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**Implication of system x_c^- in neuroinflammation during
the onset and maintenance of neuropathic pain**

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Cover illustration: Iba-1 immunostaining in lumbar spinal cord of mice following partial sciatic nerve ligation.

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List of publications

- Pierre J. Doyen, **Pauline Beckers**, Gary A. Brook, Emmanuel Hermans. Regulation of G protein signaling as pharmacological targets for the treatment of neuropathic pain. *Pharmacological Research* 2020, 160:105148.
- Mattia Ferraiolo, **Pauline Beckers**, Nicolas Marquet, Martin Roumain, Lucie Ruiz, Nadine Dupuis, Julien Hanson, Emmanuel Hermans. β -arrestin2 recruitment at the β 2 adrenergic receptor: A luciferase complementation assay adapted for undergraduate training in pharmacology. *Pharmacology Research & Perspective* 2021, 9(1):e00706.
- Mattia Ferraiolo, Hicham Atik, Romane Ponthot, Ines Belo do Nascimento, **Pauline Beckers**, Christophe Stove, Emmanuel Hermans. Receptor Density Influences Ligand-Induced Dopamine D2L Receptor Homodimerization. *European Journal of Pharmacology* 2021, 911:174557.
- **Pauline Beckers**, Olaya Lara, Inês Belo do Nascimento, Nathalie Desmet, Ann Massie, Emmanuel Hermans. Validation of a system x_c^- functional assay in cultured astrocytes and nervous tissues samples. *Frontiers in Cellular Neuroscience* 2022, 15:815771.
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- **Pauline Beckers**, Pierre J. Doyen, Emmanuel Hermans. Modulation of type 5 metabotropic glutamate receptor-mediated intracellular calcium mobilization by regulator of G protein signaling 4 (RGS4) in cultured astrocytes. *Cells* 2024, 15:98:117564.
- Olaya Lara, Pauline Janssen, Marco Mambretti, Laura De Pauw, Gamze Ates, Liselotte Mackens, Jolien De Munck, Jarne Walckiers, Zhaolong Pan, **Pauline Beckers**, Elisa Espinet, Hideyo Sato, Mark de Ridder, Daniel L. Marks, Kurt Barbé, Joeri L. Aerts, Emmanuel Hermans, Ilse Rooman, Ann Massie. Compartmentalized role of xCT in supporting pancreatic tumor growth, inflammation and mood disturbance in mice. *Brain, Behavior, and Immunity* 2024, 10.1016/j.bbi.2024.03.001.
- **Pauline Beckers**, Inês Belo do Nascimento, Mathilde Charlier, Nathalie Desmet, Ann Massie, Emmanuel Hermans. Implication of system x_c^- in neuroinflammation during the onset and maintenance of neuropathic pain. *Journal for Neuroinflammation* 2024, 10.1186/s12974-024-03112-9.

Summary

In chronic pain with neuropathic or inflammatory components, peripheral and central sensitizations stand as prominent hypotheses, characterized by an adaptation of the nervous system to persistent inflammatory states and increased synaptic excitability along pain pathways. Notably, disruptions in glutamate homeostasis, including alterations in the expression and functionality of high-affinity glutamate transporters in astrocytes, are closely associated with this phenomenon. Besides, activation of peripheral and central immune cells, such as macrophages and microglia reinforces and sustains the inflammatory component of chronic pain while also influencing neuronal transmission.

Mainly expressed on glial cells and macrophages the cystine – glutamate exchanger (also known as system x_c^-), known to reinforce glutamate transmission and support neuroimmune responses is receiving growing interest in several neurological disorders. Therefore, the present PhD thesis investigates the impact of neuropathic lesions on the regulation of system x_c^- expression and functionality, as well as the consequences of targeting system x_c^- on the development of pain hypersensitivity.

Our research reveals that the system x_c^- subunit xCT is specifically upregulated in the ipsilateral dorsal spinal cord of lesioned animals. Furthermore, by monitoring the behavioral responses to mechanical and thermal stimuli, we have demonstrated that animals lacking xCT exhibit significantly lower allodynia and hyperalgesia after sciatic nerve lesion compared to their wild-type littermates. Consistently, reduction in pain sensitization induced by the nerve lesion is observed in mice treated with the system x_c^- inhibitor sulfasalazine.

Focusing on the neuroinflammatory component associated with glial activation, we have evidenced an increased astrocytic and microglial M1 phenotype activation in the spinal cord of wild-type lesioned animals. Induction of these glial biomarkers was found significantly lower in animals lacking xCT or following the sulfasalazine treatment.

Considering the presence of system x_c^- on peripheral immune cells such as macrophages, we complemented our research using a model of chronic pain triggered by intraplantar injection of complete Freund's adjuvant. Our results demonstrate that both the pharmacological and genetic modulation of system x_c^- exhibits analgesic properties. This is correlated with a significantly reduced local and systemic inflammation in transgenic mice lacking xCT compared to their wild-type littermates.

In light of these data, we conclude that system x_c^- plays a significant role in the biochemical processes underlying chronic neuropathic and inflammatory pain. The observed analgesic effects in transgenic mice lacking xCT or wild-type mice treated with sulfasalazine appear to be mediated by a reduction in gliosis and alterations in microglial polarization towards a more anti-inflammatory state.

As chronic pain remains an important clinical issue, it appears essential to identify new effective therapeutic strategies. This study paves the way for promising treatment to alleviate chronic inflammatory and neuropathic pain in patients.

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List of abbreviations

5-HT:	serotonin
6-OHDA:	6-hydroxydopamine
AD:	Alzheimer's disease
ALS:	amyotrophic lateral sclerosis
AMPA:	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APP:	amyloid precursor protein
ASIC:	acid-sensing ion channel
ATF2:	activating transcription factor 2
ATF3:	activating transcription factor 3
ATF4:	activating transcription factor 4
ATP:	adenosine triphosphate
BBB:	blood-brain barrier
BDNF:	brain-derived neurotrophic factor
CCL2:	chemokine (C-C motif) ligand 2
CD44v:	CD44 variant
CFA:	Complete Freund's adjuvant
CGRP:	calcitonin gene-related peptide
CHO:	Chinese hamster ovary
CNS:	central nervous system
CREB:	cAMP-response element binding protein
Cx43:	connexin-43
CXCL13:	chemokine (C-X-C motif) ligand 13
CXCR5:	C-X-C motif chemokine receptor 5
DAMPs:	damage-associated molecular pattern
dBcAMP:	dibutyl cyclic adenosine monophosphate
DRG:	dorsal root ganglia
EAAT:	excitatory amino acid transporter
EAE:	experimental autoimmune encephalomyelitis
eIF2 α :	eukaryotic initiation factor 2 alpha
ENK:	enkephalin

ERK:	extracellular signal-regulated kinases
GABA:	gamma-aminobutyric acid
GCN2:	general control nonderepressible 2
GFAP:	glial-derived neurotrophic factor
GFAP:	glial fibrillary acidic protein
GLAST:	glutamate aspartate transporter
GLT-1:	glutamate transporter 1
GlyR:	glycine receptor
GSH:	glutathione
H2Bub1:	histone H2B monoubiquitination
HCA:	homocysteic acid
IASP:	International Association for the Study of Pain
Iba-1:	ionized calcium-binding adapter molecule 1
IC ₅₀ :	half maximal inhibitory concentration
iGluRs:	ionotropic glutamate receptors
IKE:	imidazole ketone erastin
IL-10:	interleukin-10
IL-1 β :	interleukin-1 β
IL-1R:	interleukin-1 receptor
IL-6:	interleukin-6
Keap1:	Kelch-like ECH-associated protein 1
LPS:	lipopolysaccharide
LTHA:	L-threo-3-hydroxyaspartic acid
MAPK:	mitogen-activated protein kinase
mGluRs:	metabotropic glutamate receptors
miR:	microRNA
MPTP:	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS:	multiple sclerosis
mSNI:	modified spared nerve injury
mTORC1:	mammalian target of rapamycin complex 1
mTORC2:	mammalian target of rapamycin complex 2
NA:	noradrenaline
Na _v :	voltage-gated sodium channels
NF- κ B:	nuclear factor-kappa B

NGF:	nerve growth factor
NMDA:	N-methyl-D-aspartate
NO:	nitric oxide
Nrf2:	nuclear factor erythroid 2-related factor 2
NSAIDs:	nonsteroidal anti-inflammatory drugs
OTUB1:	otubain-1
PBS:	phosphate buffer saline
PD:	Parkinson's disease
PGE ₂ :	prostaglandin E ₂
PI ₃ K:	phosphoinositide 3-kinase
PKC:	protein kinase C
PNS:	peripheral nervous system
PSNL:	partial sciatic nerve ligation
PWL:	paw withdrawal latency
PWT:	paw withdrawal threshold
ROS:	reactive oxygen species
RT-qPCR:	real-time polymerase chain reaction
SAS:	sulfasalazine
SNRI:	serotonin and noradrenaline reuptake inhibitor
SOCS2:	suppressor of cytokine signaling 2
SOD1:	superoxide dismutase 1
TCA:	tricyclic antidepressant
TENS:	transcutaneous neurostimulation
TGF- β :	transforming growth factor beta
TNF- α :	tumor necrosis factor alpha
TRP:	transient receptor potential
TRPV1:	transient receptor potential vanilloid type 1
USP7:	ubiquitin special peptidase 7
WDR:	wide dynamic range

INTRODUCTION

CHAPTER I: Introduction to pain

1. Generality on pain

Pain is a multifaced sensory experience perceived as a result of a noxious stimulation that is potentially harmful to the physical integrity of individuals. According to the international association for the study of pain (IASP), pain is defined as “an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage”. In physiological conditions, pain is considered a protective and adaptative response serving as a warning signal of which gradual transmission reflects the intensity of the stimuli received (Hardy et al. 1947). Pain is also essential for individuals and animals to guide their behavior in potentially harmful conditions. If this warning signal is deficient, individuals are more exposed to danger that can lead to multiple trauma as observed in patients suffering from congenital insensitivity to pain (Daneshjou et al. 2012).

According to the Institut National de la Santé et de la Recherche Médicale (Inserm), pain-related complaints account for more than two-thirds of medical consultations. These medical concerns may be from different origins and affect all categories of patients, although they are predominantly women. Indeed, more and more research describe lower pain sensitivity threshold in women as compared to men (Fillingim et al. 2009; Mogil 2012).

In addition to gender, there is an important inter-individual variability in pain perception which is heavily dependent on physiological and emotional contexts, making pain a highly complex phenomenon to study (Lumley et al. 2011; Eccleston 2001).

Before discussing the physiological mechanisms supporting the development and the transmission of pain, it is essential to clarify the distinction between

pain and nociception. Indeed, these two terms are often mishandled in the literature, particularly when referring to animal studies (Deuis et al. 2017). Nociception refers to the physiological process that facilitates the detection and the transmission of sensory information generated upon the activation of dedicated peripheral endings. Pain perception occurs only when this sensory information reaches cerebral structures. Therefore, pain is an outcome of the processing of nociceptive signals in higher brain centers, while nociception can occur independently of the sensation of pain (Mischkowski et al. 2018). Importantly, the absence of pain does not preclude the occurrence of nociception and vice versa.

2. Normal nociception

Neurons that respond selectively to stimuli generated by tissue damage are called nociceptors. The stimuli can either be chemical (e.g. presence of pro-inflammatory mediators), thermal (e.g. heat or extreme cold) or mechanical (e.g. trauma, excessive pressure). Afferent nociceptors are pseudo-unipolar neurons of which cell bodies are located in the dorsal root ganglia (DRG) or trigeminal ganglia (Dubin and Patapoutian 2010). They possess a single axon that divides into a peripheral branch, which forms free terminals in skin territories, but also in muscles, tendons, and viscera. The other branch, known as the central one, contacts the neuronal networks of the dorsal spinal cord or the brainstem (spinal trigeminal nucleus).

There are two major classes of primary afferent nociceptors capable of detecting potential harmful stimuli: A δ and C fibers (Ma 2001; Basbaum et al. 2009; Woolf and Ma 2007).

On one hand, the “fast” A δ *fibers* that have thinly myelinated axons are essentially high-threshold mecano- and thermonociceptors involved in acute, localized and transient pain (Dubin and Patapoutian 2010). They are responsible for the initial sharp pain perceived at the time of the injury (Beissner et al. 2010). A δ fibers not only establish synapses with wide dynamic

range (WDR) neurons in the deeper layers of the spinal cord (lamina V), but also with nociception-specific neurons in the superficial layers of the dorsal horn (lamina I and II) (Strominger et al. 2012; Dubin and Patapoutian 2010). WDR neurons are able to generate long range responses giving them the ability to help identify the location and intensity of painful stimulation. They use L-glutamate as the major excitatory neurotransmitter (Kendroud et al. 2023).

On the other hand, the “slow” *C fibers*, representing the largest group of nociceptors, are unmyelinated and in charge of the secondary pain sensation which is diffuse, dull and longer-lasting (Beissner et al. 2010). In this subpopulation of nociceptors, most of the afferences are polymodal, responding to thermal, chemical, and mechanical stimuli. At the spinal level, C fibers mainly project to the superficial layers of the spinal cord, laminae I and laminae II. Among C fibers, two subtypes can be distinguished: the peptidergic and non-peptidergic fibers (Basbaum et al. 2009; Woolf and Ma 2007). Peptidergic fibers communicate with second order neurons through the release of neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) as well as with glutamate. On the other hand, the non-peptidergic fibers mainly operate with glutamate and are documented to express a subclass of purinergic receptors (P_2X_3) for adenosine triphosphate (ATP) and the receptor for glial cell-derived neurotrophic factor (GDNF) (Basbaum et al. 2009).

Of note, there are peculiar $A\delta$ and C fibers, also known as “sleeping” or “silent” nociceptors, characterized by unique properties. These fibers generally remain unresponsive to noxious stimuli except at extreme range of intensity. Indeed, they are only recruited in case of prolonged or particularly intense nociceptive stimulations accompanied with the release of pro-inflammatory factors, leading to a reduction in their activation threshold (Handwerker et al. 1991; Willis and Westlund 1997; Messlinger 1997).

Between the detection of the noxious stimuli in the periphery and the perception of pain in the brain, the generation and regulation of the signal operate at four distinct stages: transduction, transmission, perception, and modulation (Figure 1).

The *transduction* or receptor activation refers to the conversion of a mechanical, thermal, or chemical stimulus into electrical signals. These signals, in the form of action potentials, can then propagate to the brain via nociceptive afferent neurons.

Tissue injury triggers a complex cascade of events that involve the release of a variety of factors which include inflammatory mediators (cytokines, chemokines, prostaglandins), growth factors, histamine, bradykinins, proteases, ATP and nitric oxide (NO) that are responsible for the initiation of an inflammatory state and pain perception (Julius 2013; Yam et al. 2018). These locally released chemicals facilitate the opening of non-selective ion channels such as acid sensing ion channels (ASIC), and diverse transient receptor potential (TRP), leading to massive entry of sodium (Na^+) and calcium (Ca^{2+}) ions in free nerve endings of nociceptors. Upon reaching a sufficient threshold, this depolarization triggers an action potential that propagates along the neuron through the activation of voltage-dependent Na^+ channels (Basbaum et al. 2009; Julius and Basbaum 2001; Yam et al. 2018).

ASICs are a group of proton-induced Na^+ channels capable of detecting changes in pH with ASIC3 and ASIC1b specifically involved in acidic nociception as observed in context of inflammation or ischemia. TRP channels represent a broader family of channels involved in several nociceptive transduction signals (Clapham 2003; Voets et al. 2005). Among them, TRPV1 is mainly activated by heat ($>42^\circ\text{C}$), acidity and molecules such as capsaicin. TRPA1 is sensitive to thermal, mechanical, and chemical stimuli. Like TRPV1, TRPV3 channels respond to heat ($>33^\circ\text{C}$) and participate in the transduction

of warm sensation. Concerning the detection of cold sensation (8 – 28°C), TRPM8 will be preferentially activated (Julius 2013).

At the central terminal of the first-order neurons, the generated action potential triggers the influx of Ca^{2+} which, in turn, facilitates the release of neurotransmitters and neuromodulators such as glutamate, substance P and CGRP (Yam et al. 2018; Basbaum et al. 2009). The glutamatergic synaptic relay of action potentials at the spinal cord primarily relies on the interaction of glutamate with ionotropic glutamate receptors (iGluRs), including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and kainate receptors, triggering the influx of Na^+ into the postsynaptic neuron and its depolarization. In case of intense or prolonged stimulation, glutamate also activates N-methyl-D-aspartate receptors (NMDAR) on the postsynaptic neuron, leading to the combined influx of Na^+ and Ca^{2+} which will contribute to more complex and persistent cell responses known to support synaptic plasticity (Basbaum et al. 2009; Tao et al. 2005). Glutamate also interacts with metabotropic glutamate receptors (mGluRs), which modulate neurotransmission more slowly and contribute to long-term changes in synaptic function (Hudson et al. 2002). Additionally, at higher stimulation frequencies, co-released substance P and CGRP act on their own receptors (neurokinin 1 receptor and CGRP receptor, respectively), modulate glutamatergic transmission, and thereby influence the overall pain signaling process (Rogoz et al. 2014).

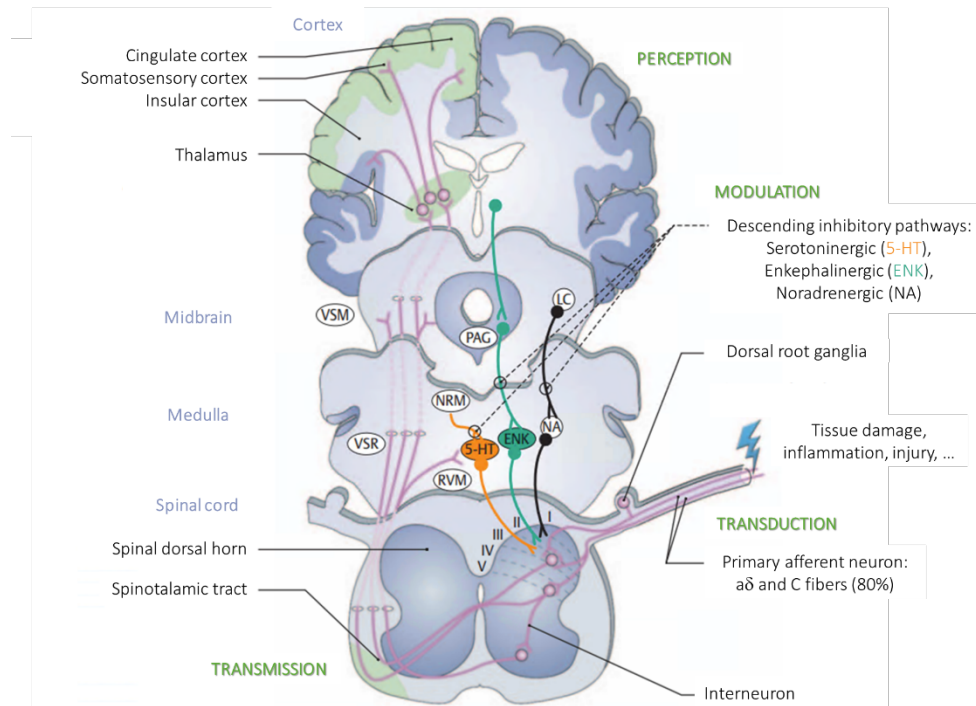


Figure 1: General pain pathways. Nociceptive nerve impulses are relayed to the dorsal horn of the spinal cord by peripheral nociceptors. Nociceptive information travels through the spinothalamic tract between the spinal cord and the brain, where it projects to the dedicated structures contributing to pain perception. Descending pain modulation systems, consisting of projections from the brain to the dorsal horn, inhibit or facilitate spinal nociceptive transmission. Connections with different brain structures allow for the integration of diverse pain components and their modulation. LC: locus coeruleus, NRM: nucleus raphe magnus, PAG: periaqueductal grey matter, RVM: rostral ventromedial medulla, VSM: spinomesencephalic tract, VSR: spinoreticular tract, 5-HT: serotonin, ENK: enkephalin, NA: noradrenaline. Figure modified from (Fields 2004), adapted from (Beaulieu 2013).

The transfer of nociceptive information from the periphery to the higher pain centers is referred to as *transmission*. The peripherally encoded information from primary afferent neurons is relayed through synaptic connections with a complex array of spinal neurons, each serving distinct roles in nociceptive

processing and pain perception. Primary afferent fibers also establish connections with motor neurons via excitatory and inhibitory interneurons in the ventral horn, contributing to the generation of nociceptive withdrawal reflexes (Derderian et al. 2023). During nociceptive transmission, *modulation* processes operate at the spinal cord and brain level to influence the intensity and the duration of the nociceptive influx. Additionally, neurons of the spinal cord transmit the nociceptive impulses to brain structures through the second-order neurons (spinothalamic tract) and then to the third-order neurons (thalamocortical pathways). A large number of studies have provided crucial insights regarding the cortical processing of nociceptive information, revealing that pain perception is not confined to a single brain region. Indeed, the nociceptive information reaches several regions of the somatosensory cortex (sensory component) and specific limbic structures (emotional and memory components). Activation of the prefrontal cortex is also related to the cognitive aspects of pain *perception* (Apkarian et al. 2005; Basbaum et al. 2009). The involvement of these distinct brain regions underlies several features of pain perception and the cognitive recall of responses or emotional reactions.

3. Pain modulation and the gate control theory

So far, the description of nociceptive system has primarily focused on the afferent excitatory component. However, nociceptive activity does not always correlate with pain perception, and conversely, pain can be perceived without evident tissue damage. Hence, a signal modulation process operates within this system, influencing the ascending pain pathway. This modulation stage refers to a set of mechanisms through which the nervous messages originating from nociceptors can be modulated or adjusted at both spinal and brain levels during the ascending transmission.

In 1965, Melzack and Wall presented the gate control theory of pain stating that the transmission of nociceptive signals can be modulated pre-

synaptically at the dorsal spinal cord level by non-painful inputs as well as by descending signals coming from the brain (Melzack and Wall 1965). Over the years, the understanding of pain modulation has largely advanced leading to revision of this theory (Woolf 2022; Wall 1996). Nevertheless, from this original model emerged the fundamental concept of modulating pain perception and the dorsal spinal cord is now considered as a key integration center for nociceptive signals. This model helps to understand the mechanisms of action of several analgesics, the most prominent being opioids that have been used for thousands of years. The gate control theory is also at the basis of various clinical applications such as the transcutaneous neurostimulation (TENS), whereby electrical stimulation of the non-nociceptive A β afferences of the skin is applied to inhibit the nociceptive A δ and C fibers (Garrison and Foreman 1994). TENS has been proven effective to relief chronic pain, at least at short-term (Richardson et al. 1981; Vance et al. 2014; Johnson et al. 2022).

As first proposed, the gate control theory consists essentially of interneurons that inhibit first-order neurons, thereby stopping or reducing signal transmission. As presented in figure 2, non-painful inputs typically “close the gate” to painful inputs, reducing pain through the activation of inhibitory interneurons. These interneurons are mainly located in the superficial layers of the dorsal horn (laminae I and II) and release inhibitory transmitters including gamma-aminobutyric acid (GABA), glycine or enkephalin (ENK). Upon activation, nociceptors stimulate second-order neurons, and at the same time, inhibit the inhibitory interneurons, thus “opening the gate” allowing transmission of pain signals. However, if non-noxious stimuli are also present, highly myelinated A β -fibers are stimulated and these reactivate the inhibitory interneurons, reducing the transmission of pain signals (Melzack and Wall 1965).

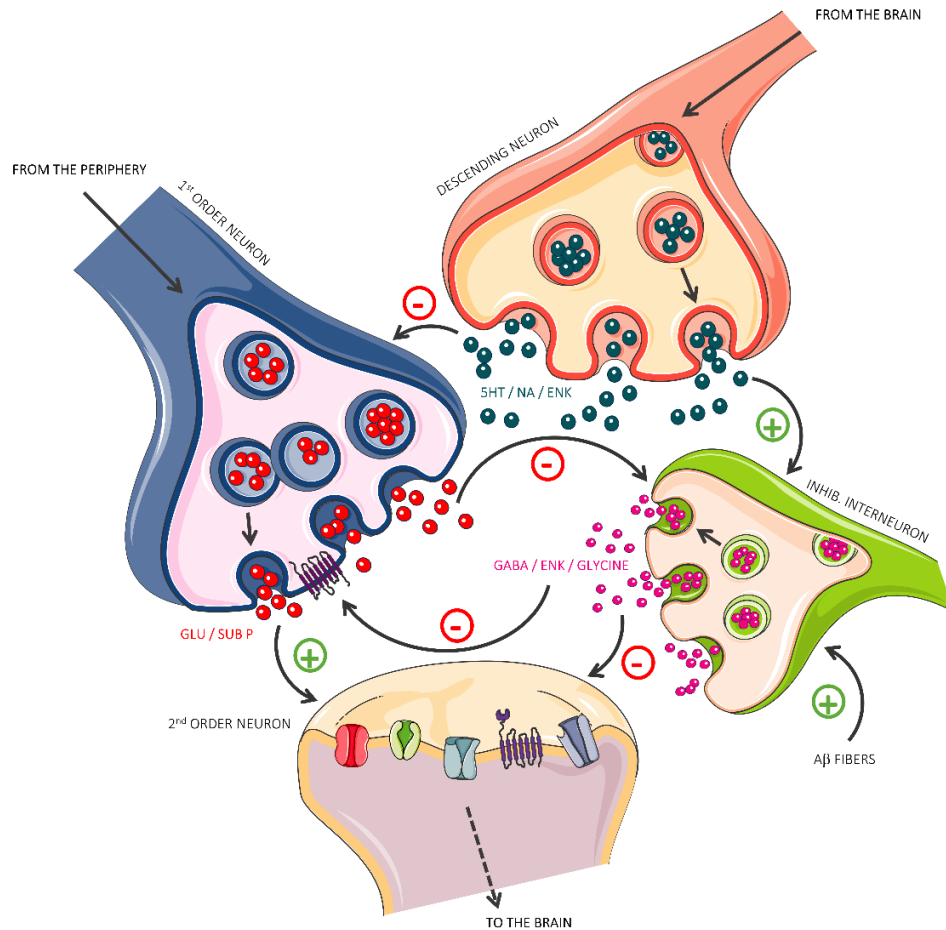


Figure 2: Spinal pain modulation. Nociceptors, mainly A δ and C fibers, activation stimulate second-order neurons and inhibit inhibitory interneurons. Additionally, A β -fibers, activated by non-noxious stimuli, reengage inhibitory interneurons, reducing pain transmission. Descending pathways from the brain modulate the pain gate, suppressing pro-nociceptive neurotransmitter release through presynaptic inhibition and influencing second-order neurons through postsynaptic inhibition via local interneurons. *Glu: glutamate, SUB P: substance P, 5HT: serotonin, NA: noradrenalin, ENK: enkephalin, GABA: gamma-aminobutyric acid.*

In addition to spinal interneurons activated by A β fibers, inhibitory descending pathways originating from several brain structures send signals

to the spinal cord to modulate the pain gate (Millan 2002). This inhibitory control involves enkephalinergic, noradrenergic and serotonergic systems (Le Bars et al. 1979; Basbaum and Fields 1978; Fields and Heinricher 1985) that respectively originate from the periaqueductal gray matter and locus coeruleus in the midbrain and the nucleus raphe magnus in the medulla. The role of these descending inhibitory pathways is to negatively control pain signals between the first- and second-order neurons of the ascending pathway.

This inhibition from the descending pathways operates both on afferent neurons and on interneurons in the dorsal horn of the spinal cord. On the first-order neurons, they reduce the release of pro-nociceptive neurotransmitters, mainly via opioid receptors densely expressed in primary terminals. This interaction is referred to as presynaptic inhibition (Kwon et al. 2014). Besides, the released serotonin and noradrenalin predominantly reduce the firing of second-order neurons by opening potassium (K^+) channels, causing hyperpolarization, known as postsynaptic inhibition (Yam et al. 2018; Kwon et al. 2014). This system is further supported by the activation of spinal inhibitory interneurons (Hughes and Todd 2020; Kwon et al. 2014).

4. Glial cells

In recent decades, it has become evident that spinal cord neurons and interneurons are not the exclusive contributors to pain. As discussed below, non-neuronal cells (glial and microglial cells) have emerged as crucial players in the development and modulation of pain at the level of the spinal cord (Xu et al. 2021; Cedeño et al. 2021).

From a classical perspective, neurons are regarded as the fundamental cellular units of the central nervous system (CNS), coordinating communication and actions within our organism. In order to effectively carry out their multifaceted tasks in the CNS, neurons rely on crucial cell partners

in their immediate environment, commonly known as glial cells. First described as “neuroglia” by Rudolf Virchow and Camillo Golgi in 1850, these cells were characterized as an important cohesive support for the brain. It wasn’t until 1919 that neuroglia was classified into distinct categories, giving rise to the recognized types of CNS glial cells: astrocytes, oligodendrocytes, and microglia. Each of these cell types fulfills specific and essential functions in supporting and safeguarding neurons including control of neurotransmission, supply of energy, elimination of metabolic waste, turnover of neurotransmitters, and establishment of the blood-brain barrier (BBB) (Allen 2009).

While oligodendrocytes play a crucial role in the formation of a laminated, lipid-rich wrapping known as myelin around specific axons, influencing the speed of action potential conduction (Allen 2009), this PhD thesis primarily focuses on the other two types of glial cells: astrocytes and microglia. These cells possess the ability to significantly influence neuronal excitability (Kimelberg and Nedergaard 2010; Beppu et al. 2021; Allen 2009). Of importance in the context of our research, both astrocytes and microglia play specific roles in the regulation of pain transmission and in neuroinflammatory processes (Chen et al. 2022; Giovannoni and Quintana 2020; Chen et al. 2018).

a. Astrocytes

Astrocytes constitute the most abundant subtype of glial cells within the CNS. Their name is derived from their recognizable stellar shape, with “astro” denoting stars and “cytes” signifying cells in Greek. Accounting for approximately 30% of the cells in the mammalian CNS, astrocytes play pivotal roles in the proper functioning of the brain and spinal cord. Their numerous cytoplasmic extensions establish multiple contacts with neighboring neurons and blood vessels, facilitating their supportive functions (Bushong et al. 2002; Halassa et al. 2007).

Astrocytes constitute a heterogeneous cell population which remains challenging to subclassify (Khakh and Deneen 2019). Traditionally, these cells are categorized into two groups: protoplasmic and fibrous astrocytes. While *protoplasmic astrocytes* are principally located in the grey matter and enwrap neuronal cell bodies and synapses, *fibrous astrocytes* are mostly found in the white matter and are associated to neuronal axons (Miller and Raff 1984). However, this classification appears to be incomplete as a number of studies have documented the large diversity in the morphology and functions of astrocytes in the brain and spinal cord (Batiuk et al. 2020). Beyond the dual classification in fibrous and stellate astrocytes, this diversity indeed reflects the complexity of functions that astrocytes fulfill across the CNS as summarized in box 1.

Within the scope of this thesis, our primary focus will be on the role of astrocytes in regulating glutamate homeostasis, glutamatergic transmission and neuroinflammation.

BOX 1. Brief, non-exhaustive overview of astrocytic functions

The proper functioning of the nervous system relies on a continuous supply of energetic substrate from the blood stream. Glucose, the primary fuel for the brain, is efficiently taken up via the glucose transporter 1 (GLUT1) expressed on endothelial cells and astrocytes at the BBB. Upon request, glucose is metabolized into lactate by astrocytes and transferred to neurons to generate ATP to ensure their high and continuous energy needs (Bélanger et al. 2011; Brown and Ransom 2007).

In addition to metabolic and energetic support, astrocytes actively contribute to the formation, maintenance and elimination of functional synapses. Through the release of gliotransmitters and growth factors, they participate in synaptogenesis, influencing the establishment and efficiency of synaptic connections between neurons (Slezak and Pfrieger 2003). This role extends to the regulation of synaptic plasticity, impacting learning and memory processes (Allen and Barres 2005).

Together with endothelial cells, astrocytes also play a crucial role in maintaining the integrity of the BBB. They contribute to its structural and functional components, regulating the passage of substances from the blood stream to the cells of the brain and spinal cord parenchyma. This selective barrier protects the neuronal environment from potentially harmful compounds circulating in the blood stream (Petzold and Murthy 2011; Abbott et al. 2006).

Furthermore, astrocytes control different chemical and biochemical characteristics of the neuronal environment. Indeed, they actively clear excess of glutamate from the extracellular space, preventing excitotoxicity and ensuring proper neurotransmission (Danbolt 2001). Astrocytes also participate in ion buffering, maintaining the balance of ions such as K^+ , which is important for neuronal signaling (Bellot-Saez et al. 2017). Expressing carbonic anhydrase enzyme and transporters involved in proton and bicarbonate shuttling, astrocytes also actively control pH (Theparambil et al. 2020).

The tripartite synapse

In the 1990s, the discovery of a bidirectional communication between neurons and astrocytes led to the formulation of the "tripartite synapse" concept (Araque et al. 1999). This concept defines the synapse as a dynamic interaction site involving three elements: pre- and post-synaptic nerve terminals surrounded by one (or more) astrocytic foot processes. Astrocytes are thus ideally positioned to detect variations in neuronal activity and respond consequently, particularly by regulating synaptic transmission. To achieve this, astrocytes express a multitude of receptors for neurotransmitters and neuromodulators, along with ion channels and transporters, enabling them to sense alterations in their environment. Importantly, the type of neurotransmitter receptors expressed by astrocytes differs across brain regions and appears to be correlated with neighboring neurons (Miyazaki et al. 2004). For instance, at glutamatergic synapses, astrocytes have been shown to express both iGluRs and mGluRs (Bradley and Challiss 2012). The release of glutamate during synaptic influx activates these receptors, triggering intracellular pathways leading to an elevation in intracellular Ca^{2+} levels within astrocytes (Nedergaard 1994; Parpura et al. 1994; Perea and Araque 2007). An important response of astrocytes to neuronal activity is the release of neuroactive molecules called "gliotransmitters" including excitatory and inhibitory transmitters (e.g. glutamate, D-serine, GABA, glycine) (Bezzi et al. 1998), ATP (Coco et al. 2003) and cytokines (e.g. interleukins, interferons, tumor necrosis factor α) (Stellwagen and Malenka 2006; Santello et al. 2012). When released, gliotransmitters influence synaptic activity by either promoting or inhibiting the release of neurotransmitters from presynaptic neurons (Perea and Araque 2007; Jourdain et al. 2007; Santello et al. 2012). However, the specific mechanisms of gliotransmitter release have not been clearly demonstrated and the concept of glial vesicle release remains controversial (Harada et al. 2015).

