels (focused on the α2δ1 subunit) and the ATP-gated ion channel P2X3. Pronounced up-regulation of the α2δ1 subunit was found in the CAIA DRGs both in terms of the proportion of α2δ1-IR neurons and signal intensity during the inflammatory and post-inflammatory phases. Furthermore, size distribution analysis also showed that CAIA led to a shift of α2δ1-IR neurons towards left, from large to medium sized neurons, which suggests there is a shift from Aβ fibered sensory neurons to Aδ fiber nociceptors after CAIA induction. We found that the percentage of P2X3- positive NPs was elevated in DRGs both 15 and 47 days after arthritis induction, but no alterations in P2X3 signal intensity or soma size distribution were observed in the CAIA mice compared to control groups. We also wanted to investigate, if CAIA evokes changes in sodium ion channels but here, again, we ran into antibody problems. Of the Novus Biologicals and Alomone Labs antibodies that were tested for Nav1.7 and Nav1.8 with immunohistochemistry, none showed reliable staining. Thus, we turned to quantitative real-time PCR. We did not observe any changes in mRNA levels of *Nav1.7* or *Nav1.8* within the three groups in either of the two phases. In conclusion, CAIA causes up-regulation of galanin, α2δ1 and P2X3 in the DRGs without altering the expression of CGRP, substance P, NPY or sodium channels.

4.3.2 Long-time joint inflammation causes changes in expression of nerve injury markers

In addition to pain-related factors we have also examined, two nerve injury markers in CAIA DRGs. While ATF3 was transiently induced (only increased in DRGs on day 15), GAP43 was up-regulated in DRGs both on day 15 and day 47. Furthermore, to investigate which subpopulation of neurons was involved in CAIA DRGs, we did double-staining of GAP43 with CGRP (peptidergic neuron marker) or isolectin Griffonia simplicifolia I-B4 (IB4, non-peptidergic neuron marker) (Donnerer et al., 1993, McMahon and Priestley, 1995). We found that GAP43 was predominantly co-expressed in CGRP-positive neurons. This indicates that damaged neurons mostly belong to the peptidergic subpopulation. Thus, it is possible that

arthritis pathology in the joint primarily affects peptidergic nociceptors, and causes stress or injury-like changes in the peripheral nervous system.

Different types of pain are often classified based on the underlying pain generating condition as being predominantly inflammatory or neuropathic. Based on the literature (Table 3), sciatic nerve transection leads to an increased expression of a variety of neurochemical markers in DRGs, including the neuropeptides galanin and NPY, sodium channel Nav1.7, and the nerve injury markers ATF3 and GAP43 (Hokfelt et al., 1984, Wakisaka et al., 1992, Nothias et al., 1993, Solway et al., 2011, Zhang et al., 2011). In contrast, expression levels of these factors are not altered in classical inflammatory pain models such as the formalin and carrageenan models (Ji et al., 1994b). On the other hand, CGRP and substance P are down-regulated after nerve injury but increased in DRGs after peripheral inflammation (Smith et al., 1991, Donnerer et al., 1993, Hanesch et al., 1993, Galeazza et al., 1995, Calza et al., 1998). Furthermore, up-regulation of the α2δ1 subunit in DRGs is detected both after nerve injury (Luo et al., 2001, Newton et al., 2001) and inflammation induced by injection of CFA to the paw (Lu et al., 2010). In addition, expression of the P2X3 receptor is elevated in the DRGs after sciatic nerve lesions (Novakovic et al., 1999, Wang et al., 2010) but is decreased in sensory neurons after CFA inflammation (Xu and Huang, 2002).

Neurochemical markers like galanin, P2X3, ATF3, and GAP43 are modulated in CAIA DRGs in a fashion similar to what is observed after nerve injury, but the expression of other factors such as NPY, CGRP and substance P are not affected by CAIA-induced joint inflammation, even though they are elevated either by nerve injury or other types of inflammatory reactions. Therefore, the present results indicate that CAIA-induced changes in DRGs has some features in common with neuropathic pain models, and suggest that antibody-mediated joint inflammation could "damage" peripheral branches of sensory neurons.

Table 3. CAIA induces a unique plastic phenotype in the DRGs.

