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PATHOLOGICAL RESPONSES OF GLIAL CELLS IN SPINAL CORD INJURY AND RHEUMATOID ARTHRITIS

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Pathological responses of glial cells in spinal cord injury and rheumatoid arthritis

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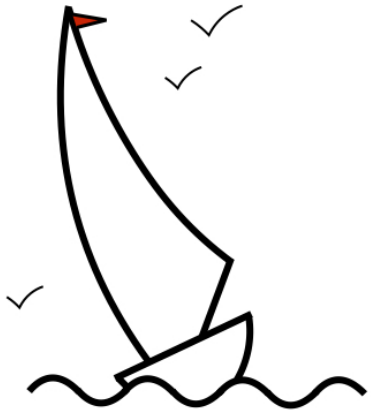


Smooth seas do not make skillful sailors

- African proverb

ABSTRACT

Scientists have considered glia as mere passive allies of neurons for a long time. As a consequence, their functions have been greatly underestimated. Major discoveries made in the last three decades have changed our view on glial cells and it is now accepted that they play important roles in health and disease. In this thesis, we have investigated the role of 3 glial cell types - astrocytes, microglia and ependymal cells - in spinal cord injury (SCI), rheumatoid arthritis (RA) and in pain-related processes.

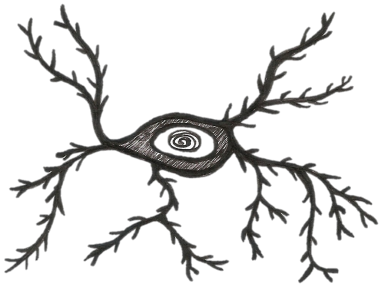
In **Study I**, we have delineated the mechanisms that regulate interleukin-6 (IL-6) expression and secretion in adult rat astrocyte cultures. We found that the PI3K-mTOR-AKT pathway negatively regulates IL-6 expression and that IL-6 secretion is calcium (Ca^{2+})-dependent. Interestingly, we observed that astrocytes express IL-6 *in vivo* after SCI, however IL-6 levels decline after 2-3 weeks. Since induction of IL-6 in reactive astrocytes could be beneficial due to the regenerative properties of this cytokine, we treated adult rats 2 weeks after SCI with mTOR inhibitors, torin2 and rapamycin, to boost astrocytic IL-6 secretion by blocking the PI3K-mTOR-AKT pathway and increasing cytosolic Ca^{2+}, respectively. This combinatorial treatment led to a transient improvement in mechanical hypersensitivity during the treatment period.

In **Study II**, we have established an adult *ex vivo* model of SCI, to facilitate the study of cellular processes that are difficult to address using animal models. In particular, we focused on assessing the ependymal cell response to injury in our model, which is based on adult mouse spinal cord cultured tissue slices. Interestingly, we found that, ependymal cells become activated, proliferate, migrate out of the ependymal layer and differentiate in a manner that fundamentally resembles their response to injury *in vivo*. Moreover, we show that these cells can respond to external adenosine triphosphate (ATP) stimulation and that some of them have spontaneous Ca^{2+} activity. We believe that this model is a useful platform to study and modulate ependymal cell responses and could contribute to the development of novel treatment avenues for SCI.

In **Study III**, we have investigated mechanisms that may participate in central sensitization in the context of RA. Here, we report for the first time the presence of disease associated autoantibodies known as ACPA (anti-citrullinated protein antibodies) in the cerebrospinal fluid (CSF) of a subset of RA patients. Moreover, we show that intrathecal injection of such antibodies into the CSF of mice led to pain-like behavior, while injection of other antibodies from RA patients or from healthy individuals did not. Furthermore, we show that co-stimulation of human astrocytes in culture with ACPA and interleukin-1beta (IL-1ß) led to IL-6 secretion in these cells, an effect that was blocked upon addition of an Fc-gamma receptor 1 (FcγRI) inhibitor. These findings support the notion that ACPA may enter the central nervous system (CNS) of RA patients, act on glial cells and activate pathways that could contribute to centrally mediated pain.

In **Study IV**, we have investigated differences between male and female spinal microglia in the context of arthritis-induced persistent pain. We focused on the late phase of the collagen type-II antibody induced arthritis (CAIA) animal model, which occurs after joint inflammation has resolved and it is characterized by persistent mechanical hypersensitivity and spinal glial activation. We found that intrathecal delivery of minocycline, often described as a microglial inhibitor, was able to revert CAIA-induced pain in male, but not female mice. Moreover, using flow cytometry we found that females had lower dorsal horn spinal microglial relative numbers as compared to males. Furthermore, genome-wide RNA sequencing results pointed to several transcriptional differences between male and female microglia, while no convincing differences were identified between control and CAIA groups. Taken together, these results suggest that during the late phase of the CAIA model changes in microglial gene expression might be highly localized or short-lasting, and that the sexually dimorphic response to minocycline might additionally involve other factors such as changes in protein expression or epigenetic modifications.

In summary, this thesis expands our understanding of mechanisms that are important in glial cell responses to pathological events and opens new avenues to explore the modulation of glial cells. The ultimate hope is that continued efforts will result in the discovery of suitable targets for therapy in individuals with spinal cord injury, rheumatoid arthritis and chronic pain.



LIST OF PUBLICATIONS AND MANUSCRIPTS

I. Codeluppi S, **Fernandez-Zafra T**, Sandor K, Kjell J, Liu Q, Abrams M, Olson L, Gray NS, Svensson CI, Uhlén P. Interleukin-6 secretion by astrocytes is dynamically regulated by PI3K-mTOR-Ca^{2+} signalling. *PLoS One* (2014) Mar 25;9(3):e92649

II. **Fernandez-Zafra T**, Codeluppi S, Uhlén P. An ex vivo spinal cord injury model to study ependymal cells in adult mouse tissue. *Exp.Cell Res.* (2017) Aug 15;357(2):236-242

III. Le Maître E*****, **Fernandez-Zafra T***, Revathikumar P, Estelius J, Rogoz K, Sandor K, Lundberg K, Hansson M, Amara K, Kosek E, Khademi M, Andersson M, Malmström V, Klareskog L, Svensson CI, Lampa P. Central nervous system autoimmunity in rheumatoid arthritis: Anti-citrullinated peptide antibodies activate human astroglial cells and induce pain behaviour in mice. *Manuscript*

IV. **Fernandez-Zafra T**, Agalave N, Sandor K, Gao T, Su J, Jurczak A, Estelius J, Lampa J, Wiesenfeld-Hallin Z, Xu XJ, Denk F, Svensson CI. Exploring the transcriptome of resident spinal microglia after collagen antibody-induced arthritis. *Manuscript*

***** Contributed equally

LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS

I. Abdelmoaty S, Wigerblad G, Bas DB, Codeluppi S, **Fernandez-Zafra T**, El-Awady el-S, Moustafa Y, Abdelhamid Ael-D, Brodin E, Svensson CI. Spinal actions of lipoxin A4 and 17(R)-resolvin D1 attenuate inflammation-induced mechanical hypersensitivity and spinal TNF release. *PLoS One* (2013) Sep 24;8(9):e75543

CONTENTS

LIST OF SELECTED ABBREVIATIONS

ACPA	Anti-citrullinated protein antibody
ALDH1L1	Aldehyde dehydrogenase 1 family member L1
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain derived neurotrophic factor
Ca^{2+}	Calcium
CAIA	Collagen antibody-induced arthritis
CIA	Collagen-induced arthritis
CII	Collagen type II
CNS	Central nervous system
CreER	Tamoxifen-dependent Cre recombinase
CSF	Cerebrospinal fluid
CSPG	Chondroitin sulphate proteoglycans
DRG	Dorsal root ganglia
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
Fab	Fragment, antigen binding
FACS	Flow associated cell sorting
Fc	Fragment, crystallizable
Fcrls	Fc receptor-like S, scavenger receptor
FcγR	Fc-gamma receptors
FKBP12	FK506-binding protein of 12 kDa
FoxJ1	Forkhead box protein J1
FPKM	Fragments per kilobase of transcript per million reads
FT	Flow through
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
HLA	Human leukocyte antigen
HMGB1	High mobility group box 1
Hsp	Heat shock protein
i.t.	Intrathecal

i.v.	Intravenous
IBA-1	Ionized Ca^{2+} binding adaptor molecule 1
Ig	Immunoglobulin
IL-1ß	Interleukin-1beta
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IP_3	Inositol-1,4,5-trisphosphate
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MHC	Major histocompatibility complex
mTOR	Mechanistic target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NF-kB	Nuclear Factor Kappa B
NMDA	N-methyl-D-aspartate
p-	phosphorylated
P2X4R	Purinergic receptor P2X4
P2X7R	Purinergic receptor P2X7
PCA	Principal component analysis
PCR	Polymerase-chain reaction
PI3K	Phosphoinositide 3-kinase
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RF	Rheumatoid factor
RNA-seq	RNA sequencing
ROS	Reactive oxygen species
RyR	Ryanodine receptors
SCI	Spinal cord injury
TLR	Toll-like receptors
TNF	Tumor necrosis factor
YFP	Yellow fluorescent protein

1 INTRODUCTION

The story of glia is often considered to have officially started in 1856, when the German scientist Rudolf Virchow identified a connective tissue surrounding nerve elements which he termed *neuroglia* (from the Greek *nerve glue*) [1]. During the second part of the 19th century, the concept of glial cells started to gain ground, as a result of the discoveries made by Otto Deiters, Camillo Golgi and Gustav Retzius among others [2-4]. Michael von Lenhossek coined the term *astrocyte* in 1893, whereas the recognition of *microglia* and *oligodendrocytes* as distinct glial cell types came in 1919-1921 by the hand of Pío del Río-Hortega, a disciple of Santiago Ramón y Cajal [5, 6].

Initially, *neuroglia* was considered to be an inert element, a mere "filling" for the area void of neurons. This concept soon evolved to the idea that glia could serve as a structural support for neurons. Later on, Golgi and Cajal postulated that neuroglia could also provide nutritional support and insulation to neurons. The overall conviction was that glia were a passive ally of the neuron: the one-and-only central and active cell type of the central nervous system (CNS). Nevertheless, it was apparent already then, that these non-neuronal cells were highly heterogeneous, and could undergo changes in pathological conditions [7]. Major discoveries made in the 20th century have changed our view about glial cells. The past few decades have experienced a rise in glial research, owing to the discovery that glial cells can be excited, albeit in fundamentally different ways than neurons [8]. Moreover, it is now well-accepted that glial cells have important active roles in health and disease [9].

In this thesis, we have investigated the role of astrocytes, microglia and ependymal cells in pathological conditions. Therefore, the scope of this dissertation will be focused on the involvement of these three glial cell types in spinal cord injury (SCI), rheumatoid arthritis (RA) or pain-related processes.

1.1 GLIAL CELLS

1.1.1 Astrocytes

Astrocytes are one of the most abundant cell types in the CNS. These cells are highly heterogeneous and their morphology, function and response to injury mainly varies depending on their developmental stage, anatomical location, gene expression profile and physiological properties [10]. Astrocytes have been classically characterized into two main subtypes. Protoplasmic astrocytes are confined to the grey matter and have a bush-like shape, with thick stem branches and numerous fine processes (Figure 2). Fibrous astrocytes are distributed along white matter tracks and have a fiber-like appearance with fewer branching processes. Both subtypes are in contact with blood vessels however, while the processes of protoplasmic astrocytes surround synapses, fibrous astrocytes contact Nodes of Ranvier [11].

Astrocytes are organized in non-overlapping domains, where only the fine processes of neighboring astrocytes interdigitate and form gap junctions, creating a highly dynamic astrocyte network [12]. Astrocytes are not electrically excitable and instead rely on calcium (Ca^{2+}) signals for communication [13]. Astrocytes can increase intracellular Ca^{2+} both in localized regions of their fine processes and in their soma [14]. Such cytosolic Ca^{2+} elevations can occur in response to external stimuli such as neurotransmitters and by Ca^{2+} release from intracellular stores [12]. Notably, Ca^{2+} signals can be oscillatory and confined to a single astrocyte or they can propagate from one astrocyte to another as a Ca^{2+} wave by diffusion of inositol 1,4,5-trisphosphate (IP_3) through gap junctions and by extracellular release of adenosine triphosphate (ATP) [15]. Astrocytic Ca^{2+} signalling plays an important role in signal transduction, for example by regulating the release of gliotransmitters and cytokines [16, 17].

The wide heterogeneity of astrocytes makes it challenging to find a unique and specific marker that can label them all. Glial fibrillary acidic protein (GFAP) has been used as the prototypic astrocyte marker for over 30 years [18]. Nevertheless, it is now clear that GFAP is not expressed by all astrocytes and that other cells in the CNS can express GFAP (see **Study II**). The most promising marker for astrocytes is perhaps aldehyde dehydrogenase 1 family member L1 (ALDH1L1), which was elucidated by transcriptomic analysis, revealing that it is highly, broadly and specifically expressed in astrocytes [19].

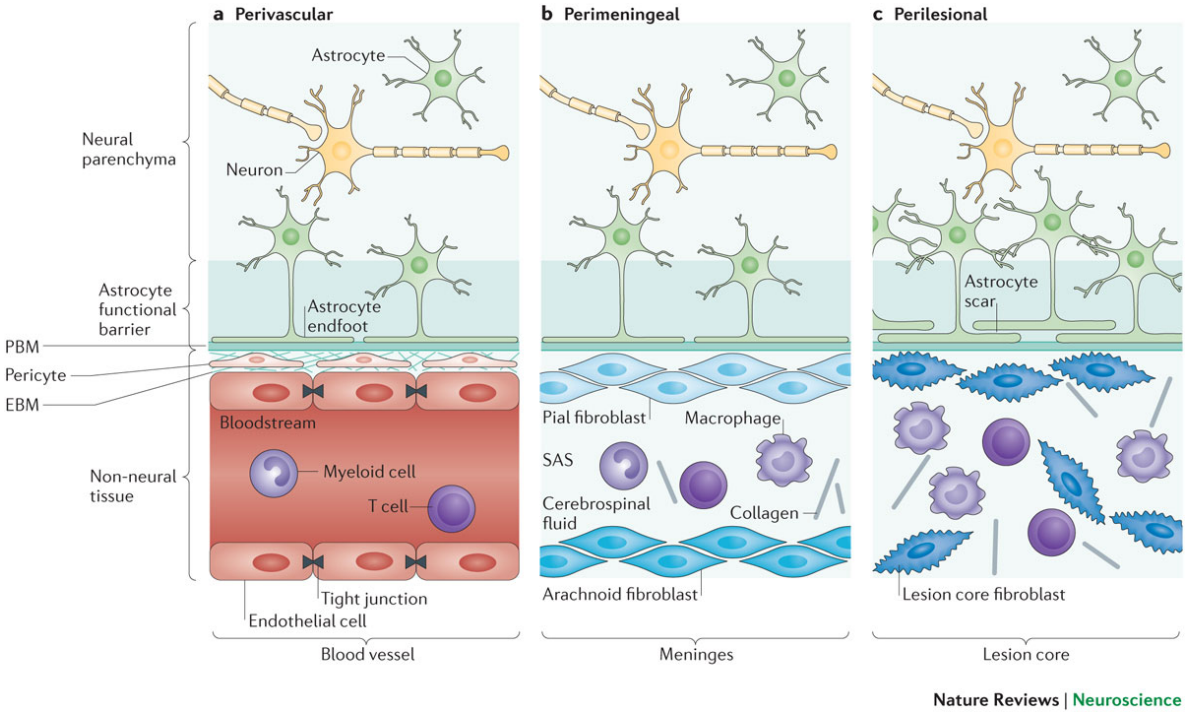


Figure 1 Astrocytes form functional barriers (*glia limitans*) that separate neural from non-neural tissue along the vasculature **(a)** and the meninges **(b)**. Astrocyte scars, which are fundamentally similar to the *glia limitans*, separate CNS lesions from the healthy parenchyma **(c)**. The *glia limitans* works in concert with other functional barriers such as the brain or spinal-cord blood barrier to restrict the entrance of leukocytes into the CNS parenchyma. PBM = parenchymal basement membrane; EBM = endothelial basement membrane; SAS = subarachnoid space. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Neuroscience, Sofroniew, M.V. *Astrocyte barriers to neurotoxic inflammation* © (2015)

Astrocytes play important roles in the formation, maintenance and pruning of synapses thus controlling the connectivity of neuronal circuits [9]. Moreover, it has been proposed that astrocytes can directly influence and regulate synaptic transmission [20]. Furthermore, astrocytes provide essential metabolic support to neurons, regulate blood flow and maintain extracellular homeostasis [11]. Astrocytes also form barriers known as *glia limitans* that separate neural from non-neural tissue along perivascular spaces and the meninges. The *glia limitans* works in concert with other functional barriers, such as the blood-spinal cord/brain barrier to restrict CNS inflammation (Figure 1) [21].

