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ANTIBODY ACTIVATION OF SENSORY NEURONS

EXPLORING NOVEL PAIN MECHANISMS IN RHEUMATOID ARTHRITIS

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Cover: Mouse sensory neurons in culture stained for β-tubulin-III (green) and FcγRIIb (red). Photo credit to Matthew Hunt.

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ANTIBODY ACTIVATION OF SENSORY NEURONS
exploring novel pain mechanisms in rheumatoid arthritis

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To my Mamma:

my strength, my guide, my LIFE

ABSTRACT

Chronic pain is a worldwide major problem that presents several challenges due to lack of treatment efficacy and/or side effects associated with long-term usage of analgesics. Autoimmune diseases such as rheumatoid arthritis (RA) are often characterized by pain components, which generally poorly respond to drug treatment. In fact, RA patients suffer from persistent pain even if the active disease and inflammation is under medical control or in remission. Moreover, pain appears years before the onset of the active disease. This indicates that RA pain components might underlie additional unknown mechanisms rather than only the classical view of pain strictly correlating with inflammation. Of note, recent studies show that RA autoantibodies are present in RA patients up to 10 years before the onset of inflammation and most of the available treatment options in the clinics do not affect antibody titers. Therefore, the aim of this thesis is to investigate possible autoantibody actions that could represent the missing link explaining pain in RA in the pre- and post-inflammatory phases of the disease.

In **study I**, we explored the role of RA-relevant autoantibodies in directly activating sensory neurons. Injection of anti-collagen type II (CII) antibodies (Abs) promoted pain-like behavior in mice in the absence of any visual, histological or molecular inflammation. This pain-like behavior was not dependent on complement activation or destabilization of cartilage structure. Instead, our data suggested a direct activation of CII-immune complexes (ICs) on sensory neurons via the activation of Fc gamma receptors (FcγRs). Indeed, we found expression of FcγRI and FcγRIIb proteins on peripheral neuronal terminals in mouse skin. In addition, CII-IC *in vitro* stimulation of cultured dorsal root ganglia (DRGs) neuronal cells promoted release of a calcitonin gene related peptide (CGRP), intracellular increase of calcium levels and membrane depolarization. Interestingly, CGRP release was prevented in cultures from FcRγ chain deficient mice (lacking activating FcγRI, III and IV, but still expressing inhibitory FcγRIIb). Accordingly, injection of anti-CII Abs failed to induce pain-like behavior in FcRγ chain deficient mice or when the Ab-FcγR interaction was altered. Instead, mice expressing activating FcγRs only on non-hematopoietic cells (including neurons), but not on hematopoietic cells, displayed similar pain thresholds to wild type mice when injected with anti-CII Abs. Altogether our data suggested a novel RA-associated pain mechanism of direct interaction between Abs and FcγRI present on sensory neurons that is independent of inflammatory functions of pathological Abs. Finally, we showed that human DRG neurons also express the activating FcγRIIIA making our data translational to clinics, possibly explaining pain in RA patients before the onset of the disease or even when it is under medical control or in remission.

In **study II**, we investigated pain-associated pathological actions of human anti-citrullinated proteins antibodies (ACPA) purified from RA-patients. Injection of human ACPA, but not non-ACPA or IgGs from healthy individuals, promoted pain-like behavior in mice in the absence of visual, histological and molecular inflammation. Furthermore, ACPA did not induce significant increase of intracellular calcium levels or membrane depolarization in cultured DRG neurons, suggesting that ACPA do not exert their nociceptive functions through a direct action of their Fab region on sensory neurons. However, ACPA bound to osteoclasts, inducing the release of the mouse interleukin-8 analogue CXCL1, which subsequentially sensitized neurons. In fact, a CXCL1 receptor antagonist or an osteoclasts inhibitor prevented ACPA-induced pain-like behavior. In conclusion, we provided evidence of novel nociceptive actions of human ACPA, offering new targets in IL-8 and osteoclasts for the pain treatment of the ACPA-positive subgroup of RA patients.

In **study III**, we characterized B35, Neuro-2a (N2a) and F11 neuroblastoma cell lines, trying to find an alternative method to primary DRG cultures from rodents for pain-related *in vitro* experiments. We compared the cell lines subjected to two differentiation media to promote the acquisition of more neuronal-like features on parameters such as morphology, proliferation, metabolic activity, expression of neuronal markers and functional activity. While B35 showed the highest neuronal-like morphological features, N2a the highest neuronal markers expression and F11 the highest neuronal excitability in functional assays, all the cell lines compared to primary DRG cultures only to some extent. Therefore, our findings indicated that neuroblastoma cell lines should be carefully selected by researchers for studying neuronal processes, as they do not represent a complete substitute of primary DRG cultures.

In summary, this thesis addresses the crucial need of better understanding the underlying pain mechanisms in RA and provides novel insights that could potentially benefit the clinical therapeutic strategies, opening new avenues for the development of innovative pain-relief drugs.

LIST OF SCIENTIFIC PAPERS AND MANUSCRIPTS

I. **Bersellini Farinotti A***, Wigerblad G*, Nascimento D*, Bas DB, Morado Urbina C, Nandakumar KS, Sandor K, Xu B, Abdelmoaty S, Hunt MA, Ängeby Möller K, Baharpoor A, Sinclair J, Jardemark K, Lanner JT, Khmaladze I, Borm LE, Zhang L, Wermeling F, Cragg MS, Lengqvist J, Chabot-Doré AJ, Diatchenko L, Belfer I, Collin M, Kultima K, Heyman B, Jimenez-Andrade JM, Codeluppi S, Holmdahl R**, Svensson CI**.
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II. Wigerblad G, Bas DB, Fernandes-Cerqueira C, Krishnamurthy A, Nandakumar KS, Rogoz K, Kato J, Sandor K, Su J, Jimenez-Andrade JM, Finn A, **Bersellini Farinotti A**, Amara K, Lundberg K, Holmdahl R, Jakobsson PJ, Malmström V, Catrina AI, Klareskog L, Svensson CI.
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Cardiomyopathy, oxidative stress and impaired contractility in a rheumatoid arthritis mouse model.
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Autoantibodies hurt: Transfer of patient-derived CASPR2 antibodies induces neuropathic pain in mice.
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LIST OF SELECTED ABBREVIATIONS

Ab	Antibody
ACPA	Anti-citrullinated protein antibodies
AIA	Antigen-induced arthritis
ASIC	Acid sensing ion channel
BDNF	Brain-derived neurotrophic factor
BM	Bone marrow
CAIA	Collagen antibody-induced arthritis
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CIA	Collagen-induced arthritis
CLAMS	Comprehensive laboratory animal monitoring system
COMP	Cartilage oligomeric matrix protein
CII	Collagen type II
DMARD	Disease modifying anti-rheumatic drug
DRG	Dorsal root ganglion
EndoS	Endo-β-N-acetylglucosaminidase
FBS	Fetal bovine serum
FcR	Fc receptor
FcγR	Fc gamma receptor
FT	Flow through
GPCR	G protein coupled receptor
GPI	Glucose-phosphate isomerase
HMGB1	High mobility group box 1
i.a.	Intra-articularly
IASP	International Association for the Study of Pain
IB4	Isolectin B4
IC	Immune complex
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IL	Interleukin

i.p.	Intraperitoneally
ITAM	Immune-receptor tyrosine-based activation motif
ITIM	Immune-receptor tyrosine-based inhibitory motif
i.v.	Intravenously
JAK	Janus kinase
KO	Knock-out
LPS	Lipopolysaccharide
NGF	Nerve growth factor
NPY	Neuropeptide Y
NSAID	Nonsteroidal anti-inflammatory drug
N2a	Neuro-2a
OVA	Ovalbumin
PAD	Peptidyl arginine deiminases
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PG	Prostaglandin
PKA	Protein kinase A
PKC	Protein kinase C
PTM	Post-translational modification
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RTK	Receptor tyrosine kinase
s.c.	subcutaneously
SP	Substance P
TNF	Tumor necrosis factor
TrkA	Tropomyosin kinase receptor A
TRPV1	Transient receptor potential vanilloid 1
WT	Wild type

1 INTRODUCTION

Nearly one out of five individuals in Europe and USA suffer from chronic pain. It is often difficult to provide adequate pain relief, which increases the risk for physical and psychological problems that dramatically reduce the quality of life for these individuals. Furthermore, chronic pain generates large socio-economical costs in the form of medical care, sick leave and loss of productivity (Andersson et al., 2013; Apkarian et al., 2009). The most common disorders associated with chronic pain are musculoskeletal joint conditions such as rheumatoid arthritis (RA) (Breivik et al., 2006; Vos et al., 2012). Although the understanding of the pathophysiology of RA has greatly improved in the last decades and many drugs that dampen the disease activity have been developed (Firestein, 2003; McInnes and Schett, 2011), pain is still a major problem for 30-40% of the patients even when the disease is under medical control or in remission (Altawil et al., 2016; Lee et al., 2011). This makes pain management in RA still an important challenge and therefore it is critical to advance our understanding for the underlying molecular mechanisms that promote and regulate pain signal transmission in these types of conditions in order to open new avenues for drug development and treatment strategies that could effectively improve the life quality of millions of patients (Davila and Ranganathan, 2011; Walsh and McWilliams, 2014).

1.1 Neurobiology of pain

Pain per definition of the International Association for the Study of Pain (IASP) is “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage”. As a physiological role, pain valuably conveys information about threats and sources of injury, detecting peripheral noxious stimuli and elaborating those signals in the central nervous system. Pain is commonly classified into acute and chronic. When the noxious stimulus is avoided or removed, *e.g*. quick removal of the hand from a hot stove, the system reverts back to homeostasis and the acute pain process is ended. However, unceasing or recurring nociceptive stimulation results into a number of complex pathophysiological modifications of pain processing at all levels (both peripherally and centrally) and eventually leads to the development of a chronic pain condition. Thus, persistent pain differs from acute pain in that it goes beyond its original useful function as a protective and warning system and instead converts to an established and debilitating disease (Dubin and Patapoutian, 2010; Talbot et al., 2016).

1.1.1 Nociceptors

Nociceptors are specialized peripheral sensory neurons, which are responsible of detecting noxious stimuli and transmitting the signal to the central nervous system. Their cell bodies are located in the dorsal root ganglia (DRGs) from which two axonal branches depart, one towards the periphery and the other towards the dorsal horn of the spinal cord. After the transduction of the noxious stimuli in the periphery an electric signal is transported towards the cell body of the neuron in the DRG and from there it reaches first the spinal cord and subsequentially the brain where it is processed and evokes a behavioral and emotional reaction (Figure 1). Nociceptors are heterogeneous and present a battery of sensory receptors and ion channels that provide the ability of detecting different types of noxious stimuli, *e.g.* heat, cold, mechanical pressure and damage associated molecules (Dubin and Patapoutian, 2010).

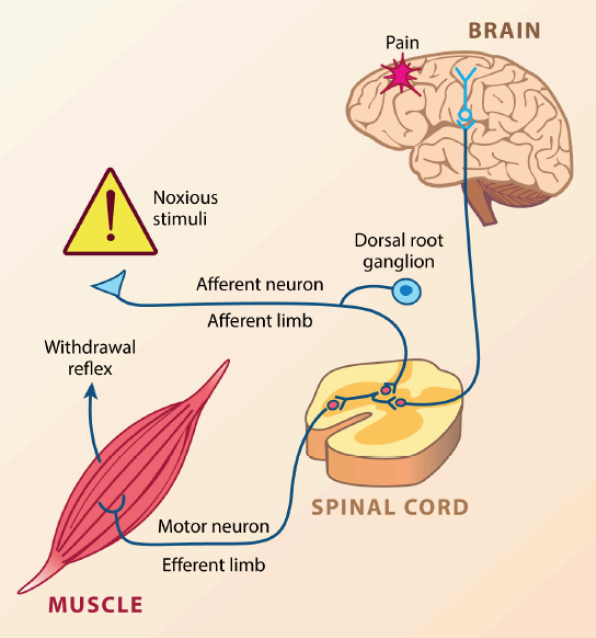


Figure 1. Nociceptive pathway, pain perception and behavioral reaction. The noxious stimuli are transduced in the periphery by sensory neurons and an electric signal is transported towards the DRG cell bodies and subsequentially to the spinal cord and the brain where the pain sensation is processed and perceived. A behavioral reaction to avoid the noxious stimuli is promoted by the brain and executed through withdrawal inputs sent to motor neurons and muscles. Reprinted with permission and adapted from (Talbot et al., 2016).

1.1.1.1 Nociceptors classification

In general, nociceptors can be classified based on their diameter and degree of myelination, which determine also their speed of signal conduction. Small diameter (0.2-1.5 µm) and unmyelinated axons are characteristics of C-fibers, which are most of the nociceptors. Their conduction velocity varies between 0.4 and 1.4 m/s and

they usually mediate poorly localized and slow pain. C-fibers are usually polymodal, meaning that they respond to more than one environmental modalities (thermal, mechanical or chemical). Importantly, one specific class of C-fibers in the joint is called “silent nociceptors”. They normally do not respond to mechanical stimuli but can become sensitized in disease or inflammatory states, firing and therefore contributing to the chronification of joint pain. Aδ fibers have medium diameter (1-5 µm) and myelinated fibers and are responsible of mediating fast and localized pain, since their conduction velocity is approximately 5-30 m/s (Djouhri and Lawson, 2004; Dubin and Patapoutian, 2010). These can be further subdivided into two different types. Type I Aδ fibers respond to mechanical, chemical and high heat threshold (around 50 °C), while type II Aδ fibers have lower heat threshold but much higher mechanical threshold. Finally, Aβ fibers are large (6-12 µm) and myelinated and are associated with fast (33-75 m/s) low-threshold mechanoreceptors.

Nociceptors can also be classified based on their neurotransmitter expression profile. All nociceptors release glutamate as principal neurotransmitter, but a specific subclass, the peptidergic neurons, also release substance P (SP) and calcitonin-gene related peptide (CGRP) and express the receptor for nerve growth factor (NGF), known as tropomyosin kinase receptor A (TrkA). The central projections of these nociceptors mostly terminate in the superficial layers of the dorsal horn of the spinal cord. In contrast, the “non-peptidergic” neurons express purinergic receptors of the P2X type, stain positive for the isolectin B4 (IB4) and their central projections terminate in the deeper layers of lamina II of the dorsal horn of the spinal cord (Basbaum et al., 2009).

The heterogeneity of nociceptors is vital for their role in responding to different noxious stimuli and, as new techniques are developed, the categorization of the nociceptors is becoming more refined. For example, based on single cell sequencing, the RNA expression profile supported classification of nociceptors into 11 distinct categories (Figure 2) (Usoskin et al., 2015).

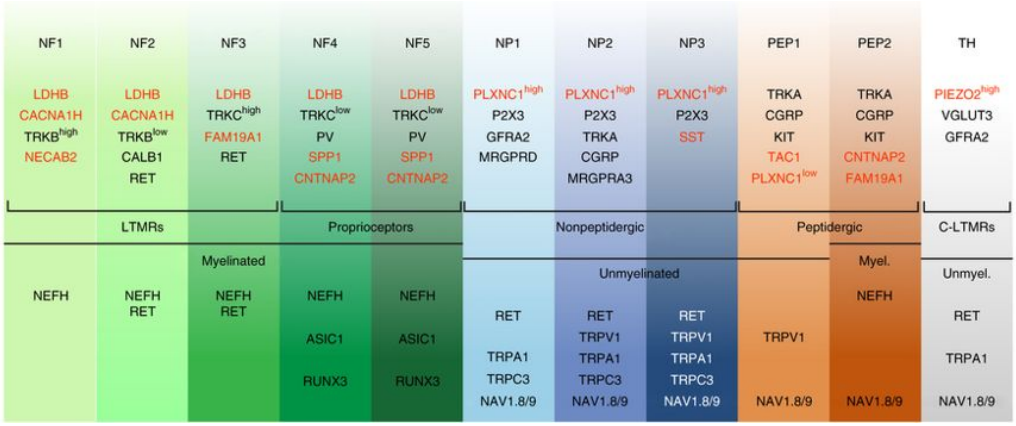


Figure 2. Unbiased classification of mouse sensory neurons based on RNA transcriptome analyses. 11 categories in total are found with the suggested molecules as markers for identification of the different subtypes. Reprinted with permission and adapted from (Usoskin et al., 2015).

1.1.1.2 Stimuli detection and signal transduction

There are three major classes of surface proteins on nociceptors responsible for the detection of noxious stimuli as well as the initiation and propagation of electrical signals towards the central nervous system: ion channels, G protein-coupled receptors (GPCRs) and receptors for cytokines and neurotrophins (Figure 3).