	CAIA	Tissue Inflammation	Nerve Injury
substance P	–	↑	↓
CGRP	–	↑	↓
NPY	–	–	↑
galanin	↑	–	↑
Nav1.7	–	↑	–
P2X3	↑	↓	↑
α2δ1	↑	↑	↑
ATF3	↑	–	↑
GAP43	↑	–	↑

'↑'means up-regualtion, '↓'means down-regulation and '–' indicates no change. Tissue inflammation (Carrageenan and CFA model) and nerve injury (sciatic nerve axotomy) data are based on the literature.

The present results demonstrate that the CAIA model generates a pain state with a unique and highly distinct neurochemical signature. Thus, antibody-driven inflammation has a disease-specific phenotype. Of note, similar "non-typical" changes in the expression of pain-related

factors in both the spinal cord and DRGs have been demonstrated in models of bone cancer (Honore et al., 2000), indicating that bone cancer pain is distinctly different from classic inflammatory or neuropathic conditions and suggesting that cancer pain is a unique type of persistent pain. Determining the disease-specific neurochemical changes involved in initiating and/or maintaining pain may provide a mechanistic platform for discovering of new agents targeting different pain states.

4.4 BLOCKAGE OF LPA REVERSES CAIA-INDUCED HYPERSENSITIVITY AND ALPHA2DELTA1 EXPRESSION IN DRGS

4.4.1 LPA is elevated in the CAIA DRGs

LPA can be synthesized by several enzymes, but it is thought that a large proportion of LPA is generated by ATX, which is released by activated platelets in the circulation and accumulates at the local site of the inflammation or injury (Inoue et al., 2008), including the arthritic joint (Barbayianni et al., 2015). Moreover, ATX is necessary for self-amplification of LPA production in the spinal cord after nerve injury (Ma et al., 2013), and this feed-forward of LPA synthesis acts as an important mechanism underlying sustained neuropathic pain (Ueda et al., 2013). Locally increased ATX and LPA were detected in arthritic synovium in both RA patients and animal models, and genetic ablation of ATX or LPA1 attenuated disease pathogenesis in the animal models (Nikitopoulou et al., 2012, Miyabe et al., 2013). We are, however, the first to examine the role of LPA in an animal model of arthritis-induced pain. We found that *Atx* mRNA levels were significantly elevated in L4/L5 DRGs 15 days after collagen antibody injection, but that there was no difference in *Atx mRNA* levels in DRGs between CAIA and the control groups in the late phase (day 45). In naïve adult mice, *Lpar1*, *Lpar5* and *Lpar6* mRNA were abundant in DRGs, while *Lpar2*, *Lpar3* and *Lpar4* were relatively sparsely expressed. Moreover, when we examined if the gene expression of *Lpars* was altered in response to induction of CAIA, we did not detect any difference in mRNA levels for any of the six *Lpars* in L4/L5 DRGs between the CAIA and control mice, neither in the inflammatory nor late phase. Though not studied directly, our data indicate the possibility that LPA levels are increased in DRGs through up-regulation of ATX in response to CAIA during the inflammatory phase, and contribute to enhancement of arthritis-induced pain in the DRGs. It will be important to assess, if ATX activity is increased also in the joint, and if LPA acts on LPA receptors expressed on the afferent terminals.

4.4.2 Systemic blockage of LPA by monoclonal antibody reverses in the CAIA-induced hypersensitivity

To investigate the effect of blocking LPA action on already established arthritis-induced pain-like behavior, we injected a murine monoclonal Ab against LPA from day 12 to day 45 subcutaneously (s.c., twice/week) after injection of CII antibodies. Control antibody without a target in the mouse was used as a control in this experiment. The CAIA mice showed a transient increase in arthritis score, peaking around day 15. There was no difference in severity of joint inflammation between the groups that received LPA Ab, control Ab or saline