1.1.2 Ependymal cells

Ependymal cells are mostly known as the glial cell type that lines the central canal of the spinal cord and the brain ventricles (Figure 2). These cells circulate cerebrospinal fluid (CSF) and regulate the bi-directional transport of molecules between the CSF and CNS parenchyma [22, 23]. Ependymal cells are heterogeneous, and have been broadly divided into three types. Cuboidal ependymal cells are multi-ciliated and are considered the most abundant cell subtype. Tanycytes have one luminal cilium and one basal process that can contact blood vessels. Radial ependymal cells are far less numerous and are found in the dorsal and ventral poles of the central canal lining, where they extend long processes along the dorso-ventral axis [24, 25].

Ependymal cells express markers characteristic of neural stem cells and precursors such as Vimentin, Nestin, Sox2, Sox9 and CD133/prominin-1 [24, 26, 27]. Some of these markers are expressed throughout the ependymal layer, while others are restricted to certain ependymal subpopulations (see for example **Study II**). In the intact spinal cord, adult ependymal cells are mostly quiescent, occasionally self-renewing to maintain their numbers [28, 29]. This proliferation is not spread evenly distributed, and it mostly occurs in ependymal cells of the dorsal region of the central canal in close proximity to blood vessels [26]. Lineage tracing experiments have demonstrated that ependymal cells hold neural stem potential, as these cells can from neurospheres across multiple cell passages and can differentiate into astrocytes, oligodendrocytes and neurons *in vitro* [24, 30]. This has prompted a vast interest on the study of these cells for regenerative therapies.

Like in astrocytes, Ca^{2+} signalling appears to play an important, albeit understudied role in ependymal cells. For instance, Ca^{2+} signalling regulates ciliary beating of brain ependymal cells, which is essential for circulating CSF [31]. Moreover, brain ependymal cells can respond to ATP via P2X7 purinergic receptors (P2X7R), leading to an increase in intracellular Ca^{2+} levels [32, 33]. The exact role that Ca^{2+}signaling plays in spinal cord ependymal cells remains unknown. Nevertheless, these cells possess gap junctions [34-36] which can potentially enable ependymal-ependymal or even ependymal-astrocyte communication.

1.1.3 Microglia

Microglia are the predominant resident immune cells of the CNS (Figure 2). In mice, they originate from primitive myeloid in the yolk-sac and invade the CNS during early embryonic development, proliferating and spreading ubiquitously across the parenchyma thereafter [37, 38]. Although this cell population was thought to be long-lived [39], new evidence has shown that microglia have a high turnover rate in both rodents and humans, allowing a complete self-renewal of their whole population one to several times across a lifetime [40-42]. Under physiological conditions, microglia exhibit a ramified morphology, with a small soma and multiple fine processes that survey the CNS parenchyma about every hour [43]. Like astrocytes, each microglial cell has its own territory, and microglial processes rarely overlap with each other [44]. Microglia have the ability to phagocytose, which is essential for clearing debris [45] and for synaptic pruning during development and the postnatal period [46, 47]. Moreover, they can also monitor neuronal activity and synaptic plasticity [48, 49] and regulate adult neurogenesis [50]. Several studies examining microglial expression profiles have reported that microglia display heterogeneity among several brain regions [51, 52]. Furthermore, microglial sex differences under physiological conditions have also been described. Of note, microglial density varies among males and females during development and adulthood, in areas such as the amygdala, hippocampus and parietal cortex [53, 54].

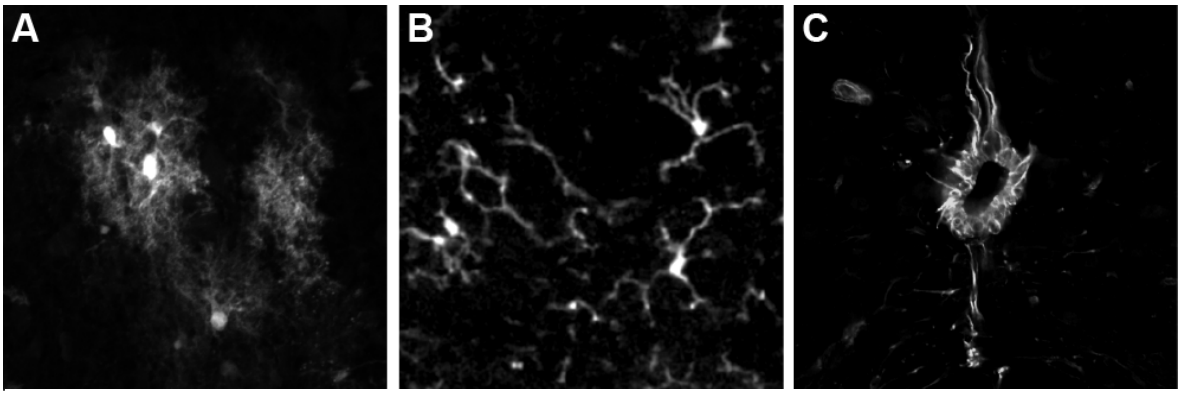


Figure 2 A) Transgenic *hGFAP*-GFP protoplasmic astrocytes **B)** Dorsal horn microglia stained for IBA-1 **C)** Ependymal cells stained for Vimentin surrounding the central canal of the spinal cord.

1.2 GLIOSIS

Glial cells can become "activated" or "reactive" in response to changes induced by trauma or pathological events [55, 56]. A large repertoire of molecules which can be released by different cell types can elicit or regulate diverse aspects of gliosis. Some of these include cytokines and growth factors such as interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) or fibroblast growth factor 2 (FGF2), neurotransmitters (norepinephrine, glutamate), purines (ATP) and reactive oxygen species (ROS) [11]. In this section, general characteristics of astrogliosis and microgliosis are described (relevant for **Study I**, **Study III** and **Study IV**), while SCI induced responses in astrocytes and ependymal cells (relevant for **Study I** and **Study II**) will be discussed in more detail further below.

1.2.1 Astrogliosis

Reactive astrogliosis (or astrocyte reactivity) is a process by which astrocytes undergo a series of morphological, cellular, molecular and functional changes in response to injury or disease [57]. These changes occur in a context-specific manner and take place in a graded fashion according to the severity of the insult, and they can affect surrounding tissue in a beneficial or detrimental way [10]. Mild to moderate reactive astrogliosis is characterized by upregulation of genes such as GFAP (the most commonly used hallmark of reactive astrocytes) and hypertrophy of the cell body and processes. Despite this, astrocytes preserve their non-overlapping individual domains and astrocyte proliferation is minimal or absent. This type of astrogliosis has the potential to resolve if the triggering insult ceases [10, 11, 57]. However, with overt tissue damage and inflammation, reactive astrogliosis can become severe, in which case astrocytic processes start to overlap into each other's domains. This event is often accompanied by extensive astrocyte proliferation and tissue re-organization. Moreover, astrocytes (including those newly proliferated) can gather around the borders of the damaged tissue and interact with other cell types to form glial scars (discussed later on). Structural changes associated with severe astrogliosis are persistent and do not resolve even if the initial insult is no longer present [10, 11, 57].

1.2.2 Microgliosis

Microglia, unlike other glial cell types, are particularly sensitive to alterations in the extracellular environment. Consequently, they are typically the first cell type to respond after injury or disease [58]. Reactive microglia undergo multiple changes, including the upregulation of surface antigens and receptors that are characteristic of innate immune responses. For example, microglial cells can take part in antigen presentation by upregulating major histocompatibility complex (MHC) class II molecules [59-61]. Reactive microglia alter their morphology and transform from a ramified to a more amoeboid shape. Cell bodies become enlarged and microglial cell processes become thicker and shorter, reducing the area that they normally cover [44]. This configuration is associated with a high degree of microglial activation, which is characterized by phagocytosis and pro-inflammatory functions [62]. Moreover, microglia markedly proliferate to increase their numbers, which potentiates their response after an insult [58, 63].

Ionized calcium binding adapter molecule 1 (IBA-1) is a common marker for resting microglia [64]. However, since this protein is well distributed around the cytoplasm and processes of microglial cells, it allows the visualization of morphological changes associated with microgliosis with ease [65]. Moreover IBA-1 reactivity increases in reactive microglia, hence this protein is often used as a marker of microgliosis [64, 65]. A similar marker used is CD11b, which in rats can be detected with a monoclonal antibody known as OX42 (see **Study I**). It is important to mention that the marker profile of microglia and blood-derived macrophages is remarkably similar. Therefore, when macrophages infiltrate the CNS, it becomes a daunting task to distinguish these two populations. The scientific community has tried to addressed this problem by identifying proteins that are more highly expressed in microglia, and to develop antibodies that can

target them specifically. Promising candidates include the Fc receptor-like S, scavenger receptor (Fcrls; used in **Study IV**), transmembrane protein 119 (Tmem119) or P2Y12 purinergic receptor (P2Y12R), which, in some contexts and with particular techniques can serve to discriminate resident microglia from blood-derived macrophages [66, 67].

1.3 SPINAL CORD INJURY

Every year, 54 people per million (mostly young or older males) suffer from traumatic spinal cord injury (SCI) in the U.S. alone, due to vehicle accidents, falls, violence and sport incidents [68]. SCI often leads to paralysis below the injury level and results in sensory and motor problems deficits of variable degree. Some of these include breathing difficulties, loss of bladder and bowel control, numbness, emotional changes and chronic pain. Despite medical advances, there is still no cure for SCI [69, 70].

Traumatic SCI occurs when an initial mechanical insult results in the compression, contusion or laceration of the spinal tissue. This initial insult damages neurons and surrounding glia, disrupts the vasculature and causes ischemia and edema [71, 72]. Tissue damage and cell death get exacerbated as a result of secondary events that include sustained inflammation, excitotoxicity and loss of blood-spinal cord barrier integrity [73-76]. Multiple cell-types get recruited to the injury site, coming both from the periphery and the spinal cord itself. Immune cells such as neutrophils, macrophages and lymphocytes infiltrate the spinal parenchyma and accumulate in the core of the lesion [77]. Moreover, through the release of pro-inflammatory factors, activated microglia/macrophages promote vascular permeability and the recruitment of more immune cells to the lesion epicenter. Even though such immune response is vital for the clearance of debris and degenerating tissue, it creates a hostile inflammatory environment that aggravates ongoing apoptosis and tissue damage [78-80]. This sustained cellular death promotes the formation of cystic cavities that are mainly comprised of extracellular fluid [72]. Additionally, activated macrophages, but not microglia, can phagocytose axonal fragments in areas of severe inflammation, contributing to the axonal dieback of injured neurons [81-83]. Following SCI, astrocytes become reactive and contribute to the formation of a glial scar (Figure 3) that surrounds the lesion core (discussed later on) [57]. Moreover, ependymal cells also get activated, increase their proliferation and migrate towards the lesion epicenter, mostly giving rise to scar-forming astrocytes (see below) [24, 30]. A fibrotic scar also develops at the lesion core, which is characterized by excess deposition of extracellular matrix molecules by stromal cells that invade the lesion epicenter [84-86]. In penetrating injuries where the meninges are disrupted, meningeal fibroblasts have been observed in the lesion core [84]. Additionally, in this type of injury, stromal cells derived from type-A pericytes can also infiltrate the lesion epicenter and contribute to the fibrotic scar. In fact, genetic ablation of type-A pericyte progeny results in an incomplete closure of the lesion [85]. Another study has reported that in response to contusion SCI, where the meninges are intact, perivascular fibroblasts are another major cell population that participates in the fibrotic scar [86]. Of note, NG2 oligodendrocyte progenitors can also proliferate and migrate towards the lesion core in response to injury [87]. For instance, it is thought that a

subpopulation of these cells is able to stabilize dystrophic axons within the aggressive inflammatory environment present in the lesion core [88, 89]. Moreover, NG2 oligodendrocyte progenitors found in demyelinated areas can differentiate into mature oligodendrocytes and contribute to axonal remyelination [90-92]. However, newly synthetized myelin sheets display immature structural properties and might not provide a complete restoration of axonal conductance [93].

Although there is no treatment for SCI, many clinical trials are ongoing. Current efforts are typically aimed at protecting spared tissue, promoting axonal regeneration and replacing lost nerve cells [71]. Indeed, experimental approaches have demonstrated that axonal regeneration through the inhibitory environment found in the lesions is actually possible [94]. Some of the most attractive regenerative strategies include stem cell/progenitor transplantations, endogenous stem cells/progenitor modulation, delivery of neurotrophic factors, peripheral nerve bridges, electrical stimulation and blockade of growth inhibiting factors [95, 96].

1.3.1 Astrocytes in SCI

Astrocytes located in the vicinity of the lesion site respond to injury-induced factors by becoming reactive. A graded degree of astrogliosis is commonly observed, which ranges from mild to severe depending on the proximity to the lesion core [11]. Astrocyte migration and proliferation around the margins of the lesion epicenter initiate the formation of an astrocytic scar [97-99]. Such astrocytic proliferation is rather moderate, peaking one week after injury and gradually declining after that [100-102]. The astrocytic scar consists of a mesh-like structure of intermixing hypertrophic cellular processes, which creates a physical

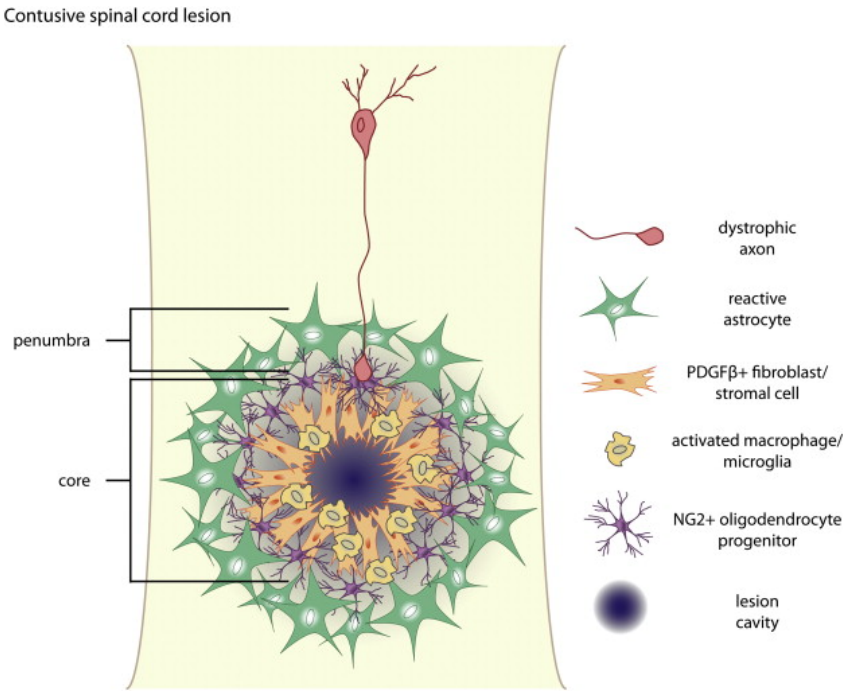


Figure 3 Diagram depicting the layered architecture of the glial scar in a contusive SCI. The lesion penumbra is comprised of reactive astrocytes (astrocytic scar) while the lesion core contains NG2 oligodendrocyte progenitors associated to dystrophic axons, leukocytes (not depicted), microglia/macrophages and fibroblasts/stromal cells. Reprinted from Cregg J.M. et al; *Functional regeneration beyond the glial scar* Experimental Neurology. 253:197-207 © 2014 with permission from Elsevier.

barrier between the core lesion and the surrounding parenchyma. This scar is typically formed two weeks after injury and matures after three weeks [57]. It has been estimated that around 85% of the astrocytes that comprise the astrocytic scar are newly proliferated [103].

The role of reactive astrocytosis and astroytic scar formation has been subject for debate over the years, and while it was first associated with detrimental effects, beneficial roles have also been described. For instance, by creating a barrier between the injured area and the healthy parenchyma, the astrocytic scar confines intense inflammatory processes to the lesion epicenter while limiting the spread of immune cells or damaging factors towards unaffected areas [57]. Certainly, inhibiting the formation of the astrocytic scar exacerbates tissue damage, cell death and demyelination, due to widespread inflammation and aberrant blood-spinal cord barrier permeability that result in larger lesions and a worse functional outcome [97-100, 104, 105]. On the other hand, reactive astrocytes can release molecules that have inhibitory effects towards axonal regeneration, such as for example, chondroitin sulphate proteoglycans (CSPG). Of note, enzymatic depletion of CSPG promotes axonal regeneration and results in improved locomotor recovery [106]. Furthermore, factors released by astrocytes, such as cytokines, can aggravate inflammatory processes and enhance the production of molecules such as ROS and glutamate that become neurotoxic at elevated levels, exacerbating tissue damage around the lesion [11].

Altogether, reactive astrocytes appear to play a crucial role in protecting spared intact tissue from inflammatory processes. However, this beneficial effect seems to come with the cost of inhibiting axonal regeneration.