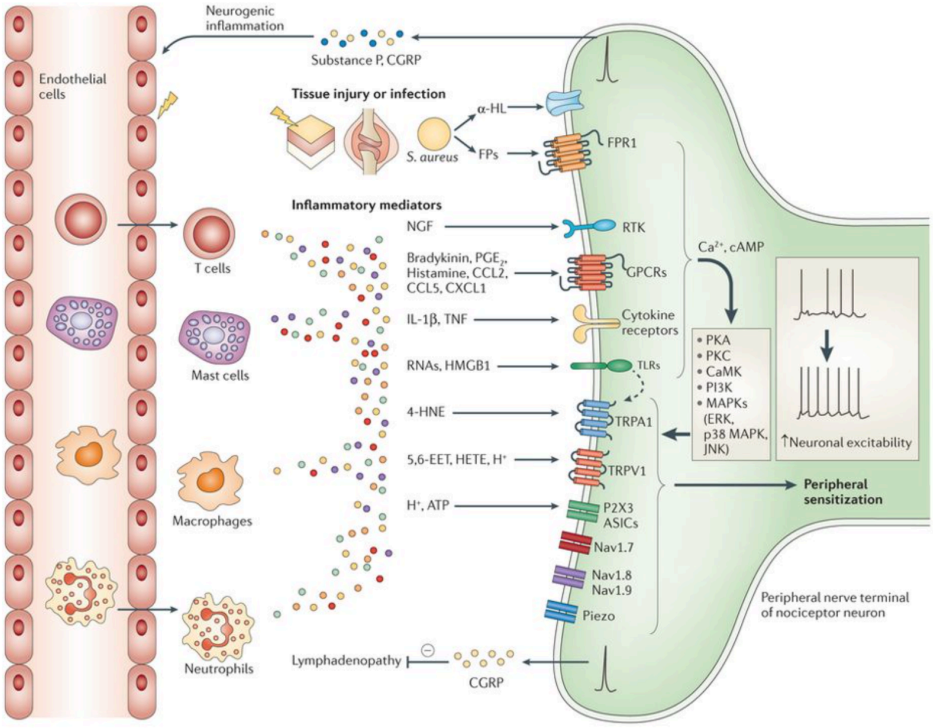


Figure 3. Nociceptor heterogeneity. Nociceptors express numerous distinct surface proteins to respond to several different noxious stimuli and interact with the immune system. Reprinted with permission and adapted from (Ji et al., 2014).

Ion channels involved in the sensory signaling are of two major different types: ligand gated and voltage gated ion channels. The first ones respond directly to the noxious input and change rapidly the membrane electric state to start the action potential that brings the information centrally. These types of channels are very common and different ones respond to different stimuli. Transient receptor potential (TRP) channels belong to the most well-known family of ligand gated ion channels. Transient receptor potential vanilloid 1 (TRPV1) responds to noxious heat, low pH, chemicals like capsaicin, the endogenous ligand N-arachidonoyl dopamine (NADA), and certain lipids, while TRPM8 detect innocuous and noxious cold and cooling chemicals like menthol (Julius, 2013). Another important class of ligand gated ion channels is the acid sensing channels (ASICs) that respond to low pH, which is characteristic of a number of noxious states like inflammation (Wemmie et al., 2013). Mechanical sensation can be detected by some TRP channels, some ASICs and another class of ion channels called Piezo. It is currently thought that pressure and

mechanical stimulus open these specific channels causing the depolarization and the initiation of the signal, but the mechanism is not yet completely understood (Basbaum et al., 2009; Ranade et al., 2015). Another group of ligand gated ion channels is the purinergic receptors, *e.g*. P2X2 and P2X3, which respond to the binding of ATP, which can be released during inflammatory processes and by damaged cells (Linley et al., 2010). In contrast, voltage gated channels do not directly transduce noxious stimuli, but have instead a modulatory effect in sensory neurons. Sodium channels, such as $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$, are important for action potential generation and levels of excitability of sensory neurons (Renganathan et al., 2001). Calcium channels with their critical subunit α2δ1 are also important voltage gated ion channels, since calcium is a crucial second messenger in many activating intracellular pathways in neurons (Grienberger and Konnerth, 2012). Finally, potassium channels usually dampen neuronal responses and excitability by regulating membrane potential, threshold of the action potential, as well as shape and frequency of the firing (Tsantoulas and McMahon, 2014).

G protein-coupled receptors are important for pain signaling since once activated they promote intracellular pathways related to adenylate cyclase with the activation of downstream kinases such as protein kinases A or C (PKA or PKC) that eventually affect neuronal properties and excitability. Ligands for these receptors are for example CGRP, bradykinin, proteases and prostaglandins. Noteworthy, a special class of GPCRs drives instead inhibitory mechanisms. Opioid receptors are members of this subgroup and their activation promotes a decrease in the level of cAMP in the cell that eventually reduces neuronal excitability (Schaible et al., 2002).

Peptidergic neurons express the receptor TrkA, which belongs to the receptor tyrosine kinase (RTK) family and binds the *neurotrophin* NGF. NGF is required for neuronal development, survival, but can be released after injury and promote neuronal sensitization. NGF can directly activate TrkA but also the low affinity receptor p75 to promote a rapid increase in TRPV1 activity. Additionally, NGF can affect neurons long-termly being internalized and inducing transcription of factors involved in the potentiation of nociception such as activating ion channels (Basbaum et al., 2009; Lewin et al., 2014). Neurons express also TrkB and TrkC, which are receptors for other important neurotrophins involved in pain processes, respectively brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (Dhandapani et al., 2018; Siniscalco et al., 2011; Tender et al., 2011).

The cross talk between the nervous and the immune systems is essential for the regulation of many of their processes. Therefore, sensory neurons express *receptors for different cytokines and chemokines* that allow them to rapidly detect ongoing inflammatory reactions through pain sensation. Receptors for the cytokines tumor necrosis factor alfa (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6) and interleukin-8 (IL-8) are among the most important ones expressed by sensory neurons. These

cytokines can either directly activate sensory neurons or promote intracellular pathways that would long-termly sensitize neurons (Schaible et al., 2010).

1.1.2 Chronic pain

When peripheral and central sensitizations occur, acute pain shifts towards a chronic pain state. Peripheral sensitization is a consequence of persistent activation of nociceptors for instance during inflammation or tissue damage. Nociceptive factors released at the site of injury over-stimulate the respective receptors on sensory neurons inducing pathological changes so that the threshold required to fire after a certain stimulus is permanently lowered, the receptive field of the sensory neuron is increased and/or non-nociceptive fibers start firing producing and amplifying pain signals. In this context, hyperalgesia, described as increased pain intensity in response to a normal painful input, and allodynia, pain due to a stimulus that normally does not induce pain, are important terms describing features of chronic pain (Grigg et al., 1986; McDougall, 2006; Schaible and Schmidt, 1985).

Central sensitization involves changes in the central nervous system and, in particular, in the dorsal horn of the spinal cord. In addition to neuronal changes taking place that facilitate and prolong the pain signal transmission, activation of glial cells is thought to contribute to the increased neuronal excitability (Tsuda et al., 2003; Woolf, 1983; Woolf and Salter, 2000). Many of the nociceptive molecules produced during inflammation are also released by microglia and astrocytes in the spinal cord and, after the neuronal activity started in the periphery activates them, they can contribute to amplify, perpetuate and facilitate pain signaling. Recent studies have also shown that factors released in the dorsal horn can promote inhibitory neurotransmitters to switch their function to become activating, contributing to the transit to chronic pain (Coull et al., 2005).

1.1.3 Model systems in preclinical pain research

Animal models and genetically modified mice have served the ground for generation of important new insights to the pathophysiological and pharmacological aspects of nociception. The use of genetically modified animals has been an important tool for researchers to decipher the contribution of different genes to specific nociceptive processes. Furthermore, experimental animal models representing different diseases or tissue injuries are crucial in pain research based on the notion that each underlying pathology leads to distinct changes in the nociceptive pathways. Several different animal models are available for investigating RA pathology and pain state and will be discussed further below (Luo, 2004).

In parallel, *in vitro* approaches can be used as well-confined systems to complement information obtained from *in vivo* models, especially for investigating and understanding molecular mechanisms of neuronal excitability and neurotransmission (Luo, 2004). In fact, primary cultured DRG cells from rodents are widely used in the pain research field. While there are many advantages with primary DRG cultures, they display limitations in terms of number of cells generated, costs and poor transfection efficiency (Posadas, 2010). Much effort has been put towards developing alternatives to DRG primary cultures. Recent studies suggest that mature sensory neuron-like cells can be obtained by inducing differentiation of neural progenitor cells and pluripotent stem cells (PSCs) (Chambers et al., 2012; Kim et al., 2017; Young et al., 2014) or by specifically reprogramming fibroblasts (Lee et al., 2015; Wainger et al., 2015). However, these approaches still present many limitations regarding cost, complicated and time-consuming generation processes and low efficiency. Considering the advantages of providing infinite number of cells and significantly high transfection efficiency, cell lines from neuroblastoma could be contemplated as substitute for *in vitro* studies in DRGs (Posadas, 2010). B35, Neuro-2a (N2a) and F11 cell lines are examples of commonly used neuroblastoma cell lines in the neuroscience field. B35 cells derive from a rat neuroblastoma and have been used in analyses of signaling pathways in cell motility and axonal outgrowth (Otey et al., 2003). N2a cells originate from a mouse brain tumor and were employed to investigate neuronal differentiation, neurite growth and neurotoxicity (Olmsted et al., 1970; Suzuki et al., 2014). F11 cells display properties of both rat and mouse cells since they are a fusion of embryonic (E13) rat DRG cells with a mouse neuroblastoma cell line (N18TG2) (Platika et al., 1985), and have been previously applied for neuro-inflammation and differentiation studies (McIlvain et al., 2006; Wang et al., 2014). However, despite the fact that these neuroblastoma cell lines have been used for nociception-related studies, *e.g.* ion channel kinetics and regulation of intracellular signaling pathways, little information about their differences and similarities to primary DRG sensory neurons is available in the literature. Therefore one part of the work in this thesis was aimed at comparing these three different cell lines to each other and to mouse primary DRG neuronal cell cultures **(Study III)**.

1.2 Rheumatoid arthritis

1.2.1 Epidemiology

Rheumatoid arthritis (RA) is an autoimmune disease that primarily targets joints causing symptoms such as pain, swelling and stiffness. RA affects 0.5-1% of the population in developed countries. There is a higher prevalence of the disease in women compared to men (ratio 3:1) and its incidence increases with age (Symmons

et al., 2002). RA frequency also changes geographically, with northern Europe and North America being the most affected areas compared to developing countries (Biver et al., 2009; Costenbader et al., 2008; Kalla and Tikly, 2003). This indicates specific differences in genetic and environmental factors that participate to its etiology.

Although research on the specific causes of the disease is still ongoing, many risk factors have already been identified. A very high percentage of the risk of developing RA (50%) is ascribable to genetic factors with 30 different regions of single nucleotide polymorphisms already classified. Among these, the most studied ones are *PTPN22* and *HLA* genes and others that often are associated with the immune system function, which could explain in some cases how the disease is initiated (Barton and Worthington, 2009; Orozco et al., 2010; Stahl et al., 2010; van der Woude et al., 2009). Besides genetics, several environmental factors have been connected to higher chances of developing RA. The clearest one is by far cigarette smoking, which doubles the risk for the disease and has been associated especially to a specific subgroup of patients (ACPA-positive, see below) (Källberg et al., 2007; Morgan et al., 2009). Moreover, exposure to silica dust or certain mineral oils is also listed as environmental causes increasing the risk for RA (Stolt et al., 2005; Sverdrup et al., 2005). Other evidences show factors like periodontitis, specific strains of gut microbiota and obesity to be involved in RA etiology (Mercado et al., 2000; Scher et al., 2013). Even though with weaker supporting indications, more potential risk factors involve alcohol and coffee intake, vitamin D levels, low socio-economical status and use of oral contraceptive (Liao et al., 2009).

1.2.2 Pathophysiology

RA is a chronic autoimmune disease characterized by the presence of several different autoantibodies that, recognizing autoantigens, drive persistent synovitis and systemic inflammation against self-structures. The continuous erosive synovial inflammation eventually leads to damage of articular cartilage, underlying bone and soft tissue that ultimately promotes joint destruction and loss of function with associated long-term disability (Lillegraven et al., 2012; van Oosterhout et al., 2008). RA mainly affects small joints of hands and feet, but with disease progression larger joints are interested too, mainly wrists, hips, knees and ankles (Firestein, 2003; McInnes and Schett, 2011).

Several cell types of both the innate and adaptive immune system are involved in RA pathophysiology, including T and B lymphocytes, monocytes, dendritic cells macrophages and mast cells. These are all stimulated and attracted to the joint site by the presence of immune complexes (antibodies bound to their antigens, ICs), which also promote extensive complement activation. As a consequence of the

immune cells trigger, a complex cocktail of cytokines and chemokines is secreted in the inflamed joint, which contributes to further exacerbate the painful inflammatory process. The primary inflammatory cytokines involved in RA are TNF, IL-1, IL-6 and IL-17 (Choy et al., 2002; Feldmann et al., 1996), but other cytokines are also contributing. With disease progression, activation of synovial fibroblasts, osteoclastogenesis and chondrocyte catabolism induces matrix degradation, bone erosion and cartilage destruction respectively, which ultimately results in articular impairment (McInnes and Schett, 2011).

In addition to joint pathology, circulating inflammatory cytokines and ICs have systemic effects, which increase the patient's risk of mortality. The main extra-articular comorbidities are cardiovascular diseases (*e.g.* myocardial infarctions, strokes and hypertension), reduced cognitive function, interstitial lung disease (ILD) and cancers (*e.g.* lymphoma, lung cancer and melanotic and non-melanotic skin cancers) (Chakravarty et al., 2005; Dougados et al., 2014; Kaiser, 2008; Levy et al., 2008; McInnes and Schett, 2011).

RA presents several different modalities when it comes to the active disease course. In most cases, it is a polycyclic disease characterized by two phases that follow each other during time: an active phase with visible inflammation in numerous joints and a dormant phase with no signs of inflammation but still some other symptoms among which pain and stiffness. Less frequently, RA can be monocyclic, with just one flare of inflammation and active phase that, after it has resolved, never awakes from the dormant phase. Finally, in some instances, it has a progressive course with the active phase that keeps on increasing in severity over time without going in the dormant phase (Graudal et al., 1998; Masi et al., 1976; Pincus and Callahan, 1993).

1.2.3 Pre-RA

In recent years more attention has been paid to a preclinical period, defined as "Pre-RA", which consists of all the events that precede the clinical occurrence of established RA with the full spectrum of symptoms and the actual diagnosis. Pre-RA is characterized by symptoms such as joint pain (arthralgia), stiffness, bone erosion and some abnormalities of the immune system without detectable inflammation (Deane and El-Gabalawy, 2014; Paul et al., 2017). Importantly, several types of autoantibodies are found in the serum of future RA patients even 10 years before the onset of the active disease (Kurki et al., 1992; Nielen et al., 2004; Rantapää-Dahlqvist et al., 2003). Thus autoimmunity represents a very early event in RA development and recent studies show that it is generated outside the joints. Little is known how the autoimmunity is then propagated to the joints where the destructive process starts and progresses. The "double-hit" theory proposes that genetic and environmental factors (*e.g.* trauma or infection) promote this transition to active RA.

Increased understanding of pre-RA and the transition to the active phase of the disease is needed in order to develop successful strategies to reduce the symptoms and ideally prevent the disease progression (Klareskog et al., 2014).

1.2.4 Autoantibodies in RA

Antibodies are proteins produced by the immune system to protect the body, neutralizing pathogenic intruders. Antibodies consist of a variable region (Fab) to recognize a particular epitope on an antigen and a constant region (Fc) that allows interaction with immune cells via Fc receptors (FcRs) expressed on their membranes. In mammals there are five isotypes of antibodies (IgA, IgD, IgE, IgG and IgM), which vary in their biological properties, function, location and ability to deal with different antigens. IgGs are the most common antibodies, accounting for around 75% of the whole antibodies pool. There are four different subclasses of mouse IgGs and each presents distinctive properties (*e.g*. affinities for the different FcRs): IgG1, IgG2a, IgG2b and IgG3. Antibodies exert their functions by binding a specific antigen with their variable region, directly promoting functional alterations of the antigen and forming immune complexes (ICs), which can further trigger the complement system and FcRs expressing cells.

Antibodies-producing cells are selected during their maturation not to produce antibodies that recognize self-structures of the individual's body so to prevent self-damage. In autoimmune diseases this regulation process, called immunological tolerance, is broken so that autoantibodies targeting self-antigens are produced and start causing tissue damage.

RA is characterized by the presence of many different types of autoantibodies (Table 1), which are also used as diagnostic criteria. The most important autoantibodies are targeting other IgGs (Rheumatoid Factor, RF), citrullinated proteins (Anti-citrullinated protein antibodies, ACPA), peptidyl arginine deiminases 3/4 (anti-PAD3/4 antibodies), carbamylated proteins (anti-CarP antibodies), glucose-6-phosphate isomerase (anti-GPI antibodies) and collagen type II (anti-CII antibodies) (Bugatti et al., 2014).

Table 1: Autoantibodies described in rheumatoid arthritis.

Rheumatoid factors
Anti-collagen type II
Anti-glucose-6-phosphate isomerase (GPI)
Anti-human cartilage glycoprotein 39
Anti-Ra33/heterogeneous nuclear ribonucleoprotein (hnRNP) A2
Anti-citrullinated fibrinogen
Anti-citrullinated vimentin
Anti-citrullinated alpha-enolase
Anti-immunoglobulin binding protein (BiP)
Anti-carbamylated proteins (anti-CarP)
Anti-peptidyl arginine deiminase (PAD)
Anti-histones
Anti-*Porphyromonas gingivalis*-derived enolase
Anti-***Porphyromonas gingivalis***-derived PAD

Table 1: Autoantibodies in RA. Reprinted with permission and adapted from (Bugatti et al., 2014).