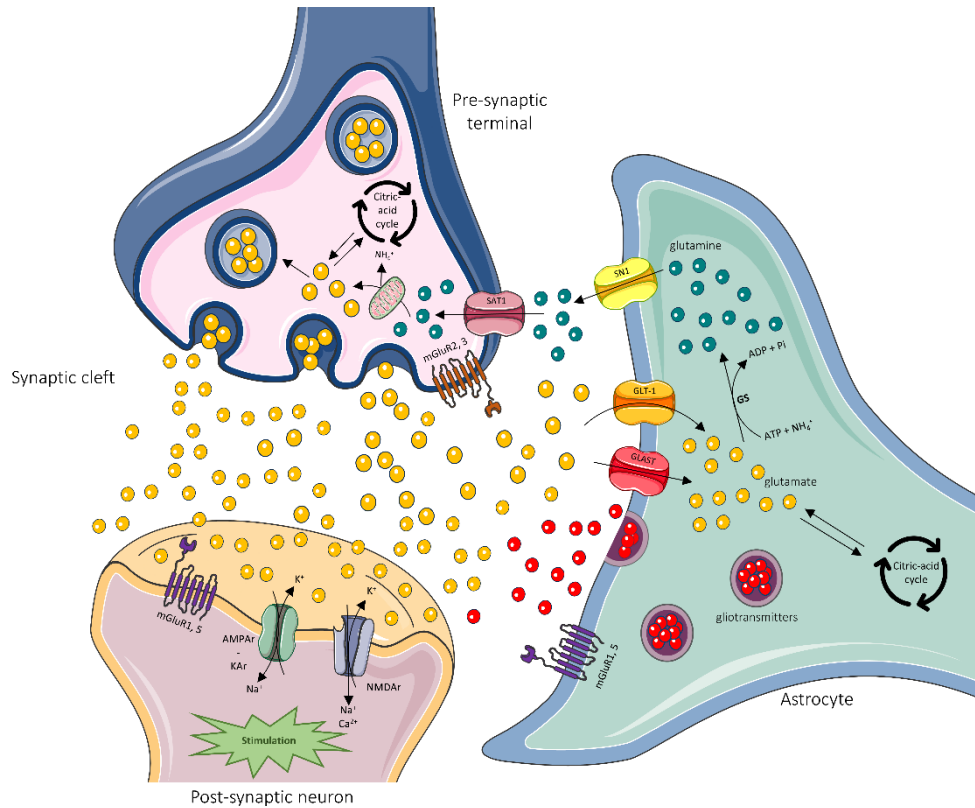


Figure 3: The glutamatergic tripartite synapse. Once released into the synaptic cleft, glutamate interacts with ionotropic and metabotropic receptors on pre- and post-synaptic neurons. Astrocytic transporters rapidly take up glutamate to prevent excitotoxicity. Astrocytes convert glutamate into glutamine via glutamine synthase. Glutamine is then transported into neurons and converted back into glutamate, which is stored in synaptic vesicles for future release. *mGluR*: metabotropic glutamate receptor; *AMPA*: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; *KAR*: kainate receptor; *NMDAR*: N-methyl-D-aspartate receptor; *SN1*: system N transporter 1; *SAT1*: spermidine/spermine N1-acetyltransferase 1; *GLT-1*: glutamate transporter 1; *GLAST*: glutamate aspartate transporter; *GS*: glutamine synthase; *ADP*: adenosine diphosphate; *ATP*: adenosine triphosphate; *Pi*: inorganic phosphate.

Glutamate homeostasis

Importantly, astrocytes are also key actors in regulating glutamate homeostasis via the clearance and recycling of neurotransmitters, such as glutamate, which account for about 90% of the excitatory synapses in the mammalian nervous system (Danbolt 2001; Cuellar-Santoyo et al. 2022). Considering that glutamate cannot be metabolized in the extracellular space, it must be removed from the synaptic cleft through cellular uptake. Indeed, an excessive glutamate concentration in both synaptic and extra-synaptic spaces can result in neuronal hyperexcitability and subsequent neuronal death. This process known as “glutamate excitotoxicity” is implicated in various inflammatory and nervous diseases of the CNS including chronic pain (Siesjö et al. 1989; Petrenko and Shimoji 2001; Clark and Vissel 2016).

To avoid this, astrocytes appear as an important contributor as neurons account for only a minor fraction of the extracellular glutamate (Anderson and Swanson 2000; Oliet et al. 2001). Indeed, astrocytes are equipped to take up, metabolize and recycle glutamate (Cuellar-Santoyo et al. 2022) (Figure 3). They express two high affinity Na^+/K^+ -dependent glutamate transporters known as glutamate aspartate transporter (GLAST in rodents, EAAT1 in human) and glutamate transporter 1 (GLT-1 in rodents and EAAT2 in human). Since intracellular concentrations of glutamate (5-10mM) are substantially higher than extracellular levels, GLT-1 and GLAST operate through a secondary active transport by using Na^+ and K^+ gradients as the driving force for transporting glutamate across the plasma membrane. Glutamate uptake is thus coupled to the Na^+/K^+ pump to restore ionic gradients. During synaptic transmission, glutamate is taken-up by astrocytes where it is converted into glutamine by the enzyme glutamine synthetase (GS). Glutamine is then released back into the extracellular space by the electroneutral, glutamine transporter SN1, from which system A transporter 1 (SAT1) take up glutamine into neurons. Glutamine is converted back into glutamate by the phosphate-activated glutaminase, which is then packed into synaptic vesicles ready for

the release and starting of a new cycle of synaptic transmission. Alternatively, glutamate can be oxidatively metabolized into α -ketoglutarate to fuel the citric-acid cycle to produce ATP (Cuellar-Santoyo et al. 2022).

Synaptic plasticity

In addition to their role in the glutamate-glutamine cycle, astrocytes actively participate in the control of synaptic plasticity. They can influence the expression and function of glutamate receptors, contributing to the overall modulation of glutamatergic signaling (Chung et al. 2015) which is essential in the context of pain. For example, the astrocytic release of tumor necrosis factor α (TNF- α) has been shown to enhance synaptic efficacy by increasing surface expression of AMPA receptors in neurons (Beattie et al. 2002). It has also been documented that the activation of astrocytic mGluR5 influences the expression and activity of glutamate transporters, suggesting an indirect regulation of excitatory synaptic transmission (Vermeiren, Najimi, Vanhoutte, et al. 2005). Synaptic plasticity and the role of astrocytes will be further discussed in Chapter II, section “central sensitization”.

Neuroinflammation

In response to various triggers, such as infection or injury, astrocytes undergo morphological and functional changes known as reactive gliosis or astrogliosis (Santello et al. 2012). Reactive astrocytes contribute to immune responses by the release of cytokines, chemokines, and growth factors, coordinating both innate and adaptive immune responses within the CNS (Giovannoni and Quintana 2020). Thus, astrocytes not only adapt their function and morphology when exposed to an inflammatory environment, but together with microglia, they actively participate to neuroinflammatory responses. The role of astrocytes in modulating neuroinflammation is quite complex, involving both neuroprotective and potentially neurotoxic effects (Liddel et al. 2017; Pekny et al. 2014). For this reason, a specific subsection will

further describe the importance of neuroinflammation and the role of astrocytes in pathological conditions such as chronic pain (see Chapter II, section “neuroinflammation”).

b. Microglia

The term “microglia” was first introduced and characterized in 1919 by Pio del Rio-Hortega. Nowadays, microglia are considered as the resident macrophages of the CNS and account for about 10-15% of cells found within the brain. They are distributed heterogeneously throughout the brain and the spinal cord (Lawson et al. 1990). Deriving from the mesoderm, it was initially suggested that microglial cells originated from invading blood monocytes during the perinatal period. However, this hypothesis was revised due to evidence revealing that microglia are derived from myeloid precursor cells originating from the yolk sac and then migrate to the developing brain and spinal cord during embryogenesis (Kettenmann et al. 2011; Ransohoff and Perry 2009). Once in the CNS, microglial progenitor cells proliferate quickly during the first two post-natal weeks, generating approximately 95% of microglia, before slowing down (Alliot et al. 1999). During early brain development, microglia undergo maturation and take on a distinctive amoeboid profile characterized by large cell bodies and short branching processes (Ginhoux et al. 2010). In the adult brain, the microglial population is long-lived and maintained by slow cell proliferation within the CNS, rather than being replaced by blood cells (Lawson et al. 1992; Perry et al. 2010).

Microglial cells exhibit remarkable plasticity and are highly dynamic, undergoing diverse morphological adaptations based on their specific locations and the CNS needs (Nimmerjahn et al. 2005; Davalos et al. 2005). This high level of plasticity is essential to fulfill the variety of functions performed by microglia such as supporting the development of neuronal circuits, synaptic remodeling, myelin turnover, neuronal excitability control and immune responses monitoring (Colonna and Butovsky 2017) (See Box 2).

BOX 2. Brief, non-exhaustive overview of microglial functions

Microglial cells play multifaceted roles beyond their well-known involvement in inflammation. These adaptive cells are crucial for the development, maintenance, and plasticity of the neuronal circuits that support brain functions.

During brain development, microglia contribute to the formation and refinement of neuronal circuits. They participate in the pruning of synapses, a critical process that eliminates weak synaptic connections, thereby optimizing neural network efficiency. This synaptic pruning is essential for normal brain development and function. Later, microglia also participate in neurogenesis, supporting the differentiation of neural progenitor cells into neurons (Guedes et al. 2022).

Microglia are also actively involved in synaptic remodeling throughout life, adjusting synaptic connections in response to experience and learning. By phagocytosing synaptic elements and releasing signaling molecules, microglia influence synaptic strength and plasticity. This dynamic process is essential for memory formation and the adaptability of the nervous system to new information (Wu et al. 2015).

Another critical function of microglia is their role in myelin turnover. Microglia can phagocytose myelin debris, facilitating the clearance and recycling of damaged myelin. This process is crucial for maintaining healthy myelination and ensuring efficient nerve conduction. In diseases such as multiple sclerosis, where myelin is damaged, microglia contribute to both injury and repair processes (Santos and Fields 2021).

Microglial cells influence neuronal excitability through their interactions with neurons and astrocytes. They can release various factors that modulate neuronal firing rates and synaptic activity. By regulating the extracellular environment and neuronal signaling, microglia help maintaining homeostasis and prevent hyperexcitability that could lead to pathological conditions (Czapski and Strosznajder 2021).

Indeed, microglia adopt specific phenotypes in response to the local conditions and chemical signals they have detected (Guo et al. 2022). Microglia were, for a long time, considered as potentially detrimental, but further studies showed that they carry both a cytotoxic and a neuroprotective function depending on their adopted phenotype that changes in physiological and pathological conditions (Guo et al. 2022; De Nicola et al. 2003).

Under homeostatic and physiological conditions, quiescent microglia adopt a “resting” or “surveilling” state (also known as M0 phenotype), characterized by a small cell body and important motile processes. Even if the cell body remains immobile within their environment, its branches are quite dynamic and constantly extending and retracting in order to efficiently monitor the surrounding environment (Nimmerjahn et al. 2005; Paolicelli et al. 2022). The role of microglia as vigilant surveillers and their responses to pathological situations are their most characterized functions (Nimmerjahn et al. 2005; Wolf et al. 2017). Resting microglia also constantly remodel neural circuits by communicating with adjacent neurons and removing dead cells (Schafer et al. 2012; Paolicelli et al. 2011).

As any potential danger is recognized, microglia change from “resting” to “activated” state and communicate essentially by cytokine release with surrounding cells and influence their functioning (Perry et al. 2010). This initiates an inflammatory state, a physiological response that is strictly regulated and silenced over time. During activation, microglia undergo several morphological changes, including the withdrawal of their processes, generating an amoeboid shape favoring their motility and phagocytic properties (Kettenmann et al. 2011). The changes in morphology are accompanied with changes in the expression of specific genes, proliferation and function (Böttcher et al. 2019; Kreutzberg 1996). Depending on the activating stimulus, reactive microglia can adopt distinct phenotypes characterized by specific changes (Gordon and Taylor 2005; Mosser and

Edwards 2008). These phenotypes have been broadly categorized into M1 (classically activated, pro-inflammatory) and M2 (alternatively activated, anti-inflammatory) (Figure 4).

M1 polarized microglia are considered as the pro-inflammatory phenotype associated with neurotoxic functions. This phenotype is induced upon activation by interferon- γ and microbial triggers such as lipopolysaccharides (LPS). M1 microglia are documented to release pro-inflammatory mediators such as interleukin-1 β (IL-1 β), TNF- α , and interleukin-6 (IL-6), contributing to the initiation and amplification of the inflammatory response. In addition, M1 microglia generate reactive oxygen species (ROS), enhancing their cytotoxic activity and participating in antimicrobial defense. If the inflammation is not silenced after the removal of the initial damage, it can lead to neuronal death.

Switching the activation phenotype to *M2 activated microglia* is assumed to have a silencing effect, favoring the reestablishment of an environmental homeostasis and promoting recovery. M2 microglia release anti-inflammatory cytokines, including interleukin-10 (IL-10) and transforming growth factor- β (TGF- β), fostering the resolution of inflammation. M2 microglia are also engaged in tissue repair and remodeling processes, phagocytosing cellular debris and promoting homeostasis.

It is essential to recognize that the M1/M2 classification represents a highly simplified model, and microglia demonstrate a spectrum of activation states. In various neuroinflammatory conditions, microglia can dynamically transit between M1 and M2 phenotypes based on specific signals within their microenvironment (Guo et al. 2022; Tang and Le 2016). The intricate balance and coordination of M1 and M2 responses are critical for maintaining tissue homeostasis during and after insults (Wolf et al. 2017).

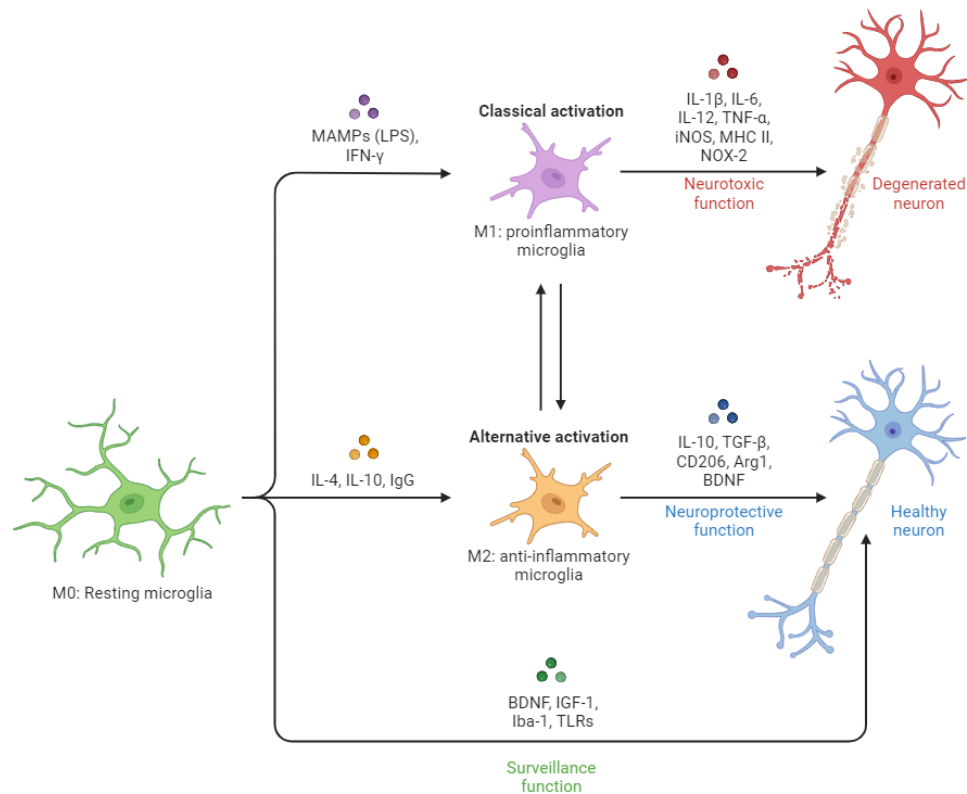


Figure 4: Polarization and phenotypic switch of microglia. Under physiological conditions, microglial cells adopt a resting phenotype with a surveilling profile to control the neuronal environment. However, various stimuli can prompt resting microglia to adopt distinct phenotypes. In general, M1 microglia represent a pro-inflammatory state, while M2 microglia represent an anti-inflammatory phenotype. M1 and M2 microglia have specific characteristics (e.g. gene expression) and are able to release different mediators. Microglia can dynamically transit between M1 and M2 phenotypes based on specific signals in their microenvironment. *MAMPs: microbial-associated molecular patterns, LPS: lipopolysaccharide, IFN: interferon, Ig: immunoglobulin, IL: interleukin, TNF: tumor necrosis factor, iNOS: inducible nitric oxide synthase, MHC: major histocompatibility complex, NOX: NADPH oxidase, TGF: transforming growth factor, CD: cluster of differentiation, Arg: arginase, BDNF: brain-derived neurotrophic factor, IGF: insulin-like growth factor, Iba-1: ionized calcium-binding adapter 1, TLRs: toll-like receptors.* Figure created with BioRender®.

Beyond their well-known role in immune responses and inflammation, microglia serve as dynamic guardians of brain health, orchestrating a range of functions. Indeed, microglial populations contribute to the maintenance of homeostasis in the CNS parenchyma through various non-inflammatory functions. Microglia are essential for synaptic pruning, a critical process for refining and optimizing neural circuits during development and in response to specific conditions (physiological or pathological) (Graeber 2010; Paolicelli et al. 2011). They actively participate in neurogenesis, supporting the generation of new neurons in certain brain regions. Additionally, microglia are engaged in the clearance of cellular debris and apoptotic cells through phagocytosis, ensuring the elimination of unnecessary elements and promoting tissue integrity (Neumann et al. 2009). Their surveillance activity helps monitor the neural environment, allowing for a rapid response to potential threats or abnormalities.

5. Pain classification

In accordance with the multidimensional nature of pain and its heterogeneity in terms of duration, etiology, intensity and pathophysiology, several classifications have been proposed. The complexity of pain also resides in the fact that the involved mechanisms depend on its origin. It is then essential to determine the underlying causal factors driving the development of pain in order to precisely adapt the treatment plan.

a. Different types of pain

In 2010, Woolf introduced a pain classification based on several criteria including the duration, symptomatology and the etiology (Woolf 2010): Firstly, there is the *nociceptive pain* identified as an early-warning physiological protective system, playing a crucial role in alerting individuals to potential harmful stimuli or noxious events. The perception of nociceptive pain triggers behaviors and reflexes geared towards avoidance of further

injury, such as withdrawing from a hot surface or adjusting posture to relieve pressure on a painful area (Derderian et al. 2023). This is further accompanied by an unpleasant emotion aimed at avoiding a similar experience in the future. Common causes of nociceptive pain include the touch of too hot, cold or sharp object. Perceived pain is typically characterized as time-limited, localized, well-defined and proportional to the extent of tissue damage (Woolf 2010). In physiological conditions, once the underlying cause of the pain is addressed and, if needed, healing occurs, nociceptive pain decreases. If needed, paracetamol or local analgesics, such as lidocaine, can be used.

Secondly, *inflammatory pain* also presents adaptative and protective characteristics, arising from the activation of the inflammatory and immune systems in response to tissue injury or infection. Inflammation is a complex biological process initiated by the body as a protective and reparative mechanism. It involves a series of events aiming at eliminating harmful stimuli, damaged cells and pathogens, while initiating the healing process. Throughout this process, released mediators such as cytokines, chemokines, and prostaglandins sensitize nociceptors, lowering their activation threshold and amplifying the pain response (Dunham et al. 2008). Pain hypersensitivity serves to discourage physical contact and movement, reducing the risk of further damage. Inflammatory pain is often characterized by redness, swelling, heat, and pain at the affected site. The management of inflammatory pain may often involve the use of anti-inflammatory medications (Scarpignato et al. 2015; Brooks and Day 1991; Tramèr et al. 1998).

Thirdly, *pathological pain*, also known as maladaptive pain, is not a direct response to a noxious stimulus but rather arises from abnormal functioning of the nervous system. This type of pain is not considered as a symptom resulting from a specific disorder but rather a disease state itself since it may persist beyond the expected duration of healing and serves no purpose in terms of defense for our body (Yam et al. 2018). Pathological pain can arise

following a lesion of the nervous system leading to neuropathic pain, or in the absence of any damage or inflammation, resulting in dysfunctional pain. It largely stems from amplified sensory signals in both the peripheral nervous system (PNS) and CNS (Wieseler-Frank et al. 2004; Willis and Westlund 1997). Pathological pain frequently manifests with abnormal sensations, including tingling, burning, or shooting pain, and may be associated with functional impairments (Willis and Westlund 1997). The treatment used for pathological chronic pain often involves a multidisciplinary approach, combining medications that modulate nerve signaling such as anticonvulsants, antidepressants, or opioids with therapies like physiotherapy, psychological and interventional procedures (O'Connor and Dworkin 2009; Shaygan et al. 2018; Bates et al. 2019). Individualized treatments are often necessary to address the specific underlying causes and to enhance the overall quality of life for patients (O'Connor and Dworkin 2009; Shaygan et al. 2018).

Understanding these different types of pain, that can co-exist, is essential for healthcare professionals. Indeed, it is fundamental for determining the most effective treatment strategies tailored to the specific nature and origin of the pain (Nalamachu 2013). It also enables healthcare professionals to offer targeted and personalized care, improving patient outcomes and minimizing unnecessary interventions or medications.

b. Dichotomous classification: acute and chronic pain

Beside this above-mentioned classification, a dichotomous distinction is commonly used, based on the duration of pain: acute pain, that mainly includes nociceptive and inflammatory pain, and chronic pain, known as pathological pain. This dichotomous classification needs, however, to be considered with hindsight, as both are intimately linked. Indeed, effective and rapid management of acute pain can reduce or prevent the development of chronic pain (Price and Ray 2019; Stephens et al. 2003). Untreated acute pain

can lead to chronic conditions that are much more complicated to treat. Therefore, efforts should be dedicated to prevent its establishment.

Acute pain is considered as a normal, predicted and valuable physiological response of the body that occurs in the presence of a noxious stimulus. It manifests suddenly and unlike chronic pain, acute pain is typically restricted in time, corresponding to the duration of the stimuli and required time for tissue healing. This form of pain plays an essential protective role by signaling the presence of a potential threat to the brain. Indeed, it triggers adaptative responses, including reflexive withdrawal, preventing further damage or contact with the painful source (Derderian et al. 2023). The daily difficulties experienced by individuals suffering from congenital insensitivity to pain, who are incapable of recognizing painful stimuli, underscore the importance of the protective function of pain. Indeed, these people are unable to feel the discomfort or the pain caused by injuries. As a result, they fail to engage appropriate protective actions and reflexes against these conditions, many of which can be life threatening (Daneshjou et al. 2012).

Another particularity of acute pain is its ability to rapidly respond to conventional analgesics. In this type of situation, the use of non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol or, in cases of more severe pain, opioids are effective, providing effective pain relief with certain acceptable side effects (Blondell et al. 2013).

In some cases, and for reasons that are still under investigation, the primary protective role of acute pain can subsequently turn into maladaptive and persistent hypersensitivity, known as *chronic pain*. In such conditions, hypersensitivity symptoms (such as allodynia and hyperalgesia) that are valuable in case of acute pain become debilitating (Campbell and Meyer 2006). Indeed, IASP defines chronic pain as “pain without apparent biological value that has persisted beyond the normal tissue healing time (usually considered to be 3 months)”. In such case, the combination of peripheral and central mechanisms results in an imbalance between pro- and

antinociceptive signals leading to an enhanced excitability along the pain pathways (Coderre and Katz 1997; Latremoliere and Woolf 2009; Colloca et al. 2017; Ossipov et al. 2014). In this pathological condition, pain does not correlate with the intensity of the stimulus, appears more diffuse and is commonly difficult to locate. More details about a subtype of chronic pain (neuropathic pain) and causal mechanisms will be further discussed (see chapter II).

Several studies highlight chronic pain as a significant public health issue, mainly because, in most cases, this persisting pain significantly affects the social, familial, and professional lives of affected people, with a considerable impact on their quality of life (West et al. 2012; Elliott et al. 2003; Azevedo et al. 2012). Indeed, it is often associated with various consequences such as anxiety, sleep disturbances, depression, and motor disabilities (Breivik et al. 2006; Elliott et al. 2003). Besides, it is crucial to highlight that chronic pain constitutes a substantial societal burden. Beyond the evident direct costs tied to healthcare services, medications, and rehabilitation, there exist numerous indirect costs linked to issues such as absenteeism, reduced workplace productivity, and overall decline in the quality of life. In a study examining the socio-economic implications of back pain in the United Kingdom, which involved 6 000 participants, the calculated total direct cost attributed to the care and treatment of back pain reached £1,632 million (€1,947 million). When these direct cost assessments are combined with the associated employment and informal care costs, the cumulative estimate reached £12,300 million (€14,674 million) (Maniadakis and Gray 2000). In the United States, the overall expenses linked to persistent pain in adults is estimated to range between \$560 billion and \$635 billion. These costs are noted to exceed those estimated for heart disease, cancer and diabetes (Gaskin and Richard 2012).

A European-wide study conducted in 2006 with over 46 000 respondents reveals an average prevalence of chronic pain of 19%, ranging from a

minimum of 12% in Spain to a maximum of 30% in Norway. Belgium ranks as the fourth most affected nation in Europe, with a prevalence close to 23% (Breivik et al. 2006). These results need to be interpreted in the context of an aging population which will increase the number of affected patients (Fitzgerald and McKelvey 2016). These epidemiological studies are not very recent, and there is reason to fear that the situation has worsened in recent years. Another major challenge with chronic pain is that analgesics classically used to treat acute pain only present a limited benefit in context of chronic pain. Not only are the conventional treatments limited in their efficacy against persistent pain, but they are also associated with significant adverse effects that restrict their clinical use, particularly when considered for a prolonged period. It is often necessary to envisage a multidisciplinary approach combining pharmacological and non-pharmacological treatment (Shaygan et al. 2018).

6. Brief overview of pharmacological pain treatments

Even if non-pharmacological approaches are sometimes essential in pain treatment, medications play a key role in alleviating symptoms and improving the quality of life for individuals experiencing acute or chronic pain (Shaygan et al. 2018). Hence, reducing the symptoms with drugs may facilitate the access and implementation of non-pharmacological approaches. The table below (table 1) provides an overview of drug families commonly employed in pain management, categorized by their application in acute and chronic (mainly neuropathic) pain.

It is worth mentioning that a multimodal pharmacological approach combining analgesics from distinct families can enhance pain relief while minimizing side effects. It is also important to assess pain intensity and determine the underlying etiology to optimize pain management outcomes.

Table 1: Overview of the main pharmacological treatments used for acute and chronic neuropathic pain.

Drug family	Mechanism of action	Clinical application	Key side effect
<u>NSAIDs</u> Ibuprofen, Naproxen, Diclofenac	Inhibit COX enzymes in charge of prostaglandin synthesis, reducing pain and inflammation	Mild to moderate pain associated with inflammation	Gastrointestinal bleeding, renal impairment, cardiovascular risks
<u>Acetaminophen</u> Paracetamol	Several mechanisms of action proposed, still unclear to date	Mild to moderate pain and fever	Liver toxicity (with high doses)
<u>Mild opioid analgesics</u> Codeine, Tramadol	Agonists of opioid receptors	Moderate to severe acute pain, post-surgical pain	Constipation, respiratory depression, risk of dependence, tolerance
<u>Local anesthetics</u> Lidocaine	Blockade of voltage sensitive sodium channels in nerve membrane, preventing the propagation of action potential	Localized pain	Skin irritation, allergic reactions
<u>Antidepressants</u> Duloxetine, Amitriptyline, Nortriptyline	Serotonin and noradrenaline reuptake inhibitors, reinforcing descending pain modulation	First line treatment for neuropathic pain, fibromyalgia	Sedation, dry mouth, constipation
<u>Anticonvulsants</u> Gabapentin, Pregabalin	Bind to voltage-gated calcium channels, stabilizing neuronal membranes and modulate the release of excitatory neurotransmitters (glutamate). Enhance GABAergic inhibition, reducing neuronal excitability	Neuropathic pain (diabetic neuropathy, postherpetic neuralgia), fibromyalgia, spinal cord injury and chronic low back pain	Dizziness, sedation, weight gain
<u>Opioid analgesics</u> Morphine, Oxycodone	Agonists of opioid receptors	Severe chronic pain not responding to other treatment	Constipation, respiratory depression, tolerance and risk of dependence.
<u>Topical analgesics</u> Capsaicin	Agonist of TRPV1 channels causing desensitization of sensory nerve endings and depletion of neurotransmitters (Substance P)	Neuropathic pain, arthritis or localized pain	Local skin reactions, irritation
<u>NMDA receptor antagonists</u> Ketamine	Antagonists of NMDA receptors, modulating pain transmission	Chronic neuropathic pain	Hallucinations, disorientation
<u>Cannabinoids</u> Cannabidiol (CBD), Tetrahydrocannabinol (THC)	Activate endocannabinoid receptors: CB1 receptors: modulation of neurotransmitters release CB2: anti-inflammatory and immunomodulatory effects	Neuropathic pain, cancer-related pain, multiple sclerosis	Dizziness, dry mouth, cognitive impairment

CHAPTER II: Neuropathic pain

As mentioned earlier, in certain cases, and for reasons that are still under investigation, normal nociception may evolve into pathological nociception. In this situation, symptoms that were valuable in case of acute pain, such as allodynia (pain due to a stimulus that does not normally provoke pain) and hyperalgesia (an increased response to painful stimuli), completely lose their primary warning function and become debilitating (Jensen and Finnerup 2014; Campbell and Meyer 2006) (Figure 5). This pathological transition from acute to chronic pain has been proposed to rely on several mechanisms within both the central and peripheral nervous systems, with the involvement of different cell types such as neurons and glial cells. This PhD thesis will primarily focus on a specific subtype of chronic pain known as neuropathic pain.

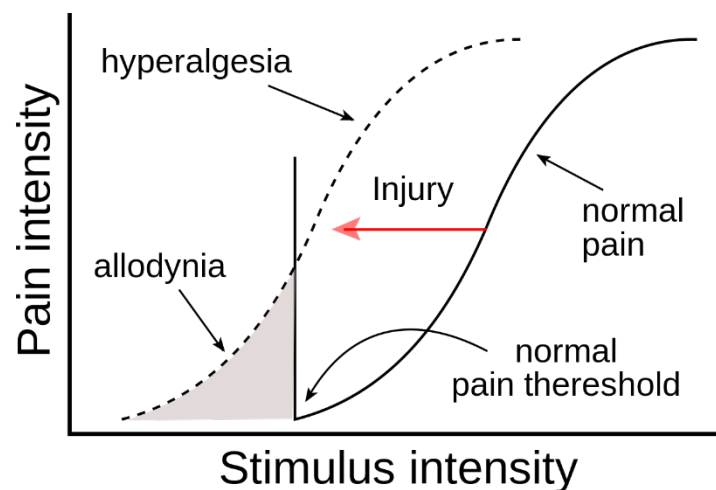


Figure 5: Allodynia and hyperalgesia, symptoms of pain hypersensitivity. The normal pain response as a function of the stimulus intensity is depicted by the solid line. Nerve injuries induce a shift in pain response leading to an intensified pain to noxious, normally painful stimuli (hyperalgesia) and a pain response triggered by usually innocuous stimuli (allodynia).

1. Background

Neuropathic pain is defined by the IASP as pain resulting from a lesion or a disease of the somatosensory nervous system, including both peripheral fibers (such as A δ and C fibers) and central neurons. These lesions or diseases disrupt the normal conveyance of sensory signals to the spinal cord and the brain.

Estimating the incidence and prevalence of neuropathic pain in the general population has historically been challenging due to the absence of clear diagnostic criteria. Consequently, prevalence rates have primarily been approximated in specialized chronic pain centers, focusing on specific conditions such as postherpetic neuralgia, multiple sclerosis (MS), painful diabetic neuropathy, cancer, spinal cord injury, and stroke. Over the past decade, the development of validated questionnaire forms has facilitated large-scale epidemiological surveys. Utilizing these reliable data collection tools, the global prevalence of chronic pain with neuropathic characteristics has been estimated to range between 6.9% and 10% (Torrance et al. 2006; Bouhassira et al. 2008; van Hecke et al. 2014). These studies also underscored that individuals experiencing neuropathic pain are more likely to be women, slightly old (> 50 years), and engaged in manual professions (Torrance et al. 2006; Bouhassira et al. 2008; van Hecke et al. 2014).

Besides, it is reported that the presence and severity of neuropathic pain considerably affect the quality of life of patients. Indeed, persistent and severe pain impair physical condition, leading to significant difficulties in mobility and coordination. Moreover, emotional well-being is frequently compromised, resulting in anxiety and depression. Sleep disturbances, cognitive impairment, and social isolation exacerbate the overall impact of neuropathic pain. Relationships may suffer, work productivity declines, and individuals may face financial stress, all of which are known to exacerbate pain perception (McCarberg and Billington 2006).

In the case of neuropathic pain, the PNS and CNS become sensitized, and pain is no longer linked to the intensity, duration, or presence of a noxious stimulus. Instead, it can occur spontaneously, either continuously or paroxysmally, highlighting the pathological nature of this pain condition (Latremoliere and Woolf 2009; Yam et al. 2018).

2. Mechanisms of neuropathic pain

While the origin of pain chronicization and the transition from acute to chronic pain remains largely unknown and debated, we will here describe the principal peripheral and central mechanisms that contribute to neuropathic pain. Although modulations in higher pain centers are important, for the scope of this thesis, we will primarily focus on spinal mechanisms.

Biochemical hallmarks of neuropathic pain include the imbalance between excitatory and inhibitory somatosensory signaling, alterations in ion channels properties and variations in the modulation of pain messages within the CNS. Those plastic changes can arise from both peripheral and central mechanisms. While some studies consider that peripheral inputs are needed to initiate and maintain central plasticity, also known as central sensitization (Devor 2009), others propose that once triggered, central changes can perpetuate, independently of the periphery (Latremoliere and Woolf 2009; Campbell and Meyer 2006).