1.3.2 Ependymal cells in SCI

Upon SCI, ependymal cells become activated and undergo transcriptional changes that result in the upregulation or *de novo* expression of proteins such as GFAP and Nestin [26, 107, 108]. Moreover, their ability to form neurospheres *in vitro* is greatly enhanced upon injury [30]. In most experimental models of SCI, the proliferation of ependymal cells begins 24 hours after SCI, peaking 3 days after injury and gradually returning to basal levels within three to four months. Of note, transection models (penetrating injuries) often present a greater albeit shorter and more localized proliferative response than contusion or compression models (non-penetrating injuries) [109]. There is conflicting evidence as to whether the ependymal layer must be directly damaged to induce ependymal proliferation. While some groups have reported ependymal proliferation exclusively after directly lesioning these cells [103, 110], others have observed ependymal proliferation when eliciting injuries that do not reach the ependymal layer [24, 28, 30, 111]. Interestingly, ependymal proliferation rates have been correlated with motor function recovery in studies carried out in rodents [108, 109] and lower vertebrates [112, 113].

In addition to their proliferative response, ependymal cells can generate progeny of the glial lineage, mainly comprised of astrocytes [24, 28, 30, 103, 111] and a small percentage of

oligodendrocytes [24, 30]. In transection models, the newly generated ependymal-derived progeny migrates towards the center of the transection gap and contribute to the glial scar with one third to half of all the scar-forming astrocytes [30]. Ependymal-derived astrocytes express lower levels of GFAP in relation to vimentin, which is indicative of a more immature phenotype. Interestingly, these astrocytes do not appear to secrete CSPGs and thus do not interfere with axonal sprouting [24]. Notably, recent reports using compression SCI have shown that ependymal cells do not migrate extensively and that their contribution to glial scar formation is much more limited than in transection models [103, 114].

Taken together, it is evident that the ependymal response differs according to the severity and the injury type. Nevertheless, these cells have the capability of proliferating, migrating and differentiating intro other cells types *in vivo*, although the proportion of the progeny produced may differ. It is important to mention that while ependymal cells *in vitro* can differentiate into neurons, this is not the case in the intact or injured spinal cord *in vivo* [24, 28, 30, 111]. This suggests that the extracellular milieu influences or may even dictate the fate of the ependymal cell progeny and may hinder ependymal differentiation into neurons. Indeed, the disparities in the ependymal cell response that are observed among injury models might also be explained by intrinsic differences in the extracellular environment.

The fact that ependymal cells are essential for the complete repair of the CNS in lower vertebrates [112, 115] has recently attracted scientists to decipher the function of these cells in adult mammals. A growing body evidence indicates that ependymal cells have beneficial effects in rodent models of SCI. Interestingly, genetic ablation of ependymal-derived progeny has revealed that these cells are important for restricting neuronal loss and secondary tissue damage that could result in larger lesions [116]. Moreover, ependymal cells appear to be essential for sealing off the disrupted central canal after SCI [103]. In addition, experimental transplantation of adult spinal cord-derived neural stem cells into the injured spinal cord can promote functional recovery [117]. These concepts demonstrate that ependymal cells have the ability to facilitate tissue repair in adult mammals, making them an attractive target for the treatment of SCI.

Strategies aimed at modulating ependymal cells *in situ* hold great promise, as ependymal cells could potentially be reprogrammed to differentiate into neurons and oligodendrocytes to enhance tissue regeneration [118]. Indeed, the study of ependymal cells in an injury setting would be essential for elucidating mechanisms that facilitate ependymal cell reprogramming in a milieu that does not favour neurogenesis. In **Study II**, we have addressed this need by establishing an accessible *ex vivo* model that allows the study and modulation of ependymal cells in an environment that resembles that of an injury state.

1.3.3 IL-6 and SCI

IL-6 is a pleiotropic cytokine that plays an important role in response to injury. Current evidence suggests that IL-6 can have a dual role in regeneration, depending on the degree, temporal expression and the balance between neurotoxic and neuroprotective effects [119-121]. During the early stages of SCI, IL-6 potentiates the inflammatory cascade and negatively affects axonal regeneration by promoting glial scar formation [122]. On the other hand, several studies have also suggested that IL-6 can act as a neuroprotective factor promoting axonal regeneration. IL-6 application has been shown to reduce ischemic brain damage *in vivo* and N-methyl-D-aspartate (NMDA) receptor mediated excitotoxicity *in vitro* [123-125]. Moreover, mice constitutively expressing IL-6 have a faster regeneration of the hypoglossal nerve after trauma [126]. Noteworthy, it has been showed that injection of a cytokine cocktail containing IL-6 in mice 1 day after SCI promoted microglia and macrophage activation and recruitment to the spinal cord, exacerbating the initial immune response. However, if the cytokine cocktail was injected 4 days after SCI, it resulted in a decreased activation of microglia and a smaller injury size [119]. Therefore, stimulation of IL-6 production in the spinal cord in a time-controlled manner could be a promising strategy to improve recovery after SCI.

1.3.4 mTOR signalling

The mechanistic target of rapamycin (mTOR) is a serine-threonine kinase that regulates fundamental cell processes such as protein synthesis, autophagy, proliferation and metabolism (see Figure 4). mTOR is the enzymatic component of two functionally distinct protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [127].

mTORC1 is a main downstream component of the phosphoinositide 3-kinase (PI3K)-AKT pathway and it regulates protein synthesis through phosphorylation of p70 ribosomal S6 kinase (p70S6K), which in turn phosphorylates the ribosomal protein S6. Consequently, phosphorylated (p-) S6 is often used as a readout of mTORC1 activity [128]. mTORC1 activity can be blocked by rapamycin, which binds FK506-binding protein of 12 kDa (FKBP12) forming a complex that inhibits mTOR only when it is part of mTORC1 [129]. Importantly, rapamycin can have additional effects that are independent of mTORC1 blockade, which are further discussed in **Study I**.

mTORC2 is also involved in the PI3K-AKT pathway although its activity is independent of mTORC1. This complex can phosphorylate and facilitate AKT activation, thus playing a fundamental role in AKT mediated cell survival [130, 131]. Currently, there are no specific blockers of mTORC2 activity. However, competitive inhibitors like torin2 act by blocking the ATP-binding site of mTOR, which inhibits its activity whether it forms part of mTORC1 or mTORC2 [132]. Nevertheless, due to the similarity between mTOR and PI3K sequences, many ATP competitive inhibitors that target mTOR, including torin2, also inhibit PI3K activity [133].

Current evidence shows that mTOR can regulate IL-6 expression negatively or positively depending on the cell type [134]. For example, in myeloid phagocytes the mTOR pathway negatively regulates IL-6 production [135] while in endothelial cells, mTOR has been found to positively influence IL-6 synthesis [136]. In **Study I**, we examine the role of mTOR in regulating IL-6 expression and secretion in astrocytes, and study the relationship between IL-6 and mTOR in astrocytes in the context of SCI.

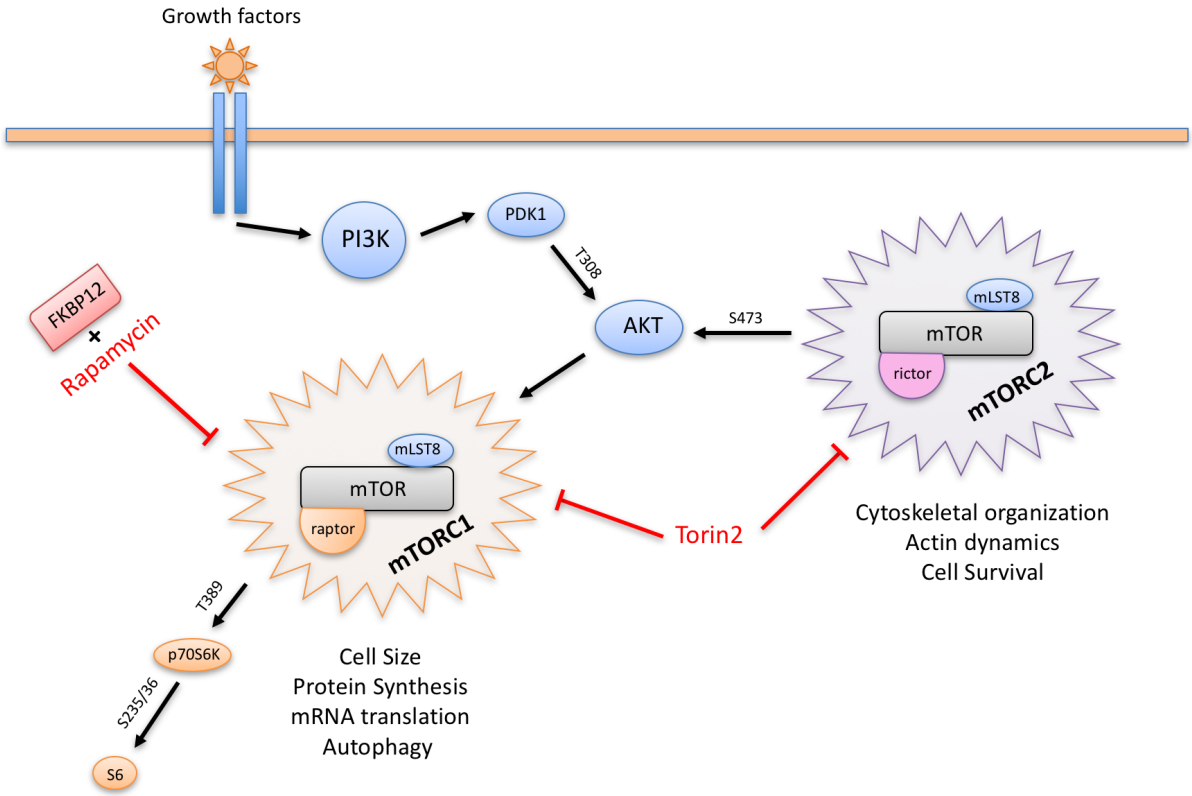


Figure 4 Schematic diagram illustrating members of the P3IK-mTOR-AKT pathway (simplified) that were investigated in Study I. Albeit not shown here, rapamycin can also induce mTORC2 and pyruvate dehydrogenase kinase isozyme 1 (PDK1) activation in astrocytes. Moreover, torin2 can also inhibit PI3K and AKT (see Study I). Relevant phosphorylation sites on serine (S) or threonine (T) are indicated on activation (black) arrows.

1.4 RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic and systemic autoimmune disease that affects approximately 1% of the population, with a higher prevalence in women than in men (3:1) [137]. The etiology of this disease remains unclear, although genetic and environmental factors such as HLA-DRB1 shared epitope alleles and cigarette smoking have been associated with a higher risk to develop RA [138-140]. This autoimmune disease affects mainly the joints and it is characterized by aggressive synovial inflammation (synovitis), local infiltration of immune cells and joint swelling, which often leads to a progressive destruction of cartilage and bone [141]. The identification of biological disease modifying anti-rheumatic drugs have led to great improvements in RA prognosis, since they can slow down or even halt disease progression in a vast majority of RA patients [142]. However, as further discussed below, chronic pain continues to be the most debilitating problem reported by RA patients [143, 144].

1.4.1 Autoantibodies in RA

A large proportion of RA patients produce autoantibodies, which can be found in synovial fluid and in blood circulation [145, 146]. Moreover, in **Study III** we also report the presence of autoantibodies in the CSF of certain RA patients. There is a large number of autoantibodies against different self-antigens that are associated with RA, including rheumatoid factor, antibodies against collagen type-II and anti-citrullinated peptide/protein antibodies. Although circulating autoantibodies are often used as diagnostic markers of RA [146], their contribution to pathology is not always clear.

Rheumatoid factor (RF) was the first autoantibody detected in RA patients, serving as the only available serological marker used to diagnose the disease. However, it is now clear that RF is not specific to RA since it has been identified in other autoimmune conditions and even in healthy individuals. RF recognizes and interacts with the Fc (fragment, crystallizable) region of Immunoglobulin G (IgG), leading to the formation of immune complexes that are thought to contribute to RA pathogenesis. The presence of RF is associated with more severe and destructive forms of RA [147-150].

Collagen type II (CII) is a major component of joint cartilage. Antibodies against CII can be found in RA patients around the onset of the disease, with a prevalence that is highly variable [151, 152]. In mice, passive transfer of anti-CII antibodies induces arthritis and contributes to synovitis, joint inflammation, pain and to the destruction of cartilage and bone, resembling the human pathology of RA [153]. These findings constitute the basis of the collagen antibody induced arthritis (CAIA) model, which is suitable for the study of RA pathology and pain aspects [154].

Anti-citrullinated peptide/protein antibodies (ACPA) are found in about 60-70% of RA patients and are routinely used as a diagnostic marker for RA [146, 155]. Citrullination is a post-translational modification that consists in the enzymatic conversion of arginine (positively charged) to citrulline (neutral). This change in amino acid net charge is thought to affect the structure and function of the protein, leading to an increase in its immunogenicity. RA is associated with aberrant levels of protein citrullination and some of the most common citrullinated proteins present in RA patients are CII, vimentin, α-enolase, fibrinogen and histone 4 [156, 157].

The role of ACPA on RA pathology has recently started to be elucidated. ACPA can be detected up to several years before the onset of RA although higher titers and epitope spreading is commonly observed when individuals approach disease diagnosis [158, 159]. ACPA positive individuals can present arthralgia (bone pain) and bone erosion before the development of RA, and these autoantibodies are also associated with a more aggressive RA course [160-162]. Such observations suggest that ACPA play an important role in driving RA pathology. Interestingly, recent experimental studies that support this notion have delineated the mechanisms behind ACPA's ability to mediate pain and bone destruction in the absence of synovial inflammation [163, 164].

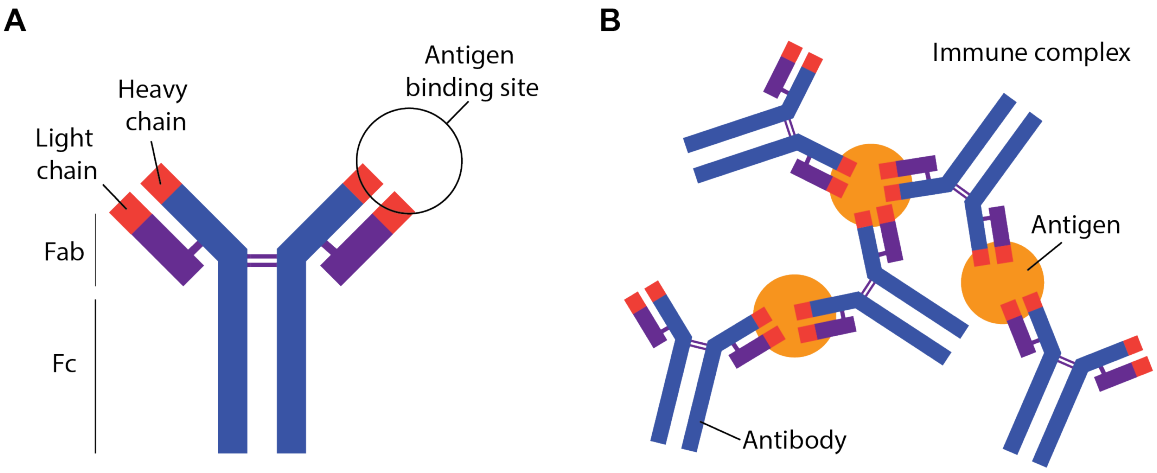


Figure 5 A) IgG structure. The variable regions (depicted in red) of the light (purple) and heavy (blue) chains form the Fab region which can recognize and bind antigens. The constant regions of the heavy chains at the other end comprise the Fc portion, which can engage in effector functions **B)** Antigen-antibody complexes, also known as immune complexes are often needed to activate certain receptors, such as FcγRs

1.4.2 Mechanisms of antibody action

Antibodies, also known as immunoglobulins (Ig), are the main effector molecules of the adaptive immune system. They can be classified into five isotypes (IgG, IgA, IgE, IgM and IgD) which have different characteristics. IgG is the most abundant antibody in circulation and the work presented in **Study III** and **IV** revolves around this particular isotype [165].

Antibodies can recognize and bind antigens with the Fab (fragment, antigen-binding) region, which is essential for mediating the clearance of pathogens. Fc-receptors found in immune cells can potentiate antibody-mediated effector functions by interacting with the Fc region of antibodies (Figure 5) [165].

Fc-receptors are classified according to the antibody isotype that they recognize. In humans, Fc-gamma receptors (FcγRs) can be further subdivided into 6 types (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, FcγRIIIA and FcγRIIIB) each of which has a different molecular structure and affinity for IgG. Notably, FcγRs can only be activated by immune complexes and not by monomeric IgG [166]. Albeit most of our knowledge regarding FcγRs come from findings made in immune cells, the presence of this class of receptors has also been reported in cell types such as neurons and glia [167-170]. Of particular interest is the FcγRI, which has the highest affinity for IgG and has been implicated in pain signalling in cultured neurons [167] and in an experimental model of arthritis [168].

1.5 PAIN

1.5.1 Acute and chronic pain

Pain is a normal physiological response that acts as a warning's system helping us to prevent tissue damage and to attend and protect wounded areas. Pain transmission is mediated by sensory neurons called nociceptors, which can convert mechanical, chemical and thermal stimuli into electrical signals. These pain signals are then relayed to the spinal

cord dorsal horn, which further convey the information to the brain, where the pain is perceived. Acute pain usually lasts until the noxious stimulus or the tissue damage have resolved. However, persistent nociceptive stimulation can result in a dysregulation of normal pain processing and lead to the development of chronic pain [171, 172].