RF mainly consists of IgM and IgA antibodies against the Fc region of IgGs, and was the first autoantibody characterized and introduced as diagnostic and prognosis tool in RA. RF was indeed shown to be present in up to 80% of RA patients and to correlate with a more severe disease phenotype. However, further studies proved that RF is not specific for RA, being present in several other autoimmune diseases, systemic infections and even in 15% of healthy individuals (Bukhari et al., 2002; Franklin et al., 1957; Nienhuis et al., 1964).

ACPA were discovered at the end of the 1990s and are present in around 60-70% of the RA patients. ACPA, which target citrullinated self-antigens, are used as diagnostic tool since they are highly specific for RA. Citrullination is a post-translational modification (PTM) that consists of the conversion of the amino acid arginine to citrulline (Figure 4). This is catalyzed by PADs in the presence of high calcium and, due to the variation of the charge of the amino acid (from positive to neutral), structural and properties changes in the target protein are promoted to regulate for instance its activation/deactivation. Although the physiological role of citrullination is not completely clear, it seems to make the target proteins more immunogenic and therefore more prone to break self-tolerance. Target proteins for ACPA are vimentin, fibrinogen, histone proteins, collagen type II and alfa-enolase, but recent studies show that many ACPA have cross-reactivity for epitopes on different antigens and therefore are able to bind more than one protein. Recent findings illustrate that ACPA have a prominent involvement in RA pathogenesis and progression by being able to directly activate osteoclasts, inducing bone erosion. (Klareskog et al., 2013; Krishnamurthy et al., 2016; Schellekens et al., 1998; Schellekens et al., 2000; Sokolove and Pisetsky, 2016; van Gaalen et al., 2004; Vossenaar et al., 2003).

In recent years more attention has been paid to a class of RA autoantibodies recognizing PAD 3/4 enzymes, which catalyze citrullination. Interestingly, studies show that these autoantibodies not only bind PAD enzymes but also activate them, partially explaining their pathological role and contribution to the expansion of autoimmunity (Darrah et al., 2013).

Another PTM important in RA is carbamylation (Figure 4), which is the conversion of the amino acid lysine to homocitrulline. Similarly to citrullination the change in charges of the amino acids promotes variations in the target proteins' functions and properties. These autoantibodies were just recently discovered and the fact that citrulline and homocitrulline present very similar structures gave rise to speculation that ACPA antibodies could bind also homocitrullinated proteins, but studies have shown that most ACPA antibodies have not such cross-reactivity. Anti-CarP antibodies are present in 45% of RA patients and interestingly around 30% of the ACPA-negative patients show reactivity to carbamylated proteins, making these autoantibodies a powerful tool for the diagnosis of this specific subgroup of patients (Shi et al., 2011).

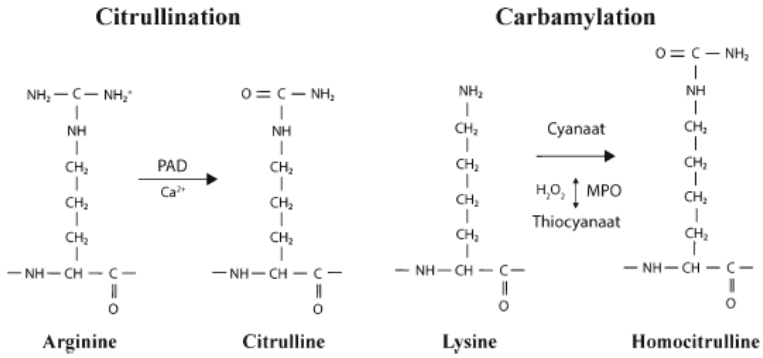


Figure 4: Important PMTs of amino acids in RA. Citrullination transforms arginine into citrulline, while carbamylation is the conversion of lysine to homocitrulline. Reprinted with permission and adapted from (Bax et al., 2014).

Anti-GPI antibodies are present in 15% of RA patients, but they appear also in other autoimmune disorders so their specificity for RA is limited. However, anti-GPI antibodies correlate with a higher disease severity, which makes them an interesting prognosis tool. GPI is an enzyme involved in the glycolytic process converting glucose-6-phosphate to fructose-6-phosphate, but it is also important for some extracellular processes, acting for example as a neurotrophic factor to promote neuronal survival. Therefore, GPI is also present extracellularly in the articular cavity, explaining the pathogenicity of its targeting autoantibodies (Matsumoto et al., 2003).

CII is a structural protein that is prominent in cartilage formation, accounting for around 50% of its all proteins. Anti-CII autoantibodies are present in almost 30% of

RA patients and their pathological role is quite clear since they are one of the main drivers of the inflammation in the joint where cartilage is largely present. For this reason, to study RA pathology in details, many animal models have been created based on immunization with CII, both in rodents and in primates (see below) (Cook et al., 1996; Holmdahl et al., 2014; Mullazehi et al., 2012; Nandakumar, 2009).

As already mentioned, these autoantibodies, especially ACPA, can be present in the pre-RA phase many years before the onset and diagnosis of the disease. Some symptoms exist in the absence of detectable inflammation and arthralgia is one of the most characteristic ones, suggesting that the nociceptive signaling starts already in this early phase. The active disease process initiates after a sequence of events that promote maturation of the autoimmunity with increase in titer and affinity for the target proteins, epitope spreading, isotype switching and changes in the glycosylation state of the autoantibodies. This stimulates their pathogenicity so that they start the active phase of the disease via forming ICs with self-antigens that activate FcRs on immune cells and give rise to the inflammatory cascades and processes (Rombouts et al., 2014; van de Stadt et al., 2011; Verpoort et al., 2006). In details, the deposition of ICs triggers inflammation via 2 specific pathways. The first one is the activation of the classical complement cascade where complement C1q binds the Fc portion of an antibody in IC formation and then activates complement components C5a and C3a. These two anaphylatoxins are able to attract and activate effector cells such as neutrophils and natural killer cells to release proteolytic enzymes and inflammatory cytokines. Alternatively, the Fc part of an antibody in IC formation can be bound by FcRs on the effector cells that are therefore directly activated to promote inflammation in the so called antibody-dependent-cell-mediated cytotoxicity (ADCC) or other cell specific processes like degranulation in neutrophils, phagocytosis in macrophages and bone resorption in osteoclasts. Moreover, antibodies in IC formation have a role in the facilitation of antigen uptake by antigen presenting cells (APCs). Monocytes, macrophages and dendritic cells can uptake antigens from ICs with the binding Fc–FcR so that it becomes ten to a hundred times easier to stimulate T-cells. Thus, autoantibodies have impact also in the break of tolerance of T-cells that is detected in autoimmune diseases such as RA. All of these activated cells at the inflammatory joint site produce several nociceptive factors that promote chronic inflammation and pain (Figure 5) (Amigorena and Bonnerot, 1999; Celis et al., 1984; Martin and Chan, 2004).

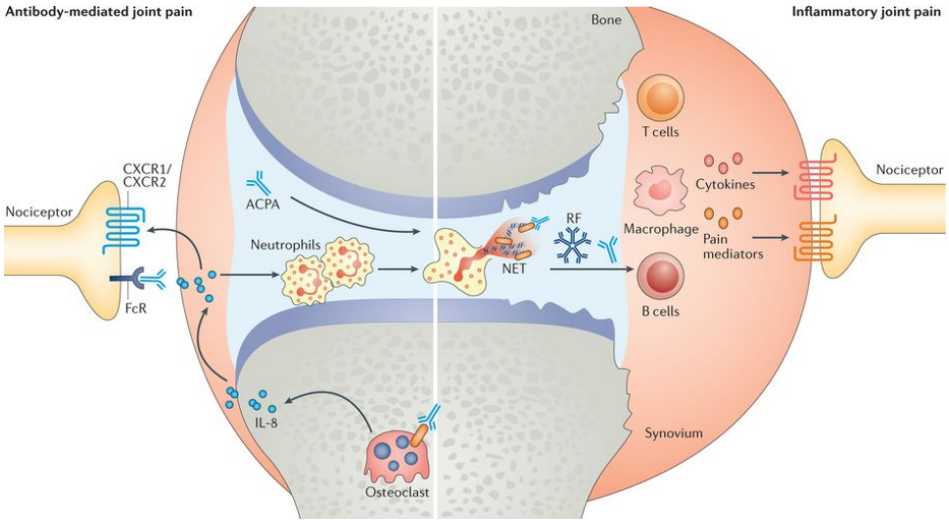


Figure 5. RA autoantibodies promote pain through direct and indirect mechanisms. Autoantibodies directly activate sensory neurons, binding to neuronally expressed FcγRs **(Study I)**, but also stimulate several different immune cells at the inflamed joint site, which contribute to pain releasing numerous nociceptive molecules. Reprinted with permission and adapted from (Catrina et al., 2017).

1.2.5 Fc-gamma receptors

Fc-gamma receptors (FcγRs) are membrane glycoproteins expressed mainly by immune cells, but also by some other cell types (*e.g.* endothelial cells and osteoclasts), that bind the constant region of IgG antibodies when forming an IC and are involved in the recruitment and activation of inflammatory cells.

There are 4 different FcγRs in rodents: FcγRI, FcγRIIb, FcγRIII and FcγRIV. FcγRs are quite conserved proteins in mammals and so the corresponding human orthologous are called FcγRIA, FcγRIIB (CD32B), FcγRIIA (CD32A), FcγRIIC, and FcγRIIIA (CD16) (Figure 6).

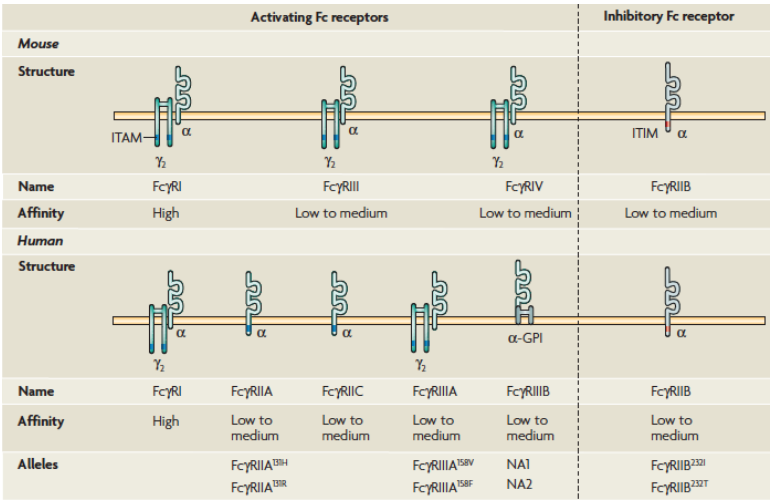


Figure 6: FcγRs in mice and humans. Reprinted with permission and adapted from (Nimmerjahn and Ravetch, 2008).

Structurally, all of the mouse FcγRs, except FcγRI, present two extracellular domains that form the ligand binding subunit, which is able to recognize the Fc part of an antibody. Instead, FcγRI has three extracellular domains, which confer it a higher affinity for IgGs. For this reason, FcγRI is the only receptor that displays also a significant binding to monomeric antibodies, while in general they only bind the Fc region of antibodies in IC formation (Daëron, 1997; Nimmerjahn and Ravetch, 2005; Nimmerjahn and Ravetch, 2006; Nimmerjahn and Ravetch, 2008).

Functionally, these receptors belong to the tyrosine kinases family, but three, FcγRI, FcγRIII and FcγRIV, are considered activating receptors and present an intracellular immune-receptor tyrosine-based activation motif (ITAM), while only FcγRIIb is inhibiting with an intracellular immune-receptor tyrosine-based inhibitory motif (ITIM). The intracellular signaling pathway activated by ITAM is a kinase cascade that through Syk, PI3K and PLCγ promotes intracellular calcium increase that triggers downstream pathways and eventually the effector functions already discussed. On the contrary, ITIM activation dampens these activation pathways through specific phosphatases like SHIP that restrict the generation of the important intermediates in the ITAM pathway such as PIP3 (Figure 7) (Dijstelbloem et al., 2001; Nimmerjahn and Ravetch, 2005; Nimmerjahn and Ravetch, 2006; Nimmerjahn and Ravetch, 2008).

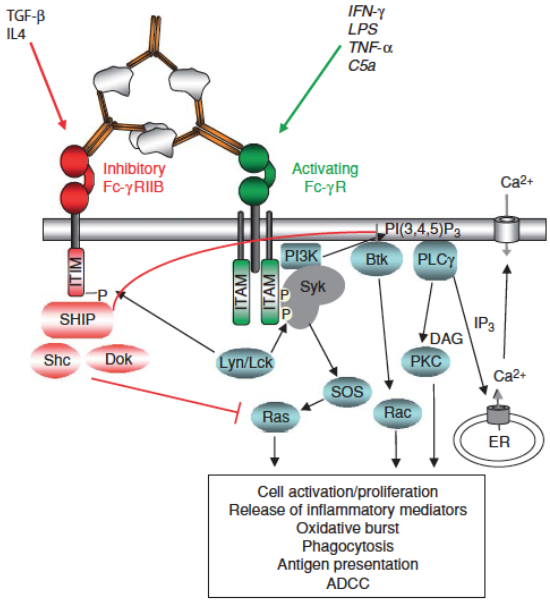


Figure 7: ITAM and ITIM intracellular pathways. After the bound of an IC, ITAM is triggered in mouse FcγRI, FcγRIII and FcγRIV, which are considered activating FcγRs, while ITIM is activated in FcγRIIb, which is the only inhibitory FcγR in mice. Reprinted with permission and adapted from (Nimmerjahn and Ravetch, 2007).

Importantly, ligands that display low affinity for the activating receptors (like monomeric antibodies or deglycosylated antibodies even if in IC formation) are not

able to promote the downstream events and act instead as receptor antagonists (Anthony et al., 2012; Nandakumar et al., 2013).

While FcγRs are predominantly expressed by immune cells, several studies now show their expression also on neurons. FcγRIIb is expressed on cerebellar and hippocampal neurons in the brain and has an important pathological role in Alzheimer's disease. In fact, it is able to bind amyloid fibers and promote neuronal apoptosis through SHIP2-GSK3β signaling pathway (Kam et al., 2016; Kam et al., 2013; Nakamura et al., 2007). Moreover, motor neurons display FcγRs in their terminals and increase their intracellular calcium after binding to ICs (Mohamed et al., 2002). Finally, in rat sensory neurons FcγRI is responsible for neuronal activation (increased calcium and release of substance P) both *in vitro* and *in vivo* after stimulation with ICs through the activation of the Syk–PLC–IP3–TRPC3 intracellular pathway (Andoh and Kuraishi, 2003; Jiang et al., 2017; Qu et al., 2012; Qu et al., 2011).

1.2.6 RA treatment

Disease-modifying antirheumatic drugs (DMARDs) are a heterogeneous group of drugs with the common denominator of reducing joint swelling, inflammation, pain and limit the progressive tissue damage in RA. The immunosuppressant methotrexate is the first line of treatment in Sweden. While its inhibitory effect on dihydrofolate reductase is utilized for cancer treatment, its anti-inflammatory properties seen with low dose treatment in RA are not fully understood, but thought to include the inhibition of enzymes involved in purine metabolism. Methotrexate is used alone or in combination with other DMARDs and provides efficient disease control in about 50% of the patients. Other small molecules that proved efficacy in treatment of RA are Janus kinase (JAK) inhibitors, such as tofacitinib or baracitinib. These compounds act on the JAK/STAT signaling pathway preventing the release of cytokines, the increase of matrix proteinases and the apoptosis of chondrocytes, which characterize the RA inflamed joints (Malemud, 2018). Furthermore, in recent years, an increasing use of biologicals for treatment of RA patients that did not respond to conventional DMARDs has developed. Biologicals differ from the "small molecule" compounds in that they are manufactured or extracted by biological sources. The most frequently used biologicals for RA treatment are soluble decoy TNF receptors (etanercept), anti-TNF antibodies (*e.g*. golimumab), IL-1 inhibitors (anakinra) and anti-IL-6 receptor antibodies (tocilizimab). Other biologicals target specific proteins on immune cells to prevent activation of the cell or deplete them. Examples of these are abatacept (protein that prevents binding between CD28 on T cells and CD80/86 on antigen presenting cells) and rituximab (antibody against CD20 on B cells) (An et al., 2009; Maxwell and Singh, 2010; Mok, 2014).

Analgesics such as nonsteroidal anti-inflammatory drugs (NSAIDs), paracetamol and glucocorticoids are used to reduce pain, joint stiffness and in some cases also synovitis and tissue damage (Kirwan et al., 2007; Wienecke and Gøtzsche, 2004). Unfortunately, due to lack of efficacy of analgesics and their side effects associated with long-term use, a frequent problem in RA is adequate pain relief (Scott et al., 2007). In fact, as already mentioned, most of RA patients suffer from pain years before the onset of the active disease and 30-40% of the patients still perceive pain even if the disease is under medical control or in remission (Lee et al., 2011; Welsing et al., 2005). The difficulty in controlling pain in RA is at least partly due to the lack of understanding of the mechanisms that induce and maintain pain in the different phases of the disease, especially in the non-inflammatory states. Thus, research on this topic, as our **studies I and II**, is of great importance and hopefully deciphering these mechanisms will open new avenues for pain management in RA and other autoimmune diseases.

1.2.7 Animal models of RA

In the pain field, the most commonly used models for studies of arthritis-associated pain are based on subcutaneous intraplantar injections of complete Freund's adjuvant (CFA) or carrageenan. These models are associated with robust and transient inflammation in the joints and pain-like behavior that lasts for days up to 1-2 weeks. From an RA perspective, the short time-span of the pain-like phenotype is not optimal, and moreover, these models lack several immunological aspects of human RA, *e.g.* bone erosion and cartilage destruction. Therefore, we argue that this type of models, while being good models of soft tissue inflammation-associated pain, may not be the best representation of arthritis-associated pain (Ahlqvist et al., 2009; Hu et al., 2013).