The following information primarily relies on preclinical studies, as there is only limited knowledge available regarding human data. While these findings offer promising avenues for understanding and treating neuropathic pain, translation to human applications requires careful consideration and validation through clinical studies.

a. Peripheral sensitization

Peripheral sensitization refers to the increased responsiveness of primary nociceptive sensory neurons, stemming from an adaptation to prolonged or intense noxious stimuli in the periphery. This phenomenon manifests as a reduction in threshold of nociceptor activation which will then respond to normally innocuous or less-painful stimuli, accompanied by an elevated firing rate at the peripheral ends of sensory nerve fibers (Basbaum et al. 2009).

Tissue damage or injuries result in the local release of various chemicals by non-neuronal cells resident or recruited at the injury site. These include immune cells, fibroblasts, endothelial cells and platelets. Particularly, the recruited immune cells (macrophages, mast cells, lymphocytes and neutrophils) release bradykinin, histamine, serotonin, prostaglandins (e.g. PGE₂), cytokines (e.g. IL-6, IL-1 β , TNF- α), protons, ATP and nerve growth factor (NGF). Together, these mediators form what is regularly called the “inflammatory soup” and facilitate pain transduction by increasing the excitability of nociceptors at the periphery (Dureja et al. 2017; Basbaum et al. 2009). Indeed, primary afferent neurons express one or more cell surface receptors for each of these pro-inflammatory agents (Basbaum et al. 2009) (Figure 6). Subsequently, nociceptors locally release glutamate, substance P, CGRP and neurokinin A (Barker et al. 2020). These substances initiate pain transduction upon binding to their respective receptors on primary afferent neurons. With a particular emphasis on glutamate, peripheral nerve terminals harbor both iGluRs and mGluRs, and activation of these receptors can reduce the activation threshold and enhance the excitability of primary afferent neurons (Miller et al. 2011).

In response to this inflammatory soup at the injury site, the activity of transduction proteins that control the excitability of nociceptor terminals is modulated at both the transcriptional and post-translational level. Inflammatory mediators produce their effect by binding to their receptors and leading to the activation of a large diversity of intracellular pathways

(Figure 6). Downstream of these cascades, TRP and voltage-gated sodium channels (Na_v) are phosphorylated, altering their activation threshold and kinetics (Woolf and Ma 2007; Gold and Gebhart 2010). Broadly speaking, inflammation typically leads to the expansion or heightened expression of nociceptive ion channels and receptors. While this increased expression is functionally significant, the underlying mechanisms remain unclear.

One of the most well-documented mechanisms involves the binding of NGF to its receptor (TrkA), which triggers the activation of protein kinase C (PKC) and phosphoinositide 3-kinase (PI_3K) (Rittner et al. 2008; Woolf and Ma 2007). NGF signaling exerts both immediate and long-term effects on the activity of nociceptive ion channels and receptors, contributing to nociceptor sensitization (Barker et al. 2020). NGF primarily acts by rapidly phosphorylating Na_v and TRPV1 (mainly via ERK and PI_3K , respectively), thus enhancing channel activation (Numazaki et al. 2003; Zhang et al. 2005; Stein et al. 2006; Barker et al. 2020). Additionally, NGF mediates long-term changes in gene expression, such as increasing the number of NMDA receptor subtype 2B (Barker et al. 2020; Wong et al. 2014). Furthermore, NGF promotes transcription of TRPV1 and Na_v genes and enhances translocation of TRPV1 to the cell surface of primary afferents through a PI_3K and/or PKC signaling downstream of TrkA (Zhang et al. 2005; Julius and Basbaum 2001). Moreover, NGF levels are rapidly increased by immune cells (lymphocytes, macrophages and mast cells), fibroblasts, or Schwann cells in inflamed tissue, thereby reinforcing the sensitization loop (McMahon 1996).

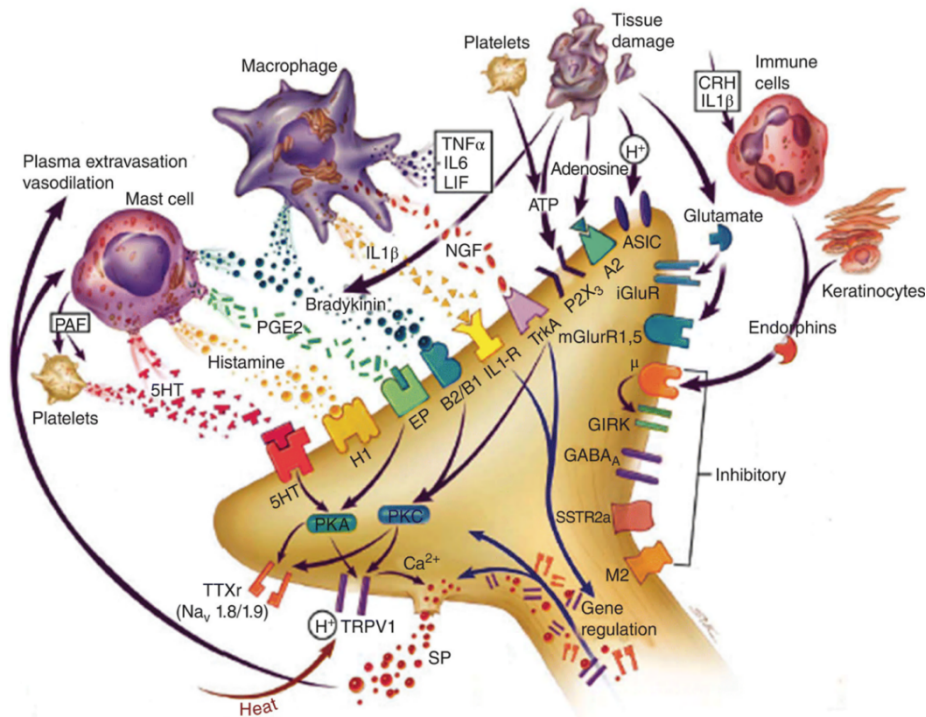


Figure 6: Nociceptor activation and peripheral sensitization. The activation of peripheral nociceptors in response to stimuli, such as injury, triggers the release of chemical mediators at the site of the lesion. Persistent pain or inflammation results in numerous plastic changes responsible for a reduction in threshold of nociceptor activation to normally innocuous or less-painful stimuli, accompanied by an elevated firing rate at the peripheral ends of sensory nerve fibers. *CRH*: corticotropin-releasing hormone; *TNF- α* : tumor necrosis factor α ; *LIF*: leukemia inhibitory factor; *IL*: interleukin; *NGF*: nerve growth factor; *ATP*: adenosine triphosphate; *PGE2*: prostaglandin E2; *PAF*: platelet-activating factor; *ASIC*: acid-sensing ion channel; *A2*: adenosine receptor 2; *P2X3*: purinergic 2X3 receptor; *TrkA*: tyrosine kinase A; *IL1-R*: interleukin 1 receptor; *B2/B1*: bradykinin 2/1 receptors; *EP*: prostaglandin E receptor; *H1*: histamine 1 receptor; *5HT*: 5-hydroxytryptamine receptor; *PKA*: protein kinase A; *PKC*: protein kinase C; *TRPV1*: transient receptor potential 1; *TTXr*: tetrodotoxin-resistant; *SP*: substance P; *M2*: muscarinic receptor 2; *SSTR2a*: somatostatin receptor 2a; *GABA_A*: gamma-aminobutyric acid; *GIRK*: G protein-gated inwardly rectifying potassium channels; *mGluR*: metabotropic glutamate receptor; *iGluR*: ionotropic glutamate receptor. Figure adapted from (Beaulieu 2013).

Early studies on peripheral sensitization have evidenced that peripheral inflammation triggers retrograde signals in nociceptors that increase the transcription of neuropeptides (substance P and CGRP), Na_v and TRP channels in the DRG (Neumann et al. 1996). If the peripheral axon of the nociceptor is physically disrupted, profound transcriptional changes occur. More recent studies indicated that negative signals such as the absence of growth factors, along with positive signals activating extracellular signal regulated kinase (ERK), stimulate the activation of transcription factors. Some of them are needed for neuronal survival and axon regrowth (e.g. CREB and STAT3 which upregulate BDNF, Sox11, Nrf2) but many of them are maladaptive and produce alterations in function that can drive neuropathic pain (e.g. NF-κB triggering pro-inflammatory cytokines production, AP-1, SP1) (Woolf and Ma 2007; Patodia and Raivich 2012). These plastic changes can lead to a phenotypic switch involving, for instance, the simultaneous downregulation of TRP and Na⁺ channels alongside with an upregulation of nitric oxide synthases and substance P production (Woolf and Ma 2007). This upregulation drives the production of NO, which is responsible for Ca²⁺ influx in primary sensory neurons (Woolf and Ma 2007). Indeed, inflammatory processes not only exert a direct impact on primary afferent neurons but also sensitize the surrounding tissues, leading to changes in the responsiveness of non-injured neurons and non-neuronal cells, such as immune and glial cells (Campana 2007).

Oxidative stress also significantly contributes to neuropathic pain by disrupting the redox balance and enhancing the production of ROS such as superoxide anions and hydrogen peroxide (Teixeira-Santos et al. 2020). These ROS interact with cellular components, leading to activation of TRP channels, lipid peroxidation, protein oxidation and DNA damage (Carrasco et al. 2018). Such molecular damage triggers and perpetuates neuroinflammation by activating pathways including NF-κB and MAPKs, which increase the expression of pro-inflammatory cytokines and chemokines. Additionally,

oxidative stress impairs mitochondrial function, further exacerbating neuronal injury thereby promoting hyperexcitability in the pain pathways.

Apart from changing the activation threshold of nociceptors which can explain hyperalgesia, nerve injury also induces some re-organization of neuronal connectivity at the level of the spinal cord, and this has been associated with the development of allodynia (Figure 7). Woolf and co-workers demonstrated that after peripheral nerve injury, the small unmyelinated primary afferent neurons (C fibers) - that typically convey nociceptive signals and terminate in the superficial dorsal horn (laminal I and II) - undergo degeneration. This is accompanied by a sprouting of larger myelinated primary afferent neurons ($A\beta$ fibers). These typically convey innocuous sensory signals into laminae II of the dorsal horn from deeper laminae where they normally terminate (Woolf and Mannion 1999; Bridges, Thompson, and Rice 2001). As $A\beta$ fibers sprout, second order neurons in the spinal cord, which typically receive high-threshold sensory inputs, become activated by low-threshold mechanoreceptors. This misinterpretation of information in the spinal cord can lead to innocuous signals being perceived as nociceptive.

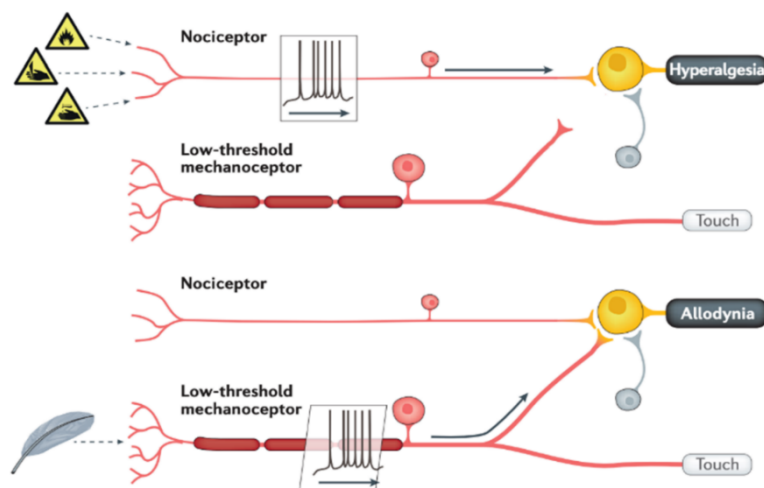


Figure 7: Allodynia and hyperalgesia. Central and peripheral sensitization leads to an intensified pain response to noxious stimuli (hyperalgesia). Following nerve injury, low-threshold $A\beta$ fibers sprout into superficial laminae

of the dorsal spinal cord. This synaptic reorganization results in an increased sensitivity of physiologically silent synapses, enabling low-threshold sensory inputs to activate the pain circuit (allodynia). Figure adapted from (Reynolds et al. 2016).

The ultimate outcome of these changes is the heightened sensitivity of primary afferent nociceptors. As the nociceptive signal reaches the dorsal horn of the spinal cord, peripheral sensitization exerts significant consequences that subsequently contribute to central sensitization (Gold and Gebhart 2010; Basbaum et al. 2009).

b. Central sensitization

Latremoliere and Woolf described central sensitization as an enhancement in the activity of neurons and circuits in nociceptive pathways caused by increases in membrane excitability and synaptic efficacy as well as by a reduced inhibition. It is a manifestation of the remarkable plasticity of the somatosensory nervous system in response to activity, inflammation, and neural injury (Latremoliere and Woolf 2009). Hence, central sensitization does not result from a unique mechanism but rather arises from a combination of processes operating in the dorsal horn of the spinal cord that heighten neuronal excitability, reinforce synaptic efficacy and decrease inhibitory signals (Figure 8). The key features of central sensitization will be further elaborated below.

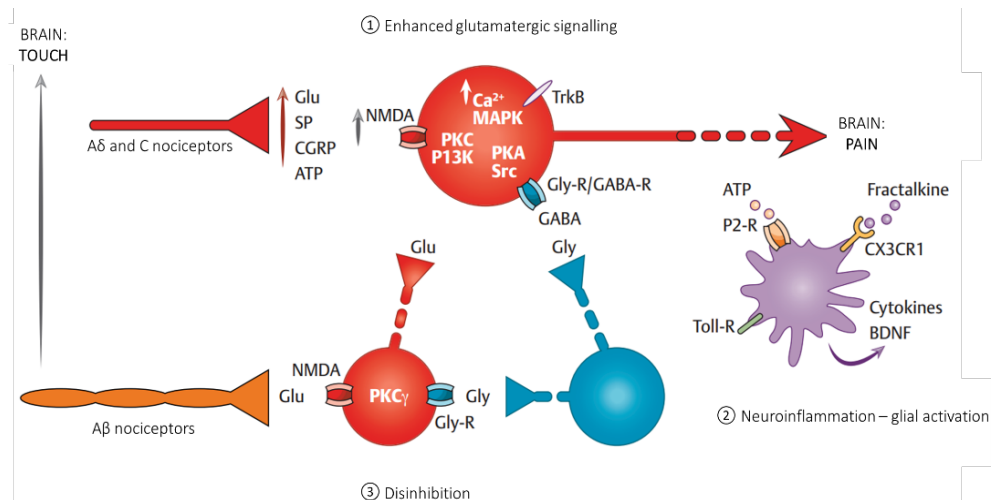


Figure 8: central sensitization in the spinal cord. Nerve injury prompts C and Aδ nociceptors to release neurotransmitters, activating NMDA receptors on postsynaptic neurons and enhancing pain transmission. Microglia activated upon nerve injury release ATP, stimulating BDNF and cytokines release. Disinhibition results from the loss of inhibitory control by GABA and glycine interneurons, causing hyperalgesia and allowing Aβ fibers to activate pain circuits. Central sensitization leads to increasing pain responses to both noxious and innocuous stimuli. *ATP: adenosine triphosphate; BDNF: brain-derived neurotrophic factor; CGRP: calcitonin gene-related peptide; CX3CR1: CX3C motif chemokine receptor 1; GABA: γ-aminobutyric acid; GABA-R: GABA receptor; Glu: glutamate; Gly: glycine; Gly-R: glycine receptor; MAPK: mitogen-activated protein kinase; NMDA: N-methyl-D-aspartate; P2-R: purinergic receptor 2; P13K: Phosphoinositide 3-kinase; PKA: protein kinase A; PKC(γ): protein kinase C (gamma); SP: substance P; Toll-R: Toll receptor.* Figure modified from (Basbaum et al. 2009), adapted from (Beaulieu 2013).

Enhanced glutamatergic signaling

As they project to the dorsal horn of the spinal cord, depolarized nociceptors release neurotransmitters, mainly glutamate, that activates a series of iGluRs and mGluRs. Following nerve injury, plastic changes occur at the spinal cord level reinforcing glutamatergic transmission at both pre- and postsynaptic levels as well as in astrocytes (Latremoliere and Woolf 2009).

- Presynaptic effects

As a consequence of peripheral sensitization and the associated intensified firing rate, voltage-gated Ca^{2+} channels are intensively activated in the presynaptic terminals of nociceptors. It induces a massive Ca^{2+} influx amplifying the release of glutamate-containing vesicles into the synaptic cleft. Besides, nerve injury leads to an upregulation of the Ca^{2+} channel $\alpha 2\delta$ -1 subunit in the DRG and the dorsal spinal cord (Li et al. 2004). This is responsible for an enhanced neurotransmitter release at the central terminals of primary afferent neurons (Campbell and Meyer 2006). Moreover, pre-synaptic NMDA receptor subunits form protein-protein complexes with $\alpha 2\delta$ -1 subunit, promoting its synaptic trafficking at the surface of both pre- and post-synaptic neurons (Liu et al. 2022). At this level, $\alpha 2\delta$ -1 subunit serves as a scaffold protein and a powerful positive regulator of NMDA receptors.

At the presynaptic side, glutamate release is negatively regulated by a multitude of G_i -protein coupled receptors including group II and III mGluRs, μ -opioid and GABA_B receptors (Campbell and Meyer 2006). Of importance, μ -opioid and GABA_B receptors are downregulated following nerve injury, with an indirect impact on the amplification of glutamate release (Kohno et al. 2005; Wang et al. 2011). A body of evidence suggests that activation of group II and III mGluRs constitutes an effective analgesic strategy in chronic pain conditions. For instance, oral absorption of LY2969822, a BBB-permeable prodrug that converts into LY2934747, a potent $\text{mGluR}_{2/3}$ receptor agonist, was proven efficient at attenuating pain behaviors across a broad range of preclinical pain models (Johnson et al. 2017; Pereira and Goudet 2018).

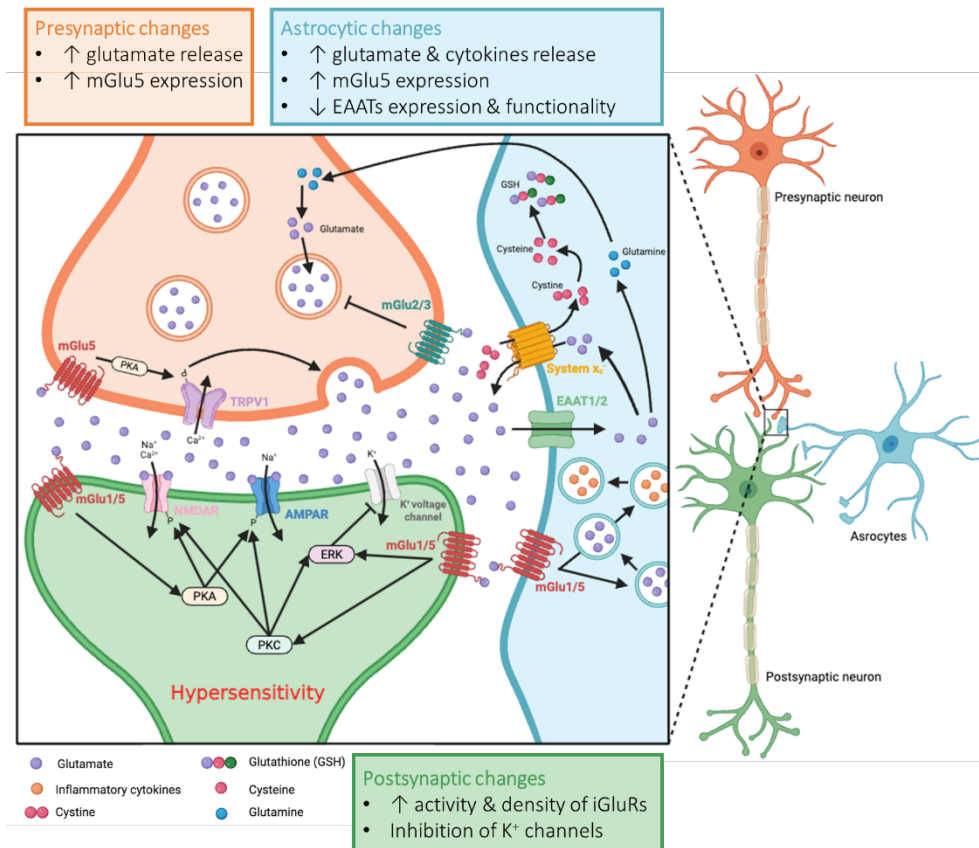


Figure 9: Glutamatergic plasticity during central sensitization. Nerve injury and the associated peripheral sensitization enhance glutamatergic transmission. Presynaptically, group I mGluRs, particularly mGlu5, which is upregulated in neuropathic pain models, sensitize TRPV1 via PKA-dependent phosphorylation, leading to increased glutamate release. Postsynaptically, AMPAR phosphorylation, GluA1 subunit insertion, and GluA2 subunit internalization result in elevated AMPAR-mediated synaptic efficacy. Membrane depolarization alleviates Mg²⁺ blockage of NMDARs, which are also upregulated via receptor phosphorylation. Group I mGluRs enhance synaptic transmission by indirectly phosphorylating NMDARs and heightening neuronal excitability through K⁺ channel inhibition. Additionally, astrocytes exhibit increased glutamate and cytokine release, heightened expression of mGluR5, and impaired EAATs expression and functionality, further contributing to the dysregulation of glutamatergic transmission and neuronal excitability in the context of neuropathic pain. *mGluR: metabotropic*

glutamate receptor; TRPV1: transient receptor potential vanilloid 1; PKA: protein kinase A; ERK: extracellular signal-regulated kinase; PKC: protein kinase C; NMDAR: NMDA receptor; AMPAR: AMPA receptor; EAAT: excitatory amino acid; GSH: glutathione. Figure created with BioRender®.

While the activation of group II and III mGluRs inhibits presynaptic neurotransmitter release, activation of group I mGluRs, especially mGluR5, sensitizes spinal TRPV1 through PKA-dependent phosphorylation, resulting in increased presynaptic release of glutamate (Jin et al. 2009; Hu et al. 2002). Furthermore, mGluR5 is known to be upregulated in dorsal horn neurons following inflammation and neuropathic lesion, thereby reinforcing the activation of spinal neurons (Hudson et al. 2002; Dolan et al. 2003; Osikowicz et al. 2008).

- Postsynaptic effects

In addition to the increased release of glutamate, alterations in the composition and the density of iGluRs and mGluRs on the postsynaptic membrane have been observed following nerve injury (Latremoliere and Woolf 2009; Pereira and Goudet 2018).

Under physiological conditions, glutamate mainly activates AMPA receptors since the NMDA receptors are non-functional and blocked by Mg^{2+} present in the receptor pore. In conditions of intense or high frequency firing of nociceptors, causing increased and sustained release of neurotransmitters, the enhanced postsynaptic depolarization triggers the release of Mg^{2+} and the activation of quiescent NMDA receptors (Basbaum et al. 2009; Baron et al. 2010). The resulting increase in cytosolic Ca^{2+} influx activates intracellular signaling pathways that enhance the synaptic efficacy of glutamate transmission. Long-lasting changes in this excitatory transmission were initially evidenced in hippocampal neurons and are considered as the fundamental biochemical mechanism supporting memory. In the pain pathways, the same mechanism is thought to contribute to the exacerbation

and prolongation of responses to noxious stimuli (Figure 9) (Jensen and Finnerup 2014; Li, Miao, and Zhuo 2019).

Beside iGluRs, group I mGluRs on the postsynaptic neuron may also contribute to the positive modulation of excitatory transmission concurring to central sensitization. First, the mGluR5-mediated increase of intracellular Ca^{2+} , promotes the activation of secondary messengers and kinases (PKC and PKA), that phosphorylate NMDA receptors and reinforce synaptic transmission (Latremoliere and Woolf 2009; Liu and Salter 2010; Ultenius et al. 2006). Indeed, phosphorylation of receptor subunits have been documented to facilitate its trafficking and increase the opening rate of the NMDA channel (Chen and Roche 2007). The NMDA receptor phosphorylation appears as a key player in the induction of central sensitization as it was correlated with the increased pain sensitivity in rodent models of both inflammatory and neuropathic pain (Guo et al. 2002; Qu et al. 2009). Supporting the same concept, Malmberg and colleagues reported that spinal administration of a selective antagonist of the NMDA receptor subunit NR2B, produced potent analgesic effects in several pain models (Malmberg et al. 2003). Secondly, activation of group I mGluRs plays a role in the inhibition of voltage-gated K^+ channels through the activation of downstream effectors including ERK. These K^+ channels are involved in the repolarization of neuronal membranes and their inhibition influences the duration and intensity of membrane depolarization of spinal cord neurons (Hu et al. 2007).

In addition to NMDA, AMPA receptors are also subject to significant regulations following nerve injury that contribute to the increased glutamatergic transmission in pain pathways. GluA1 and GluA2 subunit-containing AMPA receptors are both expressed in all glutamatergic synapses in the laminae I and II of the spinal cord (Kopach and Voitenko 2021). While AMPA receptors composed of GluA1 subunits enable rapid Ca^{2+} influx and intracellular pathway activation, the presence of GluA2 subunits, on the contrary, reduces Ca^{2+} permeability (Cull-Candy et al. 2006; Liu and Salter

2010). Following nerve injury, both subunits are highly phosphorylated but with different consequences. GluA1 subunit phosphorylation is responsible for the increased conductance, opening rate and it promotes surface insertion of AMPA receptors (Nagy et al. 2004; Wang et al. 2010). Conversely, GluA2 subunit phosphorylation following neuropathies leads to AMPA receptors internalization (Liu and Savtchouk 2012). Together, these processes significantly increase Ca^{2+} influx resulting in enhanced synaptic transmission and pain hypersensitivity.

These findings regarding the implication of mGluRs in molecular mechanisms governing chronic pain have opened new avenues for the development of pharmacological treatments. Indeed, the past decades have witnessed the accumulation of evidence indicating that iGluRs significantly contribute to the pathophysiology of neuropathic pain. This has revealed promising strategies for the management of neuropathic pain. However, targeting these receptors is responsible for severe side-effects including memory loss and hallucinations. Therefore, the attention has been progressively shifted towards mGluRs (Gass and Olive 2008; Pereira and Goudet 2018). Activation of mGluRs can either promote or inhibit nociception, depending on their anatomical localization and the signaling pathways they engage. Antagonists targeting Group I mGluRs and agonists activating presynaptic group II and III mGluRs have demonstrated therapeutic potential in animal pain models. For instance, systemic injections of a selective mGluR5 negative allosteric modulator (2-Methyl-6-(phenylethynyl)pyridine, MPEP) were found to reduce mechanical hypersensitivity induced by multiple inflammatory and neuropathic pain models (Zhu et al. 2004). A-841720, a potent and non-competitive mGluR1 antagonist, was suggested to reduce complete Freund's adjuvant (CFA)-induced inflammatory pain. It also decreased mechanical allodynia observed in different models of neuropathic pain (El-Kouhen et al. 2006). Certainly, some of the tested compounds are also responsible for side-effects and require further study to target specific signaling pathways or simply increase selectivity and thus avoid off-target effects.

- Astrocytic effects

As mentioned earlier, in the context of peripheral lesions or nerve injuries spinal astrocytes and microglia adopt an activated profile in response to centrally released neurotransmitters such as ATP, glutamate and prostaglandins. Once activated, these glial cells participate in the spinal response and reinforce central sensitization through the release of gliotransmitters including glutamate and pro-inflammatory cytokines that modulate both pre- and post-synaptic glutamate receptors (Ji et al. 2018).

Due to the presence of glutamate transporters (EAAT1; GLAST and EAAT2; GLT-1) at their cell surface, astrocytes are important players in regulating glutamate signaling. Indeed, EAATs are essential for the clearance of extracellular glutamate after synaptic transmission. They maintain a low extracellular concentration of glutamate, avoiding hyperexcitability and excitotoxicity. In a model of nervous injury, dysfunction of EAATs impairs glutamate homeostasis. Instead of removing extracellular glutamate to protect neurons, astrocytes release glutamate, triggering neuronal hypersensitivity (Rossi et al. 2000). Such deficiency or decrease in GLT-1 or GLAST expression within the rodent spinal dorsal horn has been linked to the onset of neuropathic pain resulting from peripheral nerve injury (Sung et al. 2003; Binns et al. 2005). Sung and co-workers evidenced transient upregulation of GLAST and GLT-1 during the first 4 days post-injury which is followed by a considerable downregulation that persists for weeks (Sung et al. 2003). The mechanism of spinal glutamate uptake downregulation is still not clear, but it is proposed to contribute to central sensitization. Accordingly, recent studies have indicated that the repeated administration of the β -lactam antibiotic ceftriaxone, which promotes the expression of GLT-1 via the activation of nuclear factor-kappa B (NF- κ B), efficiently reduces hypersensitivity in animal models of neuropathic pain (Lee et al. 2008; Hu et al. 2010). Worth mentioning, Macaluso and colleagues have reported on the

analgesic effect of a single preoperative administration of ceftriaxone in humans (Macaluso et al. 2013).

Astrocytes not only take up glutamate, but also release it in a regulated manner, via both vesicular (Araque et al. 1999) and non-vesicular mechanisms, through the cystine/glutamate exchanger, also known as system x_c^- (Massie et al. 2015; Malarkey and Parpura 2008). Similarly, when activated, microglia also release glutamate, employing system x_c^- or, to a lesser extent, the reversal of glutamate uptake by EAATs (Takeuchi et al. 2006; Barger et al. 2007; Noda et al. 1999; Massie et al. 2015). As for other release mechanisms, system x_c^- -mediated release of glutamate favors the activation of iGluRs and mGluRs, and thereby indirectly participates in central sensitization (Lewerenz et al. 2013). Detailed information regarding the implication of system x_c^- in the regulation of glutamate are provided in chapter III.

Aside from the finely tuned regulation of glutamate uptake and release by cell surface transporters and exchangers, astrocytes also express mGluRs (especially mGluR5) which likely serve as glial sensors of extracellular glutamate concentration (Panatier and Robitaille 2016). Indeed, mGluR5-mediated intracellular Ca^{2+} mobilization in astrocytes may trigger the release of gliotransmitters such as glutamate, ATP and cytokines that contribute to the modulation of synaptic plasticity and neuroinflammation (Gordon et al. 2009). These receptors also contribute to the functional adaptation of astrocytes to the enhanced excitatory activity in the synapse. Thus, Vermeiren and colleagues described that activation of astrocytic mGluR5 was associated with an increased GLT-1 and GLAST expression *in vitro* (Vermeiren, Najimi, Vanhoutte, et al. 2005). Even if the presence of mGluR5 in adult astrocytes in physiological conditions is debated, increased expression of astrocytic mGluR5 has been repeatedly documented in different models of nervous pathologies (Bradley and Challiss 2012; Panatier and Robitaille 2016). In particular, it has been shown that following peripheral nerve injury (using

different rodent models of neuropathic pain), astrocytic mGluR5 was upregulated in the trigeminal nucleus and in the somatosensory cortex (Michot et al. 2017; Danjo et al. 2022). Evidence suggests that the selective and non-competitive mGluR5 antagonist, fenobam, induces robust antinociceptive effects in preclinical mouse models of inflammatory and neuropathic pain (Lax et al. 2014; Montana et al. 2009; Pereira and Goudet 2018). However, only a modest analgesic effect was demonstrated in healthy volunteers compared to placebo using a well-established experimental pain model of cutaneous sensitization (capsaicin cream and heat) (Cavallone et al. 2020). This could be explained by the poor pharmacokinetic profile of fenobam after oral dose. Further studies need to be conducted to identify more suitable compounds.

In summary, in the context of chronic neuropathic pain, enhanced glutamatergic transmission during central sensitization is intricately linked to a combination of mechanisms involving all the partners of the tripartite synapse, namely the pre- and postsynaptic neurons along with astrocytes. Hence, the implication of both astrocytes and microglia in studies of CNS sensitization and chronic pain call for the concept of the tetrapartite synapse (De Leo et al. 2006).

Neuroinflammation

Growing evidence suggests that glial cells, notably microglia and astrocytes, primarily contribute to neuroinflammation during chronic pain (Cavaliere et al. 2007; Gao 2010a; Kawasaki et al. 2008). While astrocytes and microglia are undoubtedly induced and activated in the spinal cord after nerve injury, the timing of their activation differs significantly (Ji et al. 2009; Cavaliere et al. 2007). Indeed, Raghavendra and colleagues found that in a neuropathic pain model, initiating minocycline administration at the time of nerve transection (pre-emptive treatment) reduces allodynia and hyperalgesia, an effect attributed to its ability to inhibit microglial activation. However, when

administered on day 5 post-surgery (as hypersensitivity is established), it fails to alleviate behavioral hyperalgesia and allodynia, despite an effective reduction in microglial activation (Raghavendra et al. 2003). In contrast, inhibitors of astrocyte activation were proven effective in both the early and late phases of neuropathic pain onset (Chen et al. 2014), suggesting distinct roles for microglia and astrocytes in chronic pain induction and maintenance (Basbaum et al. 2009; Kobayashi et al. 2008; Campbell and Meyer 2006; Milligan and Watkins 2009; Cavaliere et al. 2007). Evidence indicates bidirectional functional interactions between microglial and astrocytic reactions (Liddel et al. 2017), where microglial activation may trigger astrocytic response, while astrocytic activation can also elicit microglial reactivity (Ovanesov et al. 2008). This results in a positive reinforcing loop in inflammation when both cell types are activated.

Following peripheral nerve injury, molecules such as damage-associated molecule patterns (DAMPs), ATP, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X-C motif) ligand 13 (CXCL13) and neurotransmitters are released by afferent neurons and trigger the activation of both glial cell types. Microglia are highly sensitive to changes in their microenvironment and can rapidly respond to these signals. For instance, ATP binds to microglial purinergic receptors (P2 subtype) leading to microglial activation and accumulation in the superficial layers of the dorsal spinal cord within hours (Kobayashi et al. 2008; Seo et al. 2008; Franke et al. 2007; Tsuda et al. 2013). Once activated, microglia start releasing a variety of molecules such as TNF- α that trigger astrocytic activation through the JNK pathway (Lu and Gao 2023; Gao et al. 2009). Concomitantly, nerve injury induces astrocytic activation through CXCL13 that binds to CXC motif chemokine receptor 5 (CXCR5), triggering intracellular pathways that will be described below.