Chronic pain refers to pain that lasts longer than 3 months and does not resolve on its own [172]. It is a debilitating condition that affects between 11.5% and 55.2% of the population and has a greater prevalence among women than men [173, 174]. Currently, treatment options for chronic pain are suboptimal, as these patients do not respond adequately to existing analgesics. As a consequence, people with chronic pain commonly have a lower quality of life with significant functional, social and emotional complications [175]. Chronic pain is characterized by processes such as hyperalgesia (increased pain sensitivity to painful stimuli), allodynia (pain sensation to a stimulus that does not normally induce pain) and spontaneous pain. The mechanisms responsible for these maladaptive pain states can be peripherally or centrally driven [176, 177]. Peripheral sensitization occurs when nociceptive terminals become hypersensitive as a result of a higher or sustained activation caused by factors in the injured area. On the other hand, central sensitization occurs as a result of changes that increase the excitability of dorsal horn neurons and amplify the pain signal (Figure 6). Different processes can lead to central sensitization. For example, persistent transmission of nociceptive signals from the periphery can facilitate glutamatergic signalling, dysregulate pain inhibitory pathways, and lead to the activation of glial cells in the dorsal horn, which further promotes the enhancement and maintenance of pain states. [175-178]

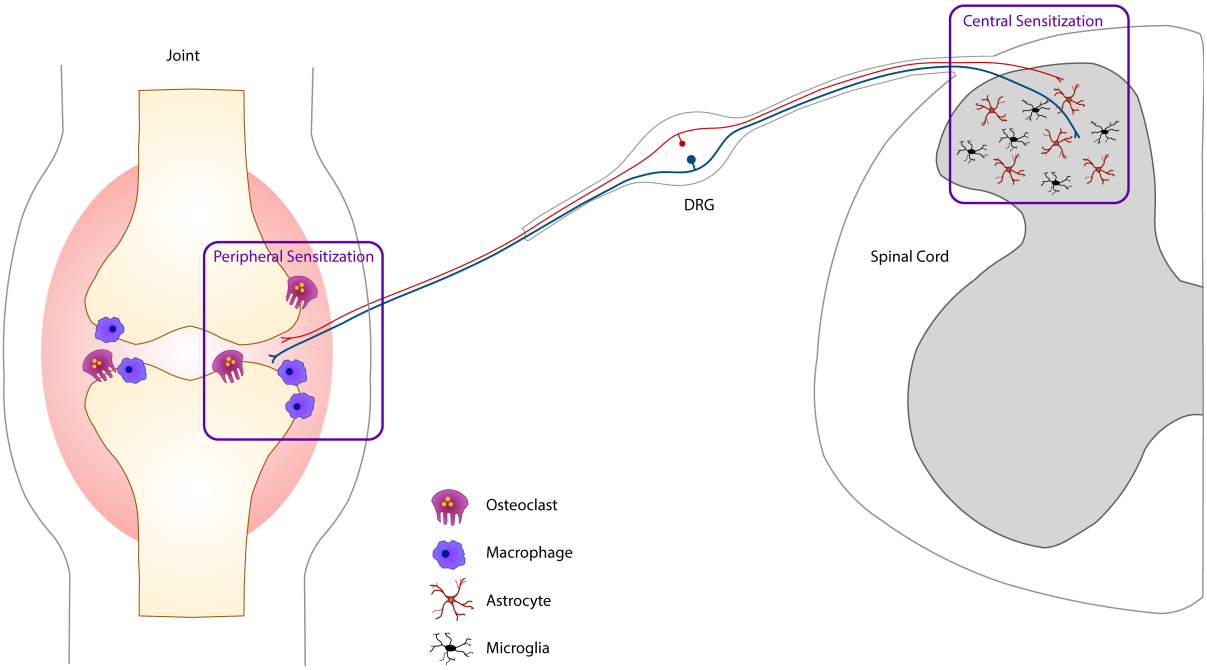


Figure 6 Schematic diagram showing peripheral and central sensitization processes in the context of RA. Nociceptive fibers that innervate bone structures include unmyelinated C-fibers (red) and myelinated A∂ fibers (blue). Persistent activation of nociceptors by inflammatory factors in the joint can lead to peripheral sensitization. Such nociceptive signals travel via the dorsal root ganglia (DRG) to the dorsal horn of the spinal cord, where they can become amplified and cause central sensitization. Glial cells such as microglia and astrocytes can contribute to central sensitization. Diagram based on Figure 1 from Jie Su's dissertation: *Chronic pain and arthritis – studies of mechanisms in the regulation of hypersensitivity*, Karolinska Institutet, 2016.

1.5.2 Neuropathic, inflammatory and arthritic pain

Chronic pain can be divided into several types according to its cause. Neuropathic pain arises when nerves in the peripheral or central nervous system become damaged by a traumatic injury or due to disease pathogenesis. Increased hypersensitivity occurs as a result of the aberrant pain signalling that takes place in the lesioned axons but also in the intact nociceptors that innervate the same territory as the injured nerve. This type of pain is commonly associated with conditions such as spinal cord injury, diabetes and autoimmune diseases [179].

Chronic pain can also be inflammatory, where pain-promoting factors found in the inflamed area can activate and sensitize nearby nociceptors. Inflammatory pain is an important component in RA. However, the pain phenotype of RA patients is complex and there is little correlation between disease activity and pain [180, 181]. For instance, a considerable proportion of RA patients continue to have pain even when the disease and the inflammation are medically under control or in remission [180, 182]. Moreover, the development of arthralgia often precedes synovitis and it is a predictive factor for RA [160, 183]. Additionally, RA patients can experience generalized hypersensitivity at distant areas from the affected joint [184-186], suggesting that central sensitization plays an important role in this condition [187]. Our current knowledge about arthritic pain has been greatly advanced with the use of poly-arthritic animals models which present pain phenotypes that closely reflect the clinical scenario. In **Study IV**, we have used the CAIA model, which is characterized by transient joint inflammation and persistent mechanical hypersensitivity. Interestingly, while it has been shown that nonsteroidal anti-inflammatory drugs can attenuate arthritis-induced hypersensitivity during the phase of joint inflammation (inflammatory phase), this is certainly not the case for the late phase of the model, when joint inflammation has resolved but mechanical hypersensitivity persists [154]. These and similar findings from other models of poly-arthritis suggest that arthritic pain has a non-inflammatory component, possibly reflecting the situation in RA patients [188]. Supporting this notion, our group has reported that passive transfer of ACPA from RA patients into the blood circulation of mice leads to thermal and mechanical hypersensitivity without causing any visual signs of inflammation [163]. Of interest, in **Study III** we investigate a possible role of these autoantibodies in mediating central sensitization by acting on glial cells.

1.5.3 Glia in neuropathic, inflammatory and arthritic pain

A growing body of evidence suggests that spinal glia play important roles in the maintenance of chronic pain. Both astrocytes and microglia can be activated in response to mediators centrally released by sensitized peripheral nociceptors, as shown in different models of neuropathic, inflammatory and arthritic pain. In turn, activated glia can synthesize and release cytokines, chemokines and other mediators that further potentiate the transmission of pain signals by increasing the excitability of dorsal horn neurons or by decreasing inhibitory neurotransmission. Moreover, glia-glia interactions can also contribute to central sensitization. Several receptors, signalling pathways and cytokines appear to be implicated in the regulation of pain transmission by glial cells [175, 189].

One of the most-well documented pathways driving pathological pain is mediated by the chemokine fractalkine, also known as CX3CL1. These chemokine is expressed by excitatory dorsal horn neurons, and, upon cleavage by cathepsin S, it becomes soluble and binds to its receptor CX3CR1, which is located in microglia. Microglial activation of CX3CR1, which is upregulated in experimental pain models, leads to the phosphorylation of p38 and a subsequent release of pro-nociceptive cytokines such as IL-6 and interleukin-1beta (IL-1ß) [190-192].

Purinergic receptors, which become upregulated upon injury, have also been associated with pain transmission [193]. For instance, ATP activation of P2X7R expressed in microglia can regulate the secretion of cathepsin S (promoting the cascade of events explained above) and also mediate the microglial release of the pro-nociceptive cytokine IL-1ß [194]. ATP can also activate P2X4 purinergic receptors (P2X4R) on microglia, and pharmacological inhibition of this receptor leads to a transient attenuation of mechanical hypersensitivity [195]. Moreover, P2X4R and P2X7R knockout mice develop less allodynia after peripheral nerve injury [196, 197]. A proposed mechanism by which P2X4R may contribute to neuropathic pain is by the release of microglial brain derived neurotrophic factor (BDNF), which binds tyrosine receptor kinase B (TrkB) and leads to the downregulation of potassium-chloride co-transporters (KCC2). This results in an accumulation of chloride ions in dorsal horn neurons, which, upon γ-aminobutyric acid binding, become depolarized instead of hyperpolarized [198].

Importantly, toll-like receptors (TLR) expressed in astrocytes and microglia including TLR2, TLR3 and TLR4 can also contribute to glial activation and to the maintenance of neuropathic, inflammatory and arthritic pain [199]. For instance, attenuation of spinal gliosis and mechanical hypersensitivity have been reported in TLR4 knockout mice subjected to K/BxN serum transfer arthritis [200]. Moreover, central administration of TLR4 ligands such as lipopolysaccharide (LPS) or high-mobility group box-1 (HMGB1) can induce mechanical hypersensitivity, and pharmacological inhibition of either of these factors in models of neuropathic or arthritic pain can reduce spinal nociceptive signalling and suppress glial activation [201-203]. Other TLR4 ligands, such as heat shock protein 90 (hsp90) have also been associated with altered pain processing and glial activation [204-206].

Of note, activation of astrocytes in models of nerve injury-induced pain has been associated with down-regulation of glutamate transporters, resulting in increased extracellular levels of extracellular glutamate that facilitate neuronal excitability and spinal nociceptive signalling [207]. However, glutamate transporter down-regulation has not been reported in inflammatory pain models, and the role of such receptors in arthritis-induced pain remains unclear.

Two different members of the mitogen-activated protein kinase (MAPK) family have been extensively studied in relation to altered pain processing; p38 and c-Jun N-terminal kinase (JNK). Even though p38 can be constitutively expressed in microglia and astrocytes, this

intracellular factor intriguingly undergoes phosphorylation (activation) exclusively in microglia in chronic pain models, including arthritis-induced pain models [208, 209]. Interestingly, p38 is involved in several signalling processes that facilitate pain transmission, such as the CX3CL1-CX3R1 and ATP-P2X4R-BDNF pathways described above [175]. Phosphorylation of astrocytic JNK has been reported in animal models of inflammatory and neuropathic pain [210, 211]. Indeed, intrathecal administration of JNK inhibitors attenuates neuropathic and inflammatory pain [211, 212]. Of note, spinal JNK blockade has also been reported to reverse mechanical hypersensitivity during the late phase of the CAIA model [154]. Nevertheless, whether p-JNK is restricted to astrocytes in this model remains to be determined. Just like p-p38, p-JNK is involved in the production and release of pro-nociceptive cytokines [199].

Lastly, the role of cytokines in facilitating and perpetuating altered spinal pain is worth mentioning. Both microglia and astrocyte can produce and secrete pro-nociceptive cytokines, including for example IL-1ß, IL-6 and TNF [175].

The pro-inflammatory cytokine IL-1ß was one of the first cytokines described to participate in the mechanisms underlying neuropathic pain [213]. Under physiological conditions, the expression of IL-1ß by glial cells and neurons is quite low, although pathological events can trigger its upregulation [175]. Importantly, IL-1ß has been detected in patients with diverse painful neuropathies and in individuals with RA [214-216]. Similarly, several models of arthritis have reported the presence of IL-1ß in the CSF of these animals, which is concomitant with glial activation and the development of hypersensitivity [216, 217]. Evidence for the pro-nociceptive effect of IL-1ß come from studies where central administration of exogenous IL-1ß led to mechanical and thermal hypersensitivity [218, 219] and from the observation that rodents lacking IL-1ß had diminished pain-like behavior after peripheral nerve injury [220]. Of interest, a recent study has suggested that collagen induced arthritis (CIA) induced pain is associated with central sensitization events that are dependent on the microglial release of IL-1ß [217]. IL-1ß can facilitate spinal nociceptive processing by activating NMDA receptors, leading to increased dorsal horn neuronal excitability. Furthermore, it is also thought that IL-1ß can also promote pain transmission by reducing inhibitory neurotransmission [221].

Mounting evidence suggests that IL-6 plays a crucial role in pathological pain processing [222-224]. IL-6 acts by binding to IL-6 receptors (IL-6R) which can be membrane bound or soluble. The pro-nociceptive effects that IL-6 exerts in neurons are caused via the soluble IL-6R, as these cells lack membrane-bound IL-6 receptors [225]. Importantly, elevated levels of IL-6 and its receptor have been found in the spinal cord of various pathological pain models, and there is evidence supporting the contribution of IL-6 to peripheral and central sensitization [222, 226]. IL-6 is also associated to glia activation, as demonstrated, for example, in transgenic mice that overexpress IL-6, which show prominent signs of astro- and microgliosis [227]. Evidence for a role of IL-6 in central sensitization come from studies which show that spinal administration of IL-6 can elicit thermal and mechanical hypersensitivity [222, 225]. Additionally, IL-6 knockout mice exhibit reduced pain-like

behavioral responses in different nerve injury models, and inhibition of IL-6 signalling with different compounds applied intrathecally can attenuate pain behavior in models of peripheral nerve injury, spinal cord injury and arthritis [228].

Lastly, intrathecal administration of compounds such as fluorocitrate, pentoxifylline or minocycline, which can inhibit glial activity despite having many off-target effects, prevent or reverse the initiation and maintenance of persistent pain states in a myriad of models of neuropathic, inflammatory and arthritic pain [154, 189, 193, 199, 229]. Thus, these findings further suggest that glial cells play an active role in spinal pain transmission.

1.5.4 Glial sex-differences in relation to pain

Currently, there is an important debate ongoing within the pain research community concerning the dimorphic involvement of glial cells in chronic pain. In 2011, an important paper by Sorge et al., described that spinal TLR4, which can be found in microglia, could mediate neuropathic and inflammatory pain in male, but not female mice [201]. In 2015, a collaborative paper that included independent observations from three different laboratories, corroborated a male specific microglial involvement in persistent pain states. A wide battery of compounds that were able to deplete microglia, inhibit microglial function or block crucial signalling molecules were tested, leading to the conclusion that the P2X4R-p38-BDNF pathway is an essential driver of inflammatory and neuropathic pain in male but not in female mice. Interestingly, in this seminal paper the authors conclude that female mice preferentially utilize cells of the adaptive immune system and not microglia to drive central sensitization [230]. These premises have led researchers to further investigate sex-differences in relation to glia and pain, to only find out that the situation is far more complex than initially suggested, with certain discrepancies among different studies.

For instance, Sorge et al., reported that intrathecal injection of LPS (which acts on TLR4) causes mechanical hypersensitivity only in male mice, despite normal expression of spinal TLR4 in both sexes [201]. On the other hand, Woller et al., have described that intrathecal LPS administration induces mechanical hypersensitivity in both sexes, albeit to a greater degree in male mice [202]. Adding to this complexity, we have previously shown that intrathecal delivery of HMGB1 elicits comparable levels of TLR4-mediated hyper-sensitivity in male and female mice [203].

Certainly, glial reactivity has been shown to occur to similar extents in male and female rodents in several chronic pain models [230, 231](see also **Study IV**). Nevertheless, increasing reports support the notion that male microglia may play a more prominent role in pain processing than females. In agreement with the initial findings by Sorge et al., a separate study has shown that spinal inhibition of p38 activity can reduce neuropathic and inflammatory pain in male but not female rodents, and this effect is likely explained by the fact that p38 activation was predominantly observed in male spinal microglia [231]. Furthermore, recent work has shown that intrathecal administration of the “microglial

inhibitors" minocycline or ZVEID (a caspase-6 inhibitor) reduced inflammatory and neuropathic pain exclusively in males [232].

It is important to note that sex-dependent glial signalling has not been examined in a systematic fashion in arthritis-induced pain models. Nevertheless, there is some evidence suggesting that microglia may mediate pain also in females. For instance, it has been shown that intrathecal injection of microglial-targeting drugs, such as P2X7R and cathepsin S inhibitors, attenuate the development of mechanical hypersensitivity and dampens microgliosis in female rats in the CIA model [12]. Furthermore, we have reported that blocking the action of spinal HMGB1 reverses mechanical hypersensitivity in the inflammatory and late phases of the CAIA model both in male and female mice [14]. Moreover, it has been shown that minocycline can reduce joint nociception when injected intrathecally in male mice after antigen-induced arthritis induction (AIA) [36], although the effect of minocycline in female mice was not investigated in this particular study. In **Study IV** we have examined male and female microglia from a global perspective, to ascertain any possible sex-differences that may be important in the context of arthritis-induced pain.