The collagen-induced arthritis (CIA) model is one of the most frequently used models in studies of RA pathology. CIA is an immunization model that promotes production of autoantibodies against CII after the inoculation of CII in CFA at the base of the tails of rats or mice. CIA is considered a good polyarthritic model of RA due to specific characteristics that resemble human RA, such as targeted cartilage autoimmunity, break of immunological tolerance and T and B cells activity. However, the production of autoantibodies is constant due to the immunization and the course of the disease is progressive with higher severity over time until the endpoint is reached and the animals are sacrificed. This does not resemble the characteristic polycyclic RA course in humans with several flares of inflammation alternated to dormant phases and can make pain like-behavior testings difficult in advance stages of the model due to the sickness of the animals (Trentham et al., 1977).

Another immunization model of RA is the antigen-induced arthritis (AIA) model, which usually involves immunization against antigens such as ovalbumin or bovine serum albumin inoculated in CFA at the base of the tail of rats and mice. A second injection of the antigen in the joints of the animals is necessary to direct the autoimmunity effects there. AIA generates a transitory local RA-like pathology that lasts for weeks/months, but, as downsides, the inflammation is very localized and it involves the usage of exogenous antigens, which are not found in the human pathology. Moreover, the duration of pain-like behavior in these animals is shorter (2-3 weeks) compared to other models (Brackertz et al., 1977).

The K/BxN serum transfer arthritis model involves the use of a specific transgenic mouse strain (K/BxN) that spontaneously develops arthritis-like joint pathology (Kouskoff et al., 1996). Serum from these mice can be extracted and when transferred to wild-type mice it induces transient inflammation and arthritis-like pathology also in the joints of the recipient animals (Ji et al., 2001). Another common passive transfer model of RA in mice is the collagen antibody-induced arthritis (CAIA) model. CAIA is induced by intravenous or intraperitoneal injection of a cocktail of anti-CII antibodies followed by a small dose of lipopolysaccharide (LPS) injection few days later (3-7), in order to synchronize and boost the immune system. The mice develop robust but transient inflammation in the joints clearly detectable between approximately days 10-25 (Nandakumar and Holmdahl, 2007; Nandakumar et al., 2003; Terato et al., 1992). In both the K/BxN and CAIA models the inflammation resolves as the antibodies are cleared, thus representing models of transient, rather than chronic, joint inflammation (Ji et al., 2001; Nandakumar and Holmdahl, 2007). Interestingly, mice lacking activating FcγRs or the complement factor C5a do not develop any inflammation when injected with anti-CII antibodies and therefore are not susceptible to CAIA even if IgGs or C3 accumulate at the cartilage and joint sites. This illustrates the relevance of the innate immune system in initiating the inflammatory process in the effector phase of RA (Grant et al., 2002; Kagari et al., 2003; Watson et al., 1987). One downside of passive transfer models is that they do not involve the full spectrum of the immune activation of RA pathophysiology such as for instance break of tolerance of T and B cells and therefore can be considered more as tool to investigate the effector phase of arthritis (Christianson et al., 2012; Kouskoff et al., 1996). However, from a pain perspective they offer the opportunity to investigate how a resolving flare of antibody-driven joint inflammation affects the sensory nervous system. Importantly, while pain-like behavior as expected is present during the inflammatory phase of the model, mechanical hypersensitivity develops days before the visual inflammation starts and also persists for weeks after the inflammation has resolved (Agalave et al., 2014; Bas et al., 2012; Christianson et al., 2012; Christianson et al., 2011). This implies that the pain mechanisms in the model could mimic not only the inflammatory phase of the disease but also the clinical situation of “pre-RA pain” and “remaining pain” in

RA. In **Study I**, we focus on exploring novel pain mechanisms that could explain pain-like behavior in the pre-inflammatory (early) phase of the CAIA model.

1.2.7.1 What have we learned about nociception from RA models?

Experimental RA animal models have been an important tool in the advancement of our knowledge regarding several molecules involved in both peripheral and central nociceptive mechanisms.

The role of cytokines in nociceptive signal transmission has been extensively studied in experimental models of RA. TNF has received most of the attention as it is one of the main driver cytokine in RA pathology. TNF does not only promote the release of other neuronal sensitizers from surrounding immune cells, but can also directly activate sensory neurons, which express TNF receptor 1 and 2. TNF has been detected in serum and joints of CAIA, CIA and AIA models and injection of TNF blockers such as etanercept attenuates mechanical and thermal hypersensitivity in these models before significantly affecting inflammatory scores. This is coherent with some patients' reports where beneficial effects of TNF-blockers on pain scores are noted earlier than observations of anti-inflammatory results. (Bas et al., 2012; Boettger et al., 2008; Hess et al., 2011; Inglis et al., 2005; Schaible, 2014; Shubayev and Myers, 2001).

IL-1β is another cytokine involved in RA pathogenesis. While sensory neurons express IL-1β receptor, IL1-R1, and their excitability can be increased *in vitro* by stimulation with IL-1β, *in vivo* injection of IL-1β blockers such as anakinra was only successful in attenuating heat, but not mechanical, hypersensitivity in the AIA model, suggesting that IL-1β involvement in joint nociception is only partial and explaining the limited clinical effects of anakinra on disease activity in RA patients (Binshtok et al., 2008; Ebbinghaus et al., 2012; McInnes and Schett, 2011).

IL-6 cytokine is elevated in the joints and in the sera of RA patients. This is coherent with data coming from CAIA and CIA models where IL-6 has also been found increased in the animals' joints. IL-6 receptor is expressed both by neurons and satellite glial cells and *in vitro* studies show that IL-6 can directly stimulate sensory neurons to release CGRP. IL-6 blockers when injected intra-articularly in the AIA model are able to decrease mechanical hypersensitivity without affecting inflammatory scores. Similarly, in the clinic some patients report effects of tocilizimab (anti-IL-6 receptor antibody) on pain scores independently of the results on disease activity and inflammation (Gardiner et al., 2002; Hirano et al., 1988; Marinova-Mutafchieva et al., 1997; Obreja et al., 2005; Opree and Kress, 2000; Vazquez et al., 2012).

IL-17 is also a critical cytokine in RA pathogenesis. Both neurons and satellite glial cells express IL-17 receptor and *in vitro* stimulation has been shown to upregulate neuronal TRPV4, which is a receptor involved in mechanical sensitivity. In the AIA model injection of IL-17 blockers has been shown to reduce mechanical hypersensitivity without affecting inflammation scores (Pinto et al., 2010; Richter et al., 2012; Segond von Banchet et al., 2013).

Prostaglandins (PGs) such as PGE2 or PGD2 are important inflammatory mediators and nociceptive factors since sensory neurons express G-protein coupled receptors that recognize PGs as ligands. PGs have been found elevated in the joints of RA patients and this is consistent with the elevation of mRNA for COX2, a prostaglandin producing enzyme, in the joints of mice in the inflammatory phase of the CAIA model. Moreover, COX-inhibitors, such as ketorolac, celcoxib and diclofenac, have shown efficacy as anti-nociceptive compounds in the CAIA, CIA and K/BxN models during joint inflammation. However, these compounds have no effect on CAIA or K/BxN late phase hypersensitivity, suggesting that PGs have a prominent role during inflammation but not after it has resolved (Bas et al., 2012; Christianson et al., 2012; Fattahi and Mirshafiey, 2012; Inglis et al., 2007; Park et al., 2016).

The expression of neuropeptides such as SP, CGRP, neuropeptide Y (NPY) and galanin is also differentially altered in DRG sensory neurons during inflammation or nerve injury states. SP and CGRP are usually elevated in inflammatory models and decreased in nerve-injury (neuropathic) states, while NPY and galanin are increased in neuropathic models, but show no changes in inflammatory states. While in the AIA and CIA models, SP receptor and CGRP are respectively elevated in the DRGs, in the CAIA model, galanin is upregulated but all the other neuropeptides mentioned above show no difference compared to controls. This is in accordance with nerve injury markers, such as ATF3 and GAP-43, found increased in the DRGs of mice both in the inflammatory and post-inflammatory phases of the CAIA model. This suggests that anti-CII antibodies-driven inflammation promotes a unique pain state that resembles for some features the classical inflammatory models and for some others the classical neuropathic ones, suggesting that long-term inflammation might promote nerve damage in sensory neurons (Bileviciute et al., 1993; Calza et al., 1998; Hokfelt et al., 1987; Ji et al., 1994; Nieto et al., 2015; Su et al., 2015; von Banchet et al., 2000).

Finally for what concerns peripheral mechanisms, the contribution to nociception of certain ion channels has also been investigated in the already mentioned models. The α2δ1 subunit of calcium voltage gated channels is usually upregulated only in neuropathic pain models. However, α2δ1 is also increased in DRG neurons in the CAIA model and gabapentin and pregabalin that are thought to act on calcium channels through direct interaction with α2δ1 subunit reverse mechanical hypersensitivity both in the CAIA and K/BxN models, suggesting once more the

neuropathic-like phenotype of arthritis-induced pain (Bas et al., 2012; Christianson et al., 2012; Rahman and Dickenson, 2013; Su et al., 2015). The TRPV1 agonist, resiniferatoxin, which acts through desensitization of the ion channel, has proven to be effective in attenuating hypersensitivity in the K/BxN model (Borbély et al., 2015). Coherently, topical application of capsaicin (TRPV1 agonist) cream has shown promising pain relief effects in RA patients (Deal et al., 1991). Mice deficient for the ASIC channel ASIC3 showed decreased hypersensitivity in the CAIA model, but more severe inflammation. This might be explained by the presence of this channel both on sensory neurons and synovial fibroblasts, and its involvement in respectively nociception and inflammatory regulation (Ikeuchi et al., 2008; Sluka et al., 2013).

Central factors involved in central sensitization mechanisms have also been studied in RA-induced pain models. Over the past decade, glial cells in the central nervous system have moved away from the concept of just being support cells or providers of protection for neurons. In fact, both microglia and astrocytes have been found responsible for pain sensitization in several distinct human pathologies and pre-clinical models (Ji et al., 2013). Even if there is still no clinical evidence of the contribution to nociception of glial cells in RA patients, activation of both microglia and astrocytes has been demonstrated in the CIA, CAIA and K/BxN models. However, some differences are found concerning the sex of the animals used for the studies, since glial central mechanisms of arthritis induced pain seem to be specific for male mice. Indeed, microglia and astrocytes can be directly stimulated by factors released by neurons, such as ATP, SP, CGRP, PGs, change their morphology, becoming activated and releasing nociceptive factor in a positive feedback that eventually leads to sensitization of central sensory neurons and chronic pain. Accordingly, the use of intrathecal glia inhibitors, such as pentoxyfilline, was proven effective in attenuating CAIA induced hypersensitivity (Agalave et al., 2014; Bas et al., 2012; Loggia et al., 2015; Sorge et al., 2015).

Cytokines such as TNF, fractalkine or high mobility group box 1 (HMGB1) have also been proven to have roles in central nociception. For instance, TNF blockers show effects on reducing nociceptive brain activity in RA patients before signs of efficacy on peripheral inflammation. This was confirmed in animal models where repeated injections of intrathecal etanercept reduce AIA-induced firing of spinal neurons (Hess et al., 2011; Konig et al., 2014). Fractalkine mediates neuron-glia interaction in the dorsal horn of the spinal cord. Microglia activated by ATP can release cathepsin S, which is responsible of cleaving neuronal membrane-bound fractalkine, releasing its soluble fragment. This can then bind to fractalkine receptor, CX3CR1, on microglia, promoting the release through p38-MAPK signaling pathway of factors that activate enhance pain transmission. This mechanism was shown to be crucial for the CIA model where a cathepsin S inhibitor is able to reduce mechanical pain-like behavior

and microglia activation, even in the absence of efficacy on peripheral inflammation (Clark et al., 2012). Disulfide HMGB1 is able to activate TLR4 receptors expressed on neurons and glial cells, promoting increase of other nociceptive factors such as COX2, TNF and IL-1β. Spinal HMGB1 has been found elevated in the CAIA model both in the inflammatory and post-inflammatory phases. Its nociceptive function was then confirmed showing that intrathecal injection of HMGB1 blockers is able to attenuate CAIA-induced hypersensitivity (Agalave et al., 2014; Kim et al., 2006; Pedrazzi et al., 2007).

Finally, spinal CGRP neuropeptide was found increased in the CIA model, where an injection of a CGRP receptor antagonist reduced both mechanical hypersensitivity and microglia activation without affecting peripheral inflammation (Nieto et al., 2015).

In conclusions, the recent knowledge provided by animal models has been often supported by clinical evidence. Strikingly, specific DMARDs appear to show direct effect on sensory neurons before even affecting the immune system. The historical concept of specific immune cells' receptors has therefore been challenged by the discovery that most of these receptors are also expressed on sensory neurons. In this thesis, we contribute to this growing field by showing how autoantibodies can directly activate sensory neurons via neuronal FcγRI, uncoupled from the inflammatory process. This can expand our views on how immune and nervous systems interact even when it comes to pharmacological treatment, providing grounds for the development of novel pain killer drugs and new therapeutic strategies in RA and other autoimmune diseases.

2 AIMS

2.1 General aim

The proposed thesis has the overall aim to investigate the involvement of rheumatoid arthritis (RA) relevant autoantibodies in promoting novel pain mechanisms, challenging the classical view of autoantibodies contributing to nociception solely by inducing the inflammatory process.

2.2 Specific aims

- To explore the mechanisms responsible for pain-like behavior in the early phase of the collagen antibody-induced arthritis (CAIA) model

- To investigate potential direct effects on sensory neurons of anti-citrullinated proteins antibodies (ACPA) purified from RA patients.

- To characterize three neuroblastoma cell lines as an alternative *in vitro* model to mouse primary dorsal root ganglia (DRGs) for nociception-related studies.

3 METHODS

3.1 Animal models

3.1.1 Animals

The local ethics committee for animal experiments in Sweden (Stockholm Norra Djurförsöksetiska nämnd) approved all the tests included in this work. This thesis work conforms to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Several distinct mouse strains were used in the different experiments and studies. For **Study I**, C57BL/6, CBA and, for certain experiments, BALB/c mice were obtained from Janvier Laboratories and Charles River. The B10Q and B10.RIII mice strains were bred at Karolinska Institutet (Stockholm, Sweden), while, for certain experiments, BALB/c mice were bred at the National Veterinary Institute (Uppsala, Sweden). Numerous genetically modified mouse lines were used (details in **Study I** paper): B10Q.C5* (B10Q background; non-functional complement 5) (Johansson et al., 2001) mice were bred at Karolinska Institutet, while FcRγ chain$^{-/-}$ mice (BALB/c background, lacking functional FcγRI, FcγRIII and FcγRIV) (Nimmerjahn et al., 2005; Takai et al., 1994) were bred at the National Veterinary Institute. The genetically modified lines were backcrossed for at least 10 generations and wild-type (WT) littermates were used as controls in all the experiments, except for FcRγ chain$^{-/-}$ mice where the WT line originated from the same breeding but was kept as homozygous animals in parallel. For **Study II**, BALB/c mice were purchased from Harlan, while B10.RIII mice were bred at Karolinska Institutet. For **Study III**, Balb/c mice were purchased from Charles River.

Mice of around 12-22 weeks of age both sexes were used. Animals were housed in standard cages (3-5 per cage) with environmental enrichments (mouse house and tissue paper) in a temperature-controlled system keeping a 12 h light/dark cycle with the possibility of accessing water and food *ab libitum*.

3.1.2 Autoantibodies

3.1.2.1 Anti-collagen type II antibodies

For **Study I**, the collagen antibody-induced arthritis (CAIA) model was achieved by injecting a cocktail of four different anti-collagen type II (CII) monoclonal Abs (mAbs) intravenously (i.v.; 4 mg in 150 µl of saline) on day 0 followed by lipopolysaccharide (LPS) intraperitoneal (i.p.) injection on day 5 (25 µg in 100 µl of saline). LPS enhances and synchronizes the inflammation, which is then rapidly detectable as arthritis score. Noteworthy, the full protocol of CAIA injection was used in few

experiments, since for most of **Study I** we concentrated on the pre-inflammatory stage of the CAIA model (first 5 days) prior to the i.p. injection of LPS, thus investigating the effect of only the mAbs injection.

When not stated otherwise all of the mAbs or modified Abs were injected i.v. in the amount of 4 mg in 150 µl of saline. The four arthritogenic anti-CII mAbs present in the cocktail used for CAIA induction, CIIC1 (IgG2a, C1 epitope), CIIC2 (IgG2b, D3 epitope), M2139 (IgG2b, J1 epitope) and UL1 (IgG2b, U1 epitope) (Nandakumar and Holmdahl, 2005) were also administered individually. As isotype control, we used mIgG2a (mouse anti-human HLA-DRa) and mIgG2b (mouse anti-human parathyroid epithelial cells). Furthermore, CIIF4 was utilized as nonarthritogenic anti-CII mAb (Croxford et al., 2010; Nandakumar et al., 2008). All antibodies were generated and purified as explained previously (Nandakumar and Holmdahl, 2005).