(Fig. 4A), indicating that LPA did not maintain CAIA-induced inflammation. In contrast, while CAIA mice injected with saline or control Ab showed a significant reduction in withdrawal threshold from day 3 through day 45, injection of LPA Ab completely reversed CAIA-induced mechanical hypersensitivity after three times of s.c. injection (10 mg/kg) (Fig. 4B). Systemic injection of a lower dose (2 mg/kg) of LPA Ab also attenuated mechanical hypersensitivity in the CAIA mice. Thus, LPA Ab blocks CAIA-induced hypersensitivity in a does-dependent manner. Moreover, LPA Ab also reversed CAIA-induced mechanical hypersensitivity even when the treatment was initiated on day 57. Notably, LPA Ab injection completely reversed CAIA-induced heat hypersensitivity in both phases and attenuated cold hypersensitivity during the inflammatory phase (Fig. 4C and D). Further, in order to examine the effect of LPA Ab treatment on the generation of RA pain, we also injected LPA Ab s.c. starting 8 days before the arthritis induction, and continued with twice/weekly administration until 15 days after CAIA injection. We found that pretreatment with LPA Ab prevented CAIA-induced mechanical hypersensitivity without any visual effect on the inflammation. Taken together, blocking LPA's actions by a neutralizing antibody both prevented and reversed CAIA-induced mechanical hypersensitivity, without reducing the joint inflammation. Also, LPA Ab treatment attenuated thermal hypersensitivity (both heat and cold) in the CAIA mice. Hence, it seems that LPA plays an important role(s) in the initiation and maintenance of arthritis-induced hypersensitivity. Of note, even though we did not couple LPA to visual signs of joint inflammation, other studies have suggested that LPA regulates RA-associated inflammatory responses and apoptosis of fibroblast-like synoviocytes (Orosa et al., 2015). Treatment with an ATX inhibitor or LPA receptor antagonist attenuates disease severity of experimental arthritis induced by K/BxN and CIA (Orosa et al., 2012, Nikitopoulou et al., 2013, Orosa et al., 2014). Thus, though not evident in the CAIA model, blocking the ATX/LPA/LPA1 pathway may serve as a therapeutic approach not only for pain relief but also for RA pathogenesis.