Once activated, glial cells display changes in their morphology and upregulate glial markers such as ionized calcium-binding adapter molecule 1 (Iba-1) and glial fibrillary acidic protein (GFAP) for microglia and astrocyte, respectively.

Astrocytic activation following chronic constriction injury of the sciatic nerve in mice is also associated with an increased level of the well-known hemichannel protein connexin-43 (Cx43, predominantly expressed in astrocytes) in the spinal cord for more than 3 weeks (Chen et al. 2014). Besides, activation of glial cells is characterized by a cascade of intracellular changes involving MAPKs pathways which are known to be associated with the synthesis and release of glial mediators (Ji et al. 2009).

Signaling molecules released by activated glial cells include IL-1 β , TNF- α , chemokines and growth factors which enhance neuronal excitability and contribute to pain hypersensitivity. In the context of this thesis, we decided to focus on the impact of inflammatory cytokines on the modulation of iGluRs.

IL-1 β recognizes two different receptors: IL-1 type 1 receptor (IL-1R₁) and IL-1 type 2 receptor (IL-1R₂) expressed on both neurons and glial cells (Li et al. 2005; Yan and Weng 2013). While binding to IL-1R₂ does not lead to any downstream response, IL-1R₁ activation is responsible for a cascade of intracellular signals (Ren and Torres 2009). These pathways regulate the expression of genes involved in inflammation, cell survival, and synaptic plasticity. Among them, activation of neuronal IL-1R₁ activates MAPK which induces NMDA receptor phosphorylation (NR1 and NR2B subunits) supporting a close communication between neurons and glial cells (Guo et al. 2007; Yan and Weng 2013). IL-1 β also binds to glial IL-1R₁ that activates transcription regulator TRAF6 triggering the synthesis of additional cytokines and chemokines which amplify synaptic transmission (Inoue et al. 1999).

TNF- α , which is primarily produced by microglia, does not only affect the membrane expression and phosphorylation of NMDA receptors but also regulates neuronal activity through modulation of the AMPA receptor trafficking (Stellwagen et al. 2005). Indeed, the activation of TNF-1R enhances the surface expression of GluA1-containing AMPA receptors through a PI₃K-dependent process. Additionally, TNF- α induces the endocytosis of GABA_A

receptors in neurons, leading to a reduction in inhibitory synaptic strength (Stellwagen et al. 2005). Besides, TNF- α can further strengthen glutamatergic transmission by decreasing the expression and activity of GLT-1 and GLAST in astrocytes (Sitcheran et al. 2005; Korn et al. 2005).

Accumulating evidence suggests that pro-inflammatory cytokines are upregulated in the spinal cord, in different chronic neuropathic pain conditions. Moreover, intrathecal injection of IL-1 β and TNF- α was documented to trigger hypersensitivity-associated responses in mice and rats (Kawasaki et al. 2008; Gruber-Schoffnegger et al. 2013). Inhibition of cytokines signaling pathways reduces glial activation and chronic pain symptoms (Sommer et al. 1999). The neutralization of TNF- α has been demonstrated to alleviate chronic pain in patients with rheumatoid arthritis more rapidly than it improves signs of inflammation, such as reducing joint swelling. This effect is likely due to the inhibition of TNF- α -mediated nociceptive neurotransmission in the spinal cord dorsal horn before inflammation shows improvement (Hess et al. 2011).

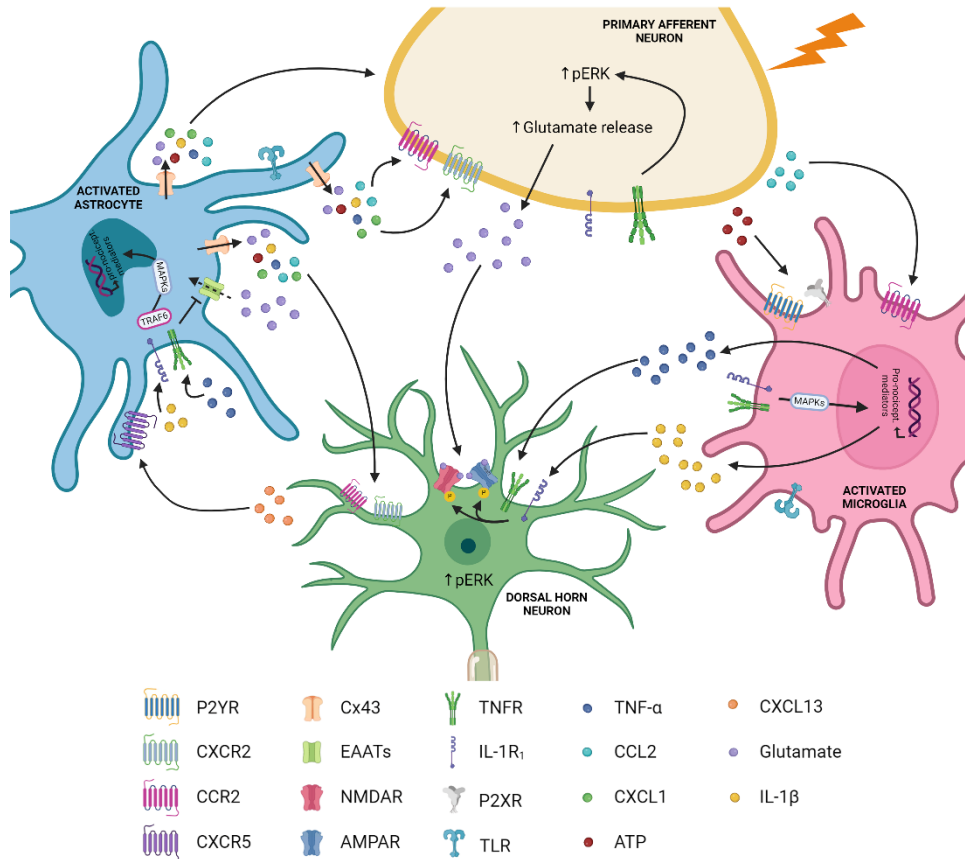


Figure 10 : Neuroinflammation during central sensitization in neuropathic pain. Nerve injury triggers astrogliosis in the spinal dorsal horn, leading to morphological changes, upregulation of GFAP and Cx43, and activation of ERK and JNK signaling. These cascades prompt the release of astroglial mediators modulating synaptic transmission, thereby promoting central sensitization and chronic pain. Additionally, astrocyte activation in neuropathic pain conditions upregulates Cx43 expression, enhancing hemichannel-mediated paracrine signaling, releasing pro-inflammatory chemokines (e.g. CXCL1 and CCL2), glutamate, and ATP. Microglial activation via ATP receptors induces the release of TNF-α and IL-1β, sensitizing dorsal horn neurons. These cytokines also activate MAPK pathways in astrocytes, enhancing the release of pro-inflammatory chemokines. Astrocytic mediators (including CXCL1, CCL2, and IL-1β) promote central sensitization via neuronal CXCR2, CCR2, and IL-1R, stimulating ERK signaling in primary afferent terminals and dorsal horn

neurons. Additionally, nerve injury-induced CXCL13 release activates astrocytes through the CXCR5 receptor, further contributing to neuropathic pain. *pERK*: phosphorylated extracellular signal-regulated kinases; *P2YR*: purinergic receptor 2Y; *P2XR*: purinergic receptor 2X; *CXCR2*: CXC motif chemokine receptor 2; *CXCR5*: CXC motif chemokine receptor 5; *CCR2*: CC motif chemokine receptor 2; *Cx43*: connexin 43; *EAATs*: excitatory amino acid transporters; *NMDAR*: NMDA receptor; *AMPA*: AMPA receptor; *TNFR*: tumor necrosis factor receptor; *IL-1R1*: interleukin 1 receptor type 1; *TLR*: toll-like receptor; *TNF- α* : tumor necrosis factor α ; *CCL2*: chemokine (C-C motif) ligand 2; *CXCL1*: chemokine (C-X-C motif) ligand 1; *ATP*: adenosine triphosphate; *CXCL13*: chemokine (C-X-C motif) ligand 13; *IL-1 β* : interleukin-1 β . Figure created with BioRender®.

Disinhibition

As previously described, the nociceptive circuit receives inhibitory control at the spinal cord level from two different origins: the descending pathway from supraspinal structures and inhibitory interneurons within the spinal cord. According to the gate control theory of pain, a loss of function in GABA- or glycine-releasing inhibitory interneurons could result in increased pain (Melzack and Wall 1965; Latremoliere and Woolf 2009). Following peripheral nerve lesions, inhibitory interneurons and descending modulatory control systems are impaired (Latremoliere and Woolf 2009; Basbaum et al. 2009). Combined with the increased activity from afferent fibers, this renders dorsal horn neurons more susceptible to activation by excitatory inputs, including non-nociceptive A β -fibers (Keller et al. 2007).

Disinhibition occurs through a multitude of mechanisms including (1) a shift in the anionic membrane gradient, (2) a reduction of inhibitory transmitter synthesis and/or release, (3) a blockade of inhibitory receptors, (4) an alteration in descending inhibitory pathways and (5) even the death of inhibitory interneurons (Todd 2015). Indeed, NMDA receptor-induced excitotoxicity accompanying nerve lesions was shown to cause the apoptosis of inhibitory interneurons in the spinal dorsal horn (Scholz et al. 2005;

Simpson and Huang 1998). Numerous studies also highlighted a depletion of GABA and glycine in the spinal cord of neuropathic animals. This is explained by the downregulation of both glutamic acid decarboxylase isoforms (GAD65 and GAD67), the key regulatory enzymes in the synthesis of GABA (Kami et al. 2016). A substantial diminution of spinal GABA in neuronal terminals has also been described as evidenced by the reduction of GABA transporters following neuropathic lesions (Miletic et al. 2003).

Another mechanism contributing to the reduction in inhibition is dependent on brain-derived neurotrophic factor (BDNF)-mediated alteration in Cl^- gradients in nerve terminals that govern the inhibitory activity of GABA_A receptors (Coull et al. 2005). Under physiological condition, intracellular concentrations of Cl^- are maintained by two anion transporters: K^+-Cl^- co-transporter KCC2 which drives Cl^- efflux and $\text{Na}^+-\text{K}^+-\text{Cl}^-$ co-transporter NKCC1 facilitates Cl^- influx. After nerve injury, BDNF released by activated microglial cells reduces KCC2 expression in dorsal horn neurons, impacting Cl^- gradients (Rivera et al. 2002; Miletic and Miletic 2008; Li, Lei, et al. 2019). This disrupted balance in the Cl^- concentration gradients leads to a reduced Cl^- entry upon GABA_A activation and subsequent disinhibition of nociceptive neurons (Latremoliere and Woolf 2009). It has even been demonstrated that activation of GABA_A receptors can lead to depolarization rather than hyperpolarization (Latremoliere and Woolf 2009; Li, Lei, et al. 2019).

Disinhibition can also occur through modulation of spinal glycinergic signaling. In physiological conditions, glycinergic interneurons innervate the so-called PKC γ excitatory interneurons, contributing to a feed-forward inhibitory circuit essential to reduce the transmission of innocuous A β fiber inputs in the spinal cord (Figure 7). However, in models of neuropathic pain, changes in glycine receptors (GlyRs) expression and function have been described. In particular, there is a switch in the subtype of GlyRs expressed on the excitatory PKC γ interneurons to GlyR α 2 (Imlach et al. 2016), the latter being less responsive to synaptic glycine as compared to GlyR α 1 and GlyR α 3

(Cioffi 2021). It has also been reported that upon binding to the EP2 receptor, PGE₂ released during central sensitization affects the expression and activity of GlyR α 3 (Ahmadi et al. 2002; Harvey et al. 2004). As a result, low threshold A β mechanonociceptor inputs activate second order-neurons in the spinal cord, leading to allodynia.

In summary, central sensitization in neuropathic pain involves a complex interplay of cell actors and mechanisms, including glutamate receptor-mediated sensitization, disinhibition, and neuroinflammation through glial activation. These processes contribute to heightened neuronal excitability and amplification of pain signals, translating into the development of hyperalgesia and allodynia.

3. Clinical treatment

Treatments for neuropathic pain are designed to normalize the nociceptive process or to prevent the development of pathological mechanisms and alleviate pain symptoms. However, despite the high prevalence of neuropathic pain, only few pharmacological agents are currently approved by US (FDA) and European (EMA) health authorities for neuropathic pain treatment. Consequently, some of the compounds are provided “off-label”. The therapeutic approaches for neuropathic pain have already been reviewed in different articles (Cavalli et al. 2019; Jongen et al. 2014). Without entering into details, we here provide a brief overview of the different existing pharmacological options that are currently used in the clinical practice. Guidelines for the management of neuropathic pain categorizes the different pharmacotherapies in first-, second- and third-line treatments.

First-line therapy

The first-line medication for neuropathic pain involves drugs that are already approved and indicated for other pathological conditions. Given the

similarities in some molecular mechanisms between these diseases and neuropathic pain, the therapeutic effects of these drug can be effectively exploited to alleviate chronic pain symptoms. Among them, the most used treatments are gabapentin and pregabalin, normally used in epilepsy, and antidepressants (tricyclic antidepressants (TCAs) and serotonin and norepinephrine reuptake inhibitors (SNRIs)).

The World Health Organization in charge of the Anatomical Therapeutic Chemical (ATC) classification system for drugs has recently reclassified all gabapentinoids in the analgesic's subgroup (N02) instead of antiepileptics (N03).

Gabapentin and pregabalin bind to the $\alpha 2\text{-}\delta$ subunit of Ca^{2+} voltage-dependent channels on central terminals of primary afferent nociceptors, leading to decreased release of excitatory neurotransmitters and reduced neuronal excitability. Gabapentin has also been documented to relieve pain by blocking the interaction between the $\alpha 2\delta\text{-}1$ subunit and the NMDA receptors (Wu et al. 2023; Fu et al. 2021). Blocking this protein-protein interaction modulates the trafficking of the NMDA receptors and can thus reduce neuropathic pain without affecting its physiological functions (Zhou et al. 2011).

The TCAs amitriptyline and imipramine as well as their metabolites, nortriptyline and desipramine are the most frequently used drugs for the treatment of neuropathic pain in first intention. They exert an analgesic effect through monoamine reuptake inhibition from presynaptic terminals in descending inhibitory pathways. They also block NMDA receptors and peripheral nerve Na^{+} channels, which may contribute to their antihyperalgesic efficacy (Dick et al. 2007; Mu et al. 2017). These analgesic mechanisms of action differ from the antidepressant effect. Indeed, analgesia is observed at lower dose as compared to the treatment of depression (Jongen et al. 2014). Nevertheless, their antidepressant properties can turn beneficial as depression is a frequent comorbidity in chronic neuropathic

pain. Unfortunately, undesirable side effects such as sedation, anticholinergic effects and orthostatic hypotension, frequently occur when using TCAs, particularly in the elderly (Mu et al. 2017). TCAs are thus contraindicated in patients presenting cardiac pathologies, glaucoma and prostate hypertrophy (Baron et al. 2010).

As they are better tolerated than traditional TCAs, SNRIs are increasingly used for the treatment of neuropathic pain. As compared to TCAs, SNRIs lack cholinergic properties leading to potentially fewer adverse effects (Watson et al. 2011). SNRIs inhibit both presynaptic neuronal serotonin and noradrenaline reuptake. This results in the accumulation of these neurotransmitters at the synaptic junction reinforcing inputs in the descending inhibitory pain pathways. While duloxetine causes a balanced inhibition of serotonin and noradrenaline reuptake, venlafaxine is more considered as a serotonin reuptake inhibitor at low dose and the noradrenaline reuptake blockade increases with drug doses (Gallagher et al. 2015). Although noradrenaline plays only an inhibitory role in pain transmission, there are inhibitory and facilitatory components related to serotonin in the descending pathways. Serotonin has multiple and often opposing effects mediated by its multitude of receptor subtypes (Cortes-Altamirano et al. 2018; Liu, Yao, et al. 2020). Indeed, 5-HT_{1A} and 5-HT₇ receptors are known to exert antinociceptive effects while 5-HT_{2B} and 5-HT₃ receptors are pronociceptive (Bardin 2011). This is one of the reasons why duloxetine is preferred in the clinical practice as compared to venlafaxine.

To determine the appropriate dosage, all of these first-line medications are typically initiated at a low dose and gradually increased until an optimal balance between analgesic efficacy and potential side effects is achieved. Tailoring treatment to individual patients is crucial during this optimization phase, and NSAIDs or opioids may be introduced to alleviate pain. It is worth mentioning that a combination of two or more drugs is often necessary in clinical practice to achieve a satisfactory pain relief.

Second-line therapy

Opioids are among the most potent available analgesics but are only recommended as second- or third-line therapy since they cause a variety of side effects and potentially lead to tolerance and dependence, even at therapeutic dosages. Since opioids present significant differences in the incidence of adverse effects, drug selection for long-term use is thus essentially based on the adverse effect profile (Jongen et al. 2014). Tramadol and tapentadol are widely employed in second-line therapy for pain management, exerting their analgesic effects by inhibiting nociceptive transmission through both presynaptic and postsynaptic μ -opioid receptors expressed in afferent fibers. Tramadol acts as a μ -opioid receptor agonist, but also exerts additional effects, such as the inhibition of noradrenaline and serotonin reuptake, which may contribute to its analgesic efficacy. Tapentadol was recently launched as a new dual opioid analgesic, combining a relatively potent agonistic activity at μ -opioid receptors with an inhibition of noradrenaline reuptake. The synergy between the two mechanisms explains the increasing use of this drug in clinical practice (Vadivelu et al. 2015; Fornasari 2017). Tapentadol is the only FDA-approved opioid for the management of neuropathic pain associated with diabetic peripheral neuropathy (Vadivelu et al. 2015; Coluzzi et al. 2017).

Topical analgesic such as lidocaine and capsaicin are also used as second-line treatment in neuropathic pain management. The use of topical analgesics provides pain relief with minimal risk of systemic toxicity or drug interactions because they are formulated to produce a local effect while avoiding plasma concentrations necessary for a systemic effect. Lidocaine is generally used in the form of 5% patches and blocks Na_v channels in primary nerve fibers, preventing nerve impulses from being generated and conducted (Davies and Galer 2004; Gudín and Nalamachu 2020). Interestingly, in neuropathies, those Na^+ channels are known to be upregulated (Dib-Hajj et al. 2010). Since the penetration of lidocaine does not exceed 10 mm, it is essentially indicated

in well-localized neuropathic pain (e.g. post-herpetic neuralgia or post-surgical pain related to skin incision) (Davies and Galer 2004; Baron et al. 2016; Gudín and Nalamachu 2020). Capsaicin is a highly potent agonist of TRPV1 receptors which are densely present in afferent C fibers and certain A δ fibers. Normally, local activation of TRPV1 receptors by noxious stimuli such as high temperature or low pH induces nerve depolarization that travels to the spinal cord and brain, eliciting pain sensations. Prolonged and repeated local exposure to capsaicin (8% patch) induces intracellular changes resulting in desensitization of TRPV1 receptors. Consequently, nerve fibers become less responsive to subsequent stimuli leading to impaired local nociception for an extended period of time (Peppin and Pappagallo 2014).

Third-line therapy

For the reasons outlined above, potent opioid analgesics like morphine, oxycodone and fentanyl are only envisaged as third-line treatment strategies (Cavalli et al. 2019). Prescription of opioids should be strictly monitored particularly for patients requiring high dosages. Except for cancer-induced pain, several guidelines state that strong opioids should be considered only when alternative treatments did not provide adequate analgesia or when patient's quality of life is severely affected and that the potential benefit balances the possible side effects (Cavalli et al. 2019; Smith 2012; Furlan et al. 2010).

Although these different treatments have shown promising efficacy in managing neuropathic pain, they are often associated with side effects that restrict their clinical use. To enhance the effectiveness of these treatments and lessen the side effects (by reducing the dosage), combinatory treatment should sometimes be privileged (Serrano Afonso et al. 2021).

Beside the well documented pharmacological treatments used in neuropathic pain, a large variety of compounds are used as fourth- or fifth-line strategy. Among them are mostly cited, NMDA receptor antagonist (e.g.

ketamine), cannabinoids (e.g. cannabidiol, δ -9-tetrahydrocannabinol), α_2 -adrenoreceptor agonists (e.g. clonidine), other SNRIs, anticonvulsants and botulinum toxin (Mu et al. 2017; Bahari and Meftahi 2019; Campos et al. 2021; Mittal et al. 2016). It is worth mentioning that non-pharmacological options such as nerve and brain stimulations, physical and psychological therapies, should also be considered for a better treatment of neuropathic pain (Cavalli et al. 2019; Remy et al. 2022).

4. Current needs

Nowadays, neuropathic pain still poses significant challenges for patients, healthcare providers, and researchers. Despite advances in our understanding of the physio-pathological mechanisms of neuropathic pain, several questions need to be addressed to improve its management. Indeed, current strategies fail to achieve adequate or satisfactory pain relief in a significant proportion of patients. Further complicating the limited effectiveness of treatments is the challenge of achieving appropriate dosage coupled with the prevalence of common side effects. The difficulty to treat neuropathic pain patients lies in the heterogeneity and complexity of the pathophysiology. A deeper understanding of the mechanisms underlying the development and the maintenance of neuropathic pain, which remain incompletely understood, undoubtedly holds the potential to ameliorate the management of this nervous disorder.

Another significant difficulty lies in the absence of accurate diagnosis criteria for neuropathic pain. Indeed, the subjective nature of pain and the absence of specific biomarkers poses significant challenges in the clinical diagnosis, often resulting in delayed intervention and suboptimal outcomes. Since chronic pain may result from early processes, prompt management of acute pain appears essential to prevent the transition into chronic pain.

Psychosocial factors contribute significantly to the challenges of managing chronic neuropathic pain. Patients often deal with anxiety, depression, and

sleep disturbances, exacerbating their pain experience (Remy et al. 2022). A holistic approach that addresses both the physical and psychological dimensions of pain is essential and could considerably improve the quality of life of patients (Fishbain 1999).

CHAPTER III: The cystine/glutamate antiporter or system x_c^-

Glutamate is commonly regarded as the primary excitatory neurotransmitter in the brain of mammals. Not surprisingly, alteration in the concentration of glutamate or in the duration of its presence in the extracellular space have been associated with a variety of neurological disorders. The concepts of hyperexcitation and neuronal excitotoxicity have been largely documented as key mechanisms participating in the development or maintenance of several diseases. This emphasizes the importance of accurately controlling extracellular glutamate levels.

Typically, excitatory signaling begins with the vesicular release of glutamate from a presynaptic terminal, followed by its diffusion across the synaptic cleft, leading to the activation of glutamate receptors on both pre- and post-synaptic neurons. Subsequently, glutamate in the synaptic cleft is cleared by Na^+/K^+ -dependent glutamate transporters (GLT-1 and GLAST) primarily found on astrocytic processes that enwrap the synapse. Nonetheless, recent insights suggest that excitatory signaling is far more complex, involving a series of release and reuptake mechanisms operated by both neurons and glial cells. These processes actively participate in the finely tuned activation of glutamate receptors located both within and beyond the synaptic cleft.

The necessity for a deeper understanding of the cellular mechanisms regulating glutamate homeostasis becomes evident when considering studies that have linked non-vesicular glutamate release to various CNS activities, ranging from transmission of excitatory inputs to neurodevelopmental functions and synaptic plasticity.

System x_c^- , also known as cystine/glutamate antiporter, is an example of an understudied source of non-vesicular glutamate release. There is accumulating evidence indicating that this antiporter constitutes the predominant source of extracellular glutamate (De Bundel et al. 2011; Sato et al. 2005; Massie et al. 2011).

1. Generality on system x_c^-

System x_c^- was first characterized in cultured human fetal lung fibroblasts by Bannai and Kitamura in 1980, when evidencing that a common carrier system transported L-cystine or L-glutamate in these cells (Bannai and Kitamura 1980). A few years later, Makowske and Christensen outlined a comparable transport system in a transformed rat hepatoma cell line (Makowske and Christensen 1982). System x_c^- belongs to the SoLute Carrier 7 (Slc7) family and is a membrane Na^+ -independent amino acid transporter that is structurally composed of two different subunits: a heavy chain common to numerous amino acid transporters, 4F2hc (Slc3a2 – 68kDa), and a light chain specific subunit, xCT (Slc7a11 – 55kDa). The heavy subunit is a single transmembrane glycoprotein with a large N-terminal domain (absent in figure 11). With a documented role in integrin signaling and cell proliferation (Feral et al. 2005; Cantor et al. 2009), it is essentially responsible for the correct trafficking of the heterodimer to the plasma membrane, whereas the light subunit confers transport function and specificity (Sato et al. 1999). In 2021, the cryo-electron microscopy structure of system x_c^- was elucidated, revealing key structural features of the binding site that enables glutamate and cystine recognition (Parker et al. 2021). Moreover, the cysteine-158 has been documented as key actor in the disulfide bond between xCT and the 4F2hc. xCT consists of 12 transmembrane domains with intracellular N and C termini (Figure 11). The re-entrant loop between transmembrane domain 2 and 3 appears to participate in the substrate binding site and/or to be linked to the permeation pathways (Gasol et al. 2004). While 4F2hc is necessary for the membrane

expression, overexpression of xCT alone has been shown to robustly increase system x_c^- activity (Shih et al. 2006; Gochenauer and Robinson 2001). Human xCT consists of 501 amino acids with 89% homology to the mouse one.

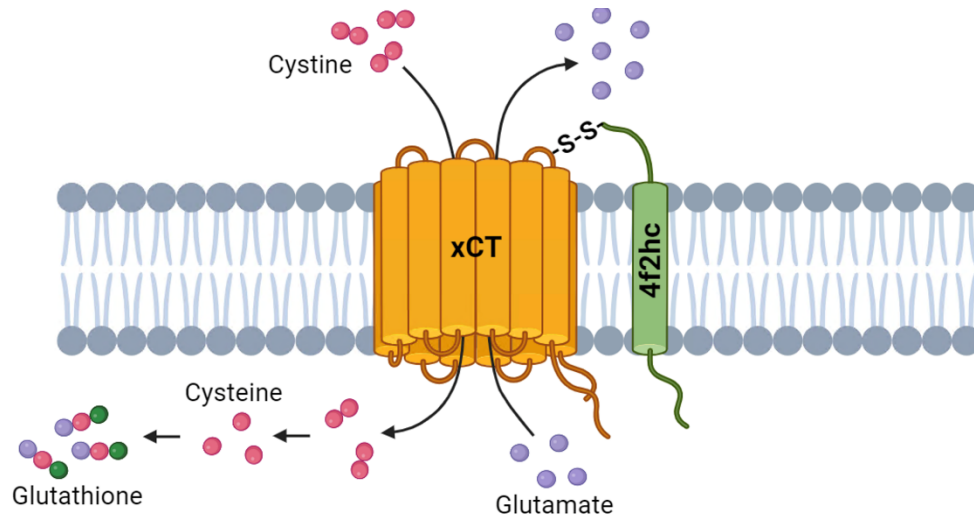


Figure 11: Cystine/glutamate exchanger, system x_c^- . System x_c^- consists of the 4F2 heavy chain (4F2hc) and the light chain, xCT, linked to each other by a disulfide bond (-S-S-). System x_c^- imports cystine in exchange for glutamate. Once in the cell, cystine is rapidly reduced into cysteine, a building block for the synthesis of glutathione (glycine-cysteine-glutamate). Figure created with BioRender®.

System x_c^- transports the anionic forms of L-cystine and L-glutamate. Although it can transport both amino acids bidirectionally, system x_c^- primarily imports cystine while exporting glutamate. This preference arises from the negligibly small intracellular pool of cystine due to its reduction into cysteine, and from the intracellular glutamate concentration which is generally higher than in the extracellular space. This transport mechanism operates with a 1:1 molecular ratio (Bannai and Kitamura 1980).

Initially, system x_c^- has received considerable attention due to its role in cystine uptake, as in many cells, this process operates as a crucial step in providing intracellular cysteine required for glutathione (GSH) synthesis and extracellular cystine/cysteine redox balance. When maintained *in vitro*, most

cell types do not survive in the absence of system x_c^- when the culture medium is not supplemented with reducing agents (Sato et al. 2005). *In vivo*, however, genetic deletion of xCT does not induce increased oxidative stress since other sources of GSH might compensate for the loss of functional system x_c^- (De Bundel et al. 2011). Within the context of the CNS, recent focus has shifted towards the other aspect of the exchange process. Specifically, attention has turned to the release of glutamate. Indeed, this exchange-mediated export of glutamate may have significant implications in modulating excitatory neuronal signaling and, potentially, exacerbating neurological disorders (see section *system x_c^- in diseases of the CNS*).

The xCT protein is constitutively expressed in immune-related organs such as the spleen, thymus, and bone marrow. In the CNS, it has been observed in most rodent brain regions, including cortex, hippocampus, striatum, substantia nigra and cerebellum with highest expression levels in the brain parenchyma, the meninges and the ependyma (Sato et al. 2002; Shih et al. 2006). As reliable tools to evaluate the protein expression of xCT have been missing for a long time, contradictory findings have been published concerning the exact localization of system x_c^- . Indeed, often studies rely on the predicted molecular weight of 55 kDa for identifying xCT on Western blots (Sato et al. 1999). However, a study from Van Liefferinge and colleagues, using brain samples from xCT-deficient (xCT^{-/-}) mice, have demonstrated that xCT migrates as a 35 kDa protein. Consequently, the reliance on predicted molecular weight alone may lead to misinterpretation concerning the true localization of xCT in the brain (Van Liefferinge et al. 2016). Moreover, it must be noted, that the detection of either xCT mRNA or xCT immunoreactivity does not necessary relate to the functionality of system x_c^- at the plasma membrane (Lim et al. 2007).

Concerning the CNS, uncertainty persists as neither the expression levels nor the distribution of xCT have been definitively determined. While xCT has been reported in astrocytes, neurons, oligodendrocytes and microglia, most of this

information derives from studies conducted on cell cultures. Despite the widespread distribution of xCT in the CNS, more recent *in vivo* studies have confirmed that system x_c^- was mainly considered as a glial antiporter. While xCT protein has been documented to be restricted to a subpopulation of astrocytes (Ottestad-Hansen et al. 2018), immunohistochemistry studies on ex vivo samples also revealed a co-localization of xCT and microglial markers (Miladinovic and Singh 2019; Kitagawa et al. 2019). Nowadays, the proportion of system x_c^- expressed in astrocytes and microglia remains a matter of debate.

It is also important to note that system x_c^- , despite its fundamental functions, is expressed at a relatively low basal level in the CNS (Kitagawa et al. 2019). Considering the existence of several regulatory mechanisms influencing the expression of system x_c^- , the inconsistent findings regarding its abundance in certain cell types may also be linked to the study conditions. For instance, microglial cells appear to express very low levels or even lack xCT under physiological conditions, but once activated, xCT is rapidly upregulated (Mesci et al. 2015). Also, it is well established that xCT is heavily induced under regular cell culture conditions (Lewerenz et al. 2013).

2. Functions

Given its widespread distribution throughout the CNS and the modulatory effect of glutamate released by system x_c^- on glutamatergic synaptic neurotransmission, it has been suggested that this antiporter is an important determinant of brain functioning under both physiological and pathological conditions (Massie et al. 2015; Lutgen et al. 2014). Since specific inhibitors of system x_c^- are not yet discovered, most of the information on system x_c^- function and localization has been obtained from studies using a genetically modified model of mice lacking xCT (Sato et al. 2005). At the same time, it was demonstrated that the subtle gray (*sut*) mouse pigmentation mutant originated from a spontaneous mutation in the *Slc7a11* gene. In this mutant

strain of C3H/HeSnJ, the phenotype results from a large 481 kb deletion that includes the last exon of *Slc7a11*, causing a low rate of cystine transport into melanocytes and reduced pheomelanin production (Chintala et al. 2005). Both strains were experimentally used as model of system x_c^- -deficient mouse, which however lead to inconsistent results. For example, $xCT^{-/-}$ mice do not display any phenotype with brain abnormalities, despite an important decrease in the extracellular level of glutamate (De Bundel et al. 2011; Massie et al. 2015), whereas *sut* mice develop brain atrophy at 3 months of age (Shih et al. 2006). These differences between $xCT^{-/-}$ mice and *sut* mice might be due to differences in their background or could be mediated by genetic drift (Massie et al. 2015). Indeed, more recent studies do not relate this difference by using littermate controls (Frare et al. 2023).

Considering its dual role as importer of cystine and exporter of glutamate, the activity of system x_c^- has been implicated in a wide array of CNS functions or in the maintenance of its homeostasis. These include neurotransmitter release, synaptic remodeling, oxidative protection, immune and inflammatory response and chemoresistance. In the context of this thesis, we will specifically focus on the role of system x_c^- in glutamatergic transmission and neuroinflammation, two main processes involved in central sensitization during neuropathic pain (for more information regarding the other functions of system x_c^- , see box 3).

a. System x_c^- participates in the regulation of glutamatergic transmission

Given the presence of system x_c^- in astrocytes and microglia, glutamate released through this exchanger represents an atypical mechanism for neuron-glia communication. As previously mentioned, while glutamate is essential for a multitude of physiological functions, an excessive extracellular concentration can lead to pathological conditions and neuronal death.

BOX 3. Brief, non-exhaustive overview of system x_c^- functions

The most obvious function of system x_c^- is the cellular supply of cystine, which, upon uptake is rapidly reduced into cysteine, a building block for GSH synthesis. GSH is essential in neutralizing free radicals and ROS. Thereby, GSH supports detoxification and prevents oxidative damage, especially in the CNS characterized by high oxygen consumption and limited antioxidant capacity. While mature neurons have minimal system x_c^- activity, they rely on astrocytes for cysteine uptake and GSH production (Shih et al. 2006). It has also been suggested that, in astrocytes, intracellular cysteine might be exported by system ASC, leading to an increase in extracellular cysteine concentration without the intermediate step of GSH synthesis (Bannai and Ishii 1982). xCT is also expressed within the BBB, in pericytes and endothelial cells. In this context, system x_c^- contributes to the regulation of the redox environment by importing cysteine from the bloodstream into the brain parenchyma.