The cocktail of four anti-CII mAbs used for CAIA induction was also used for preparing CII-immune complex (IC). CII-IC was prepared by mixing rat CII (antigen) with the anti-CII mAbs cocktail at a ratio of 1:1 at 37°C for 30 min with gentle shaking (Burkhardt et al., 2002). CII-IC was then used for cell culture experiments or intra-articular (i.a.) injections (500 ng in 5 µl of saline).

Fab fragments of the cocktail of mAbs used for induction of CAIA were produced using the Pierce Fab Preparation Kit, following the producer's guidelines.

Endo-β-N-acetylglucosaminidase (EndoS) hydrolyzes N-linked Fc-glycans and, fused with glutathione S-transferase (GST), was used to produce EndoS-treated M2139 or anti-CII mAbs cocktail, as explained earlier (Collin and Olsen, 2001). In summary, GST-EndoS was mixed with anti-CII mAbs and incubated at 37°C for 16 h. Glutathione-Sepharose 4B columns were then used to eliminate and an ion exchange column was utilized to purify the Abs.

3.1.2.2 Anti-citrullinated protein antibodies (ACPA)

For **Study II**, mice were injected i.v. with human IgGs coming from healthy individuals or RA patients (ACPA or non-ACPA IgGs (Flow Through, FT); 0.125-4 mg in 150 µl of saline).

Plasma, serum and synovial fluid samples were collected and stored at -80°C from ACPA-positive, ACPA-negative and healthy donors. RA patients were tested for anti-CCP2 (ACPA positivity) reactivity while visiting the Rheumatology clinic at the Karolinska University Hospital. In **Study II**, three distinct pools of autoantibodies were used: ACPA pool 1 (38 plasma samples), ACPA pool 2 (5 plasma and 1 serum samples) and ACPA pool 3 (15 plasma and 10 sera samples).

Human antibodies were then purified as described earlier (Ossipova et al., 2014). Briefly, from diluted plasma or serum IgGs were purified in HiTrap Proteing G HP columns. The fraction of ACPA IgGs was then purified from total IgGs using the CCP2 affinity column. The non-ACPA IgGs (*i.e*. not binding to the CCP2 column) represented the FT fraction and were used as controls in the experiments. Purity and endotoxin levels were analyzed in all the different pools of autoantibodies.

3.1.2.3 Other antibodies

For **Study I**, 15A11 was used as anti-cartilage oligomeric matrix protein (COMP) mAb (Geng et al., 2012). Similarly to the production of CII-IC, COMP-IC and IgG-IC were produced by mixing COMP or rat IgGs (respective antigens) with 15A11 anti-COMP or mouse anti-rat IgGs (respective Abs) at the respective ratios of 6:1 or 1:1 at 37°C for 1 h with gentle shaking. These ICs were used for cell culture experiments or i.a. injections (500 ng in 5 µl of saline).

3.1.3 Experimental models

For **Study I**, nerve ligation was achieved by ligating the common peroneal and tibial branches of the sciatic nerve under isoflurane anesthesia. Subsequent to the surgical procedure, animals were then given buprenorphine (0.1 mg/kg, subcutaneously (s.c.)) every 12 h for 2 days.

For chimera mice generation, recipient BALB/c FcRγ chain$^{-/-}$ or WT mice were irradiated with 750 rad. The next day, ten millions cells collected from bone marrow (BM) from tibia and femur of donor mice were inoculated i.v. in recipient mice. Irradiated WT mice received BM cells from FcRγ chain$^{-/-}$ mice, producing mice with activating FcγRs expressed solely on nonhematopoietic cells (including neurons), but not on hematopoietic cells (ko-WT). Vice versa, irradiated FcRγ chain$^{-/-}$ mice were injected with WT BM cells, producing chimera animals expressing activating FcγRs on hematopoietic cells but not on nonhematopoietic cells (including neurons) (wt-KO). Controls were generated transplanting BM from WT donors into WT recipient mice (wt-WT). Chimera mice were kept resting for 6 weeks before injecting them with anti-CII mAbs cocktail (4 mg in 150 µl of saline).

3.1.4 Assessment of arthritis

For **Study I and II**, arthritis signs in all the four paws of mice injected with anti-CII mAbs or ACPA was examined by visual inspection as explained earlier (Bas et al., 2012). In summary, scientists blinded for the origin and treatment of the animals

scored visually detectable inflammation, *i.e*. swelling and redness, on a 0-60 score scale. Each inflamed toe or knuckle was counted as 1 point, while metacarpus/metatarsus or ankle joint inflammation was counted as 5 points each, thus the maximum score reachable per paw was 15. Incidence of arthritis was estimated as percentage of animals that developed any signs of arthritis.

3.1.5 Pharmacology

In **Study I**, mice were treated with PMX53, a cyclic peptide C5a receptor inhibitor. PMX53 (3 mg/kg) was applied s.c. 1 h prior to anti-CII mAbs injection and then once per day (for 5 days) 3 h before the assessment of mechanical hypersensitivity.

In **Study II**, animals were administered reparixin, a CXCR1/2 (mouse analogues of IL-8, CXCL1/2, receptor antagonist). Reparixin was applied s.c. twice per day (30 mg/Kg) throughout the experimental period.

3.2 Assessment of pain-like behavior

In **Study I and II**, measures of evoked and spontaneous pain-like behavior were used, *e.g*. mechanical hypersensitivity and locomotion monitoring respectively. Mechanical pain-like behavior was assessed during specified test days between 10:00-17:00, while locomotor activity was always monitored during the night between day 2 and 3 of the respective model. The scientists did not know the origin and treatment of the animals throughout the experiments and their analysis.

3.2.1 Mechanical hypersensitivity

Mechanical hypersensitivity in the hind paws was assessed using von Frey filaments. Animals were habituated to the experimental environment, single units on top of a wire-mesh surface, before testing of baselines (3-5 measurements) and subsequent randomization in experimental groups. On indicated test days, mice were allowed to acclimatize to testing cages for 1 h prior to the experiment. Withdrawal thresholds were measured with von Frey OptiHair filaments of logarithmic growing stiffness (0.5, 1, 2, 4, 8, 16, and 32 mN, corresponding to 0.051, 0.102, 0.204, 0.408, 0.815, 1.63 and 3.26 g, respectively), but with the cutoff of 4 g possible tissue damage was prevented. Filaments were pressed perpendicularly against the plantar surface of mouse hind paws and a positive response was considered if a brusque removal of the paw from the filament was noted within 2-3 seconds of application. The Dixon up-down method (Chaplan et al., 1994) was used to calculate 50% withdrawal thresholds (*i.e*. filaments' force needed to induce a

response from the mouse in 50% of the solicitations). Withdrawal thresholds from both hind paws were averaged, except in monolateral experiments such as i.a. injections where only the ipsi-lateral paw was considered, and presented in grams or percentage change compared to baseline.

3.2.2 Locomotor activity

Locomotion was examined operating the Comprehensive Lab Animal Monitoring System (CLAMS). Animals were habituated to the system's enclosures and to single caging for 1 d. On the third night (12 h period from 18:00 to 6:00) of the experimental model, mice's activities in the x, y and z axes were examined by automated recording of the amount of infrared beams breaks every 20 min. Data are presented as total movement (total amount of x and y axes beam breaks) and rearing (total number of z axis beam breaks). In each CLAMS run, one or two control mice were included so that the reference control group was collected over the course of locomotor tests.

3.3 Cell cultures

3.3.1 Dorsal root ganglia (DRGs) cell culture

In **Study I, II and III**, sensory neurons cultures were used for several *in vitro* assays. DRGs (C1-L6) from BALB/c WT **(Study I, II and III)** or FcRγ chain$^{-/-}$ **(Study I)** animals were dissected and kept in cold phosphate-buffered saline (PBS) until enzymatically treated initially with papain (1.7 mg/ml; 30 min at 37°C) followed by a mix of collagenase I and dispase II (2 and 8 mg/ml respectively; 30 min at 37°C). DRG cells were then mildly triturated in Leibovitz's (L15) or F12 media added with 10 µM of the mitotic inhibitor 5-fluoro-2-deoxyuridine, 1% penicillin and streptomycin and 10% heat-inactivated fetal bovine serum (FBS). For **Study I** CGRP release trials, nerve growth factor (NGF, 30 ng/ml) was supplemented to the medium. To produce a neuronally enriched culture partially depleting satellite glia, the triturated cells were then plated on uncoated wells for 1.5 h before transfer to wells pre-coated with laminin and poly-D-lysine. Cells were then kept in 5% CO_2 environment at 37°C and medium was changed after 1 d and then every third day.

3.3.1.1 CGRP release

For **Study I**, after 6 days in culture and initial washes with Hepes buffer (25 mM Hepes, 2.5 mM $CaCl_2$, 3.5 mM KCl, 135 mM NaCl, 1 mM $MgCl_2$, 3.3 mM dextrose and 0.1% BSA; brought to pH 7.4 using NaOH), sensory neurons were put in new

Hepes buffer for 30 min at 37°C to collect pre-stimulation samples (baseline level of CGRP). The cells were then stimulated with CII-IC (0.1, 1 and 10 µg/ml), CII antigen, anti-CII mAb cocktail, control IgG2b (all 1 µg/ml) or only Hepes buffer at 37°C for 30 min. These post-stimulation samples were then collected for CGRP analysis. Capsaicin (50 nM) (10 min at 37°C) was applied as positive control. An enzyme immune assay (EIA) kit was used to measure the levels of CGRP in each sample. The percentage change before and after stimulation was then calculated for each well and plotted.

3.3.1.2 Calcium imaging

Fluo-3AM (4.4 µM) for 30 min at room temperature was used as calcium indicator to load DRG neurons (after 24 or 48 h in culture). Modified Hepes buffer (10 mM Hepes, 2 mM $CaCl_2$, 3 mM KCl, 145 mM NaCl, 2 mM $MgCl_2$, 10 mM glucose; brought to pH 7.4 using NaOH) was used to wash the cells, which were then positioned in the experimental chamber and unceasingly perfused with modified Hepes buffer at the rate of 1 ml/min. A Nikon Diaphot inverted microscope with a 40x oil-immersion objective and a diode laser (488-nm excitation) were used for the experiments. The variation in emission (506 nm), promoted by the binding of intracellular calcium to Fluo-3AM, was measured every 7-15 s using a photomultiplier tube.

Depending on the study different substances were applied as stimulation factors. For **Study I**, the cells were stimulated with CII-IC or control IgG2b (both 1 µg/ml) for 3 min. For **Study II**, the cells were challenged with human ACPA or control FT (both 1 µg/ml) for 5 min. For **both studies I and II**, the two different reagents were used in random order to the same cells and with 10 min washing between stimulations. Moreover, after each recording, the cells were stimulated with KCl (50 mM) for 1 min to identify functional and alive neurons. For **Study III**, KCl (50 mM) or Ionomycin (to enable amplitude comparisons, 5 µM) were applied for 1 min to the cells.

Modified Hepes buffer was used to prepare all the reagents used in the studies. The acquired images with around 15 cells per image were analyzed using the software ImageJ. In each image, the mean fluorescence intensity (F) was calculated for all the visible neuronal cell bodies, which were manually selected. The baseline recording (F_0) was measured as the average mean signal of the initial 5-9 images of the series before any reagents was applied. Data are presented as F/F_0 and we noted as positive, cells in which the increase of the fluorescent signal was at least ≥20% or 25% (respectively for **Study I and II** or **Study III**) compared to baseline.

3.3.1.3 Electrophysiology

DRG neurons within 24 and 48 h of culturing were picked for electrophysiological whole-cell voltage-clamp. The recordings were carried out at room temperature, using a patch-clamp amplifier and then analyzed by using Clampex 10.4 software. Patch pipettes were in house-made starting from borosilicate glass capillaries using a vertical puller. The resistance of patch pipettes was 4-5 MΩ when filled with internal solution (120 mM K^+-gluconate, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM KCl, 11 mM EGTA, 10 mM Hepes and 2 mM NaATP; brought to pH 7.15 using Tris-base). During the experiments, sensory neurons were unceasingly perfused with modified Hepes solution (see section 3.3.1.2) at the rate of 1 ml/min.

Different substances were applied to DRG neurons in the different Studies. For **Study I**, the cells were stimulated with CII-IC, IgG-IC or control IgG2b (all 1 µg/ml) for 1 min. For **Study II**, the cells were challenged with human ACPA or control FT (both 1 µg/ml) for 1 min. In **both studies I and II**, at the end of each experiment, the cells were stimulated with capsaicin (0.5 µM) for 10 s for detecting TRPV1 positive cells. In between the different applications 4 min were waited as washing period.

Cells were accepted and included in the analysis if their resting potential was lower than -40 mV. Furthermore, positive cells were counted when the detected current was at least 20 pA. All reagents were prepared in modified Hepes solution and applied via an 8-channels pressure-controlled application system.

3.3.2 Neuroblastoma cell lines

In **Study III**, F11, B35 and Neuro-2a (N2a) neuroblastoma cell lines were used for several *in vitro* assays and compared to primary DRG neuronal cell cultures on numerous characteristics.

F11 cells were gifted by Dr. Michel Pohl (Université Pierre et Marie Curie 6, Paris, France), while B35 and N2a cells were obtained from American type culture collection. The cell lines were kept in liquid nitrogen until the start of the experiments. Specific media (complete medium) were used for culturing the different cells and for promoting neuronal differentiation (serum starvation or differentiation media) (Table 1).

Cell lines	Complete medium composition	Serum Starvation medium composition	Differentiation medium composition
B35 and N2a	DMEM, 10% heat inactivated FBS, Penicillin/streptomycin/glutamine	DMEM, 0.1% heat inactivated FBS, Penicillin/streptomycin/glutamine	DMEM, 10% heat inactivated FBS, Penicillin/streptomycin/glutamine. NGF 10 ng/ml and dibutyryl cyclic AMP (db-cAMP) 0.5 mM
F11	Ham's F-12 Nutrient Mix, GlutaMAX™ supplement, 15% heat inactivated FBS, Sodium hypoxanthine, aminopterin and thymidine (HAT). Penicillin/streptomycin. Allo-4-hydroxy-L-proline 100 µg/ml.	Ham's F-12 Nutrient Mix, GlutaMAX™ supplement, 1% heat inactivated FBS, Penicillin/streptomycin.	Ham's F-12 Nutrient Mix, GlutaMAX™ supplement, 1% heat inactivated FBS, Penicillin/streptomycin. NGF 10 ng/ml and db-cAMP 0.5 mM

Table 2: Complete, serum starvation and differentiation media used for culturing B35, N2a and F11 neuroblastoma cell lines.

The cells were thawed and initially cultured in complete medium. After reaching 70-75% confluence, the cells were disattached (PBS-EDTA for F11, Trypsin-EDTA for B35 and N2a) and plated in different well-plates in accordance with experimental requirements. For calcium imaging studies, cover slips coated with poly-D-lysine and laminin were used to culture the cells. The plated cells were kept in complete media for three days before switching either to complete, serum starvation or differentiation media. The cells were maintained in those media for three further days to allow differentiation and then processed for the different experiments.

3.4 Tissue analyses

3.4.1 Joint histology

For **Studies I and II**, the degree of arthritis was also examined by histology. After deep anesthesia with isoflurane mice were perfused with saline followed by 4% paraformaldehyde (PFA). Subsequentially, PFA 4% was used to post-fix dissected hind ankle joints for 48 h, then EDTA solution for 4-5 weeks (changing the solution every 7 days) was used for decalcification, ethanol for dehydration and finally paraffin for embedding. Cut sections of 5 µm were stained with H&E and given

scores on a 0-3 scale on parameters like synovitis, bone erosion and cartilage destruction by blinded investigators as previously explained (Bas et al., 2012).

3.4.2 Mouse immunohistochemistry and immunocytochemistry

For **Study I**, immunohistochemistry (IHC) was performed on mouse lumbar DRGs, sciatic nerve and hind paw's glabrous skin, using distinct protocols. Mice were deeply anesthetized and perfused with PFA 4%. After dissection, skin, sciatic nerve and DRGs were post-fixed in PFA 4% for respectively 4, 24 and 24 h and subsequentially cryoprotected in sucrose 30% at 4°C for 48 h. Importantly, FcγRIIb mAb (Tutt et al., 2015) required a different post-fixation for a successful protocol, thus for its IHC anesthetized mice were perfused only with PBS before dissection. Collected skin and DRGs were then frozen in optimal cutting temperature (OCT) compound and kept at -80°C until cutting with a cryostat. Tissues were cryosectioned (skin 20 µm, sciatic nerve 10 µm and DRGs 14 µm) and mounted on glass slides. For IHC with FcγRIIb mAb, tissues were post-fixed on the glass slides with acetone 50% for 10 min at 4°C, directly after cutting.

For **Study I and III**, immunocytochemistry (ICC) was performed on DRG primary cell cultures or neuroblastoma cell lines. For **Study I**, after 6 days in culture sensory neurons were fixed in acetone 50% for 10 min at 4°C, while for **Study III** DRG neurons or cell lines were fixed in PFA 4% for 10 min at room temperature.

On the day of the staining protocol, tissues were permeabilized with TritonX-100 and non-specific binding was prevented using normal serum 5% (from the species of secondary antibody) in PBS. Primary antibodies (Table 2 for **Study I**) (Tutt et al., 2015) were diluted in the blocking solution and then incubated with the tissues overnight at room temperature **(Study I)** or 4°C **(Study III)** and Alexa Fluor-conjugated or cyanine (Cy)-conjugated secondary antibodies (all 1:300) were incubated for 1 h at room temperature to visualize the immunoreactivity. Coverslipping was achieved using Prolong Gold antifade mounting medium with DAPI and a confocal microscope (Zeiss LSM800) **(Study I)** was used to collect images. Figures were then composed in Adobe Illustrator CS6.