Notably, whether pain signalling in astrocytes is regulated differently in males and females has not been extensively studied. A recent study has addressed this particular question using pharmacological compounds to inhibit astrocyte function (L-α-aminoadiapate) or astrocytic signalling pathways (JNK or ERK inhibitors) involved in pain modulation, revealing that the involvement of astrocytes in chronic pain is most likely sex-independent [232].

2 AIMS

2.1 GENERAL AIM

To investigate the active involvement of glial cells and their cellular responses in pathological conditions affecting the central nervous system.

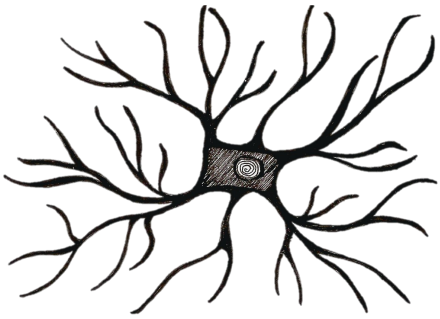
2.2 SPECIFIC AIMS

- To investigate mechanisms regulating IL-6 expression and release from cultured astrocytes in the context of spinal cord injury and rheumatoid arthritis.

- To develop an *ex vivo* model of spinal cord injury for the study of ependymal cell responses.

- To identify potential central effects of anti-citrullinated peptide antibodies found in the cerebrospinal fluid of rheumatoid arthritis patients.

- To elucidate critical differences between male and female spinal microglia in the context of arthritis-induced persistent pain



3 MATERIALS AND METHODS

3.1 PATIENTS

Informed consent was obtained from all individuals taking part in **Study IV**. All experiments were approved by the regional ethics committee at Karolinska Institutet and carried out in compliance with the Helsinki Declaration. Serum and CSF samples were collected from individuals with ACPA-positive rheumatoid arthritis, multiple sclerosis or non-inflammatory disease.

3.2 ANIMALS

All animal experiments were approved by the Northern Stockholm Animal Research Ethical Committee. Animals were housed in groups of 3-5 per cage with food and water ad libitum and kept on a 12h light/dark cycle in a climate-controlled environment. In **Study I**, adult Sprague–Dawley rats (Scanbur) were used. In **Study II**, we used several genetically modified mice: *1) hGfap*-GFP mice [233], which express green fluorescent protein (GFP) under the human *Gfap* promoter (*hGfap*); *2,3) mGfap*-Cre::GCaMP3 mice and *mGfap*-Cre::tdTomato, which express the genetically encoded Ca^{2+} indicator GCaMP3 or tdTomato under the mouse *Gfap* promoter (*mGfap*) upon Cre–Lox recombination; *4) hFoxJ1*-CreER::YFP mice [234], where yellow fluorescent protein (YFP) is expressed under the human *Forkhead Box J1 (hFoxJ1)* promoter upon CreER-LoxP recombination induced by tamoxifen injection. Adult BALB/cAnNRj mice (Janvier Labs) were used in **Study III** and **IV**. Adult CBA mice (Harlan) were also used in **Study IV**.

3.3 MODEL SYSTEMS

3.3.1 Adult rat spinal cord primary astrocyte cultures

For **Study I**, we used primary rat spinal cord astrocyte cultures which have a similar expression profile to human spinal cord astrocyte cultures [235]. Briefly, deeply anaesthetized adult rats were decapitated and spinal cords were extruded using a saline-filled syringe. After removing the meninges, spinal cords were digested with 0.25% trypsin-EDTA, mechanically triturated and plated in poly-L-lysine coated 175cm^2 flasks. Culture medium (AM medium containing 2% fetal bovine serum, 1% astrocyte growth supplement and 1% penicillin-streptomycin; ScienCell) was replaced every 3-4 days. To remove microglia, flasks were shaken for 6 hours at 450 rpm on day 12 and 13, and culture media was replaced right after. On day 14 cells were trypsinized and seeded in 6- or 24-well plates. Cells were switched to growth factor-free culture medium (DMEM containing 1% sodium pyruvate and 1% penicillin-streptomycin; Invitrogen) 48 hours prior drug treatment. Cells were kept in a humidified incubator at 37°C (95% air: 5% CO_2).

3.3.2 Human fetal cortical astrocyte cultures

For **Study IV**, we used commercially available human fetal cortical astrocytes (ScienCell) which can be passaged for up to ten times. Astrocytes were quickly thawed and plated in poly-L-lysine coated 75cm^2 flasks containing AM medium with 2% fetal bovine serum, 1%

astrocyte growth supplement and 1% penicillin-streptomycin (ScienCell). Culture medium was changed regularly until cells reached confluency. Cells were then trypsinized, seeded in 6-well plates and switched to AM medium with 0.02% fetal bovine serum, 0.01% astrocyte growth supplement and 1% penicillin-streptomycin 24 hours prior drug treatment. Cells were kept in a humidified incubator at 37°C (95% air: 5% CO_2).

3.3.3 Adult mouse spinal cord slice cultures

In **Study II**, we used adult mouse spinal cord cultured slices for the study of ependymal cells. Briefly, mice were decapitated under deep anaesthesia and spinal cords were hydroextruded with the aid of a syringe. Spinal cords were embedded in 4% low-melting agarose in PBS (Sigma) and sectioned into 350µm coronal slices with a vibratome (Leica). Slices were transferred to poly-L-lysine coated membranes (Millipore) and placed into 6-well plates with 1.5ml culture medium containing 50% Neurobasal-A, 25% heat inactivated horse serum, 25% HBSS, B-27 supplement, 0.25 mM GlutaMAX, 15 mM glucose, 15 mM HEPES and 25 µg/ml penicillin-streptomycin (Invitrogen). Culture medium was replaced three times a week (750µl). Cultured slices were kept in a humidified incubator at 37°C (95% air: 5% CO_2).

3.3.4 Contusion spinal cord injury

In contusion injury models (Figure 7), a transient force is applied in order to damage the spinal cord. To elicit a contusion spinal cord injury, animals were anaesthetized and a laminectomy of the thoracic T9 (rats) or T10 (mice) vertebra was performed to expose the dorsal surface of the spinal cord. A spinal cord injury was induced dropping a small weight from a certain distance using a custom-made (mice) or NYU (rats) impactor onto the exposed segment of the spinal cord. Pre- and post-operative care, which included analgesic treatment and bladder emptying (rats), was carried out in accordance to ethical guidelines (**Study I** and **II**).

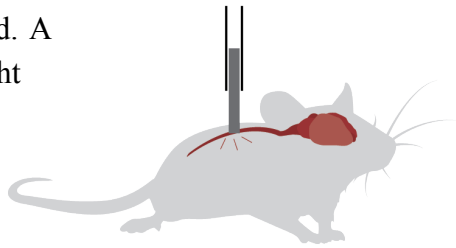


Figure 7 Schematic representation of a weight-drop induced contusion spinal cord injury

3.3.5 Collagen-antibody induced arthritis

While this model is commonly used to study RA pathology, it is also suitable for antibody-induced pain studies. For arthritis induction, mice were injected intravenously (1.25-1.5 mg) with an anti-collagen type II (CII) cocktail containing 5 different monoclonal autoantibodies (Chondrex) followed by an intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* (25 µg; serotype 0111:B4; Chondrex or 35 µg; serotype 055:B5; Sigma) 5 days after. Joint inflammation was evaluated visually and scored as previously described [154]. Briefly, 1 point is awarded for every inflamed toe or knuckle and 2.5 or 5 points for every moderately or severely inflamed ankle or wrist. The maximum arthritis score is 15 points per paw and 60 points per mouse (**Study IV**).

3.3.6 Mice injected with IgG from RA patients

To assess centrally mediated antibody-induced pain, IgG from plasma and sera of ACPA-positive RA patients and healthy controls were intrathecally injected into the CSF of anaesthetized mice. IgG from RA patients was purified into an ACPA-positive IgG fraction and a ACPA-negative IgG fraction containing all remaining IgG (denoted as flow through; FT). The purification protocol has been previously described [236] and includes a first purification step of IgG with HiTrap Protein G columns (GE Healthcare) followed by a second purification step of ACPA positive/negative IgG fractions with an CCP2 affinity column (Euro-Diagnostica) (**Study III)**.

3.4 BEHAVIOURAL ASSESSMENTS

3.4.1 Mechanical hypersensitivity

Before testing, animals were allowed to acclimatize for 30-40 min in separate Plexiglas compartments with a wire-mesh bottom. Von Frey filaments (Marstock Optihair) of incremental force were applied for 2-3 seconds to the plantar surface of the hind paw and a positive response was recorded if the paw was withdrawn (Figure 8). The 50% probability of paw withdrawal was calculated in grams for each hind paw using the "up-down" method [237, 238] and averaged into one score. Results are shown as percentage change of baseline values or as the average 50% withdrawal threshold for each group (**Study I, III** and **IV**).

3.4.2 Cold hypersensitivity

Animals were acclimatized in single wire-mesh bottom Plexiglas cubicles before testing. Cold sensitivity was assessed by applying a drop of acetone to the plantar surface of the hind paw using a 1ml syringe (Figure 8). The number and duration of nocifensive responses (lifting, biting, licking, shaking of the paw) was recorded for 60 seconds. The test was repeated three times per paw in an alternating fashion, with a 5-min rest period between each testing. Results are shown as the average of the three tests for both hind paws for every animal (**Study III**).

3.4.3 Heat hypersensitivity

Mice were allowed to habituate to the wire-mesh bottom Plexiglas cubicles and heat hypersensitivity was examined using a modified Hargreaves Box [239]. Briefly, a radiant heat stimulus was pointed at the plantar surface of the hind paw until a motion sensor detected a brusque paw withdrawal (Figure 8). The time between the start of heat stimulation and paw withdrawal was automatically recorded by the sensor. A security cut-off of 20 seconds was set up to prevent tissue damage. Results are shown as the average of three tests for both hind paws for each animal and presented as latency of the withdrawal (**Study III**).

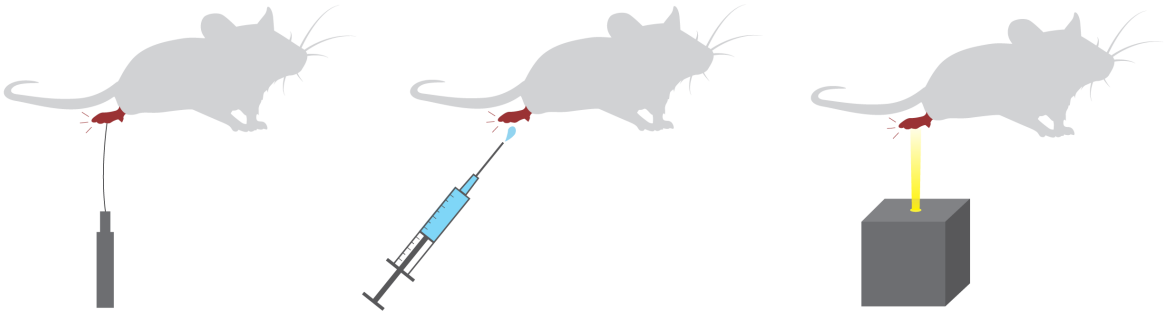


Figure 8 Behavioral tests. Left panel: Mechanical hypersensitivity was evaluated with Von Frey filaments. Middle panel: Cold hypersensitivity was assessed with the acetone test. Right panel: Heat hypersensitivity was examined using a modified Hargreaves Box.

3.4.4 Locomotion evaluation

In **Study I**, recovery of hind limb locomotor function after spinal cord injury was weekly assessed in an open field using the Basso, Beatie, Bresnahan (BBB) rating scale as described [240]. Scores 0-9 range from no hind limb movement to stationary weight support. Scores 10-21 describe plantar stepping and gait coordination parameters with score 21 representing normal locomotor function.

3.5 HISTOLOGICAL, CELLULAR AND MOLECULAR TECHNIQUES

3.5.1 Tissue processing

Deeply anaesthetized animals were transcardially perfused with saline solution followed by ice-cold 4% paraformaldehyde with or without 0.2% picric acid. Spinal cords were dissected, post-fixed and stored in 10-30% sucrose. The tissue was then cut into 14µm or 20µm coronal sections with a cryostat and mounted on glass slides. For spinal cord injury studies (**Study I** and **II**), spinal cords were divided in 7mm segments from the center of the lesion before sectioning. For studies using mice injected with anti-CII antibodies (**Study IV**), only lumbar spinal cords were processed.

3.5.2 In situ hybridization

For **Study I**, RNA probes for rat IL-6 were made as previously described [241]. Shortly, a plasmid containing the full-length cDNA clone of rat IL-6 was linearized with PstI or BamHI restriction enzymes. Anti-sense riboprobe was generated by transcription of the BamHI digested plasmid with T3 polymerase. Sense riboprobe was synthesized by transcription of the PstI digested plasmid with T7 RNA polymerase and were used as negative control. Reactions were performed in the presence of digoxigenin-labeled UTP.

For in situ hybridization (Figure 9), slides were permeabilized with PBST (PBS, 0.3% Tween-20) and hybridized at 45°C overnight with 10ng/µl of riboprobe in hybridization solution containing 4 mM Tris-HCl, 50% formamide, 20% dextran sulphate, 1X Denhard's solution, 0.5 mg/ml Poly-A, 0.5mg/ml salmon sperm, 0.1 M DTT, 0.3 M NaCl and 1 mM EDTA. After several washes, slides were incubated with 1X MABT, 2% goat serum and 2% blocking agent for 1 hour to block non-specific sites. Slides were then incubated

overnight with an anti-digoxigenin antibody conjugated to alkaline phosphatase (1:2000; Roche) and developed for 4 hours in B3 solution (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, 0.1% Tween-20) containing the chromogenic substance NBT/BCIP (1:50, Roche). Lastly, slides were mounted with glycerol and imaged with an Olympus FV1000 microscope.

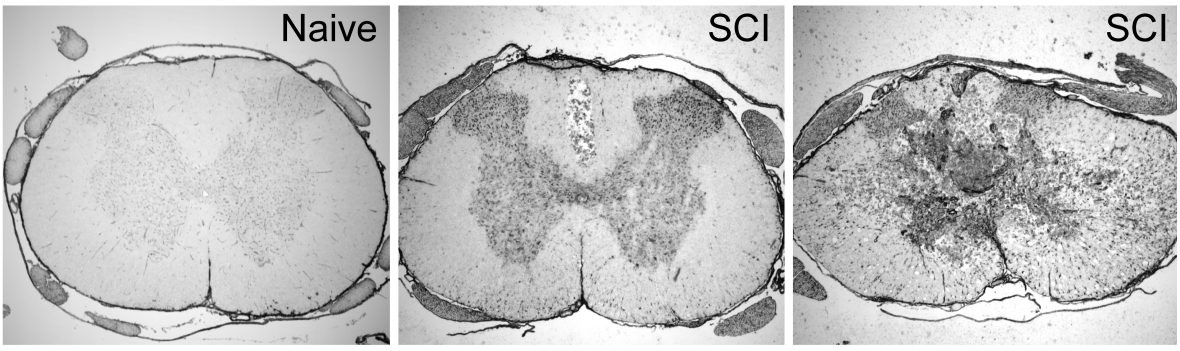


Figure 9 In situ hybridization for rat IL-6 in naïve and injured spinal cord (SCI) tissue. Figure modified from Study I.

3.5.3 Slide-mounted immunohistochemistry

In **Study I, II** and **IV,** spinal cord sections were permeabilized with PBST or TBST (PBS or TBS, 0.2% Tween-20) and incubated with 5% goat or donkey serum in PBST or TBST (blocking solution) for 1 hour to block non-specific binding. Subsequently, spinal cord sections were incubated overnight with primary antibodies at 4°C in blocking solution, washed several times and further incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen) for 1 hour also in blocking solution. In some instances, slides were additionally incubated with DAPI for 30 min in blocking solution. For **Study IV**, the TSA Plus kit (Perkin Elmer) was used as previously described [242]. Briefly, slides were blocked in TNB buffer (0.5% blocking reagent in TNT buffer) and incubated for 30 min with a horseradish peroxidase-labeled secondary antibody (1:200, DAKO) diluted in TNB buffer. Lastly, slides were incubated for 30 min with tyramide-fluorescein diluted in amplification solution (1:100). Stained sections were coverslipped with mounting media with or without DAPI and images were obtained by epifluorescent (Nikon TE300) or confocal microscopy (Olympus FV100, Zeiss LSM710, Zeiss LSM 800). For signal intensity quantification images were taken using identical settings. Image analysis and post-processing was performed using Fiji open-source software [243] or Imaris 7.3.0 (Bitplane).