Ferroptosis is a form of regulated cell death characterized by iron-dependent lipid peroxidation implicated in several neurodegenerative diseases. At variance to apoptosis or necrosis, ferroptosis does not involve caspase activation or DNA fragmentation. Instead, ferroptosis is characterized by the accumulation of ROS and lipid peroxides derived from membrane fatty acids. This process leads to membrane damage, disruption of cellular integrity and finally cell death. Dysregulation and inhibition of system x_c^- has been linked to the accumulation of lipid peroxides accompanied by ferroptotic cell death (Dixon et al. 2012).

Besides, system x_c^- has been linked to cancer invasiveness and drug resistance in chemotherapy. Indeed, in a wide variety of cancer, including lymphomas, glioma, and pancreatic cancer, xCT is upregulated. The released glutamate acts autocrinally enhancing migratory and invasive properties of tumor cells (Lyons et al. 2007). Moreover, cancer cells can maintain higher intracellular GSH level, which can neutralize the effects of chemotherapy-induced ROS and prevent apoptosis, allowing cancer cells to survive and proliferate despite treatment (Huang et al. 2005). Inhibiting system x_c^- has been proposed as a valuable approach for cancer therapy either alone or in combination with other anti-tumoral drugs (Sato et al. 2018). Indeed, inhibition of system x_c^- reduces tumor growth, tissue invasion and metastasis (Lewerenz et al. 2013; Rui et al. 2023).

Disruptions in glutamate homeostasis lead to widespread changes in synaptic activity and may be responsible for central sensitization as observed in neuropathic pain. It is therefore crucial to clearly understand the molecular mechanisms regulating the release and the removal of this neurotransmitter from the extracellular space. While the removal of glutamate is mainly driven by glutamate transporters expressed on astrocytes (GLT-1 and GLAST), the release processes are much more complex since both vesicular and non-vesicular mechanisms are involved (Danbolt 2001; Abudara et al. 2018; Massie et al. 2015). System x_c^- represents an important actor in the release process because it is known to participate in 60%-70% of extracellular glutamate concentration in certain brain regions (Massie et al. 2011; Baker et al. 2002; De Bundel et al. 2011). As for vesicular export, system x_c^- -mediated release of glutamate induces changes in iGluRs and mGluRs activation and therefore neuronal activity (Lewerenz et al. 2013).

Other evidence suggest that system x_c^- is an important regulator of glutamate homeostasis. Indeed, Baker, Moran and colleagues demonstrated that the extracellular glutamate released through system x_c^- has an essential role in the regulation of extrasynaptic type 2/3 mGluRs. Thereby, system x_c^- controls the release of neurotransmitter throughout the CNS since activation of mGluR2/3, which decreases cellular cAMP levels, inhibits the release of glutamate and dopamine (Baker et al. 2002; Moran et al. 2005). This is in line with an early report by Gochenauer, showing that prolonged treatment with dibutyryl-cAMP (dBcAMP), a cell permeable cAMP analog, induced system x_c^- expression and activity in astrocytes (Gochenauer and Robinson 2001). This pathway for glutamatergic tone regulation has been proposed to explain pathological conditions such as drug addiction (Moran et al. 2005). System x_c^- is also assumed to modulate synaptic transmission by reinforcing the activation of iGluRs. Given that, beside the synaptic location, NMDAR are also found extrasynaptically, system x_c^- -mediated extrasynaptic glutamate release

can activate mechanisms that regulate neuronal activity. It is worth mentioning that the composition and function of NMDARs differ based on their localization, leading to either beneficial or deleterious effects. Synaptic NMDARs predominantly contain the NR2A subunit, while extrasynaptic NMDARs are enriched with the NR2B subunit (Soria et al. 2014). The activation of NR2B-containing NMDARs has been linked to various neurological disorders, including neurodegenerative diseases and chronic pain (Soria et al. 2014; Qu et al. 2009). Since system x_c^- is mainly responsible for extrasynaptic glutamate release, it is believed to primarily trigger the activation of extrasynaptic NMDARs which are notably implicated in chronic pain conditions (Liang et al. 2021; Qu et al. 2009).

More recently, it has been proposed that this non-vesicular glutamate release could also reduce synaptic strength by influencing both the function (desensitization) and the localization (clustering) of postsynaptic AMPA and NMDA receptors (Augustin et al. 2007; Williams and Featherstone 2014).

Dysregulation of system x_c^- has been implicated in diverse neurological disorders characterized by glutamate excitotoxicity, including ischemic stroke, epilepsy, and neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), MS, Alzheimer's and Parkinson's disease. In these conditions, enhanced function of system x_c^- is thought to elevate extracellular glutamate levels, exacerbating excitatory transmission and possibly neuronal damage and contributing to disease progression (for more details about the role of system x_c^- in CNS disease, see section *system x_c^- in diseases of the CNS*). Nowadays, very little is known regarding the implication of system x_c^- in neuropathic pain. Nevertheless, neuropathic pain shares biochemical features with the above-mentioned diseases, in particular the dysregulated synaptic glutamatergic homeostasis and could therefore involve some modifications in system x_c^- properties (expression and/or activity).

b. System x_c^- participates in immune and inflammatory responses

Expressed both on peripheral (macrophage, lymphocytes) and central (microglia, astrocytes) immune cells, system x_c^- has emerged as a significant player in both peripheral and central inflammation. System x_c^- is also expressed in the spleen, where the activation of B lymphocyte takes place, and the thymus, the organ of the selection and maturation of T lymphocytes, suggesting that system x_c^- may modulate both innate and adaptative immune response (Taguchi et al. 2007).

In models of peripheral inflammation, xCT expression was found to be increased in macrophages, both *in vitro* and *in vivo* (Mesci et al. 2015; Fogal et al. 2007; Nabeyama et al. 2010). Upon direct activation by LPS or the pro-inflammatory cytokine TNF- α , macrophages rapidly upregulate system x_c^- (Sato et al. 1995). In line with these observations, it was shown that cultured macrophages from xCT-deficient mice die following activation with LPS (Nabeyama et al. 2010). Indeed, throughout the progression and resolution of inflammation, immune cells are exposed to numerous cytotoxic compounds, including ROS. Consequently, macrophages require protective mechanisms against oxidative stress to warrant their survival at the inflammatory site. While some papers reported that lymphocytes are the only cell type in which system x_c^- expression cannot be induced (Lewerenz et al. 2013), Castellani and colleagues showed that T-lymphocytes do not express xCT in resting condition but can rapidly induce it during inflammation (Castellani et al. 2008). The mechanism responsible for this upregulation is not yet fully elucidated but it is reasonably proposed that by increasing GSH levels, system x_c^- supports immune cell survival, activation, proliferation and facilitates the secretion of cytokines (Arensman et al. 2019).

Behaving as the resident macrophages of the CNS, microglia also demonstrate an induced glutamate release via system x_c^- following both LPS and TNF- α treatment *in vitro*. Using primary microglial culture derived from

xCT^{-/-} mouse pups, it has been confirmed that system x_c⁻ was indeed the major source of microglial glutamate (Mesci et al. 2015). Upregulation of system x_c⁻ upon activation of toll-like receptor 3 and 4 was recognized as a key mechanism involved in the enhanced release of glutamate in response to inflammatory signals. Such increase in xCT mRNA level in response to neuroinflammation was validated both *in vitro*, in mixed microglial/astrocytic cultures exposed to LPS, and *in vivo*, in a model of intraspinal injection of LPS. Interestingly, co-injection of LPS with cystine exacerbated neurotoxicity confirming the role of system x_c⁻ in neuroinflammation (Kigerl et al. 2012). Besides, the team of Ann Massie has shown that mice lacking xCT exhibited a blunted systemic inflammatory response to a sublethal dose of LPS, thereby preventing LPS-induced central neuroinflammation and depressive-like behavior to occur (Albertini et al. 2018). Later, they also documented that xCT^{-/-} mice were protected against the consequences of the typical age-related chronic inflammation (Verbruggen et al. 2022).

Activated microglia produce and release a variety of bioactive chemicals, including pro- and anti-inflammatory cytokines. Among them, IL-1 β is known to specifically increase the activity of system x_c⁻ in astrocytes but not in neurons and microglia (Jackman et al. 2010). Interestingly, a deficiency in xCT resulted in reduced release of TNF- α and, to some extent, IL-1 β in microglia both *in vitro* and *in vivo* (Mesci et al. 2015). This suggests the existence of a positive feedback loop where cytokine-induced xCT expression contributes to the release of pro-inflammatory cytokines. Supporting the same idea, Mesci and colleagues identified that the absence of system x_c⁻ result in a reduced production of specific microglial pro-inflammatory, neurotoxic factors including NO, TNF- α and IL-6 (typically associated with the M1 phenotype), whereas expression of anti-inflammatory, neuroprotective (associated with the M2 phenotype) markers was increased (Mesci et al. 2015).

While the positive outcomes of inhibiting system x_c⁻ are now well-established in neurological disorders involving neuroinflammation, the exact molecular

mechanisms underlying the associated benefits remain unclear. However, it has been demonstrated that, in a mouse model of MS, deleting system x_c^- in immune cells only is sufficient to observe beneficial effects (Merckx et al. 2017).

3. Regulation of xCT expression and system x_c^- activity

Since its discovery, system x_c^- has been considered as a strongly inducible amino acid transport system. Undoubtedly, stimuli that lead to a robust induction of system x_c^- activity include ROS, but also bacterial LPS and inflammatory cytokines. Even conventional cell culture conditions induce system x_c^- activity, probably because the ambient oxygen pressure used to maintain cell cultures is much higher than found in living tissues. Despite this, cell cultures have been successfully employed to elucidate the pathways that regulate xCT expression and system x_c^- activity. Experimental evidence strongly indicates that the transcriptional regulation of xCT expression is much more critical than the expression of the heavy chain 4F2hc, in determining system x_c^- activity (Verrey et al. 2004; Gochenauer and Robinson 2001). The two main transcription factors that have been identified as key regulator of xCT expression are nuclear factor erythroid 2-related factor 2 (Nrf2) and activating transcription factor 4 (ATF4) (Lewerenz et al. 2013), which are discussed below (Figure 12). Even if these two transcription regulators can explain the regulation of xCT in several models, some other pathways remain to be clarified. Regarding the translational regulation, limited information is presently available.

a. Transcriptional regulation of xCT

It has been documented that under various stress conditions, including oxidative stress and amino acid deficiency, xCT mRNA can be significantly upregulated. This increased level is beneficial for cells to restore their redox homeostasis, allowing them to survive and adapt to stressful environment.

ATF4 belongs to the ATF/cAMP responsive element-binding protein (ATF/CREB) transcription factor family and governs redox homeostasis and amino acid metabolism. In amino acid shortage, cells can transfer ATF4 into the nucleus via the general control nonderepressible 2 (GCN2)/eukaryotic initiation factor 2 α (eIF2 α)/ATF4 signaling axis. There, it binds to the amino acid response element in the gene promoters, thereby boosting the transcription of genes implicated in amino acid metabolism and stress response, including Slc7a11 (Lewerenz and Maher 2009; Bridges et al. 2012; Massie et al. 2015; Koppula et al. 2018).

Lewerenz and co-workers also demonstrated that, *in vitro*, during robust neuronal activity, xCT expression and system x_c⁻ activity are upregulated by growth factor signaling. This induction is mediated by glycogen synthase kinase 3 β , GCN2 and subsequently ATF4 (Lewerenz et al. 2014).

Nrf2 predominantly orchestrates the antioxidant response. Typically unstable under normal physiological conditions, Nrf2 is ubiquitinated by Kelch-like ECH-associated protein 1 (Keap1) and rapidly degraded via the proteasome pathway. However, oxidative stress blocks the Keap1-mediated Nrf2 degradation pathway, stabilizing Nrf2 and enabling its binding to the antioxidant response element. This initiates the transcription of various antioxidant defense genes, including Slc7a11 (Lewerenz and Maher 2009; Bridges et al. 2012; Massie et al. 2015; Koppula et al. 2018). There is evidence that the neuroprotective β -lactam antibiotic ceftriaxone induces xCT expression via Nrf2 in astrocytes and motor neurons (Lewerenz et al. 2009). As reported by Ye and colleagues, ATF4 and Nrf2 can interact and exhibit a synergistic effect at the Slc7a11 promoter, collectively upregulating its transcription under diverse metabolic stress conditions (Ye et al. 2014).

As previously mentioned, inflammatory stimuli have been reported to participate in xCT regulation. The specific pathway through which inflammatory mediators upregulates xCT protein remains, for some of them, to be uncovered. While, it has been indicated that LPS binds to toll-like

receptor 4 and acts independently of NF- κ B and Nrf2 (Ishii et al. 2000; Sato et al. 2001), IL-1 β -mediated xCT upregulation was attributed to the activation of NF- κ B (He et al. 2015). Moreover, IL-1 β -treated mixed cortical cell cultures containing neurons and astrocytes revealed a significant increase in the velocity (V_{max}) of cystine uptake by system x $_c^-$ without changing its affinity (K_m) (Fogal et al. 2007). Whether this increased activity is due to the upregulation of its expression or is the result of a post-translational modification that alters transporter kinetics remains to be determined.

Transcription factors can also exert a negative effect on Slc7a11 expression, with prominent actors being protein p53 and activating transcription factor 3 (ATF3) (Tu et al. 2021; Tang et al. 2022). p53, a tumor suppressor and transcription factor, has been shown to promote ferroptosis, a programmed cell death dependent on iron and characterized by the accumulation of lipid peroxides, by inhibiting Slc7a11 expression (see box 2). Conversely, p53 deficiency leads to Slc7a11 upregulation, boosting tumor cell resistance to ferroptosis. p53 inhibits Slc7a11 expression by impairing Nrf2 function. Besides, p53 enhances the translocation of the deubiquitinase ubiquitin special peptidase 7 (USP7) into the nucleus, which results in the inhibition of the histone H2B monoubiquitination (H2Bub1). Deubiquitinated H2Bub1 can bind to the gene regulatory region of Slc7a11 and represses its expression (Wang et al. 2019). Under physiological condition, ATF3, another transcription factor, forms dimers and binds to the Slc7a11 promoter retaining Slc7a11 transcription. However, under stress conditions that typically induce the expression of xCT, ATF3 does not inhibit the expression and activity of Slc7a11 (Li, Yan, et al. 2022).

Overall, even if all the underlying mechanisms are not yet fully elucidated, diverse stress conditions partially promote Slc7a11 transcription through ATF4 and/or Nrf2, while p53 and ATF3 predominantly inhibit Slc7a11 expression. These transcription factors modulate downstream biological

activities, including cysteine metabolism, by regulating Slc7a11 expression and transcriptional activity, thereby altering cell sensitivity to ferroptosis.

While most current studies have focused on transcriptional regulation of Slc7a11, its mRNA level is also regulated by post-transcriptional mechanisms. This includes regulation by microRNA (miR) such as miR-139-5p, miR-27a, miR-26b and miR-375. These microRNAs directly target Slc7a11 and alter Slc7a11 mRNA stability (Liu et al. 2011; Wu et al. 2017; Drayton et al. 2014; Zhu, De Mello, et al. 2020).

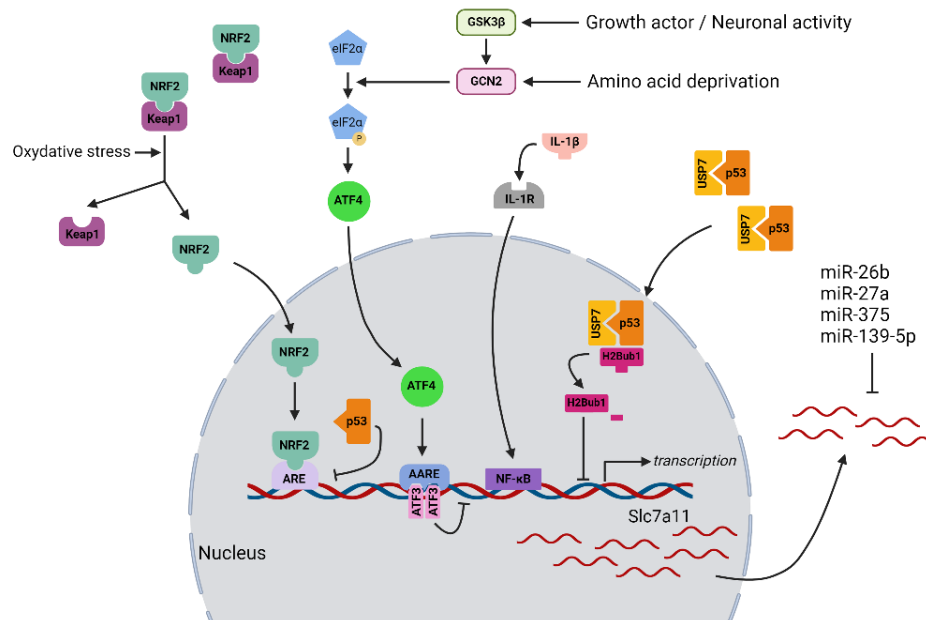


Figure 12 : Transcriptional regulation of Slc7a11, the gene coding for xCT. Under various stress conditions like oxidative stress and amino acid deficiency, xCT mRNA is upregulated, aiding cells in restoring redox homeostasis for survival. ATF4 regulates redox balance and amino acid metabolism by translocation to the nucleus via the GCN2/eIF2α/ATF4 signaling axis, enhancing the transcription of Slc7a11. Nrf2, a key regulator of antioxidant response, promotes the transcription of antioxidant genes including Slc7a11. Under inflammatory condition, IL-1β upregulates xCT through NF-κB. On the contrary, p53 and ATF3 negatively regulate Slc7a11. p53 exerts its negative effect by interfering with Nrf2-mediated transcription

and by enhancing the nuclear translocation of USP7, inhibiting histone H2B monoubiquitination. ATF3 mainly represses Slc7a11 expression through its binding on AARE. *Slc7a11*: solute carrier family 7 member 11; *Keap1*: Kelch-like ECH-associated protein 1; *NRF2*: nuclear factor erythroid 2-related factor 2; *ARE*: antioxidant response element; *GSK3 β* : glycogen synthase-3 β ; *GCN2*: general control nonderepressible 2; *eIF2 α* : eukaryotic initiation factor 2 α ; *ATF4*: activating transcription factor 4; *AARE*: amino acid response element; *ATF3*: activating transcription factor 3; *NF- κ B*: Nuclear factor κ B; *IL-1 β* : interleukin 1 β ; *IL-1R*: interleukin 1 receptor; *USP7*: ubiquitin special peptidase 7; *H2Bub1*: histone H2B on lysine 120; *miR*: microRNA. Figure created with BioRender®.

b. Translational regulation of xCT

While knowledge on transcriptional and post-transcriptional regulation of Slc7a11 increases, post-translational modification remains largely unknown.

Emerging evidence indicates that Slc7a11 is also regulated by interactions with other proteins or post-translational modifications. As previously mentioned, 4F2hc, which acts as the principal partner of xCT, is necessary to maintain xCT protein stability into the plasma membrane. Another adhesion molecule CD44 variant (CD44v) interacts with Slc7a11, enhancing the stability of the gene (Ishimoto et al. 2011). Depletion of CD44v function results in a decreased stability and altered cell surface localization of Slc7a11. CD44v is documented to regulate Slc7a11 through otubain-1 (OTUB1), a member of the ovarian tumor family acting as a deubiquinase. Indeed, OTUB1 stabilizes Slc7a11 by preventing it from undergoing ubiquitination and subsequent degradation through the proteasome pathway (Liu et al. 2019). Actually, OTUB1, CD44v and Slc7a11 form a complex in which CD44v enhances the stability of Slc7a11 by facilitating the interaction between OTUB1 and Slc7a11. Consequently, targeting CD44v or OTUB1 destabilizes Slc7a11, making cells more vulnerable to oxidative stress and ferroptosis (Liu et al. 2019). Another study showed that inhibition of CD133, decreases Slc7a11

protein stability. However, the exact mechanism has not been established (Song et al. 2019).

Moreover, mammalian target of rapamycin complex 2 (mTORC2) is a multiprotein complex with serine/threonine kinase activity that was identified as binding protein of Slc7a11. Further analyses revealed that, in response to growth factor stimulation, serine residue 26 located in the N-terminal tail of Slc7a11 is phosphorylated by mTORC2. This phosphorylation inactivates the transport activity of system x_c^- through AKT-dependent pathways (Gu et al. 2017; Koppula et al. 2018). Slc7a11 can be regulated not only by mTORC2, but also by mammalian target of rapamycin complex 1 (mTORC1). As a transmembrane protein localized in the plasma membrane, Slc7a11 is also subjected to regulation by lysosomal degradation. Gan and colleagues identified mTORC1 inactivation as an inducer of Slc7a11 lysosomal degradation (Gan et al. 2020). Suppressor of cytokine signaling 2 (SOCS2) also promotes degradation since it serves as a bridge to transfer ubiquitin to Slc7a11.

To date, apart from the above-mentioned pathways, the underlying mechanisms of Slc7a11 function and stability remain largely unclear.

4. System x_c^- in diseases of the CNS

Inflammation, oxidative stress, mitochondrial dysfunction, and glutamate excitotoxicity play crucial roles in the development of numerous neurological disorders. Extensive evidence suggests that these processes are interconnected rather than occurring independently. Two significant contributors to these pathological conditions are GSH and glutamate. Since system x_c^- exerts a dual function, which involves supporting GSH synthesis and releasing glutamate, it has been suggested that this exchanger can either protect against or contribute to diverse neurological disorders. Indeed, a neuroprotective effect is often attributed to the uptake of cystine and the subsequent increased in GSH synthesis. In contrast, excitotoxicity may arise

from glutamate release through system x_c^- . Therefore, its expression and function in various *in vitro* and *in vivo* models relevant to CNS diseases have extensively been investigated in the last decades as outlined below.

a. Alzheimer's disease

Alzheimer's disease (AD) is characterized by the accumulation of amyloid- β plaques and neurofibrillary tangles of phosphorylated Tau in the brain, leading to neuronal dysfunction and eventual cell death. Oxidative stress and inflammation contribute to disease progression, exacerbating neuronal damage. Disruption of synaptic function and neurotransmitter imbalance, particularly involving glutamate, impair cognitive function. Genetic factors, such as mutations in the amyloid precursor protein (APP) and presenilin genes, increase susceptibility to AD. Neurodegeneration in AD primarily affects regions involved in memory and cognitive function (including hippocampus and cerebral cortex), leading to progressive cognitive decline and memory loss. Increasing evidence suggests an important role of microglial cells, as key players in plaque removal as well as in the production of neuroprotective and neurotoxic factors including cytokines, chemokines and ROS (Hansen et al. 2018; Miao et al. 2023; Cai et al. 2022).

The first study evoking a potential contribution of system x_c^- in AD was performed in 2001 by Barger and Basile. They showed that microglial cultures activated by the secreted form of the β -amyloid precursor protein (sAPP) dramatically increased extracellular glutamate level leading to excitotoxicity and subsequent neuronal death. This APP-mediated glutamate release was completely blocked by the inhibition of system x_c^- (Barger and Basile 2001). Similar results were observed in $A\beta_{1-40}$ -treated microglia since the toxicity of $A\beta$ is enhanced by microglial-mediated glutamate release through system x_c^- (Qin et al. 2006). In addition to APP and $A\beta$, several inflammatory mediators that are associated with AD (e.g. TNF- α and ROS) can also enhance the

expression and activity of system x_c^- in macrophages and microglia (Decourt et al. 2017; Manoharan et al. 2016).

In vivo studies also demonstrated that in the cortex of aged APP23 mice (a mouse model of AD), xCT overexpression is accompanied by a reduced glutamate uptake and increased extracellular glutamate concentration (Schallier et al. 2011). Moreover, in other mouse models of AD (hippocampal A β injection and Thy1-APP₇₅₁ mice), reactive microglia within or surrounding the amyloid plaques have been shown to prominently express xCT (Qin et al. 2006).

In AD patients, xCT mRNA level was also higher in white blood cells as compared to healthy controls, suggesting that xCT mRNA could be an interesting biomarker in identifying AD patients (Lane and Lin 2023). However, magnetic resonance imaging data of aged patients with cognitive impairment have shown iron deposition (increased free intracellular Fe²⁺) in the hippocampus, cortex, and basal ganglia (Ghadery et al. 2015) suggesting that ferroptosis could participate in neuronal death.

Based on these results, targeting system x_c^- may represent a new potential therapeutic strategy for the treatment of AD patients. However, oxidative stress is also an important feature of AD pathology, characterized by an imbalance between ROS production and antioxidant defense mechanisms. By participating in the production of GSH, system x_c^- highly contributes to cellular antioxidant defenses. Dysfunction or inhibition of this exchanger may impair GSH synthesis, exacerbating oxidative stress and neuronal damage in AD. Nevertheless, altogether, these data strongly suggest that system x_c^- dysregulation is important in the pathophysiology of AD. However, more studies are needed to fully define the exact role of this exchanger in AD since it could have beneficial and detrimental effects.

b. Parkinson's disease

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic neurons in the substantia nigra, resulting in motor symptoms such as tremor, bradykinesia, and rigidity. Oxidative stress and mitochondrial dysfunction are crucial in PD pathogenesis, leading to neuronal damage and cell death. Notably, these biochemical alterations leading to nigrostriatal degeneration may partly be attributed to reduced GSH levels (Penugonda et al. 2005). Inflammation and dysregulation of the immune system also contribute to PD progression. While the exact cause of PD remains elusive, genetic factors, environmental toxins, and aging are believed to contribute to the disease development.

In 2008, Massie and colleagues provided the first evidence for an involvement of system x_c^- by showing increased expression of xCT in the striatum of rats following a unilateral 6-hydroxydopamine (6-OHDA) lesion of the nigrostriatal pathway (Massie et al. 2008). It has also been demonstrated that an increase in extracellular glutamate concentration could lead to GSH depletion, thereby potentially contributing to mitochondrial dysfunction and oxidative stress (Penugonda et al. 2005). Besides, it has been established that both GSH and N-acetylcysteine play a neuroprotective role, with N-acetylcysteine demonstrating efficacy in mitigating mitochondrial damage and scavenging ROS (Penugonda et al. 2005; Banaclocha 2001).

Interestingly, a few years later, Massie and co-workers demonstrated that mice lacking xCT did not develop increased oxidative stress in the striatum and substantia nigra and were unexpectedly resistant to 6-OHDA-induced nigral cell loss. Together, these data suggested a toxic contribution of increased system x_c^- activity in PD that could rely on the enhanced release of glutamate (Massie et al. 2011). Consistently, using a metamphetamine-induced dopaminergic neurotoxicity model, Dang and colleagues showed that pharmacological and genetic inhibition of system x_c^- significantly protected mice against oxidative burdens and dopaminergic loss observed in

the control/wild-type group (Dang et al. 2017). However, these findings did not extend to another PD model based on the administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a mitochondrial toxin (Bentea, Sconce, et al. 2015; Bentea et al. 2021). $xCT^{-/-}$ mice were reported to be equally susceptible to MPTP-induced pathological and behavioral changes as their wild-type counterparts (Bentea, Sconce, et al. 2015). Moreover, increased expression of xCT in the striatum but downregulation in the substantia nigra of MPTP-treated mice were observed, highlighting regional differences that may contribute to the disparate results observed between PD models (Bentea, Sconce, et al. 2015). Nevertheless, these studies called for the development of system x_c^- inhibitor as drug candidate for the treatment of PD.

c. Amyotrophic lateral sclerosis

ALS is characterized by the progressive degeneration of motor neurons, leading to muscle weakness, spasticity, and paralysis. While mainly sporadic, 10% of ALS cases are considered familial cases, of which 20% involve a gain-of-function mutation in the gene encoding Cu/Zn superoxide dismutase (SOD1) (Rosen et al. 1993). Excitotoxicity and neuroinflammation are demonstrated to contribute to the pathophysiology of ALS. Additionally, motor neuron death involves a non-cell autonomous mechanism, with neighboring non-neural cells participating in the disease (Boillée et al. 2006). While some studies have shown that reducing SOD1 mutant expression specifically in microglial cells strongly slows down the disease (Boillée et al. 2006), others have indicated a particular implication of astrocytes in the degeneration of spinal motor neurons (Nagai et al. 2007). Taken together, the combined altered level of GSH, changes in glutamatergic signaling and involvement of glial cells, support the possible implication of system x_c^- in the pathophysiology of ALS.

The first observation highlighting a potential involvement of system x_c^- in ALS pathophysiology arose from the study of Nrf2 in astrocytes. The activation of this transcription factor involved in the regulation of xCT was proven beneficial in an ALS mouse model. The authors proposed that neuronal protection depended on an increased GSH production in astrocytes (Vargas et al. 2008). A few years later, increased expression of xCT was shown to correlate with disease progression in rodent models (Mesci et al. 2015). This upregulation which was mainly observed in microglia and astrocytes but not in motor neurons was also evidenced in post-mortem tissues of patients, associated with increased inflammation (Mesci et al. 2015; Kazama et al. 2020). Based on previous findings suggesting a protective role of system x_c^- via GSH production, deleting xCT in ALS mice could lead to increased disease progression. Surprisingly, xCT^{-/-} mice exhibited earlier symptoms followed by slower disease progression, resulting in more surviving motor neurons (Mesci et al. 2015). Deletion of xCT also reduced the pro-inflammatory phenotype of activated microglia and increased expression of M2 markers, suggesting a switch toward a neuroprotective microglial phenotype (Mesci et al. 2015). M1/M2 profiles have previously been suggested to influence disease progression with the M1 phenotype linked to rapid disease evolution and the M2 phenotype associated with a slow disease advancement (Liao et al. 2012). Therefore, this effect could strongly reduce the deleterious effect of inflammation in ALS, suggesting an important contribution of system x_c^- in the pathophysiology of ALS through both glutamate excitotoxicity and microglia-associated inflammation.

There is a high medical need for ALS treatment as there are currently no effective medications, with treatments mainly focusing on symptom management and improving quality of life. Based on the above-mentioned results, system x_c^- inactivation could be a potential approach to slow down ALS disease progression once clinical symptoms have appeared.

d. Multiple sclerosis

MS is a chronic autoimmune CNS disorder characterized by inflammation, demyelination, and neurodegeneration. It is mediated by immune cells attacking the myelin sheath of nerve fibers, leading to impaired nerve transmission. Clinical manifestations vary widely and may include sensory disturbances, motor deficits, visual impairment, and cognitive dysfunction. MS can have periods of relapse and remission, and its exact cause remains unknown. However, it is well established that alterations in glutamate homeostasis contribute to oligodendrocyte death and neurological deficits.

Immunizing rodents with self-antigens derived from myelin proteins is the most extensively used animal models to study MS pathogenesis. Animals with such experimental autoimmune encephalomyelitis (EAE) show inflammatory demyelination as observed in patients (Rivers et al. 1933).

Although an imbalance in glutamatergic homeostasis has long been recognized as a major contributor in the development of MS, the molecular source of glutamate has not been fully defined. A study by Pampliega and colleagues revealed an increased level of xCT mRNA and protein in microglia, meningeal cells, and dendritic cells within the spinal cord and the brain of mice and rats with EAE (Pampliega et al. 2011; Merckx et al. 2017). Importantly, alterations in xCT expression were also observed in postmortem tissue and peripheral blood cells of MS patients (Pampliega et al. 2011; Merckx et al. 2017). While xCT^{-/-} mice showed similar susceptibility to EAE as compared to wild-type, mice lacking xCT only in their immune cells displayed reduced vulnerability to EAE (Merckx et al. 2017). Administering the non-specific system x_c⁻ inhibitor sulfasalazine (SAS) was found to reduce the clinical severity of EAE, in mice, an effect that was correlated to diminished T cell infiltration, reactive gliosis, and myelin damage (Evonuk et al. 2015). Altogether, these results indicate that immune-induced upregulation of system x_c⁻ could lead to heightened glutamate release, contributing to excitotoxic damage to oligodendrocytes (Merckx et al. 2017).

In vitro studies using co-cultures of oligodendrocytes and microglia, pointed out that upon activation, microglia show increased extracellular glutamate release, which results in oligodendrocyte excitotoxicity that is prevented by system x_c^- inhibition (Domercq et al. 2007).

There are still inconsistencies in the exact contribution of system x_c^- in the pathophysiology of MS since other studies revealed a decreased level of xCT in the spinal cord of EAE mice leading to lipid peroxidation, oligodendrocytes death and demyelination (Li, Chu, et al. 2022; Jhelum et al. 2023). Further research is thus necessary to know whether system x_c^- constitutes an interesting strategy in the treatment of MS.

e. Glioblastoma

Glioblastoma or glioma is a type of aggressive brain tumor that arises from glial cells. It is the most common and deadliest form of malignant primary brain tumors in adults. Gliomas grow rapidly and infiltrate into surrounding brain tissue, making complete surgical resection difficult. These tumors are characterized by their highly invasive nature, tendency to recur after treatment, and resistance to standard therapies such as chemotherapy and radiation.

The proliferation of gliomas is facilitated by their ability to produce and release toxic levels of glutamate. This enables the tumors to overcome physical growth restrictions by damaging neighboring tissues (de Groot and Sontheimer 2011). Significant increases in the peritumoral glutamate were indeed previously reported by microdialysis studies performed in patients with glioma (Roslin et al. 2003). It is now well established that gliomas achieve excitotoxic levels of glutamate through elevated activity of system x_c^- coupled with a relative deficiency in EAATs (Sontheimer 2008; Chung et al. 2005; Ye and Sontheimer 1999). It is noteworthy that in addition to triggering excitotoxicity in surrounding tissues, the glutamate released by system x_c^- also seems to have an autocrine effect on the tumor itself that enhances

migration and invasion (Lyons et al. 2007). These findings are consistent with clinical observations as high xCT expression observed in human glioblastoma was correlated with tumor invasiveness (Takeuchi et al. 2013). Additionally, the heightened activity of system x_c^- in gliomas also grants resistance to anti-cancer drugs or radiation therapy by maintaining high levels of GSH in tumor cells. It has even been suggested that the release of glutamate is the consequence of the import of cystine necessary for the synthesis of GSH needed for the tumor growth. Indeed, system x_c^- represents the only efficient pathway supporting cystine uptake in glioma cells (Chung et al. 2005). It was also demonstrated that pharmacological inhibition of system x_c^- by high-dose of SAS (daily i.p. injection of 16 mg/mouse) significantly suppressed the growth of glioma cells implanted in mouse brains (Chung et al. 2005). Moreover, SAS reduced peritumoral epileptic activity induced by glutamate released through system x_c^- from implanted human glioma cells in mice (Buckingham et al. 2011).