Mouse Tissue	Fixation	Primary antibody
DRG	Fresh PFA 10 min RT	Rabbit anti-NeuN (1:100, Alexa Fluor-488 conjugated, ABN78A4, Millipore)
DRG and DRG Cultures	Fresh Acetone 10 min 4°C	Rat anti-FcγRI (2 µg/ml, gift from Dr Cragg, University of Southampton, Southampton, United Kingdom)
Skin	PFA-perfused 4 h post-fixation PFA 4%	
DRG	PFA-perfused 24 h post-fixation PFA 4%	Rabbit anti-Iba1 (1:500, 019-19741, Wako)
DRG and DRG Cultures	Fresh Acetone 10 min 4°C	Rat anti-FcγRIIb (2 µg/ml, gift from Dr Cragg, University of Southampton, Southampton, United Kingdom)
Skin	PFA-perfused 4 h post-fixation PFA 4%	
Sciatic Nerve	Fresh PFA 10 min RT	Goat anti-TrkA (1:50, AF1056, R&D systems)
Skin	PFA-perfused 4 h post-fixation PFA 4%	Rabbit anti-PGP9.5 (1:500, ab37188, Abcam)
DRG and DRG Cultures	Fresh Acetone 10 min 4°C	Rat anti-FcγRIII (2 µg/ml, gift from Dr Cragg, University of Southampton, Southampton, United Kingdom)
DRG and DRG Cultures	Fresh Acetone 10 min 4°C	Rat anti-FcγRIV (2 µg/ml, gift from Dr Cragg, University of Southampton, Southampton, United Kingdom)

Table 3: Primary antibodies used for Study I mouse IHC.

For the IHC analyses of **Study III**, the subsequent primary antibodies were used: mouse anti-Peripherin (1:200, MAB1527, Millipore), mouse anti-NF200 (1:400, N0142, Sigma), mouse anti-β-III-Tubulin (1:300-1000, ab78078, Abcam), rabbit anti-PGP9.5 (1:500, ab37188, Abcam), IB4-488 (1:200, t21411, Molecular probes), rabbit anti-CGRP (1:10000, Terenius L.). For the acquisition of the images, a Nikon TE300 fluorescence microscope was used keeping fixed settings for each marker across the different cell lines and culture conditions. Six fields were randomly selected and image analysis performed with a customized python script. Briefly, the images were first thresholded with a combination of Ostu and adaptive thresholding methods. The objects smaller than 500 pixels or connected to the borders of the image were discarded. The remaining components were segmented using a watershed algorithm and area and fluorescence intensity of the isolated connected components were quantified. Data are presented as signal intensity/cell area.

3.4.3 Human immunohistochemistry

For **Study I**, IHC was performed on human DRGs (snap-frozen L4-5) harvested from brain-dead individuals after asystole (n = 4) at the University of Pittsburgh, shipped and maintained at -80°C until embedded in OCT medium.

The University of Pittsburgh Committee for Oversight of Research and Clinical Training Involving Decedents and the Center for Organ Recovery and Education approved all the procedures.

Similar, IHC protocol as the one for mouse DRGs was used, with the exception of tyramide signal amplification (cy5-TSa) with appropriate HRP-conjugated secondary antibodies to visualize the immunoreactivity.

Human Tissue	Fixation	Primary antibody
DRG	Acetone 10 min 4°C	Mouse anti-FcγRI (1:100, hCD64, clone 10.1, MCA756g, Serotec)
DRG	Acetone 10 min 4°C	Mouse anti-FcγRIIa (1:100,hCD32a, clone IV.3, Stem cell tech)
DRG	Acetone 10 min 4°C	Mouse anti-FcγRIIIa/b (1:100, hCD16 PE/cy7-conjugated, clone 3G8, Biolegend)

Table 4: Primary antibodies used for human IHC.

3.4.4 Quantitative real-time polymerase chain reaction (qPCR)

For **Study I**, after decapitation under isoflurane anesthesia, mouse ankle joints were harvested subsequently to trimming from muscle and tendons. The joints were then frozen and kept at -70°C until the experiments. For extracting the RNA, a BioPulverizer was used to macerate the joints and an ultrasonic processor was utilized to briefly sonicate the pulverized joints in TRIzol. For **Study III**, cells in the different conditions were washed in ice-cold PBS and then transferred to TRIzol.

RNA was extracted according to the producer's protocol and reverse transcribed to complementary DNA. qPCRs were run with the standard curve method using particular primers (Table 5-6) to define threshold cycle values to estimate the cell equivalents' number for each sample. The house-keeping gene Hprt1 values were used to normalize the data, which was then plotted relative expression units.

Gene	Primer
Hprt1	Mm01545399_m1
Ccl2 (Mcp-1)	Mm00441242_m1
Tpsb2 (Mcp-6)	Mm01301240_g1
Mcpt4 (Mcp-4)	Mm00487636_g1
Tnf	Mm00443258_m1
Mmp2	Mm00439498_m1
Mmp9	Mm00442991_m1
Mmp13	Mm00439491_m1
Cox2	Mm00478374_m1
Il1b	Mm00434228_m1
Il6	Mm00446190_m1

Table 5: Primers used for Study I qPCR assay.

Gene	F11 and B35 cell lines	N2a cell line
Scn8a	Rn00570506_m1	Mm00488110_m1
Scn9a	Rn00591020_m1	Mm00450762_s1
Cacna1b	Rn01643813_m1	Mm01333678_m1
Cacna2d1	Rn01442580_m1	Mm00486607_m1
P2Xr3	Rn00579301_m1	Mm00523699_m1
MrgprD	Rn01785783_s1	Mm01701850_s1
Calca1	Rn01511353_g1	Mm00801462_m1
TRPV1	Rn00583117_m1	Mm01246302_m1
TrkA	Rn00572130_m1	Mm01219406_m1
Hprt1	Rn01527838_g1	Mm03024075_m1

Table 6: Primers used for Study III qPCR assay.

3.5 Statistics

GraphPad Prism 6 software was used to run statistical analyses. For comparing changes over time, repeated measures two-way ANOVA was used followed by Bonferroni post-hoc test. For differences in three groups or more, one-way ANOVA was used followed by Bonferroni post-hoc test. For differences in two groups, Student's t-test was used. For **Studies I and II**, arthritis and histological scores were compared using the Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test. P values <0.05 were considered significant. No statistical method was applied to pre-determine sample sizes.

4 RESULTS AND DISCUSSION

4.1 Study I: Autoantibodies in immune complex formation induce pain independently of inflammation via neuronally expressed FcγRI

Pain in rheumatoid arthritis (RA) has always been attributed to the ongoing inflammatory processes affecting the patients' joints. However, RA individuals report arthralgia many years before the onset of the disease (with detectable tissue injury and inflammation) and even after the disease is under medical control or in remission, suggesting a clear disconnect between inflammation and pain in RA pathophysiology (de Hair et al., 2014; Taylor et al., 2010). Noteworthy, autoantibodies are present in future RA patients' sera up to 10 years before the RA diagnosis and most of the anti-rheumatic drugs used nowadays in the clinic to stop RA progression do not affect autoantibody titers (Bos et al., 2008; Rantapää-Dahlqvist et al., 2003; Ronnelid et al., 2005).

The aim of **Study I** was to explore mechanisms that could explain the enhancement in pain sensitivity prior to the typical signs of RA disease activity. Specifically, we hypothesized that autoantibodies could have a more prominent role in promoting nociception than only via the classical processes of inducing inflammation.

To investigate RA-induced pain, we worked with the collagen antibody induced-arthritis (CAIA) mouse model of RA. As briefly discussed in the introduction, in this model, a single dose of antibodies (Abs) against collagen type II (CII) is injected on day 0 followed by a low dose of lipopolysaccharide (LPS) on day 5 to boost the immune system and synchronize the onset of RA-like pathology. Mice develop robust but transient inflammation clearly detectable in the joints between days 10-25. Afterwards, inflammation resolves due to the clearance of injected Abs. In contrast, while pain-like behavior during the inflammatory flare is quite expected, we found that mechanical hypersensitivity develops days before the start of visually detectable inflammation and persists for weeks after inflammation has resolved (Agalave et al., 2014; Bas et al., 2012; Fernandez-Zafra et al., 2019; Su et al., 2015). This implies that the pain mechanisms driving CAIA hypersensitivity could well mimic the pain profile experienced by RA patients.

In this study, we explored the underlying pain mechanisms of the early phase of the CAIA model (after the injection of anti-CII Abs up to day 5, before LPS injection), where both evoked and spontaneous pain-like behaviors appear in mice before any signs of inflammation detectable by visual inspection (Figure 8).

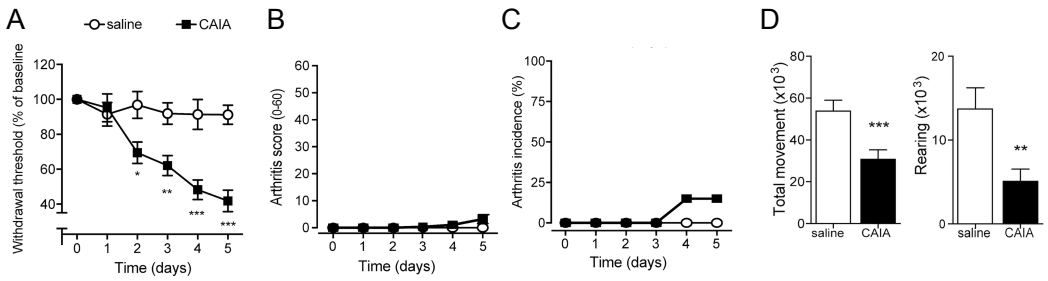


Figure 8: Evoked and spontaneous pain-like behavior in the early phase of the CAIA model. Mice injected with anti-CII Abs develop evoked pain-like behavior measured with von Frey filaments detectable as early as day 2 after injection (A), in the absence of significant visual signs of inflammation measured by arthritis score and incidence (B and C, n = 9-10). Mice subjected to CAIA display also spontaneous pain-like behavior measured with comprehensive lab animal monitoring system (CLAMS) during the third night after the anti-CII abs injection (D, n = 15-19). Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ compared to saline controls.

During the first 24 h after the injection, anti-CII Abs rapidly bind to CII in the articular joints of the animals' joints and form immune complexes (ICs) (Jonsson et al., 1989). ICs attract and activate immune cells, which promote the development of a joint pathology that resembles clinical features of RA in humans. Our initial hypothesis for explaining early CAIA hypersensitivity involved the possibility of CII-ICs driving low-grade inflammation, which would not be visually detectable as redness or swelling, but could induce the release of neuronal-sensitizing factors and promote pain-like behavior. Therefore, we examined ankle joints of mice subjected to CAIA both at an early (day 5) or inflammatory (day 15) phase stages both at histological and molecular levels. No significant histological signs of synovitis, bone erosion and cartilage destruction or increase in mRNA of several commonly known inflammatory and pain-related factors were detected in ankle joints at day 5 of the CAIA model, while all of these parameters were prominent at the peak of inflammation (day 15). Moreover, ICs also activate the complement cascade, releasing C5a peptides, which, binding to their receptor C5aR, have a critical role in the genesis of acute and chronic pain states (Jang et al., 2010; Ting et al., 2008). Thus, we explored the possible involvement of complement activation in our model and found that both using a C5aR antagonist or C5a-depleted mice we could not reverse early CAIA pain-like behavior. Taken together these data suggest that other factors rather than classical inflammatory molecules are mediating early CAIA hypersensitivity.

Importantly, the four different Abs in the anti-CII Abs cocktail have distinct arthritogenic potencies and when injected alone they can induce different degrees of arthritis scores and incidence (Nandakumar and Holmdahl, 2005). Therefore, if early CAIA hypersensitivity was connected to the actual pathology promoted by the anti-CII Abs, we would expect the pain-inducing properties of each anti-CII Abs in the cocktail to be proportional to their pathological potential. However, when we injected

the four anti-CII Abs alone they all induced the same degree of pain-like behavior, comparable to the full Abs cocktail. Noteworthy, injection of isotype controls (IgG2a and IgG2b Abs), which would not bind and form ICs with any mouse protein, was not able to promote any pain-like behavior.

Another common feature of arthritogenic anti-CII Abs is to cause denaturation of CII and loss of both CII and proteoglycans from cartilage *in vitro* and *in vivo* even in the absence of inflammation (Nandakumar et al., 2008). To test if these actions could have pro-nociceptive properties, we injected a non-arthritogenic antibody CIIF4 that binds to CII (forming therefore ICs), but does not lead to any cartilage damage and it is instead protective when injected together with pathogenic anti-CII Abs both *in vitro* and *in vivo* (Croxford et al., 2010; Nandakumar et al., 2008). Strikingly, mice injected with CIIF4 developed robust mechanical hypersensitivity comparable to all pathogenic anti-CII Abs (Figure 9).

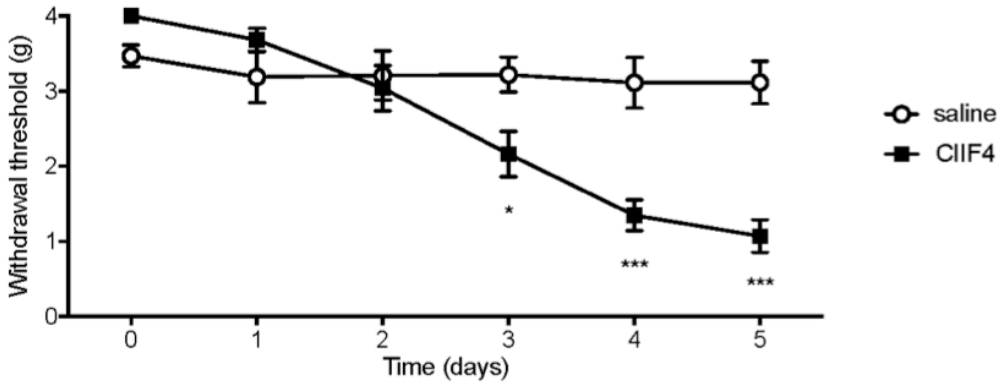


Figure 9: The non-arthritogenic anti-CII Ab CIIF4 induces pain-like behavior in mice comparable to pathological anti-CII Abs. Mice injected with CIIF4, which binds to CII and forms ICs, develop evoked pain-like behavior measured with von Frey filaments even if CIIF4 does not induce any pathology (n = 7-8). Data are presented as mean ± SEM. *, $p < 0.05$; ***, $P < 0.001$ compared to saline controls.

The fact that a non-pathogenic Ab could have similar effects on pain-like behavior as arthritogenic Abs led us to conclude that the mechanistic explanation for early CAIA hypersensitivity could not be found on any strictly pathological feature of the Abs, but rather on their capacity to bind epitopes on the CII molecule, thus forming ICs. Prompted to explore other mechanisms we investigated possible direct actions of anti-CII Abs and ICs on peripheral sensory neurons, turning our attention to the IgGs receptors, Fc gamma receptors (FcγRs).

Using several techniques, we evaluated the expression pattern of the four FcγRs in mouse sensory neurons. With single molecule fluorescence *in situ* hybridization, we detected mRNA molecules for *Fcgr1, Fcgr2b* and *Fcgr3* in the soma of primary afferents located in the dorsal root ganglia (DRGs). At a protein level, DRG neuronal

cell bodies expressed only FcγRIIb protein, while FcγRI was present exclusively in Iba1-positive resident macrophages. On a side note, this differs from rat FcγRs expression pattern, where rat FcγRI is the only FcγRs expressed in DRGs and it is found in sensory neurons (Qu et al., 2012; Qu et al., 2011). Importantly, mRNAs can be transported and translated *in loco* at the neuronal peripheral terminals, since the machinery responsible for the translation process can be found along the axons of sensory neurons and it has been shown that peripheral expression of specific proteins is involved in regulating neuronal plasticity (Jimenez-Diaz et al., 2008; Obara et al., 2012; Price and Geranton, 2009). In fact, following ligation of the sciatic nerve, we could detect accumulation of mouse *Fcgr1* and *Fcgr2b* mRNA molecules at the ligature site in fibers positive for TrkA, which is a marker highly expressed in nociceptors innervating the joints (Mantyh et al., 2011). This suggested mRNA transport and possible local translation at the peripheral terminals of sensory neurons, which we later confirmed by detection of FcγRI and FcγRIIb proteins in skin neuronal fibers. Both FcγRI and FcγRIIb were expressed also in non-neuronal cells in the skin (Figure 10). Instead, FcγRIII and FcγRIV proteins were not detected in any of the analyzed tissues.