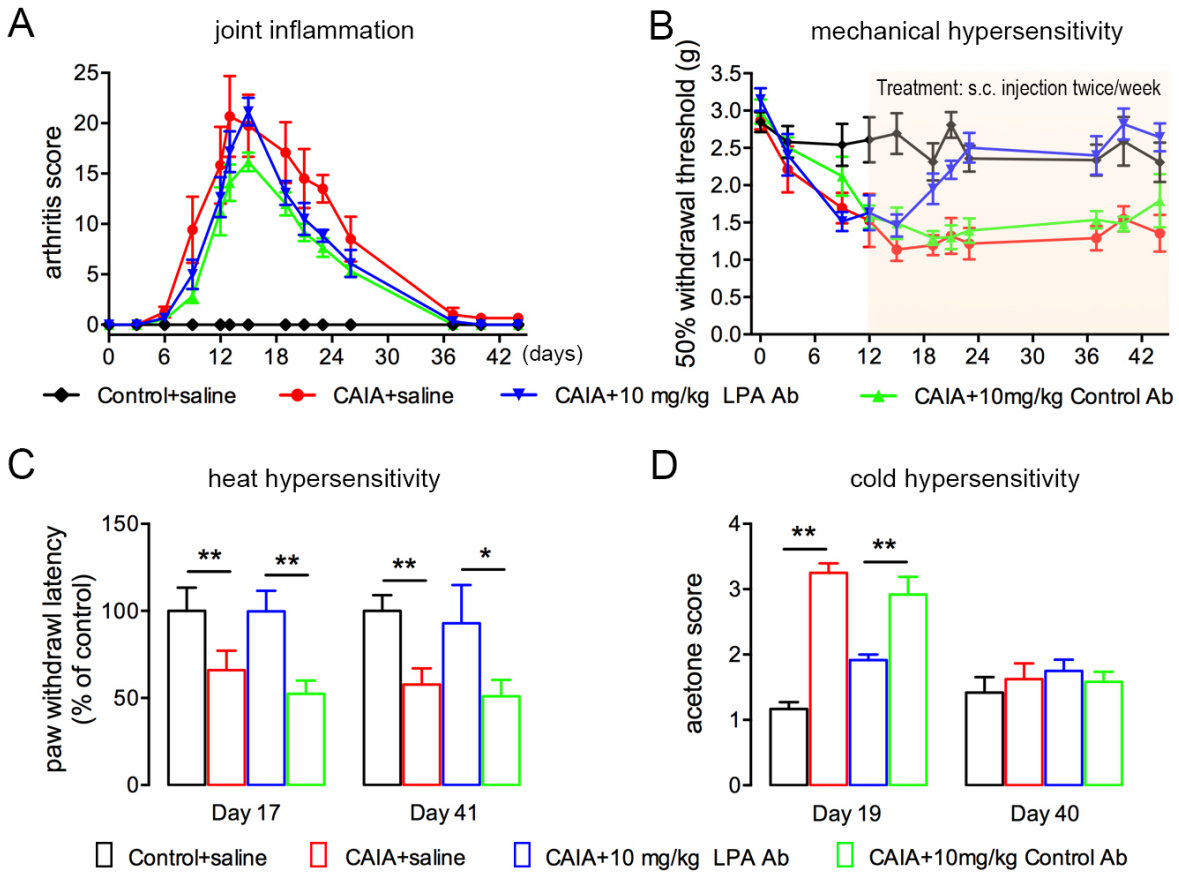

Figure 4. Systemic neutralization of LPA reverses CAIA-induced pain-like behavior. (A) Subcutaneous (s.c.) LPA antibody treatment twice per week day 12-45 does not influence arthritis scores at any time point but (B) reverses mechanical hypersensitivity, and (C) paw withdrawal latency for heat hypersensitivity both in the inflammatory and late phases. (D) Cold hypersensitivity only develops in the inflammatory phase and is alleviated by LPA antibody treatment. Mechanical hypersensitivity expressed as mean ± SEM, other data expressed as mean ± SD (from Paper IV).

4.4.3 Blockage of LPA action reverses CAIA-induced α2δ1 up-regulation

It has previously been reported that LPA1-mediated up-regulation of α2δ1 in the peripheral nervous system is prominent in experimental models of neuropathic pain (Inoue et al., 2004). In agreement with the study described above, we found that both the percentage and signal intensity of α2δ1 expression was increased in the cytoplasm of DRG neurons, especially in medium to large-sized neurons on day 47 in CAIA mice compared to saline controls. Of note, the up-regulation of α2δ1 in CAIA DRGs was not present in CAIA mice treated with the LPA Ab compared to CAIA mice receiving the control mAb. Furthermore, the up-regulation of α2δ1 was totally abolished in *Lpar1*$^{-/-}$ mice injected with CAIA, both in terms of the number and fluorescence intensity of α2δ1-IR neurons. Meanwhile, there was no change in intensity of LPA1-LI signal in CAIA DRGs by immunohistochemistry, which confirmed lack of change in mRNA levels of *Lpar1* in the CAIA animal compared to the control group. Up-reguation of α2δ1 has been observed after nerve injury and i.th. injection of LPA 18:1, and usually associates with hypersensitivity. Moreover, blocking of LPA1 by antagonists or using transgenic animals can reverse both the α2δ1 expression and pain-like behavior. It indicates that CAIA-induced up-regulation of α2δ1 can be blocked with LPA Ab treatment and abolished in *Lpar1*$^{-/-}$ transgenic mice.

To examine the direct effect of LPA on expression of nociceptive factors in the DRG neurons, we applied three different species of LPA into DRG primary culture of adult mouse. Western blotting data showed that the protein expression of α2δ1 was significantly up-regulated 4 hours after application of LPA 18:1 (highest affinity for LPA1, 10 μM) but not LPA 18:2 (highest affinity for LPA3, 10 μM) or cLPA 18:1 (highest affinity for LPA5, 10 μM) compared to vehicle. Moreover, *α2δ1* gene expression was transiently elevated in the cultured DRGs after LPA 18:1 stimulation, peaking at 4 hours. Interestingly, no difference of *galanin* or *Gap43* expression was found in the cultured DRG stimulated by LPA 18:1.

LPA1 expression in mouse DRGs has been reported previously based on qPCR and western blotting methodology (Inoue et al., 2004). However, the cellular location of LPA1 in the DRGs has not been examined. Therefore we assessed the cellular distribution of LPA1 by riboprobe *in situ* hybridization in both human and mouse DRGs. In mouse DRGs, *Lpar1* mRNA was exclusively expressed in non-neuronal satellites cells. And LPA1 antibody also showed staining in satellite cells surrounding the neurons, which was completely abolished in the *Lpar1* KO DRGs. In human ganglion, *Lpar1* mRNA showed a ring-like distribution surrounding neurons, which was confirmed at the protein level by the LPA1 antibody. Confocal images showed cytoplasmic immunostaining of α2δ1 in neurons and a surrounding LPA1-positive satellite cell staining both in the mouse and human DRGs (cover photo). Further studies are warranted in order to dissect how LPA1 activation of satellite cells leads to increased α2δ1 expression in neurons. Intriguingly, hitherto unexplored communications between these two cell types may play an important role in chronic pain.

5 CONCLUDING REMARKS

This thesis focuses on studying molecular mechanisms underlying RA-induced pain with special attention to involvement of neuropeptides, ion channels, and spinal glia activation.