Temozolomide stands as the gold standard chemotherapeutic agent in treating glioblastoma. *In vitro* studies revealed that exposure to temozolomide prompts heightened levels of GSH in glioblastoma cell lines, likely through the upregulation of system x_c^- activity (Chen et al. 2015). Pharmacological inhibition of system x_c^- by erastin prominently exacerbated the sensitivity of tumor cell lines to temozolomide, indicating that the upregulation of xCT may contribute to the acquired resistance to temozolomide in glioma. Unfortunately, clinical trials using combination of temozolomide, radiotherapy and SAS fail to increase efficacy of antitumor therapy and was poorly tolerated (Takeuchi et al. 2014; Robe et al. 2009).

f. Epilepsy

Epilepsy stands as a neurological disorder characterized by the occurrence of seizures, stemming from perturbations in the excitatory and inhibitory balance. In most cases, epilepsy arises from developmental causes, genetic

predispositions or develops after an initial insult, such as a brain injury, brain tumor or stroke. Subsequently, synaptic maladaptation occurs, reducing the threshold for neuronal activation. This heightened excitability can lead to uncontrolled neuronal firing with local synchronization causing the manifestation of seizures within the epileptic brain. Advancements in the research field of epilepsy highlighted the importance of astrocyte-mediated glutamate release in the modulation of synaptic transmission and seizure threshold (Coulter and Steinhäuser 2015). It is indeed noteworthy that several antiepileptic drugs aim at decreasing glutamate transmission.

With regard to system x_c^- , its involvement in seizure development during epilepsy appears more straightforward as compared to other CNS disorders. In a mouse model of generalized seizures (EL strain), increased expression of xCT was documented, stressing on its potential role in seizure susceptibility (Takaki et al. 2008). Additionally, in patients with temporal lobe epilepsy, there was an increase in hippocampal xCT expression linked to the activation of the PI3K/GSK-3 β /eIF2 α /ATF4/xCT pathway explained earlier (Lewerenz et al. 2014). Further insights were gained from xCT^{-/-} mice, which exhibited reduced susceptibility to seizures (Leclercq et al. 2019). It was attributed to reduced extracellular glutamate levels (De Bundel et al. 2011) which could be due to a reduced activation of astrocytes and microglial cells. These observations are in line with the above-mentioned contribution of xCT to tumor-induced epileptic seizures.

Intriguingly, the link between increased xCT expression and lowered seizure threshold aligns with the pro-convulsive properties attributed to N-acetylcysteine (De Bundel et al. 2011). Conversely, repeated administration of SAS conferred protection against chemically-induced seizures in epileptic mice as compared to controls (Leclercq et al. 2019).

In summary, it is clear from most of the studies mentioned above that in most animal models of several neurological disorders, xCT expression is upregulated. It may seem intuitive that enhancing system x_c^- activity would

be beneficial for protecting the CNS against oxidative stress commonly associated with those diseases. However, upregulation of xCT might also contribute to injury or disease progression as documented before. Most likely, the enhanced release of glutamate via system x_c^- overrides any potential protective effects, as it may lead to excitotoxicity through the activation of iGluRs. These effects might even be amplified in several diseases of the CNS as downregulation of EAATs is commonly observed.

Hence, employing pharmacological inhibitors targeting system x_c^- could present an intriguing avenue for treating CNS pathologies. However, it is crucial to consider the potential adverse effects associated with reducing the GSH pool. This effect might have been masked in animal models with constitutive suppression of xCT as compensatory mechanisms could develop to counter oxidative stress.

5. Pharmacological inhibitors

As illustrated before, over the past years, research has highlighted the importance of system x_c^- in a wide range of CNS pathologies, including neurodegenerative disorders and brain tumors. Its involvement in modulating oxidative stress, inflammation, and excitotoxicity makes it a promising therapeutic target for treating these conditions. In this context, it is not surprising that progress has been made in the research and development of system x_c^- inhibitors.

While analogs of the natural substrates of system x_c^- were used for their inhibitory potential, in the scope of this thesis, we will mainly focus on describing the most used inhibitors of system x_c^- known to date, highlighting their mode of action, experimental activity, and therapeutic potential. Other compounds discovered as system x_c^- inhibitors are illustrated in figure 13.

a. Sulfasalazine

SAS is an EMA- and FDA-approved drug initially prescribed for inflammatory bowel diseases like ulcerative colitis and Crohn's disease (Gout et al. 2001; Patel et al. 2019). Effectiveness in these disorders arises from its breakdown by gut microbiota azoreductases, generating sulfapyridine and 5-aminosalicylic acid. These metabolites possess anti-inflammatory properties by inhibiting NF- κ B activation (Wahl et al. 1998). Additional research has indicated that while SAS was effective in inhibiting system x_c^- ($IC_{50}=30\mu M$), its metabolites show very limited inhibitory activity on system x_c^- ($IC_{50} > 1$ mM) (Gout et al. 2001; Patel et al. 2015; Chung et al. 2005). Despite being considered as a non-transportable inhibitor interacting with the lipophilic domains adjacent to the substrate site, the precise mechanisms responsible for its inhibitory property remain unclear (Sontheimer and Bridges 2012).

With a moderate potency, but an apparent lack of significant adverse effects, this molecule has initially been seen as a promising drug candidate (Kusuhara et al. 2012; Rains et al. 1995). However, the use of SAS is limited by its poor systemic bioavailability after oral administration and rapid degradation by colonic gut microbiota into inactive constituents (Patel et al. 2019). Additionally, its BBB-permeability is not fully elucidated and considering its hydrophilic and acidic properties (Figure 13), one would predict a limited penetration in the CNS. Despite these challenges, SAS represents a potential lead compound for structural optimization to improve its pharmacokinetic properties. Indeed, studies have demonstrated beneficial effects in neurological disorders affecting both the brain and the spinal cord, such as reduced microglial activation and tumor growth inhibition in mouse models of glioblastoma (Chung et al. 2005; Buckingham et al. 2011; Miladinovic and Singh 2019). Nonetheless, we cannot exclude the possibility that in such pathologies, BBB integrity may be compromised, potentially facilitating the passage of molecules, including SAS (Wolburg et al. 2012; Archie et al. 2021). However, Lutgen and co-workers found that SAS produced a dose-dependent

reduction in extracellular glutamate in the brain of healthy rats (Lutgen et al. 2014). Therefore, another possible explanation for the passage of SAS into the CNS is its transportation via the organic anion transporting peptides 2b1 (Oatp2b1) transporter present in the BBB (Kusuhara et al. 2012).

Despite its off-target effects and poor pharmacokinetic profile, SAS remains the most commonly used molecule to inhibit system x_c^- and evaluate its potential as a drug target in both preclinical and clinical studies. Additionally, given its FDA- and EMA-approved status, a rapid transition to clinical setting can be envisaged. Considering the abundant research on system x_c^- in cancer, SAS was largely tested in models of tumors, but promising results also emerged from models of CNS diseases. Among them, it has been shown that SAS administration could prevent seizures in different preclinical models of epilepsy (Leclercq et al. 2019; Alcoreza et al. 2022) and attenuate autoimmune demyelination in a mouse model of MS (Evonuk et al. 2015). SAS has also been shown to reduce brain tumor growth both *in vitro* and *in vivo* and so decrease tumor-associated seizures in animal models of glioblastoma (Chung et al. 2005; Gout et al. 2001; Guo et al. 2011; Hu et al. 2020; Buckingham et al. 2011). It has been demonstrated that the anti-tumoral effect observed was entirely attributable to system x_c^- inhibition, rather than its reported anti-inflammatory and apoptotic influence through NF- κ B inhibition (Chung et al. 2005). However, clinical trial using SAS in combination with other chemo- and radiotherapy did not result in increased antitumoral activity and was poorly tolerated (Takeuchi et al. 2014). Another clinical study using SAS monotherapy had to be interrupted prematurely as severe effects were encountered (Robe et al. 2009). Similar observation has been documented for trials in MS in which SAS failed to slow progression of the disease as compared to placebo (Noseworthy et al. 1998). Additional studies are therefore needed to understand why the transition to clinical phases does not yield equally the promising results observed on preclinical studies. This could also pave the way for exploring new SAS derivatives with a more favorable pharmacokinetic profile, thereby resulting in improved outcomes.

b. Erastin

More recently, erastin was identified in a high-throughput screening of synthetic lethal compounds targeting engineered tumorigenic cells (Dolma et al. 2003). Hence, the term erastin refers to “eradicator of RAS and ST-expressing cells”. Subsequently, Dixon and colleagues demonstrated that erastin prevents Na⁺-independent cystine uptake through the inhibition of system x_c⁻ function (Dixon et al. 2012). They further discovered that erastin-induced cell death was mediated by lipid peroxidation and sustained iron-dependent ROS formation, a process they called ferroptosis (Dixon et al. 2012). Indeed, erastin operates by inhibiting cystine import, resulting in intracellular GSH depletion, a crucial cofactor for the enzyme glutathione peroxidase 4 (GPX4) in neutralizing lipid peroxides (Dixon et al. 2012). While the precise mechanisms by which erastin inhibits system x_c⁻ are still being elucidated, research suggests that erastin directly binds to the xCT subunit. By interacting with specific residues and altering its conformation, erastin blocks the transport of cystine by system x_c⁻ (Yan et al. 2022). Additionally, it has been recently demonstrated that part of the response to erastin is achieved by upregulating ATF3 and promoting cell ferroptosis (Wang et al. 2020; Li, Yan, et al. 2022). Another mechanism regularly evoked is that erastin negatively regulates Slc7a11 through an upregulation and activation of the p53/USP7/H2Bub1 pathway (Wang et al. 2019; Huang, Yang, et al. 2018). Overall, the inhibition of system x_c⁻ by erastin likely involves a combination of direct and indirect mechanisms that disrupt the normal function or regulation of the antiporter complex, ultimately leading to impaired cystine transport and induction of the ferroptotic cell death. However, further research is needed to fully elucidate the precise molecular mechanisms underlying erastin's inhibitory effect on system x_c⁻.

When compared to other biologically validated inhibitors of system x_c⁻, erastin appears the most potent inhibitor with a submicromolar activity (IC₅₀ = 0.20 μM) (Patel et al. 2015). To date, erastin has been extensively

investigated in cancer research due to the heightened oxidative stress and susceptibility to redox imbalance commonly observed in cancer cells. Multiple *in vitro* studies have validated that erastin treatment inhibits system x_c^- function and causes ferroptotic cell death in various cancer types (Shiromizu et al. 2019; Dixon et al. 2014; Nie et al. 2018; Sato et al. 2018; Dolma et al. 2003). It has also been shown that short exposure of tumor cells to erastin strongly potentiated the cytotoxic effects of cisplatin to eradicate tumor cells (Sato et al. 2018).

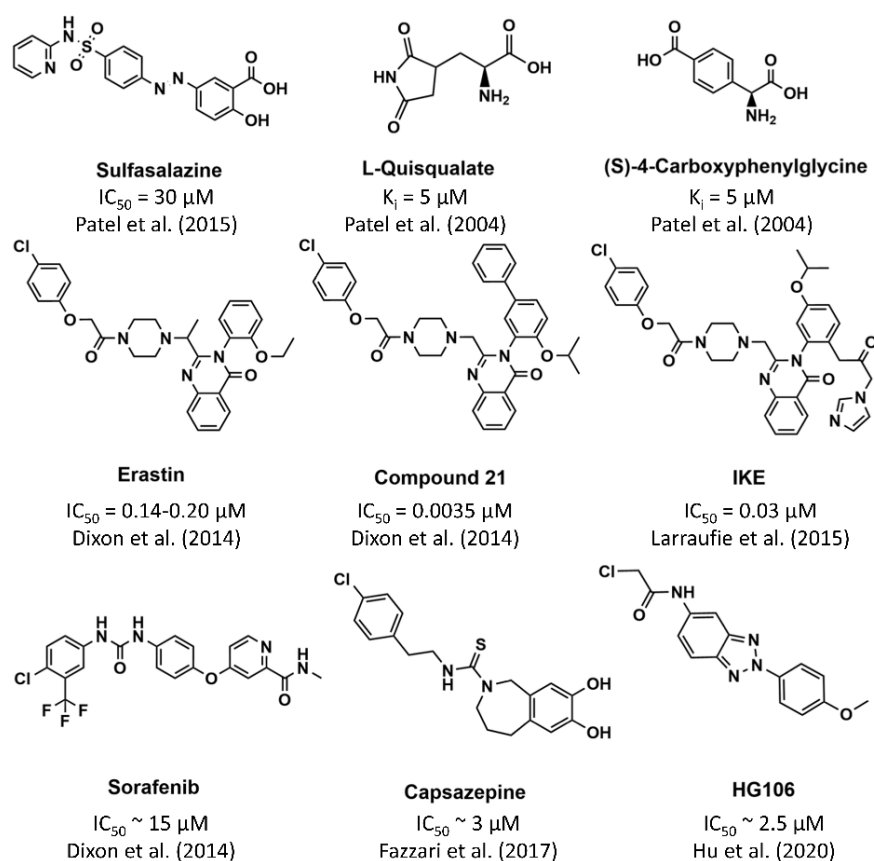


Figure 13 : Overview of the structure of xCT inhibitors and their related potency. *IKE: imidazole ketone erastin.* Figure adapted from (Jakobsen and Nielsen 2024).

Despite its potential for cancer therapy, however, erastin has not reached the clinic due to its low water solubility and unstable metabolism when administered *in vivo*. Therefore, two erastin analogues imidazole ketone erastin (IKE) and piperazine erastin with improved water solubility, potency ($IC_{50} = 0,03 \mu M$ for IKE) and metabolic stability have been developed (Larraufie et al. 2015). These derivatives however still await further characterization to consolidate their biological activity.

c. Sorafenib

Sorafenib, an FDA-approved inhibitor targeting multiple oncogenic kinases, has been found to induce ferroptosis in diverse human cell lines. Unexpectedly identified as a system x_c^- inhibitor by Dixon and colleagues in 2012, this finding holds relevance due to its recognized anti-cancer effects and established safety profile (Dixon et al. 2012). While sorafenib robustly inhibits system x_c^- activity ($IC_{50} \sim 15 \mu M$), its induction of ferroptosis occurs within a narrow range of concentrations. Since other drugs from the same class do not induce ferroptosis, the inhibition of system x_c^- by sorafenib was shown to be independent of kinase inhibition (Dixon et al. 2012; Lachaier et al. 2014). However, at higher concentrations, sorafenib appears to trigger additional lethal mechanisms that operate in parallel to the ferroptotic response (Dixon et al. 2014).

While the complete mechanism of action of sorafenib is not yet fully elucidated, as for erastin, it has been documented that sorafenib partly exerts its inhibitory effect on xCT through the upregulation of ATF3 which results in GSH depletion and ferroptosis (Wang et al. 2020; Li, Yan, et al. 2022). More recently, Xu and colleagues also identified ATF2 as an important regulator of sorafenib-induced ferroptosis in gastric cancer cells (Xu et al. 2023).

In vitro studies documented a pro-ferroptotic effect of sorafenib in various human tumor cancer cells including lung carcinoma, fibrosarcoma, gastric cancer, pancreatic adenocarcinoma, kidney cancer (Lachaier et al. 2014;

Dixon et al. 2014; Xu et al. 2023). Roh and co-workers demonstrated that sorafenib, in combination with aspirin potentiates cisplatin cytotoxicity in resistant head and neck cancer cells through xCT inhibition both *in vitro* and *in vivo* (Roh et al. 2017). Currently, the clinical opportunities offered by sorafenib in targeting system x_c^- remain untested (Lin et al. 2020). However, data from phase I clinical trials indicated that at recommended doses of sorafenib (400 mg), plasma concentration reaches 5-20 μ M, overlapping with the concentrations known to induce ferroptosis (Awada et al. 2005; Strumberg et al. 2005). Additionally, patients treated with sorafenib exhibit protein oxidation in their sera, with higher oxidation levels correlating with better outcomes (Coriat et al. 2012), suggesting a potential *in vivo* effect of sorafenib on system x_c^- inhibition and ROS generation.

Apart from the aforementioned molecules, which are not entirely selective for system x_c^- , research is being conducted to identify new specific and potent system x_c^- inhibitors, such as SAS and erastin analogues. Moreover, molecules inhibiting the exchanger by targeting upstream regulators (e.g. p53) of xCT, immunotherapy and DNA-based vaccines against xCT are emerging (Lin et al. 2020).

HYPOTHESES AND OBJECTIVES

At the cellular level, neuropathic pain emerges as a disrupted interplay between excitatory and inhibitory controls of pain transmission. This dysregulation leads to reinforced glutamatergic signaling and consequently to an enhanced synaptic excitability in the pain pathways. Together with a persistent neuroinflammation, this imbalance leads to a plastic adaptation within the nervous system known as central sensitization.

Despite concerted efforts, current therapeutic modalities targeting specific glutamate receptors or other mediators to restore the balance between excitatory and inhibitory pathways have only yielded modest benefit. Consequently, other approaches focusing on physiological systems governing extracellular glutamate levels or neuroimmune signals appear as an attractive alternative. Notably, studies have underscored the impaired capacity of astrocytes to take up glutamate through EAATs (GLT-1 and GLAST), implicating these membrane proteins in neuropathic pain pathogenesis. However, the regulatory mechanisms governing another critical glutamate transporter, the cystine/glutamate exchanger, also known as system x_c^- , remain relatively unexplored.

Predominantly expressed in glial cells, system x_c^- orchestrates glutamate release concomitantly with cystine uptake, thereby providing the cells with this rate limiting amino acid for GSH synthesis and reinforcing glutamate signaling. Therefore, as for EAATs, a tight regulation of its expression and activity is essential to maintain a low extracellular glutamate concentration and avoid hyperexcitability. Besides, system x_c^- has been demonstrated to participate in and regulate neuroinflammatory responses. Microglia can present a multitude of phenotypes ranging from an anti-inflammatory, neuroprotective (M2-microglia) to a pro-inflammatory, neurotoxic (M1-microglia) state. In different animal models of neuropathic pain, a modification in the phenotype polarization towards predominant M1-microglia has been documented at the spinal cord level. Previous work has also highlighted an enhanced system x_c^- expression and activity on activated

microglia and an altered polarization of microglial response in pathological conditions in the absence of system x_c^- . Indeed, it may shift the polarization of microglia towards a protective anti-inflammatory phenotype (M2). Since activated microglia can also release glutamate via system x_c^- and therefore favor excitotoxicity and ROS synthesis, both parameters need to be addressed in the context of neuropathic pain.

In several neurological disorders characterized by excitotoxicity and neuroinflammation such as PD, ALS, glioma and AD, xCT mRNA has been shown to be upregulated. However, the pivotal role for this exchanger in a context of neuropathic pain has never been addressed. Regarding its involvement in the control of extracellular glutamate concentrations and neuroimmune responses, it is certainly essential to evaluate its putative implication in the processing of nociceptive signals and its involvement in central sensitization.

We hypothesized that modulation of system x_c^- in neuropathic pain could be beneficial in two ways. First, by reducing the release of glutamate into the synaptic cleft and thus decreasing excitatory neurotransmission in the pain pathways. Second, by shifting the microglial activation toward the neuroprotective phenotype to reduce persistent inflammation during the progression of neuropathic pain and avoid plastic adaptation in the CNS. We thus propose that the pharmacological inhibition of system x_c^- could prevent, reduce, or alleviate neuropathic pain symptoms such as allodynia and hyperalgesia. Such research could thus pave the way for successful chronic pain treatments.

To test these hypotheses, the current manuscript is structured into three interconnected workpackages.

EXPERIMENTAL WORKPACKAGE I: Validation of a system x_c^- functional assay in different cultured cell lines and nervous tissues samples.

While previous studies on system x_c^- have primarily focused on measuring the expression of its specific subunit xCT through RT-qPCR or Western blotting, the activity of this exchanger in tissue samples remains largely unexplored. The aim of this project was to optimize and validate a reliable and cost-efficient method for assessing the specific activity of system x_c^- under both physiological and pathological conditions. To achieve this, we used radiolabeled glutamate as a substrate for the reversed transport mediated by system x_c^- . By working with buffers containing defined concentrations of Na^+ , we were able to differentiate the glutamate uptake supported by system x_c^- or by EAATs. Furthermore, the validity of this technique was then confirmed by using pharmacological and genetic approaches.

EXPERIMENTAL WORKPACKAGE II: Implication of system x_c^- in neuropathic pain process

The well-characterized model of peripheral nerve lesions was used and consists of a partial sciatic nerve ligation (PSNL). This neuropathic pain model induces gliosis, disrupts glutamate homeostasis and triggers neuroinflammation, all of which correlate with the persistence of pain behaviors such as allodynia and hyperalgesia.

Within this part of the project, our primary objectives were: (1) characterizing xCT expression and system x_c^- activity in the spinal cord following sciatic nerve injury, (2) assessing the impact of the genetic inactivation of system x_c^- on the development of neuropathic pain symptoms, (3) comparing spinal gliosis and neuroinflammatory responses between wild-type and $xCT^{-/-}$ mice following nerve injury, (4) investigating the efficacy of SAS, a system x_c^- inhibitor, by evaluating neuropathic pain behavior and associated neuroinflammation.

EXPERIMENTAL WORKPACKAGE III: Role of system x_c^- in chronic pain after peripheral inflammatory insult

Building upon the findings obtained in the second study, we aimed at specifically examining the involvement of system x_c^- in the inflammatory component of chronic pain. To achieve this, we utilized an animal model of chronic inflammatory pain induced by unilateral intraplantar subcutaneous injection of a well characterized inflammatory chemical trigger, the complete Freund's adjuvant (CFA).

Within this part of the thesis, our main objectives were: (1) determining the impact of genetic inactivation of system x_c^- on the development of inflammatory pain symptoms, (2) comparing systemic peripheral inflammation between wild-type and transgenic mice lacking xCT following CFA injection, (3) assessing the responsiveness to both preventive and curative treatment of SAS by evaluating inflammatory pain symptoms.

By pursuing these objectives, our aim is to obtain insights into the role of system x_c^- in the pathogenesis of neuropathic pain and to propose potential therapeutic strategies for alleviating neuropathic pain symptoms. These strategies may involve targeting glutamate-mediated hyperexcitability and/or neuroinflammation.

RESULTS

EXPERIMENTAL WORKPACKAGE I:

Validation of a system x_c^- functional assay in cultured astrocytes and nervous tissue samples

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Abstract

Disruption of the glutamatergic homeostasis is commonly observed in neurological diseases and has been frequently correlated with the altered expression and/or function of astrocytic high-affinity glutamate transporters. There is, however, a growing interest for the role of the cystine/glutamate exchanger system x_c^- in controlling glutamate transmission. This exchanger is predominantly expressed in glial cells, especially in microglia and astrocytes, and its dysregulation has been documented in diverse neurological conditions. While most studies have focused on measuring the expression of its specific subunit xCT by RT-qPCR or by Western blotting, the activity of this exchanger in tissue samples remains poorly examined. Indeed, the reported use of sulfur- and carbon-radiolabeled cystine in uptake assays shows several drawbacks related to its short radioactive half-life and its relatively high cost.

We here report on the elaborate validation of a method using tritiated glutamate as a substrate for the reversed transport mediated by system x_c^- . The uptake assay was validated in primary cultured astrocytes, in transfected cells as well as in crude synaptosomes obtained from fresh nervous tissue samples. Working in buffers containing defined concentrations of Na^+ , allowed us to differentiate the glutamate uptake supported by system x_c^- or by high-affinity glutamate transporters, as confirmed by using selective pharmacological inhibitors. The specificity was further demonstrated in primary astrocyte cultures from transgenic mice lacking xCT or in cell lines where xCT expression was genetically induced or reduced. As such, this assay appears to be a robust and cost-efficient solution to investigate the activity of this exchanger in physiological and pathological conditions. It also provides a reliable tool for the screening and characterization of new system x_c^- inhibitors which have been frequently cited as valuable drugs for nervous disorders and cancer.

1. Introduction

Initially proposed in the early fifties, the role of glutamate as a neurotransmitter was demonstrated twenty years later. Since then, this rather simple amino acid has been unveiled as one of the most essential bioactive chemicals in the CNS of mammals. In most excitatory synapses, glutamate is released from presynaptic nerve terminals to activate a large diversity of iGluRs and mGluRs glutamate receptors. As major excitatory neurotransmitter, glutamate plays a crucial role in complex physiological processes including synaptic plasticity, learning and memory (Malenka and Nicoll 1999). Glutamate also participates in the formation of GSH, the most abundant antioxidant in the CNS (Had-Aissouni 2012) and may serve as an alternative source of energy for diverse cells of the nervous system (McKenna 2013). Besides these roles in physiological activities in the CNS, glutamate is also known for its implication in a large variety of nervous disorders (Lewerenz 2015). High concentrations of this excitatory transmitter can cause damage to neuronal cells, a process known as excitotoxicity (Dong et al. 2009).

The control of glutamate transmission in the CNS does not solely depend on the interplay between neuronal cells, but also on closely associated glial cells in what is known as the tripartite synapse (Perea et al. 2009). Indeed, astrocytes play multiple roles in the control of glutamate homeostasis, in particular because they express EAATs, that ensure the clearance of extracellular glutamate and thereby protect neurons against excitotoxic insults (Anderson and Swanson 2000; Oliet et al. 2001). Hence, impaired glial function and the associated excitotoxicity is frequently proposed as an important mechanism involved in several neurological disorders (Beart and O'Shea 2007) and this has been validated in animal models of human diseases. Besides, astrocytes contribute to synaptic activity through the non-vesicular release of substantial amounts of glutamate by the cystine/glutamate exchanger, also known as system x_c^- (Massie et al. 2011;

De Bundel et al. 2011). Its widespread distribution throughout the CNS and the critical roles played by this exchanger suggest that it could be an important determinant of brain functions under physiological and pathological conditions (Lutgen et al. 2014; Lewerenz et al. 2013). There is a growing interest in the study of its physiological regulation in glial cells and in the possibility to develop specific drugs to pharmacologically manipulate its activity.

The cystine/glutamate exchanger is a Na^+ -independent transporter that is constituted of a heavy chain subunit common to several amino acid transporters, 4F2hc (encoded by the *Slc3a2* gene), and a specific light chain subunit, xCT (encoded by the *Slc7a11* gene). While the 4F2hc subunit ensures the trafficking and the cell surface expression of the heterodimer, xCT confers transport function and substrate specificity (Sato et al. 1999). Under physiological conditions, system x_c^- supports the exchange of intracellular L-glutamate for extracellular L-cystine at a 1:1 molecular ratio, driven by their concentration gradients across the plasma membrane (Bannai 1986). Once taken up by the cell, cystine is intracellularly reduced into cysteine, a building block for GSH synthesis which is essential to face oxidative stress (Bannai and Kitamura 1980). Astrocytic GSH can be transferred to neurons and therefore, system x_c^- contributes to the antioxidant protection throughout the CNS. Furthermore, through its glutamate release activity, the exchanger constitutes a substantial source of extracellular glutamate in several brain regions, likely impacting on the glutamatergic neurotransmission across the CNS (Massie et al. 2015; Barger and Basile 2001).

The implication of system x_c^- in essential functions of astrocytes has encouraged the study of its modulation in diverse conditions both *in vivo* and *in vitro*. Exposing cultured astrocytes to the pituitary adenylate cyclase-activating polypeptide (Kong et al. 2016), IL-1 β (Shi et al. 2016; Jackman et al. 2010) or substance P (Johnson and Johnson 1993) has been shown to regulate the exchanger. Besides, impaired expression or activity of system x_c^-

was documented in several animal models of neurological disorders such as PD, ALS and in glioblastoma (Lewerenz et al. 2013). So far, most of these studies have examined the regulation of system x_c^- by RT-qPCR to quantify xCT mRNA. For a while, the lack of reliable anti-xCT antibodies has limited the study of its protein expression, yielding rather inconsistent published data (Van Liefferinge et al. 2016). Functional characterization of the cystine/glutamate exchanger is limited and frequently poorly described. In these studies, [^{14}C] and [^{35}S] radiolabeled-cystine is used as substrate in uptake assays (Lewerenz et al. 2006; Liu et al. 2009; Bridges, Kekuda, et al. 2001; Merckx et al. 2015; Massie et al. 2011), but its high cost and relatively limited shelf-life constitute experimental drawbacks.

These issues have prompted us to validate a robust and cost-effective uptake assay for the functional study of system x_c^- in physiological and pathological conditions. We here report the use of tritiated L-glutamate as a substrate for system x_c^- (Bridges, Kekuda, et al. 2001). Monitoring the reverse uptake of glutamate was already reported for measuring the activity of system x_c^- on models of cell cultures (Gochenauer and Robinson 2001; Shih and Murphy 2001; Webster et al. 2014; Shih et al. 2003; Patel et al. 2004). In the current study, the specificity of this assay was strongly consolidated by using cell lines with induced or repressed xCT expression as well as crude synaptosome preparations and primary cultures of astrocytes derived from transgenic mice lacking xCT. Furthermore, the use of selective pharmacological inhibitors allowed us to distinguish the uptake supported by system x_c^- or by EAATs.

2. Materials and Methods

a. Animals and ethics statement

All experiments were conducted in strict accordance with the recommendation of the European commission and with the agreement of the Belgian Ministry of Agriculture (code number LA 1230618). The ethical

committee of the Université catholique de Louvain (UCLouvain) for animal experiments specifically approved this study (code number 2019/UCL/MD/033). xCT^{+/+}, xCT^{+/-} and xCT^{-/-} mice used in this study were high-generation descendants of the strain previously described by Sato et al. (Sato et al. 2005).

One to 3 days old pups and adult female mice (wild-type or xCT transgenic with a C57BL/6 background (Sato et al. 2005)) and rats (Sprague Dawley) aged between 10 and 12 weeks were used and kept in groups of maximum six per cage. All animals were accommodated under standard laboratory conditions, receiving food and water ad libitum, and were housed in the animal facility at the UCLouvain (Brussels), in controlled light/dark cycle, temperature and humidity conditions. xCT transgenic mice were genotyped by PCR on genomic DNA extracted from a tail biopsy using specific primers for Slc7a11 gene in order to identify bands corresponding to wild-type (xCT^{+/+}), homozygous (xCT^{-/-}) or heterozygous (xCT^{+/-}) genotypes (Sato et al. 2005; De Bundel et al. 2011).

b. Primary cultures of cortical astrocytes

Cortices from rats or mouse pups were collected at postnatal day 2 and mechanically dissociated. Meninges were carefully removed, and astrocytes were separated from other cell types using 30% and 60% Percoll gradient (GE Healthcare) and seeded into coated flasks. Cells were left to proliferate at 37°C in a humidified atmosphere containing 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM-glutaMAX, Thermofisher Scientific) supplemented with 10% fetal bovine serum (FBS) (VWR), 100 µg/mL penicillin-streptomycin (Thermofisher Scientific), 2.5 µg/mL fungizone (Thermofisher Scientific), 50 µg/mL L-proline (Thermofisher Scientific) and 50 µM of β-mercaptoethanol (Gibco). The medium was renewed on day 7, and on day 14, astrocytes were collected by trypsinization (trypsin-EDTA, Thermofisher Scientific) and seeded at 70 000 cells per well in 6-well plates for Western blotting and into poly-L-lysine coated 24-well plates at 35 000 cells per well for uptake

measurement. On day 16, serum concentration was reduced to 3% as culture medium was renewed. When indicated, a supplement of 250 μ M N⁶,2'-O-dibutyryl adenosine 3',5'-cyclic monophosphate (dBcAMP) (Sigma-Aldrich) was added on day 16. For all the experiments, the astrocytes were used after 23 days in culture.

c. CRISPR/Cas9-induced xCT deletion in C6 astrocytoma cells

Guide RNA targeting the mouse xCT sequence (5'-CACCGTCATTACACATACATTCTGG-3') was cloned into the lentiCRISPRv2-PURO vector (Backx et al. 2021). Lentiviral transduction and clone selection were performed as previously described by Backx and colleagues (Backx et al. 2021) with little modifications. Briefly, cells were seeded in a 6-well plate at a density of 100000 cells per well. After 4 hours, cells were transduced with lentivirus-containing supernatant supplemented with protamine sulphate (10 μ g/mL, Sigma-Aldrich) and 50 μ M β -mercaptoethanol for 24 hours. Before renewing the medium (DMEM medium supplemented with 10% FBS, 100 μ g/mL penicillin-streptomycin, 2.5 μ g/mL fungizone and 50 μ M β -mercaptoethanol), cells were washed with PBS. After expanding the cells to T25 flasks and proliferation for 1 week, cells were selected with 5 μ g/mL puromycin (InvivoGen) for at least 1 more week before any experiments were performed.

d. Overexpression of xCT in transfected cells

The cDNA sequence encoding xCT (Slc7a11) was amplified from primary cultures of rat astrocytes and cloned into pcDNA3.1 (Invitrogen). Chinese hamster ovary (CHO) cells were cultivated at 37°C in a humidified environment (5% CO₂) with Ham's F-12 medium supplemented with 10% FBS and 100 μ g/mL penicillin-streptomycin (Thermofisher Scientific). 2 x 10⁶ cells were seeded in a 6-well plate a few hours before transfection with a mix consisting of 200 μ L Ham's F-12 medium, xCT DNA and X-tremeGene™ HP

DNA transfection reagent according to the manufacturer's instructions. Selection with geneticin (G418) (1000 µg/mL, Gibco) was initiated 72 hours after transfection, resulting in a stable transfected cell line, herein referred to as CHO-xCT. These cells were routinely passaged and grown in the presence of geneticin (250 µg/mL), except when plated for dedicated experiments.

e. Crude synaptosome preparation

Mice were euthanized with CO₂ and the spinal cord was immediately flushed out with phosphate-buffered saline (PBS) (Meikle and Martin 1981). Freshly isolated tissues were used for synaptosome preparation as previously described by Berger et al. (Berger et al. 2011), with little modifications. Briefly, the samples were homogenized in an ice-cold solution of 320 mM sucrose by up-and-down movements with a pre-chilled Teflon/glass potter followed by up-and-down movements with a tight Downs. The homogenates were centrifuged at 1000g for 10 min at 4°C and the supernatants were carefully collected and stored on ice. The pellets were resuspended in ice-cold 320 mM sucrose solution and centrifuged again at 4°C at 1000g for 10 min. The two supernatants were combined and finally centrifuged at 4°C for 30 min at 17500g. The final pellet containing the crude synaptosomes was suspended in 1 mL of ice-cold appropriate buffer (see below). Protein concentration was determined by the Bradford method with the Bio-Rad protein assay (Bio-Rad Laboratories) and samples were diluted to 33.33 µg/mL for uptake assay.

f. [³H]-L-glutamate and [³H]-D-aspartate uptake assays

On crude synaptosome preparations: To assess the uptake, [³H]-L-glutamate and [³H]-D-aspartate (with specific activity of 48.6 Ci/mmol and 12.2 Ci/mmol, respectively, Perkin Elmer) were used as substrates at tracing concentrations of 20 nM and 50 nM, respectively. In a total volume of 500 µL, 10 µg of

protein from the synaptosome preparation was incubated with the substrate at 37°C in a 96-well Masterblock (Greiner Bio-one). The assay was performed in a Na⁺-free buffer containing 140 mM *N*-methyl-D-glucamine (NMDG, as a substitute for NaCl), 5.4 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 0.4 mM KH₂PO₄, 10 mM HEPES and 5 mM D-glucose, pH 7.4 adjusted with HCl, or with a Na⁺-containing buffer composed of 120 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 120 mM NaCl, 1.3 mM CaCl₂, 25 mM HEPES pH 7.4 and 6 mM D-glucose. When indicated, homocysteic acid (HCA, Sigma-Aldrich) and L-threo-3-hydroxyaspartic acid (LTHA, Tocris) were included in the assay. After 20 min incubation at 37°C, the suspension was filtered through a GF/B glass fiber filter adapted to a 96-well plate (UniFilter GF/B, PerkinElmer), and washed three times with the ice-cold Na⁺-free buffer. After drying overnight at room temperature, Microscint 20 (PerkinElmer) was added to each well of the filter plate. Plates were shaken for 2 hours and then counted with a Topcount[®] NXT Microplate scintillation and luminescence counter (PerkinElmer). Results are expressed as cpm per µg of protein.