3.5.4 Free-floating immunohistochemistry

Before immunostaining, spinal cord cultured slices were briefly post-fixed in 4% paraformaldehyde and incubated for 2 hours with blocking solution (1% goat or donkey serum, HEPES buffered HBSS, 0.3% Triton X-100) to mask non-specific binding sites. Next, slices were incubated with primary antibodies for 48 hours at 4°C in blocking solution, washed several times, and incubated with by Alexa Fluor-conjugated secondary antibodies (Invitrogen) for another 48 hours at 4°C in blocking solution. In some cases, slides were further stained with DAPI for 1 hour in blocking solution. After several washing steps, stained slices were incubated in U2 scale clearing solution (4 M Urea, 30%

glycerol, 0.1% Triton X-100) for 1 week at 4°C and mounted onto glass slides. Image acquisition and analysis was performed as described in the section above (**Study II**).

3.5.5 Calcium imaging

For **Study I**, adult rat primary astrocyte cultures were loaded with the ratiometric fluorescent dye Fura-2 AM and 0.1% pluronic acid (Invitrogen) in growth factor-free culture medium for 30 min, washed several times and imaged in Ca^{2+}-free KREBS-Ringer´s solution at 37°C with an upright wide-field microscope (AxioExaminer A1, Zeiss) equipped with a 20x/1.0 NA water immersion objective. Images were acquired at a frequency of 0.5 Hz (Figure 10).

For **Study II**, adult mice expressing a genetically encoded Ca^{2+} indicator (GCaMP3) under a specific promoter were used, enabling Ca^{2+} imaging in specific cell types. Spinal cord slices were attached to the imaging chamber with Matrigel (BD Biosciences) and allowed to recover for 30 min before imaging. Slices were perfused with KREB-Ringer's solution at 37°C throughout the experiment and images were taken every 15-20 seconds using 40x or 63x water immersion objectives mounted on a Zeiss laser scanning microscope (LSM510 META NLO) equipped with an Argon laser tuned at 488 nm (confocal imaging) or a Ti:Sapphire Chameleon Ultra2 laser tuned at 810 nm (two-photon imaging).

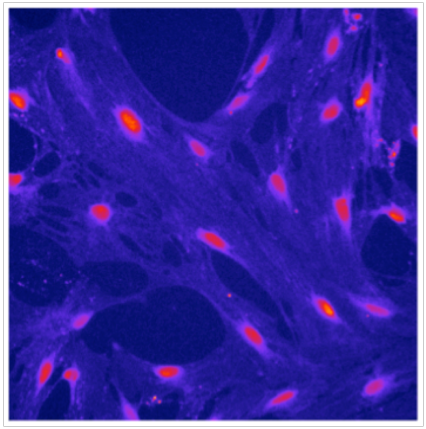


Figure 10 Adult rat primary astrocyte culture loaded with the Ca^{2+} indicator Fura-2 AM (shown as a heat gradient).

Ca^{2+} responses were quantified using Fiji [243] (**Study II**) or with custom scripts written in Phyton (**Study I**). Basal signals were determined by averaging the pixel intensity of the regions of interest for each time point (t) before stimulation (F0). Signal intensity after stimulation was determined by dividing the relative changes in fluorescence by F0 for each time point (dF/F0).

3.5.6 Live imaging

In **Study II**, long term imaging of spinal cord slices kept in culture was carried out using an automated cell culturing system equipped with a 10X air objective (Cell-IQ, Chip-Man Technologies). Briefly, freshly prepared slices were positioned inside the Cell-IQ device and imaged under stable humidity and temperature conditions for 7 days. Bright-field images were taken every 15 hours while fluorescent images were acquired every 8 hours. Images were post-processed with the Cell-IQ analyzer software.

3.5.7 Western blot

Astrocyte cultures were homogenized in protein extraction buffer containing protease and phosphatase inhibitor cocktails (Sigma, Roche) and protein concentration was determined

using the BCA Protein Assay Kit (Pierce). Proteins were heat-denatured, separated by gel electrophoresis (Figure 11; NuPage 4-12% Bis-Tris gel, MES or MOPS running buffer; Invitrogen) and transferred to nitrocellulose membranes. Non-specific binding was blocked for 1 hour with 5% low-fat milk in TBST (TBS, 0.1% Tween-20). Membranes were incubated overnight at 4°C with primary antibodies in 5% bovine serum albumin (BSA) in TBST, washed several times, and further incubated with horseradish-conjugated secondary antibodies (Cell Signalling) for 1 hour in 5% BSA TBST. After a few washes, membranes were developed with Pierce ECL chemiluminescence substrate (Thermo Scientific) using a Bio-rad ChemiDocXRS+ (Bio-rad). Membranes were stripped with Reblot Western Blot Recycling Kit (Millipore) before re-probing with a different antibody. Bands were normalized to their respective loading controls (e.g. GAPDH) and results presented as a percentage of the control experimental condition. Signal intensity was quantified using Fiji open-source software [243] (**Study I** and **III**).

Figure 11 Protein separation by gel electrophoresis (SDS-PAGE)

3.5.8 ELISA-based cytokine and antibody measurements

The detection of IL-6 from cell supernatants and plasma was carried out using a rat IL-6 Ultra-Sensitive Kit (Mesoscale Discovery) and a human IL-6 DuoSet ELISA Kit (R&D Systems) according to manufacturer's instructions. 96-well plates were read in a MSD Sector Imager 2400 (rat IL-6) or on a Molecular Devices (human IL-6) plate reader. The concentration of each sample was calculated based on the standard curve generated from the recombinant protein provided in the kit (**Study I, III**).

For **Study III**, human ACPA-IgG levels were determined with a commercial anti-CCP2 ELISA (Euro-Diagnostica) according to the manufacturer's instructions. IgG reactivity to human influenza was evaluated by H1N1 ELISA as described [244]. Serum was diluted 1:50 or 1:500 for ACPA or influenza antibody measurements respectively. CSF samples were used undiluted.

3.5.9 Citrullinated peptide microarray

In **Study III**, ACPA-IgG binding specificity to the citrullinated peptides α-enolase (CEP-1), vimentin (Vim60-75), fibrinogen (Fib36-52) and collagen type II (CII C1) as well as to their native arginine-containing counterparts was assessed with a custom-made microarray based on the Immune CAP ISAC system (Phadia Laboratory Systems) as previously described [245].

3.5.10 Fluorescence-activated cell sorting

Deeply anaesthetized animals were perfused with ice-cold HBSS and lumbar spinal cords were flushed out by hydroextrusion. Dorsal horns were dissected and homogenized into a cell suspension and myeloid cells were extracted using a 37%/70% Percoll® density gradient (Sigma). For some experiments, cells were stained with anti-Fcrls (gift from Dr. Butovsky) primary antibody and an APC-conjugated secondary antibody before staining with CD45-PE and CD11b-FITC directly conjugated antibodies. Dead cells were stained with Sytox blue (Invitrogen). Unstained cells and single staining controls including Sytox blue dead stained cells and BD Comp beads (BD Biosciences) for FITC, PE and APC fluorochromes were used for compensation. Microglial cells were sorted with a BD Influx flow cytometer (BD Biosciences) into RTL lysis buffer (Qiagen) with 1% β-mercaptoethanol for RNA extraction (**Study IV**).

3.5.11 RNA sequencing

For **Study IV**, RNA from sorted microglia was extracted using a Qiagen RNeasy® Micro Kit and its quantity and integrity was assessed in an Agilent Bioanalyzer (Agilent Technologies). cDNA library preparation and RNA sequencing were performed by the High Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics (Oxford University). The cDNA libraries were synthetized with a SMARTer® Ultra® Low Input RNA Kit (Clontech). Samples were amplified and multiplexed on an Illumina HiSeq4000 platform to yield 75bp fragments at a depth of at least 27 million reads per sample. Quality control was performed using the RSeQC algorithm [246] and reads were aligned to the mm10 mouse genome using STAR [247]. Fragments per kilobase of transcript per million reads (FPKM) values were obtained using the *cufflinks* algorithm [248] on the Galaxy Freiburg server [249]. Differential gene expression data was generated and analyzed with the *featurecounts* [250] and *Deseq2* [251] algorithms. Lastly, interaction networks were produced using STRING [252] and edited with Cytoscape [253].

3.5.12 Real-time PCR

For **Study I** and **III**, adult rat and human fetal astrocyte cultures were lysed in Trizol® (Invitrogen) and RNA was extracted using a phenol-chloroform protocol according to the manufacturer´s instructions. cDNA was synthesized by reverse transcription using random hexanucleotide primers. PCR amplification reactions were performed using TaqMan master mix with the primer/probe of interest using a StepOne real-time PCR system (All from Applied Biosystems). The standard curve method was used to quantify IL-6. Results were normalized to GAPDH values and expressed as relative expression or as a percentage of the control experimental condition.

3.5.13 Luciferase gene reporter assay

In **Study I**, cultured rat astrocytes were transfected with a plasmid containing the firefly luciferase gene under the control of the wildtype hIL-6 promoter (p1168huIL6P-Luc+) or the hIL-6 promoter with a mutated nuclear factor-kappa B (NF-kB) binding site (p1168Hil6mNFkB-Luc+). Astrocytes were also co-transfected with a plasmid consti-

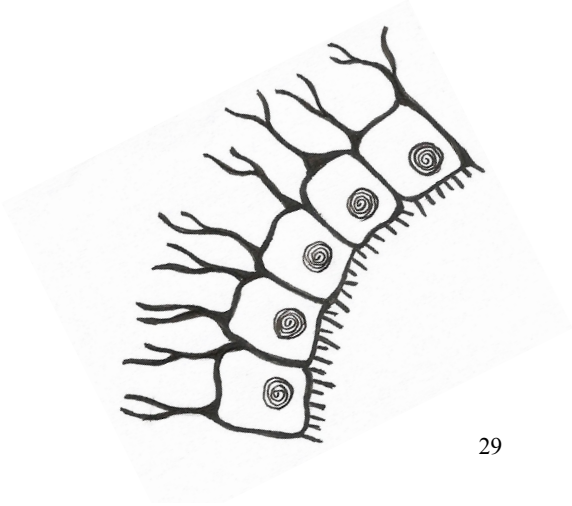
tutively expressing renilla luciferase (pRL-TK). Astrocyte transfection was carried out with the Amaxa nucleofector kit (VPI-1006, Lonza) and gene reporter activity was measured with the Dual-Luciferase Reporter Assay Sytem (Promega), both according to the manufacturer's protocol. Luciferase activity was measured with a VICTOR2 plate reader (Perkin Elmer Wallac). Firefly luciferase activity was normalized to that of the Renilla luciferase, to control for transfection efficiency.

3.5.14 TUNEL assay

Apoptosis of cultured rat astrocytes was assessed using the Click-iT TUNEL Alexa Fluor Imaging Assay (Thermo Scientific) according to manufacturer's intructions. Treatment with DNAse I was used as a positive control. Cells were imaged using a Cell Observer (Zeiss) and quantified with a custom pipeline made in CellProfiler [254] **(Study I)**.

3.5.15 Statistical analysis

Differences between two groups were performed using the Mann-Whitney U test (non-parametric, two-tailed) or the unpaired Student's t-test (two-tailed). Differences between more than two groups were analyzed using one-way ANOVA for one independent variable (i.e. treatment) and two-way ANOVA for two independent variables (i.e. treatment and time). Linear correlation between variables was calculated with the Pearson correlation coefficient. Multiple comparison corrections were adjusted using the Bonferroni *post-hoc test*. A P-value < 0.05 was considered significant in all cases. Results are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Prism 6.0 (GraphPad).



4 RESULTS AND DISCUSSION

4.1 STUDY I: INTERLEUKIN-6 SECRETION BY ASTROCYTES IS DYNAMICALLY REGULATED BY PI3K-mTOR-CALCIUM SIGNALLING

It has been suggested that the induction of factors promoting regeneration, such as IL-6, in reactive astrocytes could be beneficial for tissue recovery. The main goal of this paper was to elucidate signalling pathways that regulate IL-6 release in primary rat astrocyte cultures (Figure 12), in order to identify potential targets for the modulation of IL-6 *in vivo*.

Several reports have shown that the mTOR pathway regulates pro-inflammatory cytokine production in myeloid phagocytes [255]. Particularly, mTORC1 inhibition by rapamycin was reported to increase the levels of cytokines such as IL-6 and TNF-α [135]. In cultured astrocytes, we did not observe mRNA IL-6 expression after mTORC1 inhibition by rapamycin. However, inhibiting both mTORC1 and mTORC2 using torin2, resulted in IL-6 induction. Of note, torin2 also inhibited PI3K and AKT signalling cascades, indicating that mRNA IL-6 expression is negatively regulated by the PI3K-mTOR-AKT pathway in cultured astrocytes.

Next, we aimed to determine whether IL-6 expression was dependent on NF-kB activity, since this transcription factor is a potent inducer of IL-6 [256]. Transfection of astrocytes with a luciferase gene under the control of a wildtype IL-6 promoter led to higher gene reporter activity in torin2-treated astrocytes, as compared to vehicle- or rapamycin-treated astrocytes. In contrast, when astrocytes were transfected with an IL-6 promoter containing a mutated NF-kB binding site, luciferase expression levels were similar among all conditions. Therefore, our data indicates that the PI3K-mTOR-AKT pathway regulates IL-6 transcription via NF-kB.

Previous work has shown that MAPKs can control IL-6 mRNA expression via NF-kB activation [257]. Based on this finding, we examined MAPKs activation and found higher phosphorylation of p38, but not ERK1/2 nor JNK, after torin2 treatment in our astrocyte cultures. Inhibition of p38 abolished the increase in mRNA IL-6 levels observed in torin2-treated astrocytes, indicating that p38 activation is a crucial component in the cascade that triggers mRNA IL-6 expression upon PI3K-mTOR-AKT inhibition.

As a following step, we went on to investigate the mechanisms controlling IL-6 secretion in astrocytes. Despite increasing IL-6 mRNA levels, blockade of the PI3K-mTOR-AKT pathway did not result in IL-6 protein secretion. Of interest, increased cytosolic Ca^{2+} caused as a result of "leaky" ryanodine receptors (RyR) facilitates IL-6 release [258]. Rapamycin, apart from blocking mTORC1, can also act by increasing the permeability of RyR [259]. Thus, we treated astrocytes with torin2 and rapamycin simultaneously, to increase IL-6 mRNA and intracellular Ca^{2+} levels, respectively. Indeed, such approach lead to an astrocytic release of IL-6 in the culture media. Pre-treatment of astrocytes with the Ca^{2+} chelator BAPTA-AM prevented the IL-6 secretion mediated by the combined torin2 and

rapamycin treatment. Taken together, these findings demonstrate that IL-6 secretion from astrocytes requires both IL-6 mRNA expression and increased cytosolic Ca^{2+} levels.

Next, we performed Ca^{2+} imaging and stimulated astrocytes with the RyR agonist 4-chloro-m-cresol (4-cmc) to assess Ca^{2+} release from the ER. Interestingly, we observed that astrocytes treated with torin2 alone or together with rapamycin presented Ca^{2+} responses that were of smaller amplitude than in vehicle-treated astrocytes, suggesting a decrease in Ca^{2+} content from ER stores. Of note, the 4-cmc-induced Ca^{2+} responses were significantly lower in astrocytes treated with torin2 and rapamycin than if treated with torin2 alone.