Using LCMS we have identified 50 peptides, which are only expressed in the dorsal region of the spinal cord, and 38 peptides that are predominately expressed in this region. One of these peptides, desCER, is explored in behavioral experiments and shown to induce a dose-dependent mechanical hypersensitivity but not heat or cold hypersensitivity. This study provides evidence for involvement of desCER in nociception and forms the basis for continued exploration of involvement of novel neuropeptides in the regulation of nociceptive transmission. Thus, we have a window of opportunity to study novel peptides expressed in spinal cord, and we will focus especially on dorsally expressed peptides, as they are more likely to be involved in pain processing, compared to ventrally expressed peptides. Furthermore, in an ongoing LCMS analysis we are examining how the expression of peptides, including desCER, is changing in the spinal cord after induction of CAIA.

We report that CAIA evokes a transient joint inflammation and cold allodynia but persistent mechanical and heat hypersensitivity. We have characterized the CAIA model from the pain perspective by using pharmacological and histochemical phenotypic profiling: i) While gabapentin and buprenorphine attenuate mechanical hypersensitivity both in the inflammatory and post inflammatory phases, the analgesic effect of diclofenac is confined to the inflammatory phases. ii) Microglia and astrocytes are activated in the spinal dorsal horn, and inhibition of glia activation reverses CAIA-induced mechanical hypersensitivity. iii) CAIA causes up-regulation of galanin, α2δ1, ATF3 and GAP43 in the DRG neurons. These data indicate that long-term, antibody-driven joint inflammation causes a nerve injury-like phenotype, which gives a deeper understanding of the complexity of the mechanisms underlying pain in RA. According to our finding, broader therapy strategies might be explored for treatment of pain in RA, like glial inhibition, blocking the action of galanin and preventing activation of P2X3 (which are up-regulated in the CAIA DRGs). Moreover, since CAIA induced a neurochemical phenotype that resembles nerve damage, analgesic drugs used for neuropathic pain probably could probably be used for treatment of RA pain, particularly in the chronic phase.

We show that systemic treatment of CAIA with an LPA Ab completely reverses arthritis-induced mechanical and thermal pain-like behavior. The LPA antibodies also block the up-regulation of the voltage-gated calcium channel subunit α2δ1 in peripheral sensory neurons, which is also abolished in the *Lpar1* deficient mouse. Thus, blockage of LPA by applying therapeutic antibodies may represent an efficacious approach to treat arthritis-induced pain via the LPA1/α2δ1 pathway, that is via a peripheral site of action. In this project, we are using murine monoclonal antibody against LPA to neutralize the action of LPA in initiation and maintenance of arthritis-induced pain. Further, based on the ATX/LPA/ LPA receptor pathway, new drugs could be developed and tested for pain relief in RA patients. For instance, ATX inhibitors and several different LPA receptors antagonists show anti-

nociceptive effect in neuropathic pain models, which raises the possibility to treat RA pain. Moreover, compared to animal experiments, very few studies explore human DRGs as we have done in the present study. Including human samples seems relevant and offers a valuable translational perspective.

To conclude, I show desCER as a novel modulator in spinal nociception, and that CAIA-induced hypersensitivity is associated with a time-dependent activation of prostaglandin mechanisms and spinal glia activation. In addition, I describe neurochemical changes of neuropeptides and ion channels in the CAIA DRGs and spinal cord. Last but not least, I show an antinociceptive effect of an LPA Ab in the CAIA mouse with involvement of the LPA1/α2δ1 axis. These results may help to understand pain mechanisms in RA diseases, and identify molecules involved in arthritis-induced pain, in particular the chronic state. Hopefully, my work will help answering some questions related to arthritis-induced pain and bring light to potential new targets for pain relief in RA.

6 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all those who have inspired, supported and encouraged me during my years of doctoral studies. Especially, I would like to thank:

My main supervisor, **Associate Professor Camilla I. Svensson**, for giving me the opportunity to pursue this project and introducing me to the field of joint pain. Thanks for your scientific guidance and for helping me to become a more mature and independent scientist! For supporting me to attend several international conferences and interact with the scientists all over the world. I greatly appreciate your patience, motivation and enthusiasm.

My co-supervisor, **Professor Tomas Hökfelt**, it is lucky for me to be under the guidance of a great scientist and person like you. Under your mentoring, I have started the anatomy and morphology journey in your lab. Thanks for teaching me other fundamental things in science and life: open-mind, patience and full energy.