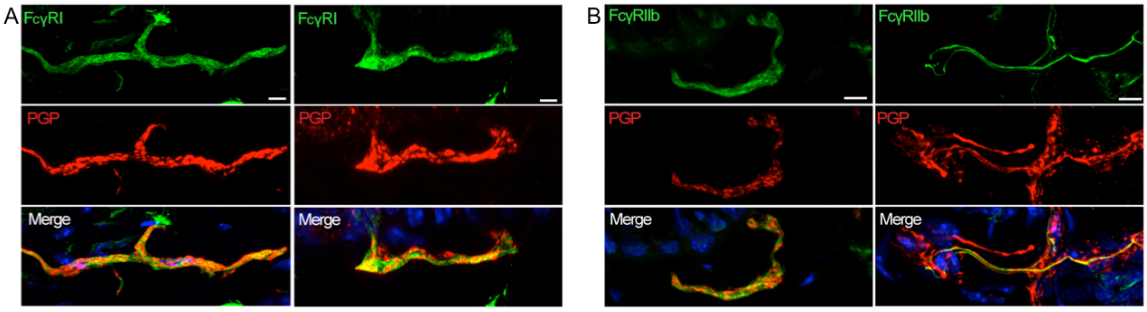
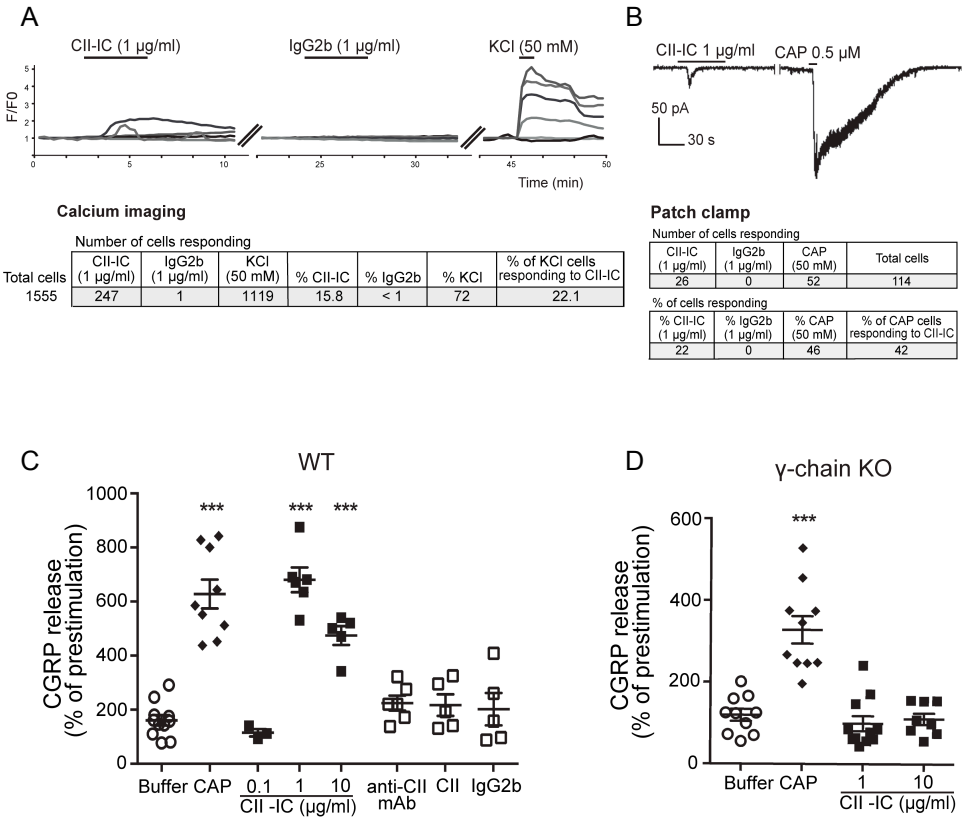


Figure 10: FcγRI and FcγRIIb are present on sensory neurons in mouse skin. FcγRI and FcγRIIb immunoreactivity co-localizes with the neuronal marker PGP9.5 in sections of mouse skin. Non-neuronal cells are also stained positive for FcγRI and FcγRIIb.

Based on FcγRI and FcγRIIb expression on sensory fibers, we speculated that ICs could directly activate neurons therefore acting as pain-inducing molecules completely uncoupled from the inflammatory process. We tested this hypothesis in experiments *in vitro*, using DRG cell cultures, which were neuronal-enriched due to a pre-absorption step in the culture protocol to remove of most of the satellite glial cells. Interestingly, we found that a RA-relevant IC, CII-IC (CII as antigen and antibodies anti-CII), could promote calcitonin gene related peptide (CGRP) release, increase of intra-cellular calcium levels and positive inward currents when applied to cultured DRG neurons from wild type (WT) mice (Figure 11A-C). Thus, CII-IC can directly activate sensory neurons *in vitro* in the absence of any immune or accessory cells, further strengthening the link between autoantibodies and their pro-nociceptive properties. Moreover, when CII-IC was applied to cultured DRG neurons from FcRγ-

chain$^{-/-}$ mice, which lack the activating FcγRs (I, III and IV) but retain the inhibitory FcγRIIb, CGRP release was prevented (Figure 11D), suggesting that FcγRI, and not FcγRIIb, is the receptor responsible for CII-IC activation of sensory neurons *in vitro*. This is in accordance with previous work showing generic IgG-ICs promoting increase of calcium levels, membrane depolarization and release of substance P from cultured rat sensory neurons via FcγRI and the Syk–PLC–IP3–TRPC3 intracellular pathway (Qu et al., 2012; Qu et al., 2011).



Calcium imaging

Number of cells responding

Total cells	CII-IC (1 µg/ml)	IgG2b (1 µg/ml)	KCl (50 mM)	% CII-IC	% IgG2b	% KCl	% of KCl cells responding to CII-IC
1555	247	1	1119	15.8	< 1	72	22.1

Patch clamp

Number of cells responding

CII-IC (1 µg/ml)	IgG2b (1 µg/ml)	CAP (50 mM)	Total cells
26	0	52	114

% of cells responding

% CII-IC (1 µg/ml)	% IgG2b (1 µg/ml)	% CAP (50 mM)	% of CAP cells responding to CII-IC
22	0	46	42

Figure 11: *In vitro*, CII-IC activates DRG sensory neurons from WT, but not from FcRγ-chain$^{-/-}$ mice. CII-IC, but not controls, promotes increase of intracellular calcium levels (A), membrane depolarization (B) and CGRP release (C) in WT DRG neurons in culture. Neurons from FcRγ-chain$^{-/-}$ mice are protected from CII-IC activation (D). IgG2b, CII and anti-CII Abs are used as negative controls, while capsaicin (CAP) and KCl as positive controls. Data are presented as mean ± SEM. ***, P < 0.001 compared to saline controls.

Next, we confirmed that autoantibodies could also act on neuronal FcγRI *in vivo*. Therefore, we injected CII-IC in the intra-articular space of the ankle joint of WT and FcRγ-chain$^{-/-}$ mice and, while WT mice developed pain-like behavior, FcRγ-chain$^{-/-}$ mice were protected. Moreover, injection of Fab fragments (Abs depleted of their Fc region) or EndoS-treated anti-CII Abs (presenting reduced affinity for FcγRs) failed to induce any pain-like behavior, indicating that the Fc-FcγRI interaction is also necessary for developing anti-CII Abs induced hypersensitivity *in vivo*.

As FcγRs detect the constant region of Abs in IC formation, the observed pain-like behavior should not depend on the ICs constituents. Therefore, we tested if other ICs could promote similar results as CII-IC and found that both a generic IC (rat IgGs and mouse anti-rat IgGs) or cartilage oligomeric matrix protein (COMP)-IC (COMP and anti-COMP Ab) were able to promote pain-like behavior in WT mice when injected intra-articularly in the ankle joint. Importantly, while we used another RA-relevant IC to confirm our results, since it has been shown that COMP is released from cartilage during development of RA (Saxne and Heinegard, 1992), work from other groups, in which ovalbumin (OVA)-ICs induced pain-like behavior in rat when injected intraplantarly, also supported our findings (Jiang et al., 2017).

However, none of our previous experiments excludes the role of immune cells in participating to the induction of pain-like behavior *in vivo*. Therefore, we used chimera mice as an approach to address the contribution to early CAIA hypersensitivity of FcγRI of hematopoietic cells as compared to non-hematopoietic cells (including neurons). Hence, mice were irradiated to deplete hematopoietic cells and then transplanted with bone marrow from either WT or FcRγ-chain$^{-/-}$ mice. Mice lacking activating FcγRs solely on non-hematopoietic cells were protected from early CAIA hypersensitivity, while mice lacking activating FcγRs on hematopoietic cells but still expressing them on non-hematopoietic cells (including neurons) developed mechanical hypersensitivity indistinguishable from control mice (Figure 12). These data provide evidence that FcγRs on immune cells are not essential for promoting early CAIA hypersensitivity, supporting the *in vivo* role of FcγRI on neurons, although the involvement of other non-hematopoietic cells cannot be excluded.

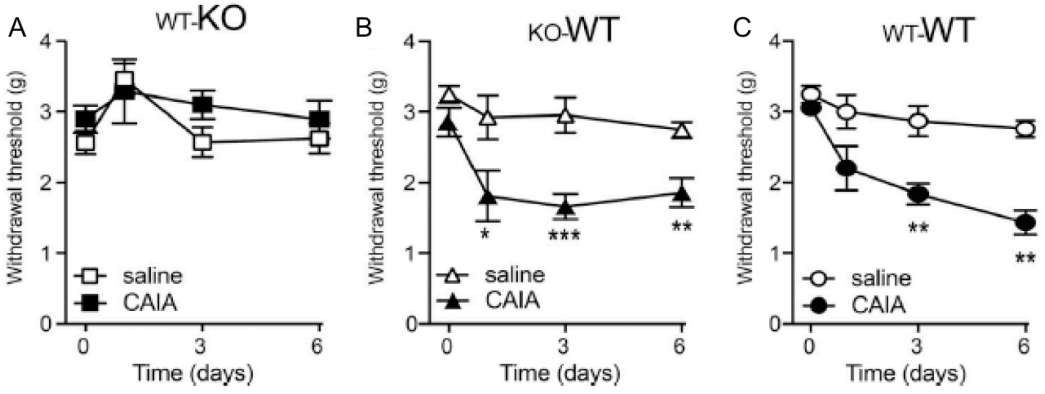


Figure 12: FcγRI on non-hematopoietic cells is essential for early CAIA hypersensitivity. Chimera mice injected with anti-CII Abs develop evoked pain-like behavior only when FcγRI was expressed on non-hematopoietic cells (B; ko-WT), but not when it was present solely on hematopoietic cells (A; wt-KO). Control mice (C; wt-WT) for the irradiation and bone marrow transfer processes still develop early CAIA hypersensitivity. Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ compared to saline controls. n = 8-9.

Finally, to examine the translational potential of our study we characterized the FcγRs expression pattern in human DRGs. While human FcγRI is solely expressed on macrophage-like cells in the DRGs, similarly to mouse but not rat FcγRI, we discovered that the activating FcγRIIIA is expressed in human sensory neurons and could theoretically drive similar mechanisms in clinical autoimmune diseases, such as RA.

Together our data suggest that local ICs directly interact with FcγRI present on sensory neurons, leading to pain-like behavior independently of inflammation. As cartilage is not innervated, it is more likely that early CAIA hypersensitivity is driven by the injected anti-CII Abs bound to soluble CII, rather than the Abs bound to CII on the cartilage surface. Soluble CII is known to be present in human synovial fluid (Lohmander et al., 2003; Yoshida et al., 2006), and is also likely to be present in rodents. CII-ICs would then be responsible of inducing and maintaining pain-like behavior, first interacting with FcγRI expressed on sensory neurons innervating the synovial tissue and bone structures and subsequentially promoting the development of the inflammatory process with all the connected nociceptive factors released by activated immune cells. While the latter represents the classical concept on how autoantibodies contribute to nociception, the first is a novel view defined in this study for which autoantibodies are functionally coupled to pain transmission, even in the absence of inflammation. This might explain initial and persistent pain in RA patients, since autoantibodies are present many years before the onset of the disease and nowadays available treatment options often do not affect antibody titers in the patients (Bos et al., 2008; Rantapää-Dahlqvist et al., 2003; Ronnelid et al., 2005). Therefore, targeting this novel mechanism may represent a new strategy for development of disease-related pain-relieving therapies, not only for RA, but also for other autoimmune diseases, which are associated with IC formation in innervated tissues.

4.2 Study II: Anti-citrullinated protein antibodies induce nociception via release of CXCL1 from osteoclasts, but not through direct stimulation of sensory neurons

Anti-citrullinated protein antibodies (ACPA) are used as diagnostic marker since their presence is very high and specific for RA patients (Schellekens et al., 1998). Importantly, ACPA are associated with arthralgia before the onset of the inflammatory phase of RA and predict a worse prognosis since they correlate with a more destructive disease phenotype (van de Sande et al., 2011). Moreover, ACPA titers remain high even after successful treatment (Bos et al., 2008; Ronnelid et al., 2005). However, if and how ACPA present pathological properties or contribute to pain in RA is still unknown.

In **Study II**, we aimed at investigating the pro-nociceptive and pathological roles of human ACPA purified from RA patients using a pre-clinical approach.

Mice injected with ACPA, but not non-ACPA IgGs (flow through, FT) or IgGs purified from healthy individuals, developed evoked and spontaneous pain-like behavior that persisted for at least 28 days without any signs of joint inflammation (visual, histological or at a molecular level) (Figure 13).

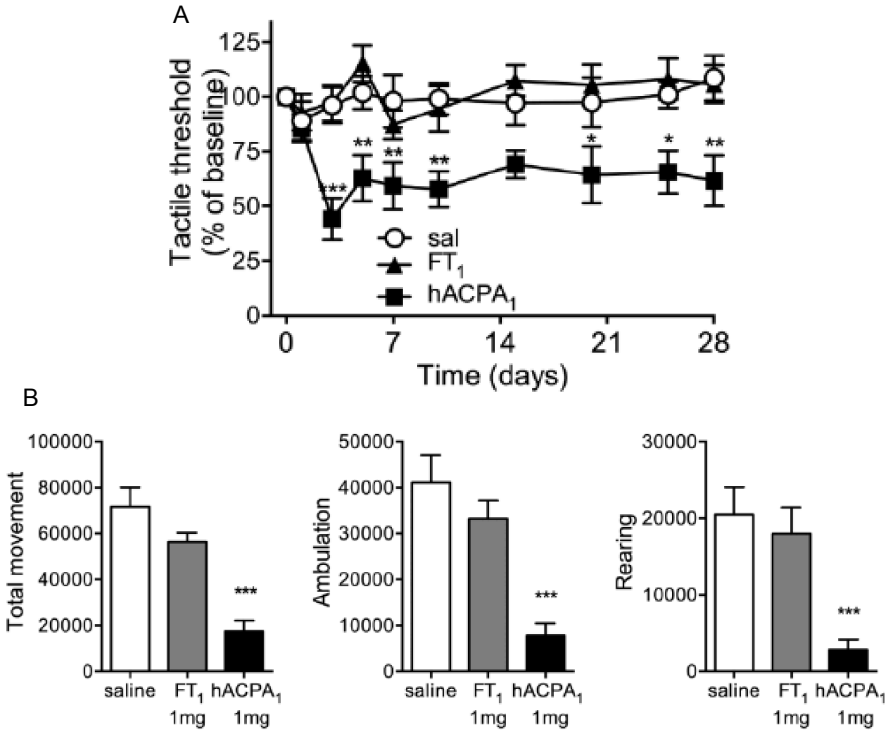
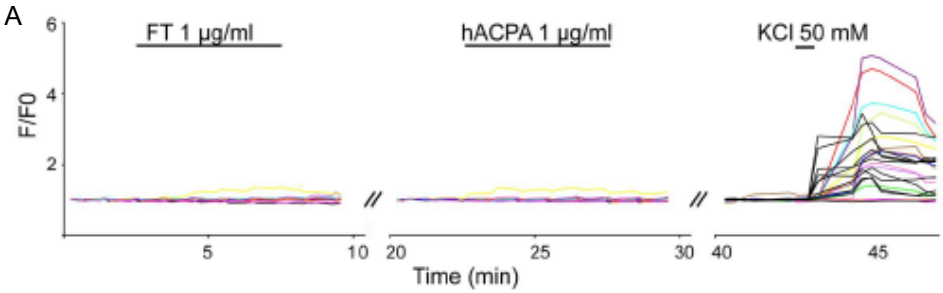


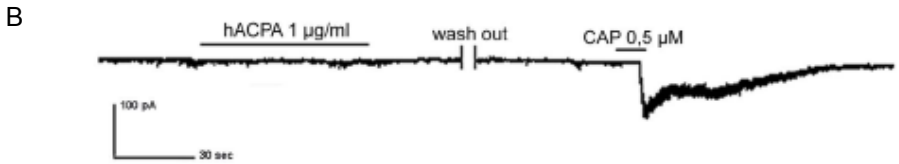
Figure 13: Evoked and spontaneous pain-like behavior in mice after the injection of human ACPA. Mice injected with ACPA, but not FT, develop evoked pain-like behavior measured with von Frey filaments detectable for at least 28 days (A, n = 4-6), in the absence of significant visual signs of inflammation (data not shown). Moreover, ACPA also induce spontaneous pain-like behavior measured with CLAMS during the third night after the anti-CII abs injection (B, n = 4-6). Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ compared to saline controls.

Similarly to anti-CII Abs from **Study I**, ACPA-ICs in the animals' joints could directly participate in inducing nociception via neuronal FcγRI uncoupled from the inflammatory process. Unfortunately, at the time of the study, there was no soluble ACPA-IC (with any citrullinated antigens) available for *in vitro* testings. However, autoantibodies can exert their pathological functions not only in IC formation with their Fc portion, but also through their antigen binding region (Fab). For instance, antibodies targeting a potassium channels complex or specifically contactin-associated protein 2 (CASPR2) have been related to neuropathic pain via actions promoted by their Fab regions (Dawes et al., 2018; Klein et al., 2012). Therefore, the focus of my project was to examine if ACPA could directly activate DRG neurons in culture through their Fab regions, potentially explaining *in vivo* ACPA effects

uncoupled from inflammation. Importantly, ACPA failed to promote significant increase in intracellular calcium levels or positive inward currents in sensory neurons, proving that the pro-nociceptive actions of ACPA *in vivo* are not due to an acute direct effect on sensory neurons (Figure 14).