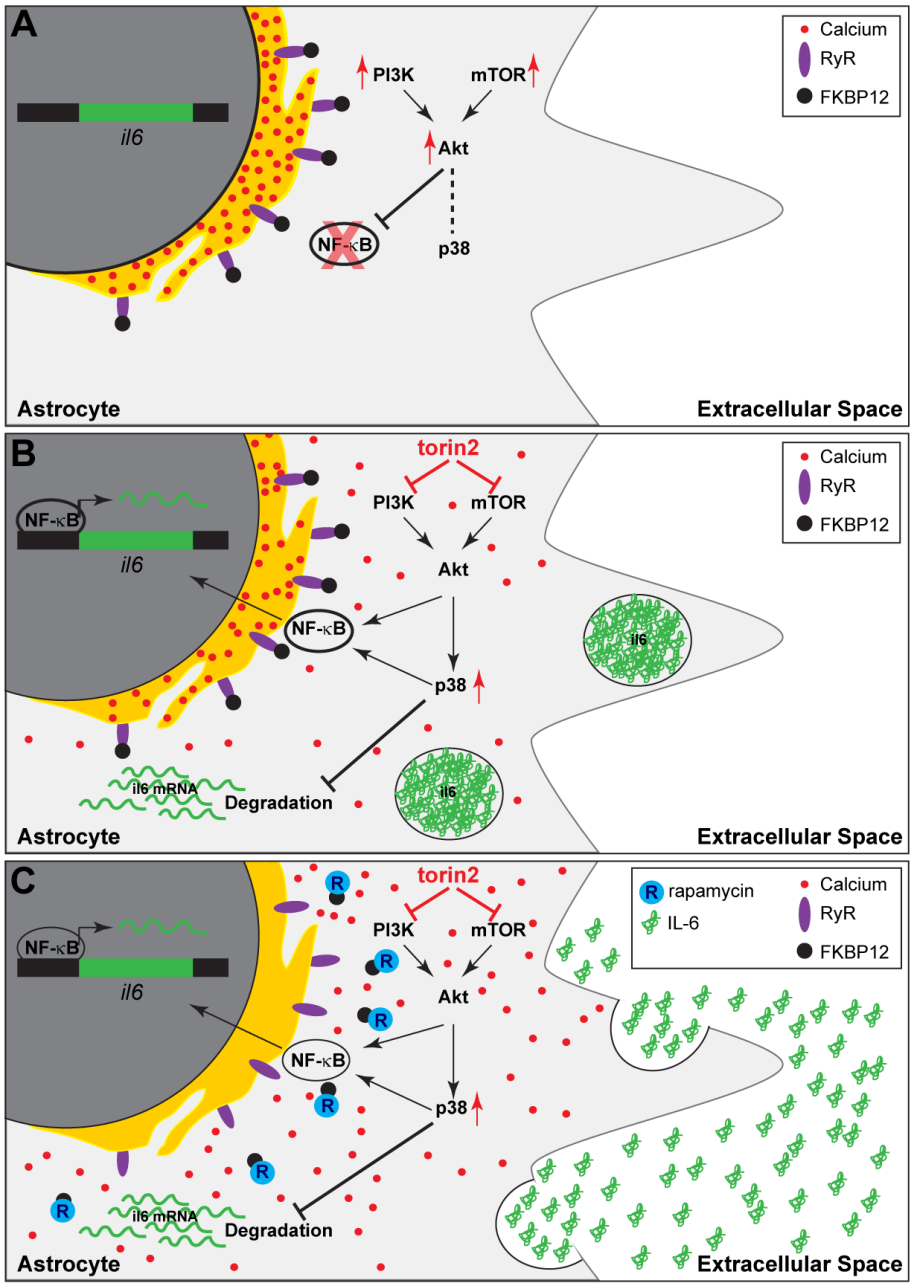


Figure 12 Diagram depicting IL-6 regulation in cultured astrocytes. **A)** In basal conditions the PI3K-mTOR-AKT pathway is active and prevents IL-6 expression in astrocytes. **B)** Inhibition of the PI3K-mTOR-AKT pathway by torin2 results in p38 activation and NF-kB mediated IL-6 transcription. Torin2 also decreases the amount of Ca^{2+} stored in the ER, suggesting an increase is cytosolic Ca^{2+} levels. This increase in cytosolic is not sufficient to cause IL-6 secretion. **C)** When astrocytes are treated with torin2 and rapamycin, cytosolic Ca^{2+} levels increase sufficiently (due to RyR opening) to trigger IL-6 secretion. Figure modified from Study I.

Thus, we hypothesized that torin2 treatment not only led to a depletion in Ca^{2+} stored in the ER, but presumably to an increase in cytosolic Ca^{2+} levels. Although this hypothetical increase in cytosolic Ca^{2+} might have not been sufficient to facilitate IL-6 secretion in astrocytes treated exclusively with torin2, in the presence of rapamycin, enhanced Ca^{2+} release from the ER into the cytosol could have been enough to trigger IL-6 secretion. Importantly, additional experiments are required to confirm our premise.

IL-6 mRNA and protein expression has been previously reported in acute stages after SCI [260]. Particularly, at 12 hours after SCI, IL-6 mRNA expression seems to be mainly controlled by microglia/macrophages, astrocytes and neurons [261]. Despite this, the expression of IL-6 in later stages of SCI remains controversial. In agreement with the studies above, we found IL-6 expression acutely after injury, specifically at 6 hours post-injury. Moreover, IL-6 was also detected at later stages, one to two weeks after SCI. Interestingly, we found that cells morphologically classified as neurons, astrocytes, immune cells and ependymal cells were able to synthesize IL-6 mRNA after SCI. We confirmed this observation by immunohistochemistry, which revealed that IL-6 co-localized with neuronal, microglial/macrophage and astrocytic cell markers after SCI.

Additionally, we found that after SCI, the vast majority of astrocytes expressing IL-6 did not show any mTORC1 activity as indicated by p-S6 immunoreactivity, a downstream target of mTORC1 which is often used as a readout [127]. Notably, we found an increase in mTORC1 activity in astrocytes three weeks after injury, when IL-6 levels return to baseline and the glial scar is fully formed.

Interestingly, several studies have suggested that IL-6 can act as a neuroprotective factor [124, 126]. Moreover, a study published earlier this year has reported that IL-6 can promote axonal regeneration and locomotor recovery after SCI in rats [262]. Therefore, stimulation of IL-6 production in the spinal cord in a time-controlled manner could be a promising strategy to improve recovery after SCI. Based on our *in vitro* and *in vivo* observations, we hypothesized that we could promote IL-6 secretion in the spinal cord of injured rats by blocking the PI3K-mTOR-AKT pathway during the sub-acute phase of SCI, when intrinsic levels of this cytokine are back to basal. Upon oral administration of torin2 and rapamycin, we found a transient reversal on mechanical hypersensitivity during the treatment window, despite a lack of improvement in locomotion recovery. Interestingly, several groups have reported that mTOR inhibition can have positive effects after SCI, including reduction of inflammation, tissue damage, glial scar formation, increased axonal regeneration as well as sensory and locomotor improvements [263-268]. On the other hand, other groups have reported positive effects upon mTOR activation after SCI [269, 270], indicating that the field is still quite controversial and that further investigations are required.

Of note, we cannot rule out that the positive sensory effect observed in this study might have been mediated by other cell types, as the drugs were administered systemically. Furthermore, such effect could have been due to the regulation of additional mechanisms by mTOR, like autophagy or anti-inflammatory actions, which have been previously

reported [271-273]. Since some studies have reported a pro-nociceptive role of IL-6 in SCI [224, 228], it would have been interesting to assess whether IL-6 levels were elevated when mechanical hypersensitivity was attenuated. However, we lost such opportunity since we decided to continue the treatment regime to explore whether the beneficial effect could be maintained over time. Moreover, it could have been interesting to investigate if the treatment led to any changes in the glial scar or promoted axonal regeneration.

In summary, in this study we have described that the inhibition of the PI3K-mTOR-AKT pathway is required for IL-6 production in astrocytes, an event that also relies on p38 and NF-kB activation. Moreover, we found that administration of torin2 and rapamycin in rats with SCI led to a transient improvement in mechanical hypersensitivity during the treatment period. Further work is needed to verify that IL-6 released by astrocytes contributed to the transient beneficial effect reported here.

4.2 STUDY II: AN EX VIVO SPINAL CORD INJURY MODEL TO STUDY EPENDYMAL CELLS IN ADULT MOUSE

Spinal cord injury (SCI) is a complex condition in which the interplay between cells and its surrounding environment influence disease progression. In this study, we aimed to develop a model of SCI that could serve as an intermediary step between *in vitro* and *in vivo* systems. We found organotypic slice cultures to be an attractive tool, since they retain the cytoarchitecture of the tissue of origin while allowing the study of cellular and molecular processes in an accessible way [274]. As traumatic SCI occurs mainly in adult individuals, we decided to use adult mouse spinal cord to set up our model. However, the use of adult tissue for preparation of organotypic slices can be quite challenging, due to a higher vulnerability to tissue damage upon slice sectioning and culturing conditions [275-277]. Taking this to our advantage, we postulated that slicing and culturing adult spinal cord could serve as an *ex vivo* model of SCI (Figure 13), which is characterized by an initial traumatic insult followed by secondary tissue damage.

We originally intended to use this injury model to study astrocytes, and build-up on the findings presented in **Study I**. Thus, we decided to prepare spinal cord slices from transgenic mice which allow the visualization of astrocytes that express GFP under the control of the human *Gfap* promoter (*hGfap*-GFP) [233]. However, when imaging *hGfap*-GFP cultured spinal cord slices over 7 days, we noted that the majority of astrocytes underwent cell death. On the contrary, we found that cells lining the central canal began to express GFP after 2-3 days in culture. By performing SCI in *hGfap*-GFP transgenic mice and staining for various ependymal markers, we corroborated that GFP expression was also taking place on the ependymal layer of SCI animals, thus ruling out that this phenomenon was an artefact of our *ex vivo* preparation. Moreover, we also performed immuno-histochemistry in the spinal cord slices and found out that ependymal marker expression was maintained upon culturing. This prompted us to think that ependymal cells might be undergoing some kind of "activation" and that our *ex vivo* SCI model could then be well-suited to study ependymal cell responses to injury. To confirm that, we assessed ependymal proliferation, migration and differentiation, which are characteristic events occurring after

traumatic SCI in different animal models [24, 108, 111]. Indeed, we found that ependymal cells could proliferate in culture, leading to an enlargement of the ependymal layer. However, while in the *in vivo* setting ependymal cells are usually arranged in 1-2 layers after SCI, in our *ex vivo* model they were arranged in multiple layers. This was possibly as a result of the culturing conditions, which lead to slice flattening over time. Despite this, we found very low variability in ependymal cell numbers across slides, indicating that proliferation can be quantified in a reproducible manner in this model.

Using cultured spinal cord slices made from *hFoxJ1*-CreER::YFP mice, which label ependymal cells and their progeny in a specific, inducible and heritable manner [234], we found that ependymal cells gave rise to progeny that could migrate away from the ependymal layer (Figure 13). In animal models of SCI, ependymal progeny commonly migrates in a polarized manner towards the lesioned area [24, 111]. Here, we observed that ependymal progeny migrated radially in all directions, most likely as a result of the transectional damage inflicted on the slices upon sectioning. In agreement with the literature, we observed that some of the ependymal progeny expressed GFAP and Sox9, indicating a possible differentiation of the ependymal progeny towards the astrocytic lineage [24, 111]. While we did not assess whether these cells could give rise to oligodendrocytes, we found no evidence of ependymal-derived neurons in our model. This is an interesting finding since spinal cord ependymal cells can give rise to neurons when cultured *in vitro*, while this phenomenon does not seem to take place *in vivo* [118]. This indicates that the surrounding environment can significantly influence ependymal cells, which should be taken into account when studying injury-related cellular responses.

Lastly, we performed Ca^{2+} imaging on ependymal cells, in order to verify that these cells were functionally active and viable in our SCI model. We used spinal cord slices cultured for 4-7 days, made from transgenic mice expressing the genetically encoded Ca^{2+} indicator GCaMP3 under the control of the mouse *Gfap* promoter (*mGfap*-Cre::GCaMP3). In contrast to the *hGfap*-GFP line, ependymal cells expressed *mGfap* in homeostatic conditions, as demonstrated by co-localization of ependymal markers with a tdTomato reporter driven by the *mGfap* promoter (*mGfap*-Cre::tdTomato). Since astrocytes also express *mGfap*, we selectively analyzed Ca^{2+} recordings from cells located in the

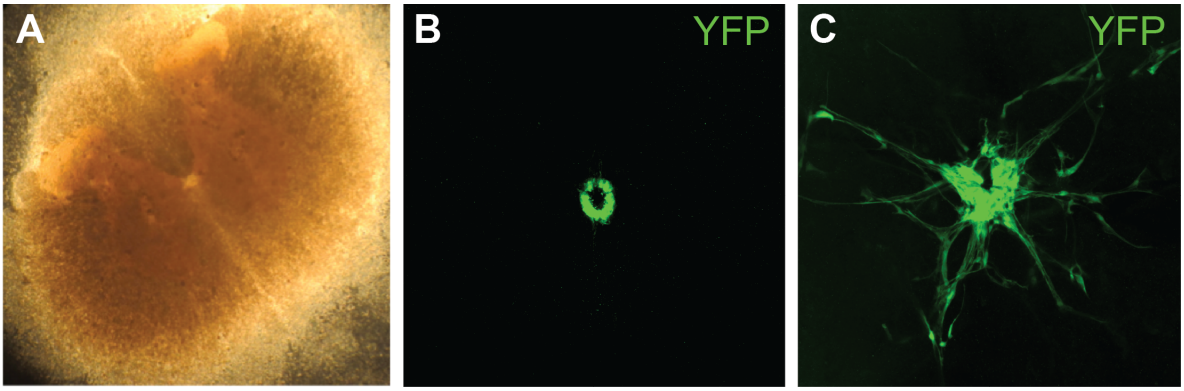


Figure 13 A) Adult mouse spinal cord cultured slice. **B)** *hFoxJ1*-CreER::YFP labelled ependymal cells in the intact spinal **cord C)** *hFoxJ1*-CreER::YFP labelled ependymal cells proliferate and migrate out of the ependymal layer in adult mouse spinal cord slices kept in culture for 7 days.

ependymal layer and in proximal migrating cells, to minimize the inclusion of possible astrocytic responses. Interestingly, we found that the majority of ependymal cells were able to respond to ATP stimulation, while a small proportion of ependymal cells exhibited spontaneous Ca^{2+} activity. While the functional implications of these Ca^{2+} responses were not investigated in this study, we can conclude that ependymal cells are functionally active and viable in our SCI model. Nevertheless, we believe that such Ca^{2+} transients could be involved in the proliferation, migration and differentiation of ependymal cells, since such cellular events can be regulated by Ca^{2+} [278]. Of note, ependymal cells have purinergic receptors and gap junctions [34, 279], indicating that Ca^{2+} could be an important messenger mediating ependymal-astrocyte communication. The fact that spontaneous Ca^{2+} activity was only observed in about 15% of the recorded cells could indicate that *1)* these cells comprise a particular sub-population of ependymal cells *2)* they may be ependymal-derived progeny on their way to astrocytic differentiation *3)* they represent astrocytes, which are known to exhibit spontaneous Ca^{2+} activity [13]. Future studies using transgenic lines specifically allowing the study of Ca^{2+} responses in ependymal cells would be ideal to gain a better understanding of the role of Ca^{2+} signalling in the ependymal cell response to injury and to corroborate our observations.

The model described here can serve different potential applications. For instance, it can facilitate the study of injury-related cellular processes with live imaging techniques in cells that are normally found at a considerable depth *in vivo*. In addition, it could also serve as a platform to reprogram ependymal cells into suitable progeny cell types in an injury setting, or to modulate the ependymal response to injury by testing different external effectors in a high-throughput manner.

Taken together, in this study we have demonstrated that cultured spinal cord slices are an excellent model of *ex vivo* SCI for the study of ependymal cells. We have shown that, in this model, ependymal cells recapitulate the main events that occur after injury in the *in vivo* setting, including proliferation, migration and differentiation. Furthermore, we suggest that ependymal cells can exhibit spontaneous Ca^{2+} activity and respond to ATP stimulation. Moreover, we propose the use of *hGfap*-GFP transgenic mice as a practical tool to visualize and track ependymal cells after injury *in vivo* and *ex vivo*. To conclude, we believe that our model provides an attractive platform to study and modulate ependymal cell responses that could complement *in vivo* studies and possibly contribute to the development of novel treatment avenues for SCI.

4.3 STUDY III: CENTRAL NERVOUS SYSTEM AUTOIMMUNITY IN RHEUMATOID ARTHRITIS: ANTI-CITRULLINATED PEPTIDE ANTIBODIES ACTIVATE HUMAN ASTROGLIAL CELLS AND INDUCE PAIN BEHAVIOUR IN MICE

Pain experienced by individuals with RA have been often attributed to inflammatory processes in the affected joints. However, there is current evidence indicating that central sensitization can also contribute to pain in RA [187]. Here, we went on to study whether anti-citrullinated peptide antibodies (ACPA), which have pro-nociceptive effects when injected systemically [163], could be found in the CSF of RA patients and be involved in centrally-mediated pain signal transmission by acting on resident cells of the CNS.

In this study, we describe, for the first time, the presence of ACPA in the CSF of RA individuals with high levels of ACPA in serum. ACPA was undetectable in CSF samples from patients with multiple sclerosis or non-inflammatory disease, which served as negative controls (Figure 14A). To ascertain the potential origin of the ACPA found in the CSF, we carried out several experiments. Firstly, we assessed the pattern of ACPA specificity towards common citrullinated epitopes and found that ACPA from serum and CSF samples had strong affinity to the same antigens. Secondly, we compared the serum/CSF ratios of ACPA and systemically produced anti-influenza antibodies, revealing no differences among the two. Taken together, the findings above point to a systemic rather than intrathecal production of ACPA, which most likely enter the CSF by redistribution from the periphery. Nevertheless, we cannot exclude the possibility that these antibodies might have been locally produced within the CNS.

Under pathological conditions, antibodies can penetrate blood-CNS-barriers as a result of local or systemic inflammation [280]. Interestingly, RA patients present elevated levels of IL-1ß in the CSF [216], and this particular cytokine can lead to alterations in blood-brain-barrier (BBB) permeability in several experimental studies [281-284]. Intriguingly, none of the RA patients examined had altered blood-CNS-barriers, as indicated by normal albumin indexes. Although it may seem controversial, antibody redistribution from the blood to the CSF/CNS over intact barriers has been previously reported, both by active transport or through the circumventricular organs, which lack a fully functional BBB [285, 286].