On cell cultures: Primary cultured astrocytes or CHO cells were grown on poly-L-lysine coated 24-well plates. Culture medium was removed and immediately replaced with preheated Na⁺-free or Na⁺-containing buffers. After a brief incubation of 10 min, the plate was placed on the surface of a 37°C water bath and the buffer was removed and replaced with the same buffer supplemented with either [³H]-L-glutamate or [³H]-D-aspartate at a final concentration of 20 and 50 nM, respectively. When indicated, specific inhibitors (see above) were added to the assay. After 20 min ([³H]-L-glutamate) or 10 min ([³H]-D-aspartate), the uptake was stopped by three rinses with ice-cold buffer and cells were lysed with 0.1 M NaOH. A fraction of the lysate was collected, and the radioactivity content was measured using the liquid scintillation solution Microsint 40 and the Topcount[®] NXT Microplate scintillation and luminescence counter. Another fraction of the lysate was used for protein quantification using the Bradford method with

the Bio-Rad protein assay. Results are expressed as pmol of radiolabeled substrate transported per min per mg of protein.

g. Western blotting

Cells seeded in 6-well plates were rinsed with PBS and scraped in ice-cold lysis buffer (100 mM Tris, 150 mM NaCl, 1mM ethylene diamine tetra acetic acid, 1% Triton-X100, 0,1% SDS, 1% deoxycholic acid sodium salt 99%, pH 7.4) while synaptosome samples where directly resuspended in 1 mL of the same lysis buffer after the last centrifugation. Protein concentration was determined by the PierceTM BCA method (Thermofisher Scientific) and samples were diluted to a concentration of 1µg/µL. After adding Western blot loading buffer to the samples (final concentrations: Tris-HCl 60 mM pH 6.8, glycerol 10%, sodium dodecyl sulfate 2%, β-mercaptoethanol 5% and bromophenol blue 0.01%), proteins were separated through a 10% SDS-PAGE and transferred to nitrocellulose membrane by electroblotting. Membranes were then incubated for 1 h in Tris-buffered saline (50 mM Tris pH 7.4, 150 mM NaCl) containing 0.05% Tween-20 and 5% bovine serum albumin (BSA, Carl Roth) to reduce non-specific labeling. Immunoprobng was carried out by incubating membranes overnight at 4°C with primary antibodies recognizing xCT (rabbit polyclonal antibody, 1:1000, (Van Liefferinge et al. 2016; Massie et al. 2008)) or EEF2 (rabbit monoclonal antibody, 1:1000, Thermofisher Scientific). Membranes were rinsed in TBS-Tween 0.05% and then incubated for 1 h with a peroxidase-conjugated secondary antibody (goat anti-rabbit IgG, 1:5000, Jackson ImmunoResearch Laboratory). Immunoreactive proteins were detected with enhanced chemiluminescence reagent (Clarity, Bio-Rad Laboratories). Densitometric analysis of the signal was performed using ImageJ (Broken Symmetry Software).

h. Statistical analysis

Data were presented as means of three different experiments with the standard error of the mean (SEM). All statistical analyses were performed using GraphPad Prism version 5.01 (GraphPad Software, CA). For multiple comparisons, data from distinct experimental conditions were analyzed using a one-way ANOVA followed by a Dunnett's or a Bonferroni test. A p-value < 0.05 was considered significant for all statistical analyses.

3. Results

a. System x_c^- and EAAT-dependent uptake in cultured astrocytes and C6 astrocytoma cells

Primary cultures of astrocytes derived from the cortices of rat and mouse pups were used to measure the uptake of [^3H]-L-glutamate and [^3H]-D-aspartate. Indeed, astrocytes are best known for their capacity to take up excitatory amino acids through diverse EAATs which recognize both glutamate and aspartate whereas system x_c^- only achieves the transport of glutamate. Another noticeable feature of EAATs is their dependency on the Na^+ gradient across the cell membrane that provides the driving force for the uptake. Thus, in contrast to system x_c^- of which activity is Na^+ -independent, EAAT activity requires the presence of a high concentration of Na^+ in the buffer. To distinguish the [^3H]-L-glutamate uptake supported by EAATs or by system x_c^- , the assays were therefore performed both in Na^+ -containing or Na^+ -free buffers.

As shown in figure 14A and C, both substrates were taken up by rat derived astrocytes in the buffer containing Na^+ . On the other hand, only [^3H]-L-glutamate was taken up in the Na^+ -free buffer (Figure 14B and D).

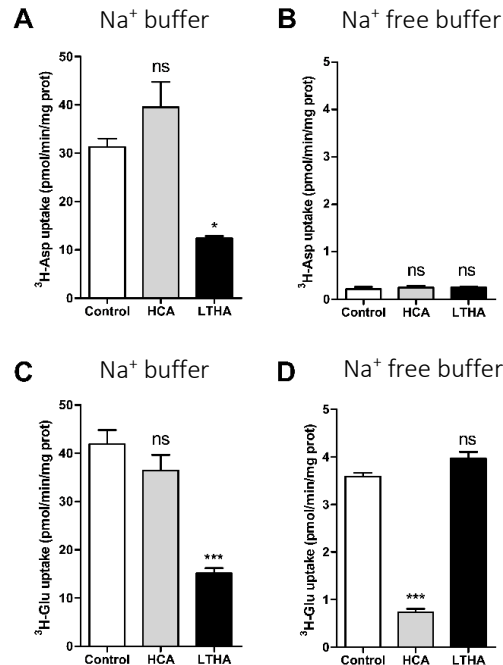


Figure 14: Influence of extracellular Na⁺ on [³H]-D-aspartate and [³H]-L-glutamate uptake in primary cultured rat astrocytes. The [³H]-D-aspartate (A, B) or [³H]-L-glutamate (C, D) uptake assays were performed in Na⁺-containing (A, C) or Na⁺-free (B, D) buffers. HCA (10⁻⁴M) and LTHA (10⁻⁴M) were used to discriminate the involvement of EAATs or system x_c⁻ in the uptake. Data shown are the mean with SEM of the uptake capacity from three independent experiments performed in quadruplicate. Statistical analyses were performed using a one-way ANOVA followed by a Dunnett's test for multiple comparisons (ns p>0.05, * p<0.05, *** p<0.001 when compared to control). *HCA*, homocysteic acid; *LTHA*, L-threo-3-hydroxyaspartic acid.

The implication of EAATs and system x_c⁻ in the uptake of the two substrates was further examined using specific inhibitors of these transporters. The system x_c⁻ inhibitor HCA did not affect the uptake of [³H]-D-aspartate whereas a large proportion of this uptake was inhibited by LTHA (Figure 14A), an EAATs inhibitor. Regarding the influence of these inhibitors on the transport of [³H]-L-glutamate, data presented in figure 14C showed that LTHA considerably reduced the uptake in the Na⁺-containing buffer whereas only HCA was

inhibiting the uptake in the Na⁺-free buffer (Figure 14D). Together, these data confirm that astrocytes achieve glutamate uptake through both system x_c⁻ and EAATs. Comparing the absolute uptake values in the different conditions indicates that in the Na⁺-containing buffer, EAATs predominantly contribute to the uptake of [³H]-L-glutamate that reaches up to 40 pmol/min/mg of protein. Conducting the assay with [³H]-L-glutamate in a Na⁺-free buffer appears as the optimal experimental condition to characterize the activity of system x_c⁻ without any interference of EAATs. The uptake through system x_c⁻ represents approximately one tenth of the total uptake in rat astrocytes (approx. 3.5 pmol/min/mg of protein).

The [³H]-L-glutamate uptake assay evaluating system x_c⁻ activity was also performed in primary cultures of astrocytes derived from the cortices of xCT^{+/+} and xCT^{-/-} mouse pups. In astrocytes derived from xCT^{+/+} mice, the uptake of [³H]-L-glutamate measured in the Na⁺-free buffer was particularly low (Figure 15A). Exposing the cells during the period of maturation (7 days in a 3% FBS medium) to dBcAMP (250 μM) significantly increased the uptake values by 10-fold, reaching up to 2 pmol/min/mg of protein. Confirming the sole involvement of system x_c⁻, this uptake was almost completely inhibited in the presence of HCA whereas LTHA was without effect. As shown in figure 15B, the uptake of [³H]-L-glutamate was barely detectable in astrocytes derived from the transgenic mice lacking xCT, even when the culture was exposed to dBcAMP. Similarly, [³H]-L-glutamate uptake measured in the Na⁺-free buffer in the C6 astrocytoma cells was considerably reduced in the presence of HCA whereas LTHA was without such influence on the uptake capacity (Figure 15C). In the C6 cell line where xCT expression was specifically targeted using CRISPR-Cas9, the uptake of [³H]-L-glutamate was hardly detectable and was insensitive to HCA (Figure 15C). Together, these data

validate the measure of [^3H]-L-glutamate uptake in the Na^+ -free buffer as a selective functional monitoring of system x_c^- activity.

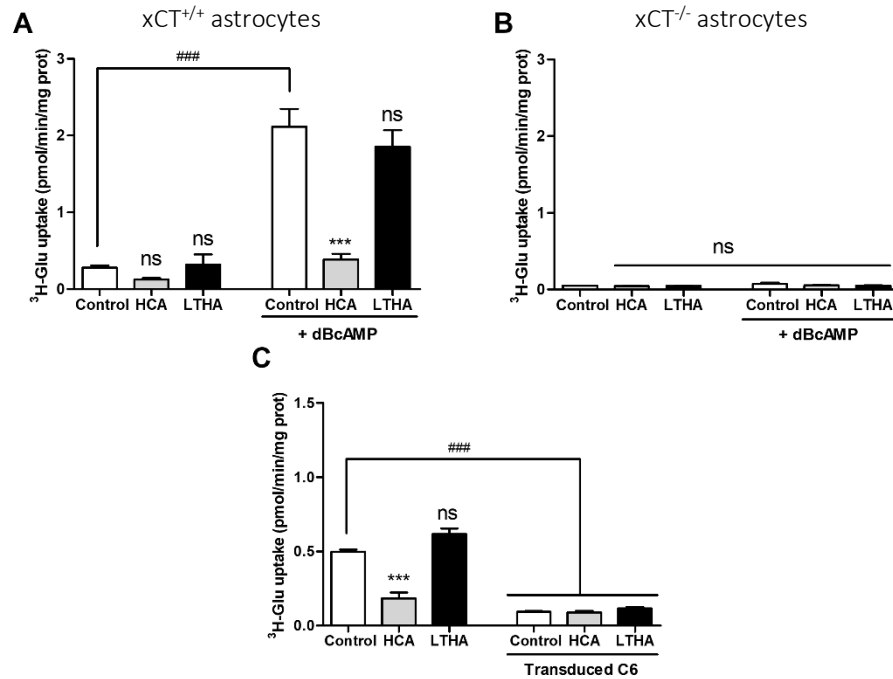


Figure 15: Influence of genetic xCT deletion on [^3H]-L-glutamate uptake supported by system x_c^- in cultured astrocytes and C6 astrocytoma cells. [^3H]-L-glutamate uptake was quantified in primary cultured astrocytes derived from wild-type (A) or $xCT^{-/-}$ mice (B) matured for 7 days in the presence or the absence of dBcAMP, as well as on C6 astrocytoma cells with and without CRISPR/Cas9-mediated xCT deletion (C). Specific pharmacological inhibitors were used (10^{-4}M) and added to the radioactive preparation when indicated. Histograms represent the mean with SEM of the uptake capacity from three independent experiments performed in quadruplicate. Statistical analyses were performed with a one-way ANOVA followed by a Dunnett's test for multiple comparisons (ns $p > 0.05$, *** $p < 0.001$ refer to the comparison within the same cellular population (vs control). ### $p < 0.001$ in panel A and B refers to the comparison between dBcAMP-matured and non-matured astrocytes or in panel C between C6 cells with and without xCT deletion). HCA, homocysteic acid; LTHA, L-threo-3-hydroxyaspartic acid; dBcAMP, dibutyryl cyclic adenosine monophosphate.

b. xCT expression in CHO cell increases glutamate transport supported by system x_c^-

CHO cells were stably transfected with the eukaryotic expression vector pcDNA3.1 carrying the rat xCT coding sequence. While some expression of xCT was detected by immunoblotting in samples from non-transfected cells, geneticin selected transfectants showed a higher expression level of the immunoreactive signal (Figure 16A). Accordingly, [^{3} H]-L-glutamate uptake measured in the transfected cells was significantly higher as compared to non-transfected cells (Figure 16B). The specificity of this [^{3} H]-L-glutamate uptake was confirmed using the system x_c^- inhibitor HCA which causes a significant decrease in the uptake in both transfected and non-transfected cells. Non-linear analysis of the concentration-dependent uptake inhibition curves allowed to determine a half-maximal inhibitory concentration (IC_{50}) of HCA in control and CHO-xCT cells in the 10 μ M range (pIC_{50} values of 5.01 ± 0.12 and 5.24 ± 0.11 respectively) (Figure 16C). In contrast, LTHA did not influence the [^{3} H]-L-glutamate uptake in these experimental conditions (Figure 16B).

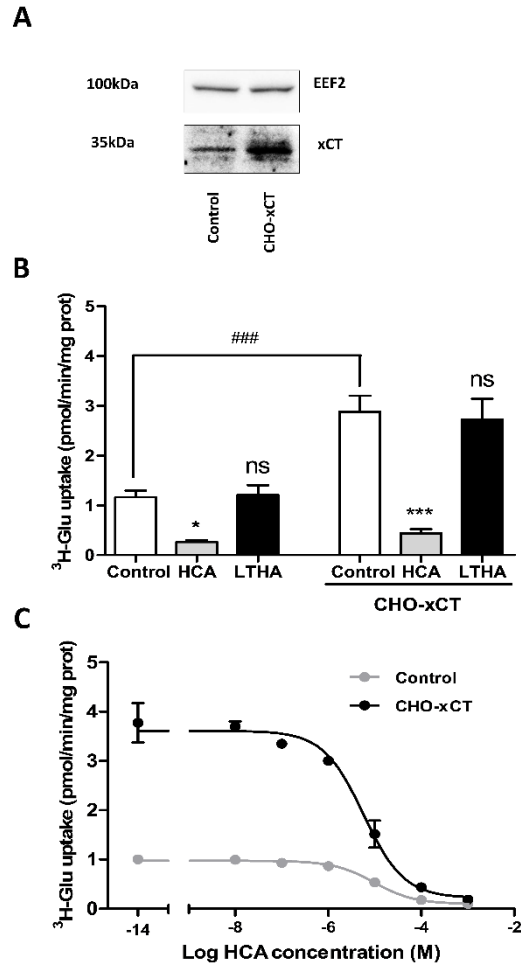


Figure 16: Influence of xCT overexpression on ^3H -l-glutamate uptake capacity of CHO cells. The increased expression of xCT was validated by Western blotting comparing protein samples from control and CHO-xCT cells using a specific rodent xCT antibody. Detection of the EEF2 was used as a control for sample loading and protein blotting. The blot shown is representative of three independent experiments (A). ^3H -l-glutamate uptake capacity was measured on control and CHO-xCT cells. Pharmacological inhibitors (10^{-4}M) were added to the preparation to further confirm the nature of the transporter involved (B). The potency of HCA at inhibiting system x_c^- -dependent ^3H -l-glutamate uptake capacity was examined by performing the assay in the presence of increasing concentrations of HCA (C). Data represent the mean of the uptake capacity

with SEM from three independent experiments performed in quadruplicate. Statistical analyses were performed using a one-way ANOVA followed by a Dunnett's test for multiple comparisons (ns $p > 0.05$, * $p < 0.05$, *** $p < 0.001$ refer to the comparison within the same cellular population (vs control). ### $p < 0.001$ refers to the comparison between CHO and CHO-xCT cells). *EEF2*, Eukaryotic elongation factor 2; *HCA*, homocysteic acid; *LTHA*, L-threo-3-hydroxyaspartic acid; *CHO*, Chinese hamster ovary; *CHO-xCT*, Chinese hamster ovary transfected with xCT.

c. System x_c^- -dependent glutamate uptake and xCT expression in crude spinal cord synaptosomes

As previously described by Berger et al. (Berger et al. 2011), crude synaptosomes from spinal cord samples were obtained after several steps of homogenization and centrifugation in ice-cold isotonic conditions. For the functional characterization of system x_c^- , crude synaptosomes from $xCT^{+/+}$, $xCT^{+/-}$ and $xCT^{-/-}$ mice were prepared and resuspended in Na^+ -free buffer before the uptake experiment. The respective decreased expression or suppression of xCT in the heterozygous and xCT knockout animals was first validated by Western blotting on synaptosome samples submitted to detergent lysis (Figure 17A). In synaptosomes from $xCT^{+/+}$ animals, a substantial [^3H]-L-glutamate uptake was measured in the Na^+ -free buffer, which was almost completely inhibited in the presence of HCA (100 μM) but entirely preserved in the presence of LTHA (100 μM) (Figure 17B). Synaptosome samples prepared from the spinal cord of $xCT^{-/-}$ mice presented a minimal glutamate uptake capacity as compared to $xCT^{+/+}$ mice. This modest uptake was not affected by the pharmacological inhibitor HCA. In samples prepared from $xCT^{+/-}$ mice, the substrate uptake value was estimated at 60% of the uptake measured in samples from the wild-type animals. In the conditions tested (Na^+ -free buffer), this uptake was completely inhibited in the presence of HCA and not influenced by LTHA.

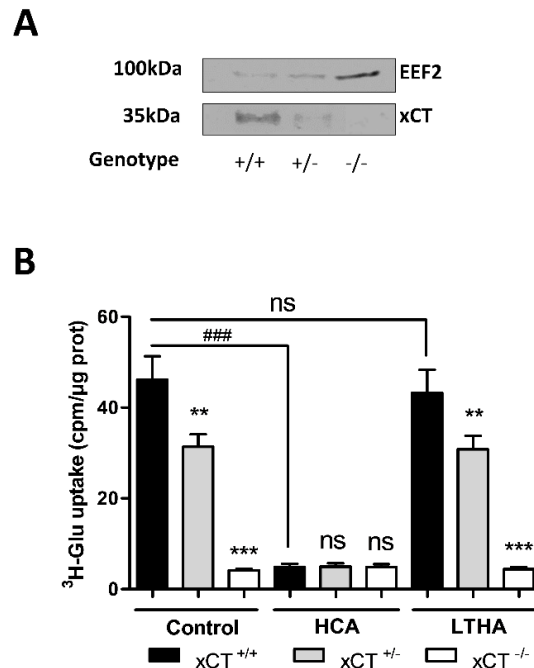


Figure 17: xCT expression and system x_c^- -dependent [^{3} H]-L-glutamate uptake using spinal cord synaptosome preparations. xCT expression (A) and [^{3} H]-L-glutamate uptake (B) in crude synaptosomes prepared from freshly isolated spinal cord samples. Tissues were obtained from transgenic xCT^{+/-} and xCT^{-/-} C57BL/6 mice and from wild-type littermates (xCT^{+/+}). The genetic deletion of xCT was validated by Western blot using a specific antibody against rodent xCT. EEF2 detection was used as a control for sample loading and protein blotting. The blot shown is representative of three independent experiments. Histograms represent the mean with SEM of the uptake capacity from three independent experiments performed in quadruplicate. Statistical analyses were conducted using a one-way ANOVA followed by a Dunnett's test for multiple comparisons (ns $p > 0.05$, ** $p < 0.01$, *** $p < 0.001$ refer to the comparison between genotypes (vs xCT^{+/+}). ns $p > 0.05$, ### $p < 0.001$ refers to the comparison within the same genotype). *EEF2*, Eukaryotic elongation factor 2; *HCA*, homocysteic acid; *LTHA*, L-threo-3-hydroxyaspartic acid.

4. Discussion

While the implication of astrocytic EAATs in the control of glutamate homeostasis and the protection against excitotoxicity is well characterized (Kanai et al. 1993), accumulating reports identify system x_c^- as another essential actor in the regulation of excitatory glutamate transmission in the CNS (Lewerenz et al. 2013; Bentea et al. 2020; Williams and Featherstone 2014). By ensuring the uptake of cystine in exchange for glutamate, the activity of system x_c^- provides astrocytes with cysteine that is incorporated into GSH which serves as an antioxidant in the nervous tissues. This however concurs with the release of glutamate in the synapse.

While several studies have repeatedly documented the importance of EAATs dysfunction in the alteration of glutamate homeostasis (Rodríguez-Campuzano and Ortega 2021), the impact of physiological and pathological changes affecting system x_c^- should not be overlooked. So far, most studies have focused on assessing the expression of its specific subunit xCT by RT-qPCR or by Western blotting. Importantly, different studies have revealed an upregulation of xCT expression in animal models of several nervous system disorders, including PD (Massie et al. 2008), epilepsy (Lewerenz et al. 2014), or ALS (Mesci et al. 2015). This increased expression promotes the release of glutamate, and it is indeed noteworthy that alteration in the glutamate homeostasis is commonly cited amongst the pathogenic mechanisms of these disorders (for review, see (Massie et al. 2015)). Beside these reports focusing on the regulation of xCT expression, only few studies have considered changes in the activity of the exchanger in neurological disease models. Functional studies are however essential in order to appreciate the consequences of the altered expression of the exchanger on the glutamate/cystine handling. Some laboratories have reported on *in vitro* and *ex vivo* experiments evaluating system x_c^- activity using radiolabeled cystine ($[^{35}\text{S}]$ -cystine or $[^{14}\text{C}]$ -cystine) as substrate (Merckx et al. 2015; Massie et al. 2011; Resch et al. 2014; Murphy et al. 1989; Fogal et al. 2007). Previously

validated on cell cultures, cystine uptake has been conducted on spinal cord and brain slices to monitor the activity of system x_c^- (Huang, Lin, et al. 2018; Albano et al. 2013; Kato et al. 1993; Melendez et al. 2005). This approach is however not routinely exploited as it presents several drawbacks including the high cost of these cystine radiochemicals and the limited radioactive half-life ($[^{35}\text{S}]$ -cystine). Siska and colleagues have recently reported on the use of a fluorescent derivative of cystine (fluorescein isothiocyanate - FITC) as a non-radioactive alternative to characterize system x_c^- activity (Siska et al. 2016). The use of the cystine-FITC probe was validated on T-cells in flow cytometry, but its use for large scale pharmacological screening or functional studies on tissue samples requires further optimization. Moreover, the use of radiolabeled substrate in biochemical assays offers a higher sensitivity that is essential for quantitative studies on small biological samples.

For *in vitro* experiments, functional studies have been conducted using tritiated glutamate as substrate for a reverse transport supported by the exchanger (Gochenauer and Robinson 2001; Shih and Murphy 2001; Shih et al. 2003; Patel et al. 2004; Webster et al. 2014). Several transporters and ions channels, including system x_c^- operate according to the concentration gradient of the transported chemicals across the cell membrane. For numerous transporters, a reversed activity has been documented in pathological conditions (GABA and glutamate transporters in epilepsy and ischemia (Rossi et al. 2000; Szatkowski et al. 1990; Wu et al. 2003)), in response to drugs (influence of psychostimulants on the dopamine transporter (Sulzer et al. 1995)) or at some stages of the development (chloride channel in the GABA_A ionotropic receptor (For review, see (Peerboom and Wierenga 2021))). In biochemical assays, the transport direction can be manipulated by changing the extracellular concentrations of the transported chemicals and this is the case for system x_c^- for which a reverse transport activity can be exploited. In the absence of extracellular cystine, the exchanger operates in a reverse direction and takes up glutamate

(Bannai 1986), which allows the use of radiolabeled glutamate to monitor system x_c^- activity.

The use of glutamate as substrate for functional studies of system x_c^- , inevitably leads to difficult data interpretation related to the implication of other glutamate carriers. In particular, GLT-1 (EAAT2), which represents up to 1% of total proteins in the nervous system of mammals (Lehre and Danbolt 1998). This transporter, commonly detected in matured primary cultures of astrocytes, is recognized as the major contributor to glutamate uptake in most of the experimental models. The evaluation of the glutamate uptake specifically achieved by other mechanisms, including system x_c^- , necessitates to develop and validate experimental conditions that exclude the activity of GLT-1 and other EAATs. The strict Na^+ dependency of EAAT, which is not shared by system x_c^- , offers the possibility to focus on the latter by removing Na^+ from the assay buffers.

Even though a few publications have already exploited this approach (Bridges, Kekuda, et al. 2001; Patel et al. 2004; Shih et al. 2003; Shih and Murphy 2001; Melendez et al. 2016; Melendez et al. 2005; Mysona et al. 2009), we here report on the validation of the assay and its selectivity using pharmacological inhibitors and genetically modified models, and its adaptation to samples of the nervous tissues where both glutamate transporters and the exchanger are expressed. Thus, in the absence of Na^+ , the uptake of radiolabeled glutamate was almost completely inhibited by HCA with an IC_{50} value consistent with a previous report on the inhibition of cystine uptake by system x_c^- (Patel et al. 2004). In these conditions, the pan-EAAT inhibitor LTHA was without any significant impact on the glutamate uptake. Also, in the absence of Na^+ , the glutamate uptake measured in primary cultures of astrocytes derived from transgenic mice lacking xCT was barely detectable and unchanged in the presence of HCA. Together, these experimental observations validate the robustness and selectivity of the functional assay used for the evaluation of system x_c^- activity.

EAATs and system x_c^- also differ in their specificity for amino acid substrates. While EAATs equivalently transport the L- and D- enantiomers of both glutamate and aspartate, system x_c^- only recognizes L-glutamate as substrate (Bridges, Kekuda, et al. 2001; Patel et al. 2004). As a result, only EAAT-associated activity is assessed when examining the uptake of radiolabeled D-aspartate, as indicated here by the lack of its uptake in the absence of Na^+ . Quantitative analysis of the uptake values in these different experimental conditions allowed to clearly confirm that in primary cultured astrocytes, the glutamate uptake is predominantly assured by EAATs whereas system x_c^- only partially contributes to the uptake (10%). Several protocols for the maturation/differentiation of cultured astrocytes derived from new-born rodent brain have been proposed (decrease in serum concentration, supplementation with a cocktail of growth factors or addition of dBcAMP) (Vermeiren, Najimi, Maloteaux, and Hermans 2005; Moonen et al. 1976; Sensenbrenner et al. 1980; Prah et al. 2019). These procedures commonly increase the expression and activity of EAATs (Vermeiren, Najimi, Maloteaux, and Hermans 2005; Eng et al. 1997; Schlag et al. 1998). Similarly, exposing the primary cultured astrocytes to dBcAMP during the differentiation step considerably increased the system x_c^- -dependent glutamate uptake, in accordance with the documented increased expression of the exchanger in the same conditions (Gochenauer and Robinson 2001). The absence of dBcAMP-induced increase in Na^+ -independent glutamate uptake in astrocyte cultures derived from transgenic mice lacking xCT further consolidates the specificity of the assay.

The growing interest for the study of system x_c^- as a major actor in the control of glutamate transmission is largely supported by reports highlighting the regulation of this exchanger in models of diseases both in peripheral tissues and in the CNS. While the characterization of system x_c^- function in cell cultures may contribute to study some molecular mechanisms of its regulation, the possibility to examine the function of this exchanger in tissue samples is essential. We here successfully applied the reverse transport of

radiolabeled glutamate to monitor the activity of system x_c^- in crude synaptosomes prepared from the mouse spinal cord. These subcellular fractions are prepared from nervous tissues by homogenization and centrifugation in an isotonic buffer, yielding a suspension of large, resealed cell vesicles with preserved membrane integrity and functional properties. The assay was adapted to a 96-well plate format allowing the harvesting of several samples simultaneously and limiting the amount of tissue needed to less than 10 μ g of protein per sample. Initially performed on crude synaptosomes prepared from freshly dissected spinal cord, the experiment was successfully reproduced on hippocampal tissue as well as on frozen samples without loss of uptake activity (data not shown). Considering the heterogeneity of CNS samples and the large diversity of transporter systems in these tissues, the specificity of the assay appears essential. Our data evidence a substantial Na^+ -independent glutamate uptake in synaptosomes that was inhibited by HCA and totally absent in synaptosomes prepared from tissues of transgenic mice lacking xCT. Obviously, performing functional studies on synaptosomes appears far less quantitatively restrictive than on freshly prepared tissue slices, as frequently used for diverse transmitter uptake assays in the literature (Albano et al. 2013; Melendez et al. 2005). While an elaborated fractionation procedure can be used to enrich synaptosome preparations with nervous terminals, crude synaptosome samples are known to contain a large proportion of glial contaminants (Henn et al. 1976). As demonstrated for EAATs (Petr et al. 2015), system x_c^- is predominantly expressed in glial cells but also detected in neurons (Jackman et al. 2010) and the possibility to discriminate the activity of the exchanger and other glutamate transporters is essential to study their specific regulation.

The documented increase in the expression of system x_c^- in several neurological and non-neurological disorders, in particular, in contexts of inflammatory insults should encourage to consider this exchanger as a valuable pharmacological target. The protocol that we here describe and

validate, appears as a robust, cost-effective, and reliable biochemical method for the screening and characterization of new system x_c^- inhibitors both in cell culture models and in tissue samples. Combined with protein/mRNA expression studies it offers access to a comprehensive toolbox for the study of this exchanger in models of diseases.

EXPERIMENTAL WORKPACKAGE II:

Implication of system x_c^- in neuroinflammation during the onset and maintenance of neuropathic pain

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Abstract

Background: Despite the high prevalence of neuropathic pain, treating this neurological disease remains challenging, given the limited efficacy and numerous side effects associated with current therapies. The complexity in patient management is largely attributed to an incomplete understanding of the underlying pathological mechanisms. Central sensitization, that refers to the adaptation of the central nervous system to persistent inflammation and heightened excitatory transmission within pain pathways, stands as a significant contributor to persistent pain. Considering the role of the cystine/glutamate exchanger (also designated as system x_c^-) in modulating glutamate transmission and in supporting neuroinflammatory responses, we investigated the contribution of this exchanger in the development of neuropathic pain.

Methods: We examined the implication of system x_c^- by evaluating changes in the expression/activity of this exchanger in the dorsal spinal cord of mice after unilateral partial sciatic nerve ligation. In this surgical model of neuropathic pain, we also examined the consequence of the genetic suppression of system x_c^- (using mice lacking the system x_c^- specific subunit xCT) or its pharmacological manipulation (using the pharmacological inhibitor sulfasalazine) on the pain-associated behavioral responses. Finally, we assessed the glial activation and the inflammatory response in the spinal cord by measuring mRNA and protein levels of GFAP and selected M1 and M2 microglial markers.

Results: The sciatic nerve lesion was found to upregulate system x_c^- at the spinal level. The genetic deletion of xCT attenuated both the amplitude and the duration of the pain sensitization after nerve surgery, as evidenced by reduced responses to mechanical and thermal stimuli, and this was accompanied by reduced glial activation. Consistently, pharmacological inhibition of system x_c^- had an analgesic effect in lesioned mice.

Conclusion: Together, these observations provide evidence for a role of system x_c^- in the biochemical processes underlying central sensitization. We propose that the reduced hypersensitivity observed in the transgenic mice lacking xCT or in sulfasalazine-treated mice is mediated by a reduced gliosis in the lumbar spinal cord and/or a shift in microglial M1/M2 polarization towards an anti-inflammatory phenotype in the absence of system x_c^- . These findings suggest that drugs targeting system x_c^- could contribute to prevent or reduce neuropathic pain.

1. Introduction

Neuropathic pain largely arises from a pathological imbalance between excitatory and inhibitory controls of pain transmission, especially at the level of the dorsal spinal cord (Colloca et al. 2017; You et al. 2010; Campbell and Meyer 2006; Saadé and Jabbur 2008). Together with a persistent neuroinflammation, this imbalance promotes plastic adaptations in the CNS that alter membrane excitability of neurons. This leads to an enhanced synaptic efficacy in pain pathways and is commonly referred as central sensitization (Latremoliere and Woolf 2009). Pro-inflammatory cytokines released by glial cells have been suggested to enhance neuronal and glial release of glutamate, the major excitatory neurotransmitter in the CNS (Vezzani and Viviani 2015; Wu et al. 2008). Neuroinflammation also promotes a transcriptional up-regulation of glutamate receptors, which leads to a persistent facilitatory influence on excitatory signals supporting pain transmission (Carlton and Coggeshall 1999).