	Number of cells responding					
Total cells	ACPA (1 µg/ml)	FT (1 µg/ml)	KCl (50 mM)	% ACPA	% FT	% KCl
234	6	4	188	2,5	1,7	80,3



	Number of cells responding		
Total cells	ACPA (1 µg/ml)	CAP (0,5 µg/M)	% CAP
24	0	8	33

Figure 14: *In vitro*, ACPA does not directly activate sensory neurons. ACPA stimulation of sensory neurons promotes minimal increase of intracellular calcium levels, comparable to FT controls (A). Moreover, none of the cells stimulated with ACPA was depolarized in electrophysiological recordings (B). FT is used as negative control, while capsaicin (CAP) and KCl as positive controls.

Prompted to find other cell targets that could be involved in explaining ACPA pro-nociceptive properties, we stained mouse bone and joints with ACPA and indeed found that they bind to osteoclasts. Moreover, mouse osteoclasts stimulated with ACPA, but not FT, released one of the mouse interleukin-8 (IL-8) analogues (CXCL1) *in vitro*. This is in accordance with another work from our collaborators where they showed that human osteoclasts release IL-8 when stimulated by ACPA *in vitro* (Krishnamurthy et al., 2016).

Since sensory neurons express CXCR2, the receptor for CXCL1/2, they can be sensitized by these chemokines (Qin et al., 2005; Wang et al., 2008; Zhang et al., 2013). In fact, CXCL1/2 have been shown to promote neuronal sensitization *in vitro*, increasing ion currents and TRPV1 activity, and to induce pain-like behavior when injected peripherally or centrally into mice (Cunha et al., 2005; Dong et al., 2012; Guerrero et al., 2012; Yang et al., 2009; Zhang et al., 2013). To test the role of CXCL1/2 *in vivo* after the injection of ACPA, we used reparixin, a CXCR1/2 receptor

antagonist. In the published paper, we showed that monoclonals Abs produced from synovial B cells sorted from RA patients, induced hypersensitivity, which was partially reversed by reparixin (Figure 15).

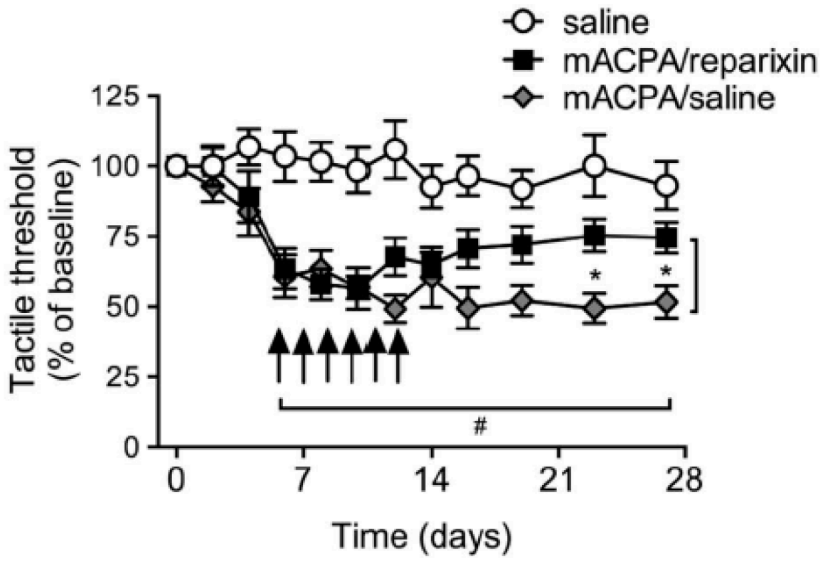


Figure 15: Antagonizing CXCL1/2 receptor partially reverses monoclonal "ACPA"-induced mechanical hypersensitivity. Monoclonal Abs, initially thought as ACPA, but later proven not to have specific citrulline reactivity, induced hypersensitivity measured with von Frey filaments, which is partly reversed by several injections of reparixin (n = 9). Data are presented as mean ± SEM. * or #, $p < 0.05$; comparing saline and mACPA/saline (#) or mACPA/reparixin and mACPA/saline (*).

However, while the monoclonal Abs used for this experiment were initially reported to have citrulline peptide reactivity, later developed assays showed that they do not actually display such reactivity and cannot therefore be considered ACPA. A correction statement has been published to inform the scientific community that the effects promoted by these Abs cannot be attributed to citrulline reactivity. However, we repeated the same experiment using polyclonal ACPA (total IgGs isolated from patients), similar as the one used for figures 13 and 14, and found that reparixin as well as zoledronate, an osteoclasts inhibitor, prevent ACPA-induced hypersensitivity (Figure 16). Although these data are not yet published, the results support a functional connection between ACPA, CXCL1/2, osteoclasts and nociception *in vivo*.

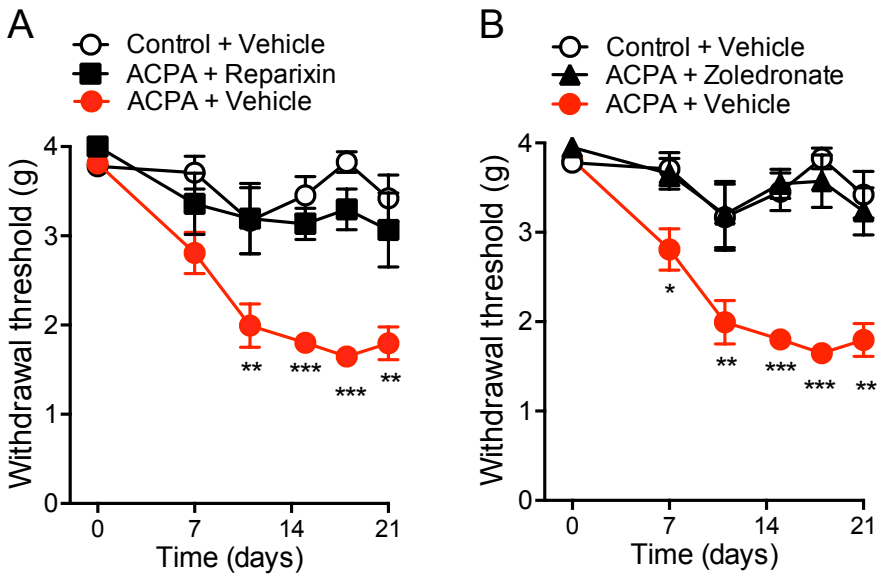


Figure 16: Antagonizing CXCL1/2 receptor or inhibiting osteoclasts prevent ACPA-induced mechanical hypersensitivity. ACPA induced hypersensitivity measured with von Frey filaments is prevented by pre-treatment with reparixin (A; n = 6-9) or zoledronate (B; n = 6-9). Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ compared to saline controls. Unpublished data.

In conclusion, here we showed the possible pathological role of human ACPA in driving pain in RA through osteoclasts activation and CXCL1 release, which subsequentially sensitizes neurons. However, we cannot exclude that also other pathological mechanisms could be promoted by ACPA, such as ACPA-IC stimulation of neurons via FcγRI or direct effect on neurons through ACPA's Fab region site in a long-term stimulation setting.

While more studies are needed to further elucidate even more deeply the pathological role of ACPA, we here show another novel indirect mechanism by which autoantibodies contribute to neuronal sensitization other than the classical inflammatory pathways. This could majorly change the way we treat the ACPA-positive subgroup of RA patients, providing new cells of interest in osteoclasts and novel potential therapeutic targets in IL-8 and all the connected pathways. Most importantly, these clinical implications affect even the early stages of the disease and/or when the disease is under control or in remission, which nowadays represent the main challenge in RA pain-management.

4.3 Study III: Characterization of neuroblastoma cell lines as possible alternative to primary DRG neuronal cell cultures for nociception-associated studies

Rodent primary cultures of sensory neurons are often employed to address mechanistic aspects in pain studies. In this thesis, we used mouse DRG cultures for *in vitro* assays both in **Studies I and II**. Primary neuronal cultures present not only experimental difficulties (for instance poor transfection efficiency) and high costs, but also an ethical dilemma for the elevated number of animals sacrificed. Thus, during the past decades in the neuroscience field, many approaches to find viable alternatives to primary neuronal cultures were developed. Examples of these strategies include stem cells differentiation or fibroblasts reprogramming into neuronal-like cells, which still exhibit several limitations for cost and feasibility matters.

Furthermore, rodents' neuroblastoma cell lines, such as B35, Neuro-2a (N2a) or F11, have been widely used in pain-related studies. Although they pose several advantages, such as low costs, high cell numbers and transfection efficiency, little information is available in the literature about how these cell lines compare to primary DRG cultures regarding overall similarity and therefore if they really represent a valuable alternative *in vitro* method.

In **Study III**, we aimed at testing how B35, N2a and F11 cell lines relate to each other and to mouse primary sensory neurons cultures concerning several pain-associated features. We further evaluated if and how two differentiation protocols (serum starvation or a cocktail of differentiation factors) would promote a more neuronal-like phenotypical change in the cell lines.

Initially, we examined the effects of the above mentioned differentiation media on the cell lines' morphology. While N2a and F11 cells exhibited no obvious morphological changes, B35 cells assumed a more neuronal-like phenotype, such as longer neurites and more complex network structure, upon differentiation. However, proliferation and/or metabolic activity, which if reduced are known to correlate with a more neuronal-like phenotype (Cho et al., 2001; Shea et al., 1985), were partially reduced in both B35 and F11 cells using one or the other differentiation media. Surprisingly, N2a cells showed an increase in both these parameters upon differentiation.

Next, we examined the expression of several neuronal markers highly expressed in DRG neurons at both mRNA and protein levels. Details about these results can be found in the correlated **Study III** manuscript.

Importantly, at a protein level, for all of the markers tested, N2a displayed the highest signal intensity followed by F11 compared to B35 cells. In all the three cell lines,

differentiation protocols generally improved the markers' expression, but N2a cells still remained the most neuronal-like on this aspect. Noteworthy, none of the cell lines expressed detectable CGRP levels. Thus despite their high *TrkA* mRNA expression and previous reports showing F11 cells releasing substance P (Puttfarcken et al., 1997), these cells cannot be considered peptidergic.

Finally, we evaluated functional activity in the cell lines measuring their excitability after stimulation with a depolarizing agent as KCl. The readout of intracellular increase of calcium was utilized to analyze cellular activation and compare the cell lines and the differentiation protocols to mouse primary DRG cell cultures. For this assay, F11 showed both higher number of cells and amplitudes of responses to KCl followed by N2a compared to B35 cells, which in fact displayed minimal and under-threshold responses. Interestingly, upon differentiation F11 cells showed lower number of responding cells and therefore a less neuronal-like phenotype. These data correlate with voltage-gated calcium channels' expression where F11 showed the highest expression of *Cacna2d1* mRNA and with previous studies showing higher calcium responses in F11 compared to N2a after ATP stimulation (Vetter and Lewis, 2010). However, while F11 cells' results seem to be the best among the three cell lines, they were still significantly lower, both in number of responses and amplitudes, compared to those of primary DRG cells (Figure 17). This could be explained with a limited and overall lower ion channels' expression in the cell lines compared to mouse DRGs.

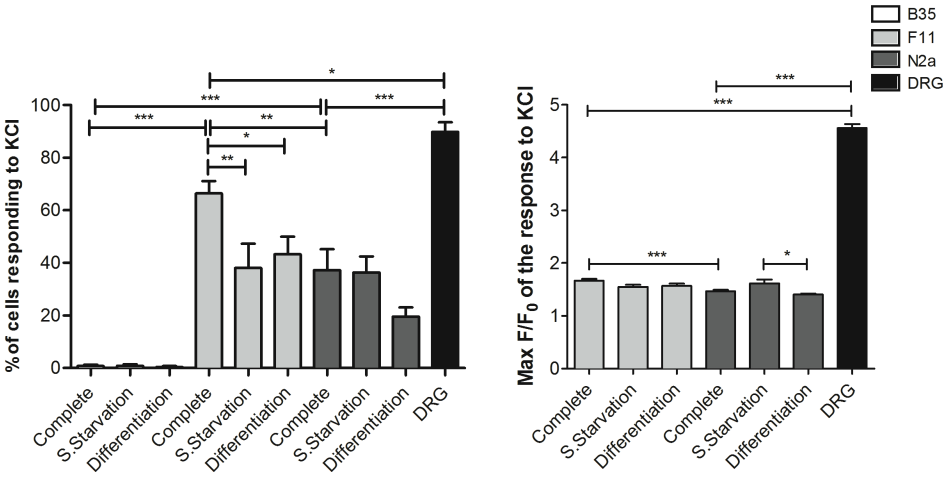


Figure 17: Number and amplitude of B35, N2a and F11 cells' responses to KCl-induced depolarization. In calcium imaging experiments, F11 in complete medium showed the highest number of responses and amplitudes, followed by N2a cells among the different cell lines. B35 displayed minimal responses to KCl application. Primary DRG neurons showed significantly higher responses both in number and amplitudes compared to any of the cell lines in any of the culture conditions. Data are presented as mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$ compared to saline controls.

Taken all the data together, while all of the cell lines with or without the differentiation protocols showed particular neuronal-like properties in some of the analyzed parameters, they overall compare only to some extent to mouse primary DRG cultures and hence cannot replace them completely. We consider these data a valuable source of information to enable neuroscientists to carefully select a particular cell line or culture condition for a specific experiment. However, the take-home message of **Study III** is that, even though primary DRG cultures also do not mimic completely the *in vivo* pain processes, they are still the most preferable choice for *in vitro* nociception-associated experiments.

5 FUTURE PERSPECTIVES AND CONCLUSIONS

In this thesis we focused on exploring novel mechanisms involved in nociception in autoimmune diseases, specifically rheumatoid arthritis (RA).

In **Study I**, we showed in a mouse model of RA that autoantibodies in immune complex (IC) formation can directly activate neuronal FcγRI and therefore promote pain independently of inflammation. Noteworthy, a paper published by an independent group (at the same time as ours) shows data completely in line with our results. They found that IC-induced pain was decreased in mice with a conditional deletion of FcγRI in Nav1.8 expressing nociceptive neurons (Wang et al., 2019). Moreover, our work is the first demonstration that FcγRIIb is also present in sensory neurons. However, its functional role in regulating IC-induced hypersensitivity is unknown and should be explored. Indeed, future studies are warranted to examine changes in neuronal FcγRs expression in reaction to inflammation or disease states, since this could potentially enable an increased capability to respond to IC.

In order to increase the translational aspects of our studies, we strive to use disease-related animal models and when possible to validate our findings in human material. For instance, our discovery of a homologue receptor, FcγRIIIa, on human sensory neurons could provide support for the strategy of targeting FcγRs, antibody production or antibody recycling as avenues to approach for treatment or prevention of the early and persistent pain in RA. Most of the currently used drugs do not affect the FcγRs signaling pathway directly, but target the immune system through other types of intervention. Therefore, autoantibody activation of sensory neurons would not be inhibited and this could explain why pain is such a difficult symptom to treat in these patients. However, drugs targeting FcγRs or their intracellular pathway in immune cells could potentially have similar effects in the neuronal setting. Several drugs recently developed following this approach are currently studied in clinical trials for several autoimmune diseases (Zuercher et al., 2019). Our hope is that acting on neuronal FcγRs, these drugs could promote also a better pain-relief outcome.

Furthermore, our ambition to undertake studies with high disease-relevance is demonstrated in **Study II,** where we use anti-citrullinated protein antibodies (ACPA) purified from RA patients to examine their possible link to nociception. We found that ACPA induce pain-like behavior when injected into mice without generating activation of classical inflammatory processes. Thus, also in this case our work suggests that RA-associated autoantibodies can be coupled to pain through novel mechanisms. Importantly, further studies are needed to deepen our understanding of how ACPA activate sensory neurons, in particular in the light of the first generation monoclonal “ACPA” not having the citrulline reactivity that was initially proposed. Nonetheless, we were first to show that a reverse translational approach, using

human antibodies in a serum transfer pre-clinical model, can be successfully implemented to study RA pain-pathology. This is an expanding field as the same approach has been used for other diseases with pain components (Dawes et al., 2018; Tekus et al., 2014). We strongly believe that this new concept of performing pre-clinical research will enable a stronger connecting bridge between researchers, doctors and patients, which could potentially provide more valuable data and clinical impact.

Finally, in **Study III**, we showed the importance of characterizing alternative *in vitro* methods compared to primary cultures. Although neuroblastoma cell lines cannot be considered a complete substitute to dorsal root ganglia cultures, they can still be utilized for specific experiments in the investigation of particular nociceptive processes. Continuing to invest in this field will eventually identify an equivalent strategy to substitute primary cultures hence reducing the animal use in scientific experiments.

In conclusions, the pre-clinical work showed in this thesis has provided several novel molecular insights that could explain unknown pain mechanisms in autoimmune diseases. While further studies are needed to know if the translational impact of this work will be substantial, several indications hinted the potential of this work to positively affect pain management in RA, with the ultimate hope of improving patients' quality of life.

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