In this study, we also report that mice intrathecally (i.t.) injected with ACPA (30 µg) showed cold and mechanical hypersensitivity as compared to animals i.t. injected with ACPA-negative IgG from RA patients (referred to as flow through, FT, 30 µg), healthy IgG (30 µg) or PBS (Figure 14B, FT not shown). This is in contrast to mice that were intravenously (i.v.) injected with ACPA (125 µg to 4 mg), which also display signs of heat hypersensitivity [163]. However, while there are apparent differences in some aspects of the ACPA-induced pain observed in i.t. and i.v. injected animals, we cannot rule out that the pro-nociceptive effects described here are partially due to a redistribution of ACPA from the CSF to the periphery. Further studies are warranted to determine if this is the case. One possibility could be to inject ACPA i.v. at the dose used in this study (30 µg), and see whether hypersensitivity is induced or not. Alternatively, it would also be interesting to

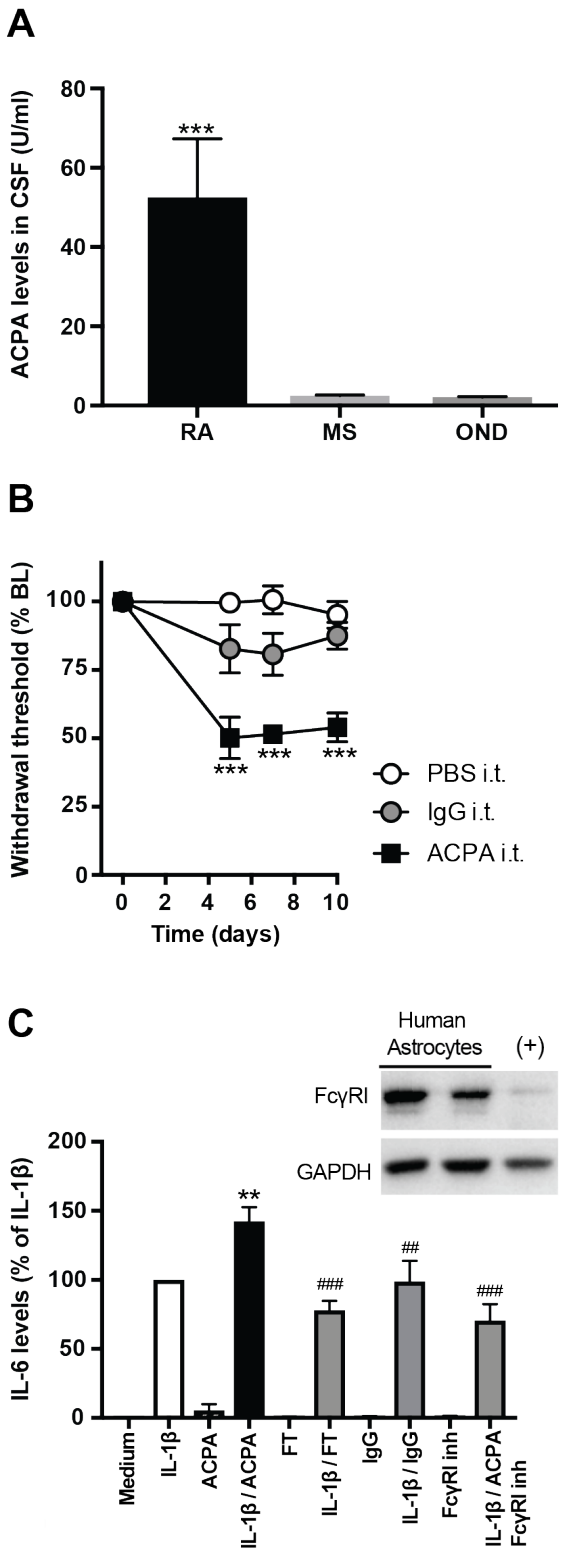


Figure 14 A) ACPA were detected in the CSF of rheumatoid arthritis (RA) patients in contrast to individuals with multiple sclerosis (MS) and non-inflammatory disease (OND). **B)** Mice i.t. injected with ACPA show mechanical hypersensitivity as compared to mice i.t. injected with healthy IgG or PBS. **C)** Co-stimulation of human fetal astrocytes with IL-1ß and ACPA significantly increases IL-6 release as compared to other conditions. This effect is abolished in the presence of an FcγRI inhibitor. **C, insert)** Human fetal astrocytes express FcγRI. Figure modified from Study III.

investigate whether pro-nociceptive effects are observed upon injection of ACPA directly into the CNS parenchyma. Certainly, it is tempting to speculate that ACPA found in the CSF (and potentially in the CNS) could be responsible for the residual pain that only a subset of ACPA-positive RA patients experience, despite taking appropriate medication for their condition [182].

Next, we decided to investigate whether ACPA could potentially interact with resident cells of the CNS. We focused on potential effects ACPA could exert on astrocytes, as they form a functional barrier that separates the CNS parenchyma from the vasculature and the meninges [21]. Most importantly, there is increasing evidence showing that astrocytes play an important role in the induction and maintenance of chronic pain [154, 188, 203, 217, 287]. Therefore, we reasoned that, if ACPA were to enter the CNS, they could initially act on astrocytes to drive central pain processes. We found that co-stimulation of cultured human fetal astrocytes with ACPA and IL-1ß, factors that are present in the CSF of RA patients, led to an increase in IL-6 mRNA levels and IL-1ß-induced IL-6 release, as compared to astrocytes co-stimulated with healthy IgG and IL-1ß. Stimulation with ACPA alone failed to induce IL-6 mRNA transcription and IL-6 release from astrocytes. This observation prompted us to think that ACPA might be forming immune complexes which could act on astrocytes by binding FcγRs. Of interest, FcγRI has been linked to pro-nociceptive signalling [167, 168]. Notably, we found that astrocytes in culture expressed FcγRI and that incubation of astrocytes with an FcγRI inhibitor abolished the enhanced IL-6 release caused by ACPA and IL-1ß co-stimulation (Figure

14C). In the future, we aim to determine whether astrocytes express citrullinated epitopes that could be involved in immune complex formation or serve as antigens for ACPA. Interesting candidates include vimentin and GFAP, proteins that are expressed and upregulated upon astrocyte activation. Particularly, citrullinated vimentin is an important factor for immune complex formation in the synovial fluid of ACPA-positive RA patients [288], and both vimentin and GFAP levels are elevated in synovial fluid and serum of individuals with RA [289]. Of note, citrullinated GFAP has been reported in the brain of patients with multiple sclerosis, and it is thought to play a role in the pathophysiology of the disease [290].

To conclude, our findings favour the idea that ACPA may potentially reach the CNS of RA patients, act on glial cells and activate mechanisms that could promote central sensitization. Future work should aim at elucidating whether ACPA can certainly be detected in the CNS of RA patients. Moreover, it could also be interesting to investigate whether ACPA could mediate central effects by interacting with other cell types in the CNS/CSF.

4.4 STUDY IV: EXPLORING THE TRANSCRIPTOME OF RESIDENT SPINAL MICROGLIA AFTER COLLAGEN ANTIBODY-INDUCED ARTHRITIS

The contribution of male and female microglia to experimental chronic pain states is currently under debate. While some studies have provided evidence for an exclusive role of male microglia, others have reported that female microglia might also be involved [230, 231, 291].

In this study, we went on to study possible differences in male and female lumbar dorsal horn spinal microglia in the context of arthritis-induced pain. We used the CAIA animal model, which is characterized by transient joint inflammation and persistent mechanical hypersensitivity [154]. These animals display a concomitant increase in male and female microglial IBA-1 reactivity in the dorsal horns of the lumbar spinal cord (where hind limb nociceptive processing occurs), suggesting that microglia could be involved in the maintenance of arthritis-induced pain in both sexes (Figure 15A). To test if this was the case, we injected minocycline (often described as a “microglial inhibitor”) intrathecally into male and female mice during the late phase of the CAIA model (Figure 15B,C), when joint inflammation has resolved but mechanical hypersensitivity and microglial activation are still present. In agreement with data from neuropathic pain models, we found that minocycline was only able to revert CAIA-induced pain to control levels in male mice [230, 232]. Although these findings point to a sexually dimorphic role of microglia in the maintenance of persistent pain states, it is important to note that minocycline is not a selective microglial inhibitor [292]. Hence, we advise for a prudent interpretation of these results and those in the existing literature with regards to the cell type mediating such effects.

In light of the findings described above, we decided to take a global approach to explore microglial transcriptional sex-differences in relation to CAIA-induced persistent pain.

Initially, we investigated whether macrophages could infiltrate into the lumbar spinal cord after CAIA induction, as this would require the use of additional markers to discriminate them from resident microglia. Since we found no signs of macrophage infiltration at the different time points examined after CAIA induction, we decided to sort resident lumbar dorsal horn microglia by FACS using CD45 and CD11b as markers. We found no differences in relative microglial numbers between saline and late phase CAIA conditions, however, when comparing male and female microglia, we observed a lower proportion of spinal microglia in female mice. In line with our findings, sex differences in microglial cell numbers have also been reported in brain areas such as the hippocampus or the amygdala [54].

Upon sequencing, we found 11,567 genes expressed in our lumbar dorsal horn microglia samples. Of note, there was an 85% overlap between our dataset and other RNA-seq studies performed on microglia isolated from the brain or spinal cord [170, 293-296]. We compared microglial transcriptomes by principal-component analysis (PCA) and found that male and female samples were well separated into two distinguishable groups (Figure 16A). We detected 21 differentially expressed genes among both sexes, 5 of which were X or Y linked genes. When re-running the PCA analysis without the differentially expressed sex-linked genes, male and female samples were no longer separated into groups, indicating that microglial transcriptional sex differences are limited and mostly accounted for by sex-linked genes. Despite this, we went on to explore putative interactions between the top 46 male and female dysregulated genes (unadjusted p-value <0.001) and found that members of the heat shock protein (Hsp) family comprised most of the gene network generated (Figure 16B). Hsp were mostly upregulated in male microglia and were highly interconnected. Notably, sex differences in Hsp have been reported in the literature [297-299]. Moreover, Hsp have been associated with microglia activation [206, 300] and pain processing [204, 205], making them an attractive target for future studies and for independent validation of our RNA-seq results.

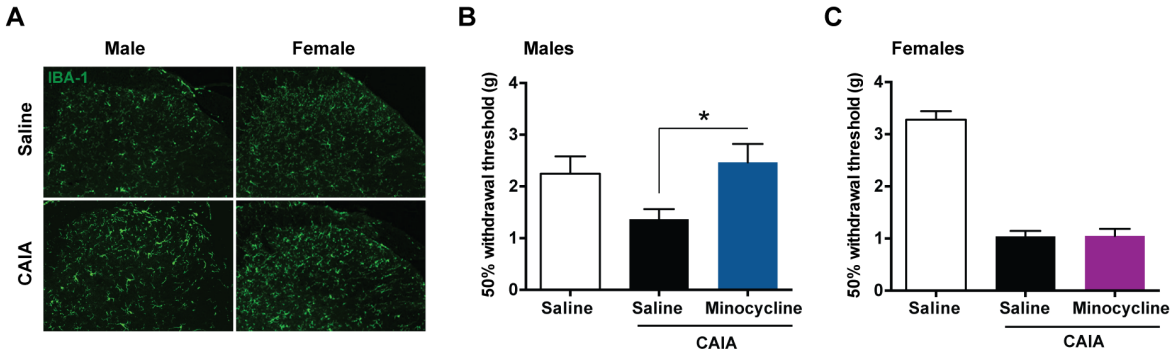


Figure 15 A) Male and female mice injected with CII antibodies (CAIA model) show microglia reactivity as compared to saline injected mice. **B,C)** Minocycline injected intrathecally reverts CAIA-induced mechanical hypersensitivity in males but not females. Figure adapted from Study IV.

Intriguingly, we did not identify any convincing transcriptional differences between the saline and late phase CAIA groups, despite being well-powered to detect 1.5-2.0 fold changes in gene expression in the top 3,000 expressed genes. This observation may indicate that *1)* transcriptional changes during the late phase of the CAIA model are either subtle and/or highly localized, and hence difficult to detect using bulk RNA-seq *2)* transcriptional changes after CAIA induction are transient and short-lasting *3)* the microglia separation protocol might have induced similar activation in microglia from saline and CAIA groups, masking any transcriptional differences that may be present *in vivo*. We believe this last point to be unlikely, as other RNA-seq studies which have used similar microglial isolation protocols still identify transcriptional differences between conditions [301-303]. The fact that no transcriptional changes were detected between the saline and CAIA groups suggests that late phase CAIA microglial activation and the potential involvement of microglia in CAIA-induced pain could be mediated by other factors, like changes in protein expression or epigenetic modifications.

Taken together, our findings provide evidence for subtle but significant sex differences in spinal microglia isolated from lumbar dorsal horns. The extent to which such differences could bring about the dimorphic effect of minocycline in CAIA-induced pain remains to be elucidated. Although we observed signs of microglia activation in the late phase of CAIA, no transcriptional changes were detected when compared to controls. Therefore, it seems likely that changes in gene expression were subtle, highly localized or short-lasting. Complementary behavioral studies using tools that target specific microglial pathways could be a suitable strategy to get a clearer picture as to the role of male and female microglia in CAIA-induced pain.

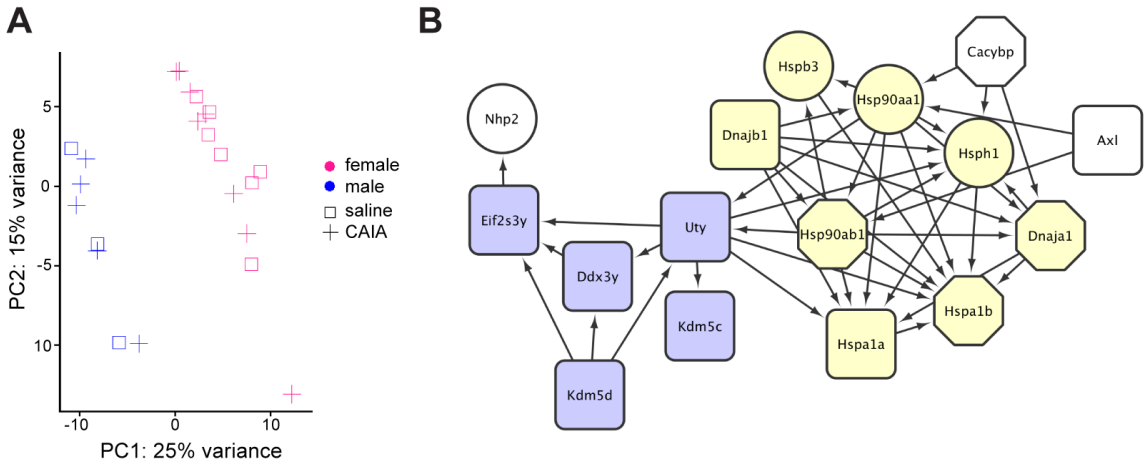


Figure 16 A) Principal component analysis comparing male and female microglia in mice i.v. injected with saline or subjected to CAIA. **B)** Gene network showing differentially expressed genes in male vs female microglia using different p-values as a cut-off. Circles: unadjusted p<0.001; rectangles: adjusted p<0.05; octagons: adjusted p<0.1. Blue genes are also differentially expressed in male vs. female sensory neurons. Yellow genes belong to the family of heat shock proteins. Figure modified from Study IV.

5 CONCLUDING REMARKS

In this thesis, we have focused on deepening our understanding of glial pathophysiological events in relation to spinal cord injury, rheumatoid arthritis and pain-related processes. A running theme across our work has been to make our studies as translational as possible, either by incorporating animal models and patient material or by developing a model that could serve as a bridge between *in vitro* and *in vivo* platforms to study glial cell responses. When possible, we have also tried to go from single molecules to cellular responses all the way to finding behavioral outcomes.

Importantly, the role that glial cells play on pathological process is by no means static. Glia are very dynamic and can respond differently according to the environment. It is therefore that the study of glial cells at different stages of disease becomes critical to understand how to harness their full potential while avoiding any associated detrimental consequences. Indeed, the modulation of glial cells as a strategy to improve the outcome of SCI is capturing the attention of the scientific community. In **Study I**, we went from deciphering molecular pathways in cultured astrocytes to applying such concepts *in vivo* to try and modulate astrocytes after SCI. Moreover, in **Study II**, we have described an attractive platform to modulate ependymal cell responses in an injury setting, which could aid in the development of novel treatment avenues for SCI.

It is an intriguing thought that difficulties in developing new pain therapeutics may in part be due to an overseen role of glia. Hence, it is critical to explore the role of these cells in pain transmission to determine the feasibility of targeting astrocytes and/or microglia in conditions of chronic pain. In **Study III**, we have provided an interesting hypothesis as to how central sensitization could be driven in RA, which may depend on the interaction of autoantibodies and astrocytes. In **Study IV** we decided to study the role of transcriptional changes in microglia in arthritis-induced persistent pain paying particular attention to sex-differences. While transcriptional changes did not drive persistent pain in at the time point studied, we found some differences in male and female microglia. Further investigations are warranted to clarify whether the sex-related role of glia is restricted to selected mechanisms, pain modalities or animal models, and how these observations could be relevant to clinical pain.

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