My co-supervisor, **Professor Zsuzsanna Wiesenfeld-Hallin**, for being so enthusiastic and encouraging. For interesting and useful discussion and positive attitude all the time. I appreciated you allow me to start animal work in your lab for learning the CAIA model from the help of **Xiaojun Xu** and **Jinxia Hao**.

My co-supervisor, **Docent Kim Kultima**, for helping me to think out of the box and refresh my head, your passion of science and one and half pages of email from midnight. Thank you for introducing me to mass spectrometry and statistical analysis.

My mentor, **Dr. Xiaoqun Zhang**, for your interest, time and good advice.

Camilla, Tomas and Mingdong, many thanks for your help in revising my thesis.

My close collaborators:

Azar barhapoor - for your help and supporting from lab work to lifetime. I love our close talk as roommates. Thank you for all those things that just appears when I need them. I've been so lucky to work with you!

Ada Denaley - for primary culture and behavior experiment from early morning till late evening, and also for deep discussion especially LPA project and sharing your experience. I enjoy the journey together with you in Cambridge and for science also.

Duygu Bas – Grandma of CAIA model, thanks for introducing CAIA model to me and supervising me of DRG cultures. For your passion in science. Stable!

Katalin Sandor – for introducing the Molecular Pain lab to me, and teaching me behavior testing, qPCR and a lot of other experiments. For your supporting and understanding.

Tianle Gao – for starting using CBA mice, this cute even though smelly strain, and it is good time for me to talk with you about experiment and science with you.

Swapnali Barde – thank you for *in situ* hybridization and sharing ups and downs of science and life.

Anna Finn - thanks for your kindly help for application of defense in all details, I could not imagine how can I manage these documents without your guide.

All the present and former colleagues in **the Molecular Pain Group** for help and support, friendship, and laugher. **Alex Bersellini Farinotti**, my neighbor Mr. Alex, it is great to work, teach and talk with you. **Gustaf Wigerblad**, for involving me in your project and your spark in the research and science. **Nilesh Agalave**, very helpful all the time! **Jungo Kato**, for your help of the experiments with your magic hands. **Katarzyna Rogoz**, always there to help for resolving the problems of anything. **Kristina Ängeby Möller**, really enjoy the lunch talk with you about science, life, news and everything. **Sally Abdelmoaty**, for helping and supporting during my stay, and making lab easier to work under your organization. **"**New" colleagues**: Alexandra Jurczak, Resti Rudjito**, **Shibu Krishnan, Teresa Fernandez Zafra**. Thanks for bringing the fresh air and ideas to the lab. Other friends working around, **Camilla Ultenius, Aisha Ahmed**, **Funda Orhan**, **Xicong Liu.** Thank you all for making the lab a nice place to work in.

I enjoy the atmosphere in Tomas lab in Neuroscience. **Karin Lagerman**, for all the help from all the parts. **Blanca Silva-Lopez**, for teaching me the experiments of immunohistochenmistry and in situ hybridization. **Csaba Adori**, for involved me in your study and suggestions of experiments. **Joanne Bakker**, enjoy the talk and discussion with you.

The colleagues in C3 corridor, from **Jorge Ruas group**, **Elisabet Stener-Victorin group, Johanna Lundberg group and Lennart Lindbom group.** Thanks for the professional and lovely environment of solidarity, support and friendship.

My friends in Pharmacology building, **Shijin Zhang** and **Na Guan**, I really enjoy the chatting and appreciate all the suggestions.

I really appreciate **Dr. Tiejun Shi** for introducing me to join Camilla's group and supervision for animal work, dissection, immunohistochemistry and in situ hybridization.

I want to thank **Prof. Manying Xu,** my master supervisor, for guiding me into pain research.

I also want to thank **Prof. Yu Li**, for supervising me in her Cancer biology lab and teaching me knowledge and technics in molecular cancer field.

I ought especially thank D**r. Jilai Liu**, for mentoring me the molecular and cellular methods and suggesting and advising for all the things.

I would like to thank all my half-time review examiners, **Gunnar Schulte, Igor Adameyko**, and **Olivier Peyruchaud** for the excellent suggestions helping my research during the last years.

I am grateful to all my collaborators that contributed to the work presented in this thesis.

Family,

My parents, thanks for all your support and giving encouragement, always let me do whatever I want!

Mingdong Zhang, my dearest husband, soul mate and best friend. Thanks for your support, help, understand and “suffer” me these years, especially the last period of PhD. Thank you all the things.

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