In different models of neuropathic pain, the capacity of astrocytes to rapidly clear glutamate from the synaptic cleft was shown to be compromised (Guo et al. 2019; Cavaliere et al. 2007; Gegelashvili and Bjerrum 2017). Accordingly, pharmacological and genetic interventions aiming at increasing the expression or the functionality of high-affinity glutamate transporters (EAATs) have demonstrated promising analgesic effects in animal models of neuropathic pain (Gunduz et al. 2011; Hobo et al. 2011). To date, research on neuropathic pain and the development of novel treatments targeting glutamate transmission have primarily focused on receptors and EAATs. As such, other molecular actors known to play an important role in the control of glutamate transmission, such as the cystine/glutamate exchanger (system x_c^-) have been overlooked.

Mainly expressed in glial cells, system x_c^- is a transmembrane protein composed of two different subunits (Ottestad-Hansen et al. 2018). While the heavy chain, 4F2hc (SLC3A2), is shared among various plasma membrane

solute carrier proteins, the light chain xCT (SLC7A11) determines substrate specificity and transport activity. System x_c^- mediates the exchange of cystine for glutamate in a 1:1 molecular ratio, releasing glutamate into the extracellular space. Consequently, it provides cells with the cysteine, the rate-limiting amino acid residue for synthesizing antioxidant GSH, but also indirectly reinforces glutamate signaling (Lewerenz et al. 2013; Bridges et al. 2012). Hence, system x_c^- has even been identified as the major source of extracellular glutamate in several structures of the CNS (Massie et al. 2011; De Bundel et al. 2011). As for EAATs, a tight regulation of its expression and activity is essential to support protection against oxidative stress, but also to control extracellular glutamate concentration and neuronal excitation.

Considering its biochemical functions and its widespread distribution in the CNS, system x_c^- has been studied in several neurological disorders in which dysregulated excitatory transmission contributes to nervous insults and neurodegeneration. In particular, increased xCT expression has been documented in models of PD, ALS, MS as well as in stroke or glioblastoma (Mesci et al. 2015; Pampliega et al. 2011; Robert et al. 2015; Merckx et al. 2017; Chung et al. 2005; Soria et al. 2014). A role for system x_c^- was also documented in the modulation of inflammatory responses, raising further interest for its implication in pathologies combining neuroinflammation and increased neuronal excitation (Mesci et al. 2015). Considering this, the present study aimed at examining a putative role for system x_c^- in the modulation of nociceptive signals and associated neuroinflammation. To address this question, we have used a validated surgical mouse model of neuropathic pain in which the expression and activity of system x_c^- were manipulated by genetic and pharmacological approaches.

2. Materials and methods

a. Animals and Ethical statement

All experiments were conducted in strict accordance with the recommendations of the European commission and with the agreement of the Belgian Ministry of Agriculture (code number LA 1230618). The ethical committee of the Université catholique de Louvain for animal experiments specifically approved this study (aggregation number 2019/UCL/MD/033). Wild-type (xCT^{+/+}) and xCT knock-out (xCT^{-/-}) mice used in this study were high-generation descendants (with C57BL/6J background) of the strain previously described (Sato et al. 2005). These mice lacking xCT were generously provided by Pr. Hideyo Sato (Department of Medical Technology, Niigata University, Japan) and bred in the animal facilities of the Vrije Universiteit Brussel and the Université catholique de Louvain. All experiments combined homozygous animals obtained both from the breeding of heterozygous animals (F1) and from a second generation using homozygous parents (F2).

All animals were accommodated under standard laboratory conditions, receiving food and water ad libitum, and were housed in the animal facility at the Université catholique de Louvain, in a 12-12h light-dark cycle, controlled temperature and humidity conditions. xCT knock-out mice were genotyped by end-point PCR on genomic DNA extracted from an ear biopsy using specific primers for the Slc7a11 gene. PCR amplification products were analyzed by agarose-gel electrophoresis to identify bands revealing the intact or truncated gene (Sato et al. 2005). Since pain hypersensitivity in model of neuropathic pain is sex-dependent, only female mice were herein used to reduce variability.

b. Neuropathic pain model – Partial sciatic nerve ligation (PSNL)

Adult female mice aged between 10 and 12 weeks were randomly subjected to PSNL or sham surgery using a procedure described earlier with minor modifications (Malmberg and Basbaum 1998). Briefly, mice were anesthetized with sevoflurane (4% for induction and 3% for maintenance with oxygen as a carrier gas). After isolation from the surrounding connective tissue, the left sciatic nerve was exposed. With an 8-0 suture, one half to one third of the nerve was tightly ligated above its trifurcation. Muscle and skin layers were finally closed with a 6-0 suture. Sham surgeries were performed by simply exposing the nerve before suturing the wound without performing the sciatic nerve ligation.

c. Mechanical hypersensitivity – von Frey hair filament test

Animals were placed in transparent chambers positioned on an elevated mesh floor. Acclimatization was allowed for 20 min after which the von Frey test was performed. Herein, a set of 10 calibrated von Frey hair filaments (Stoelting Co., Wood Dale, IL) was used (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, 4 and 6 g). Filaments were applied perpendicular to the plantar surface of the hind paw starting with the 0.4 g filament.

To determine the mechanical sensitivity, the “up and down” method was used (Deuis et al. 2017). Briefly, the choice for the following filament was based on the previous filament response, being the closest-lower filament in case of a positive response or the closest-higher filament in case of a negative response. A positive response was defined by a paw withdrawal associated with aversive behavior, such as licking and/or shaking the stimulated paw. This method of von Frey filament application was continued until a sequence of nine filament applications was completed. The 50% paw withdrawal threshold (PWT) was then calculated using the formula previously described

(Chaplan et al. 1994). The experimenter responsible for conducting the behavioral tests was blind to the treatment, the genotype and the surgery performed on the mice. The cumulative changes in PWT from baseline values throughout the entire experiments have been computed and are depicted as the area under the curve (AUC).

d. Thermal hypersensitivity – Hargreaves Test

The thermal paw withdrawal latency (PWL) was used to assess hyperalgesia (Hargreaves et al. 1988). Briefly mice were acclimated for 20 min in separated transparent, bottom-free plastic chambers placed over the glass enclosure of the Hargreaves paw thermal stimulator (University of California, San Diego, CA). A heat source was positioned underneath the plantar surface of the hind paw (both ipsi- and contralaterally) and the time taken to withdraw from the heat source was automatically recorded by the apparatus. Each animal was stimulated 3 times with a 3 min inter-stimulation period. The PWL is averaged per animal and per paw. A cut-off was fixed at 20 seconds of stimulation to avoid any tissue damage. The experimenter responsible for conducting the behavioral tests was blind to the treatment, the genotype and the surgery performed on the mice. As mentioned before, PWL data have been computed as the AUC.

e. Sulfasalazine administration

Animals were randomly assigned to either the treatment or control group. An 80 mg/mL stock solution of sulfasalazine (SAS, Sigma-Aldrich, St. Louis, MO) was prepared by dissolving the powder in DMSO. The final solution was daily prepared by adding saline to reach the desired concentration of SAS. The pH was adjusted using a small volume of NaOH 0.1 M. Mice were daily injected intraperitoneally (i. p.) with 200 mg/kg of SAS (or vehicle that includes the same dilution of DMSO for the controls) (Verbruggen et al. 2021; Tsuchihashi

et al. 2016) starting 2 days prior the sciatic nerve ligation and during the entire experiment until sacrifice, 3 or 7 days after surgery.

f. Tissue collection

Mice were euthanized 3 or 7 days after sciatic nerve or sham surgery with CO₂ and the spinal cord was immediately extruded with phosphate-buffered saline (PBS) as previously described (Meikle and Martin 1981). The tissue from the lumbar enlargement of the spinal cord was isolated. A first longitudinal cut was performed along the ventral-median fissure exposing two halves corresponding to the ipsi- and contralateral sides. Another longitudinal cut along the central canal of each halves was performed to collect four quadrants. The dorsal quadrants were rapidly frozen in liquid nitrogen and finally stored at -80°C before being processed for RNA extraction or synaptosome preparation. For immunohistochemistry, animals were transcardially perfused with PBS before lumbar spinal cord collection.

g. Total RNA extraction and real-time quantitative PCR (RT-qPCR)

Ipsi- and contralateral lumbar dorsal spinal cord samples were frozen and mechanically dissociated using a pre-chilled Teflon/glass homogenizer in TriPure isolation reagent (Roche, Bâle, Switzerland), followed by RNA extraction according to the manufacturer's protocol. Reverse transcription was carried out with 1 µg of extracted RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA) in a total volume of 20 µL. Real-time qPCR amplifications were then performed using a 3-steps protocol with the Bio-Rad CFX Connect™ real-time PCR detection system (Bio-Rad Laboratories), in a total volume of 20 µL containing iTaq Universal SYBR® Green Supermix (Bio-Rad Laboratories), cDNA (equivalent to 10 ng retrotranscribed RNA) and a final concentration of 0.5 µM of each primer. Quantitative analysis was performed using the delta-delta Ct method,

normalized to the relative expression of 3 different housekeeping genes (Ywhaz, RPL-19 and HPRT-1) previously validated for RT-qPCR studies on injured nervous tissues (Xu, Liu, et al. 2018; Košuth et al. 2019). The sequences of the primers used in this study are indicated in Table 2.

Table 2 : RT-qPCR primer sequences.

Gene	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>xCT</i>	GATGCTGTGCTTGGTCTTGA	GCCTACCATGAGCAGCTTTC
<i>GFAP</i>	GAGGGACAACCTTGCACAGG	TCCTCCAGCGATTCAACCTT
<i>Iba-1</i>	GGATTGCAGGGAGGAAAAG	TGGGATCATCGAGGAATTG
<i>Nox-2</i>	TGAATGCCAGAGTCGGGATTT	CCCCCTTCAGGGTTCTTGATTT
<i>Arg-1</i>	CGTGTACATTGGCTTGCGAG	ATCACCTTGCCAATCCCCAG
<i>HPRT-1</i>	CCTAAGATGAGCGCAAGTTGAA	CCACAGGACTAGAACACCTGCTAA
<i>RPL-19</i>	TGACCTGGATGAGAAGGATGAG	CTGTGATACATATGGCGGTCAATC
<i>Ywhaz</i>	TAGGTCATCGTGGAGGGTCG	GAAGCATTGGGGATCAAGAACTT

h. Crude synaptosome preparation and [³H]-L-glutamate uptake assay

Frozen dorsal spinal cord samples were used for synaptosome preparation and for the measurement of system x_c⁻ driven [³H]-L-glutamate transport. The method is based on the reversal uptake of glutamate when performed in the absence of extracellular cystine and in the absence of Na⁺, as validated in our previous study (Beckers et al. 2021). Briefly, the tissue was homogenized and

centrifuged in ice-cold isotonic solution. The final pellet containing the synaptosomes was diluted in ice-cold buffer lacking Na^+ , in order to avoid any detection of EAAT-mediated glutamate uptake. Protein concentration was determined, and samples were diluted to achieve a final protein concentration of 33.33 $\mu\text{g}/\text{mL}$.

To assess the uptake, [^3H]-L-glutamate (with specific activity of 48.6 Ci/mmol, Perkin Elmer, Waltham, MA) was used as substrate at a concentration of 20 nM. In a total volume of 500 μL , 10 μg of the synaptosome preparation was incubated with the substrate at 37°C in a 96-well Masterblock (Greiner Bio-one, Kremsmünster, Austria). When indicated homocysteic acid (HCA, H9633, Sigma-Aldrich), a system x_c^- inhibitor, was added to evaluate the HCA-dependent uptake. After 20 min incubation at 37°C, the suspension was filtered (UniFilter GF/B, PerkinElmer), and washed with ice-cold Na^+ -free buffer. After drying overnight, a liquid scintillation solution, Microscint 20 (PerkinElmer) was added to each well and the radioactivity measurements were done using the Topcount® NXT Microplate scintillation and luminescence counter (PerkinElmer). Results are expressed as a percentage of the respective [^3H]-L-glutamate uptake in the contralateral side.

i. Immunohistochemistry

Lumbar spinal cords were used for immunohistochemistry as previously described by Gallo et al. with minor modifications (Gallo et al. 2017). Briefly, after tissue collection, lumbar spinal cords were fixed overnight in 4% paraformaldehyde in PBS. Cryoconservation was achieved by successively incubating the samples in 10%, 20% and 30% sucrose/PBS solution. Afterwards, spinal cords were frozen and stored at -80°C until further use. For cryosectioning, samples were embedded in tissue Tek O.C.T (Sakura, Osaka, Japan) and transversal sections of 20 μm were cut using a CM3050S Leica cryostat (Leica Microsystems, Wetzlar, Germany). Cryosections were collected on Superfrost Plus object glass slides (Thermo Fisher Scientific,

Waltham, MA) and stored at -20°C until immunohistological staining. After a dry time of 1 h at room temperature (RT), sections were washed in PBS and incubated in a blocking buffer (5% normal goat serum, 0.5% Triton in PBS) for 1 h at RT. Sections were then incubated overnight at 4°C with either of the following primary antibodies diluted in the blocking buffer: anti-Iba1 (rabbit, 1:1.000, Wako, Osaka, Japan, cat. no. 019/19741 provided at 0.5 mg/mL) or anti-GFAP (chicken, 1:1.000, Abcam, Cambridge, UK, cat. no. ab4674 provided at 1 mg/mL). The next day, sections were rinsed in PBS, and were incubated for 2 h at RT with anti-rabbit or anti-chicken secondary antibodies conjugated to Alexa Fluor 647 (Thermo Fisher Scientific A21245 and A21449, respectively, both provided at 2 mg/ml and diluted 1:500 in PBS). Afterwards, sections were again washed with PBS and mounted on glass slides using EverBrite mounting medium (Biotium, Fremont, CA). Images were examined with an EVOS fluorescence microscope (AMG EVOS fl, Westburg, Leusden, Netherlands). The fluorescent intensity was quantified using the mean grey value of the defined area (ImageJ software).

j. Statistical analyses

Data from biochemical studies (uptake assay, RT-qPCR, immunohistochemistry, and AUC), statistical analyses were performed on the difference in log-transformed ipsilateral versus contralateral measures for each mouse in order to control for individual variability. Normality was assessed using the Shapiro-Wilk normality test, and equality of variances was tested using the Levene test. Graphs depicting the back-transformed data are provided for all experiments to facilitate interpretation. As such, results are presented as geometric mean for biochemical endpoints and as arithmetic mean for behavioral endpoints. Statistical analyses were performed using one or two-way ANOVA model, depending on the experimental design. In case where a significant interaction was observed in the two-way ANOVA model, Tukey's pairwise comparison method was employed. When appropriate and

identified by the Grubb's test, significant outliers were removed from the analysis set. For all analyses, a nominal two-sided Type I error of 0.05 was considered for statistical significance. Statistical analyses were conducted using GraphPad Prism 9.5.1 and JMP Pro 17 software.

3. Results

a. PSNL triggers increased xCT expression and system x_c^- activity in the ipsilateral spinal cord

To examine the contribution of system x_c^- in neuropathic pain, the impact of a peripheral nerve lesion on the expression of the specific subunit xCT and system x_c^- activity was assessed by RT-qPCR and ^3H -glutamate uptake, respectively. Animals were sacrificed 3 days or 7 days after surgery and quadrant at the ipsi- and contralateral lumbar dorsal spinal cord were dissected and processed for analyses. Three days after PSNL, xCT mRNA level was significantly increased in the ipsilateral dorsal quadrant of the lumbar spinal cord. As shown in figure 18A and B, a 70% and 20% increase in lesioned mice as compared to the sham-operated mice was measured for xCT expression and system x_c^- activity, respectively. This change in xCT expression appeared transient as it was absent from samples collected 7 days after surgery (Figure 18C). However, the increased system x_c^- activity was even more important in synaptosomes prepared from spinal cord samples 7 days after surgery (130% increase in the ipsilateral dorsal quadrant of lesioned mice as compared to the sham-operated mice) (Figure 18D).

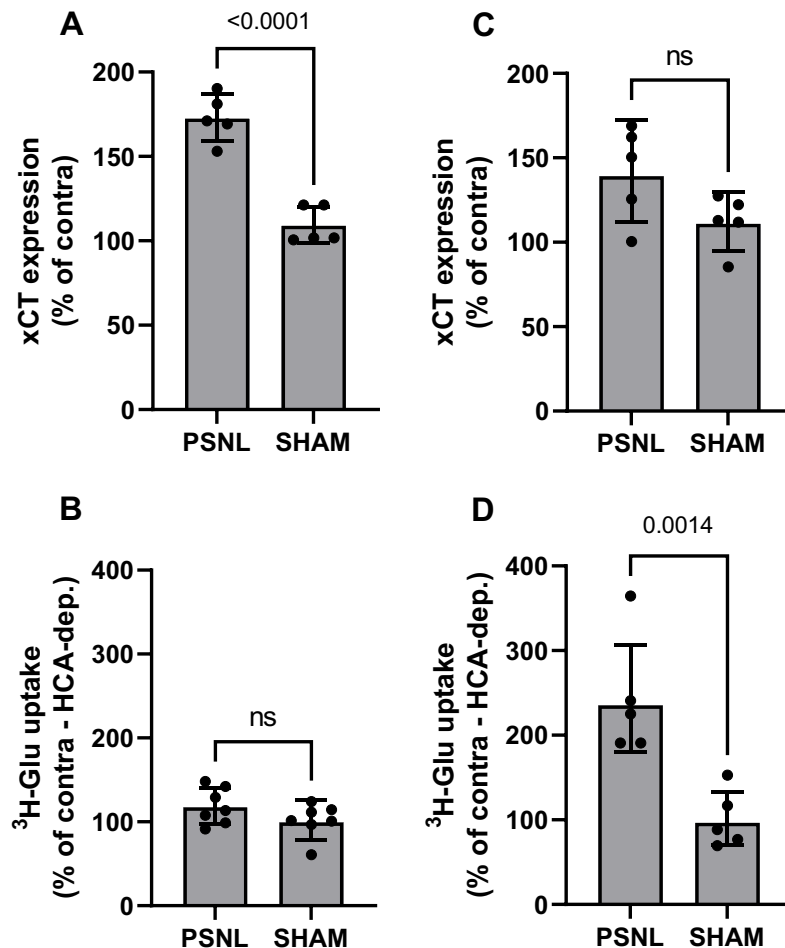


Figure 18: xCT expression and activity in the lumbar dorsal spinal cord following PSNL or sham surgery. Three (panel A & B) and seven (panel C & D) days post-surgery, the ipsilateral and contralateral dorsal horn quadrants of the lumbar spinal cord were dissected and used for RT-qPCR or functional assay. xCT mRNA was quantified, normalized to the mean of 3 housekeeping genes (RPL-19, Ywhaz, HPRT-1) and expressed as a percentage of the respective contralateral mRNA level (A, C). Uptake of $^3\text{H-L-glutamate}$ on crude spinal synaptosomes was used to determine system x_c^- functionality (B, D). Ipsilateral system x_c^- specific uptake (HCA-dependent) is expressed as relative (%) to the respective contralateral side. As shown on the figure, data were obtained from 5-7 different animals per group and are presented as

geometric mean with SD. Statistical analyses were conducted using a one-way ANOVA model. *PSNL*, *partial sciatic nerve ligation*; *Glu*, *glutamate*; *HCA*, *homocysteic acid*.

b. Mice lacking xCT present a higher pain tolerance following PSNL

The role of system x_c^- in the development of pain sensitivity was examined by subjecting wild-type ($xCT^{+/+}$) and xCT -deficient ($xCT^{-/-}$) mice to unilateral PSNL. Female mice were monitored for 7 days post-injury using a von Frey (Figure 19A-C) and a Hargreaves test (Figure 19D-F) to respectively evaluate lesion-associated allodynia and hyperalgesia. In the absence of nerve lesion, mice lacking xCT did not present any signs of hypersensitivity to mechanical or thermal stimuli compared to their wild-type littermates. Also, sham-operated $xCT^{-/-}$ mice showed similar PWT (Figure 19A-C) and PWL (Figure 19D-F) as compared to non-lesioned $xCT^{+/+}$ mice. As shown in figure 19A, nerve-lesioned $xCT^{+/+}$ animals exhibited prolonged and strong mechanical allodynia starting from day 1 after surgery. The 50% PWT reached the minimum value of 0.19 ± 0.04 g in injured $xCT^{+/+}$ animals compared to 3.49 ± 0.26 g in the sham-operated animals. In mice lacking xCT , we also observed a decreased threshold following the sciatic nerve ligation (1.39 ± 0.21 g compared to 3.22 ± 0.32 g for sham-operated animals). However, this decrease was less pronounced compared to what was observed in $xCT^{+/+}$ operated animals (Figure 19A&B). Moreover, the PWT to mechanical stimulation returned to baseline values as from the sixth day after surgery, indicating that the hypersensitivity observed in $xCT^{-/-}$ animals was transient. At variance, the decreased pain threshold observed in the $xCT^{+/+}$ injured mice persisted for the entire duration of the experiment. No changes were observed in the contralateral paw when tested for mechanical sensitivity (Figure 19C).

When testing the response to a thermal stimulus using the Hargreaves test, $xCT^{+/+}$ mice undergoing PSNL developed a strong and persistent hyperalgesia as indicated by the decreased latency for ipsilateral paw withdrawal reaching

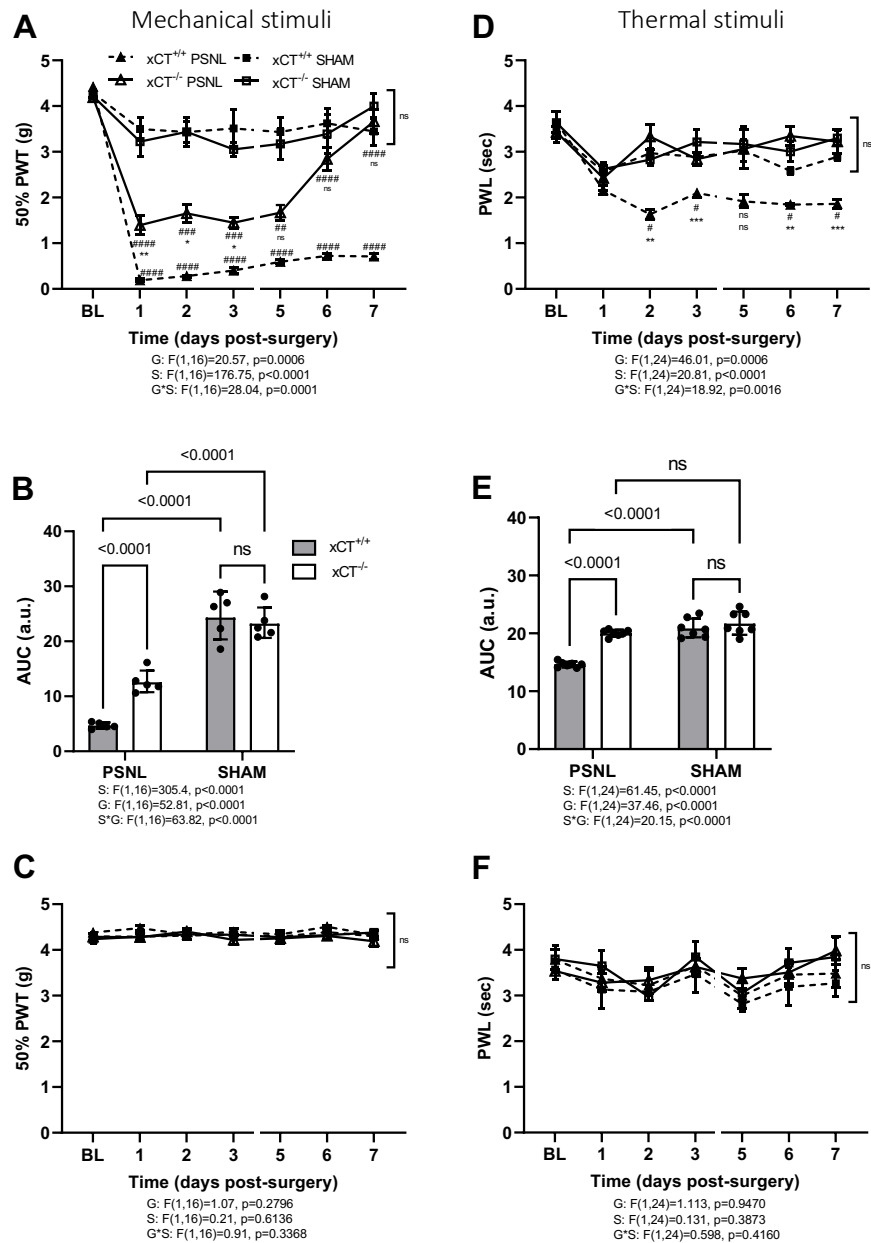


Figure 19 : Allodynia and hyperalgesia in $xCT^{+/+}$ and $xCT^{-/-}$ mice following PSNL or sham surgery. Pain hypersensitivity was assessed upon stimulation of the ipsilateral (A, B, D, E) and contralateral (C, F) hind paw at baseline (BL) and for up to 7 days after surgery. The 50% PWT to mechanical stimulation

was used to determine allodynia (A-C), whereas the PWL to thermal stimulation was used as a read-out of hyperalgesia (D-F). Panel B and E depict the area under the curve (AUC in arbitrary unit a.u.) of the ipsilateral 50% PWT and PWL, respectively. Data are presented as arithmetic mean with SEM (A, C, D, F) or geometric mean with SD (B, E) of 5 animals per group for allodynia and 7 animals per group for hyperalgesia. Statistical analyses were performed through a two-way ANOVA model (B, E) with repeated measures (A, C, D, F). Main and interaction effects are indicated below the graphs. When statistically significant interaction was detected, Tukey's pairwise comparisons were conducted. P-values for significant effects are reported on the graphs (B, E). In panel A and D, * indicates a significant difference between lesioned wild-type (PSNL xCT^{+/+}) and transgenic mice lacking xCT (PSNL xCT^{-/-}), # indicate a significant difference between lesioned and non-lesioned animals (within the same genotype). *PSNL*, partial sciatic nerve ligation; *PWT*, paw withdrawal threshold; *PWL*, paw withdrawal latency; *AUC*, area under the curve; *S*, surgery effect; *G*, genotype effect; *S*G*, interaction effect.

1.63 ± 0.11 s at 2 days post-injury (Figure 19D). In contrast, mice lacking xCT did not develop any thermal hyperalgesia. Indeed, at all tested time points following the surgery, the PWL of injured xCT^{-/-} mice was similar to what was observed in sham-operated animals (AUC of 19.98 ± 0.39 for xCT^{-/-} injured mice and 21.75 ± 0.75 for uninjured xCT^{-/-} mice) (Figure 19E). No changes were observed when testing the contralateral hind paw for thermal sensitivity (Figure 19F).

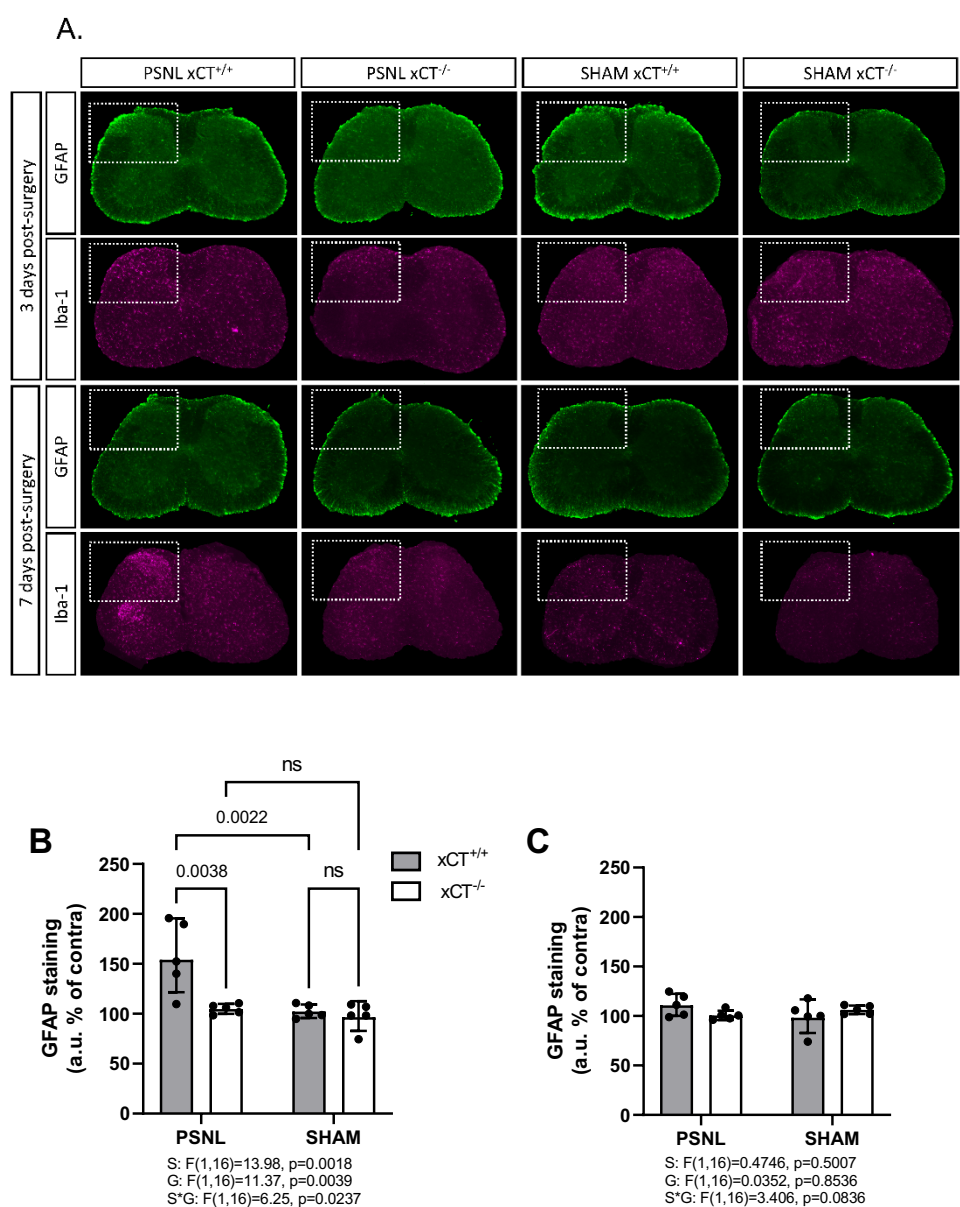
c. xCT^{-/-} mice show reduced astrogliosis and a distinct microglial polarization after PSNL

Pain sensitization following peripheral nerve injury often coincides with pronounced glial activation in the ipsilateral spinal cord. Astroglial and microglial activations are commonly authenticated by evidencing an increased expression of the typical markers glial fibrillary acidic protein (GFAP) and ionized calcium-binding adapter molecule 1 (Iba-1), respectively.

Changes in the protein expression (Figure 20) and mRNA (Figure 21) levels of both glial markers were therefore examined by immunohistochemistry on lumbar spinal cord sections and RT-qPCR on the dorsal quadrants of the lumbar spinal cord of xCT^{+/+} and xCT^{-/-} mice undergoing PSNL or sham surgeries. As expected, GFAP was significantly upregulated (1.5-fold-increase for protein level and up to 3-fold-increase for mRNA) in the spinal cord of xCT^{+/+} mice 3 days after unilateral surgery as compared to the contralateral side or to sham-operated animals (Figure 20A&B & 21A). This was however not observed in samples collected 7 days after the surgery (Figure 20C & 21B). The early upregulation of GFAP was less pronounced (1.6-fold-increase for mRNA) in xCT^{-/-} mice indicating a lower astrocytic activation following nerve surgery (Figure 20B & 21A).

Regarding microglial activation, a 2-fold increase in Iba-1 mRNA level was observed in the ipsilateral spinal cord of both xCT^{+/+} and xCT^{-/-} mice, 3- and 7-days post-surgery compared to the contralateral side or to the sham-operated animals (Figure 21C&D). Iba-1 immunoreactivity was found to be increased (1.2-fold after 3 days and 1.6-fold after 7 days) in the ipsilateral spinal cord of xCT^{+/+} animals following PSNL compared to sham-operated animals or to the contralateral side. This, however, was not observed in transgenic animals lacking xCT where no alteration was detected (Figure 20D&E). Upon gliosis, microglial cells change their expression of several inflammation-related genes, and thereby adopt predominantly pro- or anti-inflammatory phenotypes (Zhang, Wei, et al. 2018). The expression of NADPH oxidase 2 (NOX-2) and arginase 1 (Arg-1) was examined to monitor the polarization of reactive microglial cells in the spinal cord of mice after PSNL (Figure 21E-H). In the ipsilateral dorsal quadrant of the lumbar spinal cord of xCT^{+/+} mice, 3 days after surgery, the expression of the pro-inflammatory marker NOX-2 was upregulated (2.8-fold increase) while the anti-inflammatory gene Arg-1 was downregulated (2-fold decrease). At variance, a trend for an upregulation of NOX-2 (1.5-fold increase) was detected in the ipsilateral spinal cord of xCT^{-/-} mice after PSNL, while no changes were

observed regarding Arg-1 mRNA level (Figure 21E&G). Additionally, in the lumbar spinal cord of $xCT^{+/+}$ and $xCT^{-/-}$ mice the expression of both inflammation-related microglial markers were unchanged when examined 7 days post-surgery (Figure 21F&H).



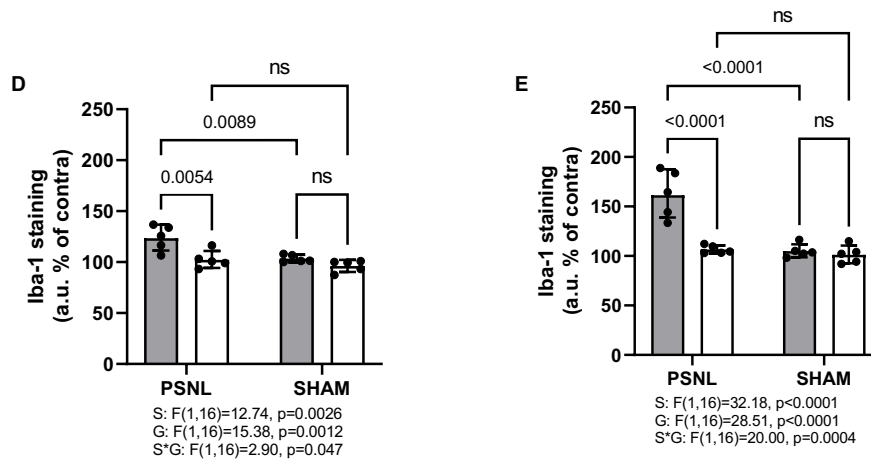
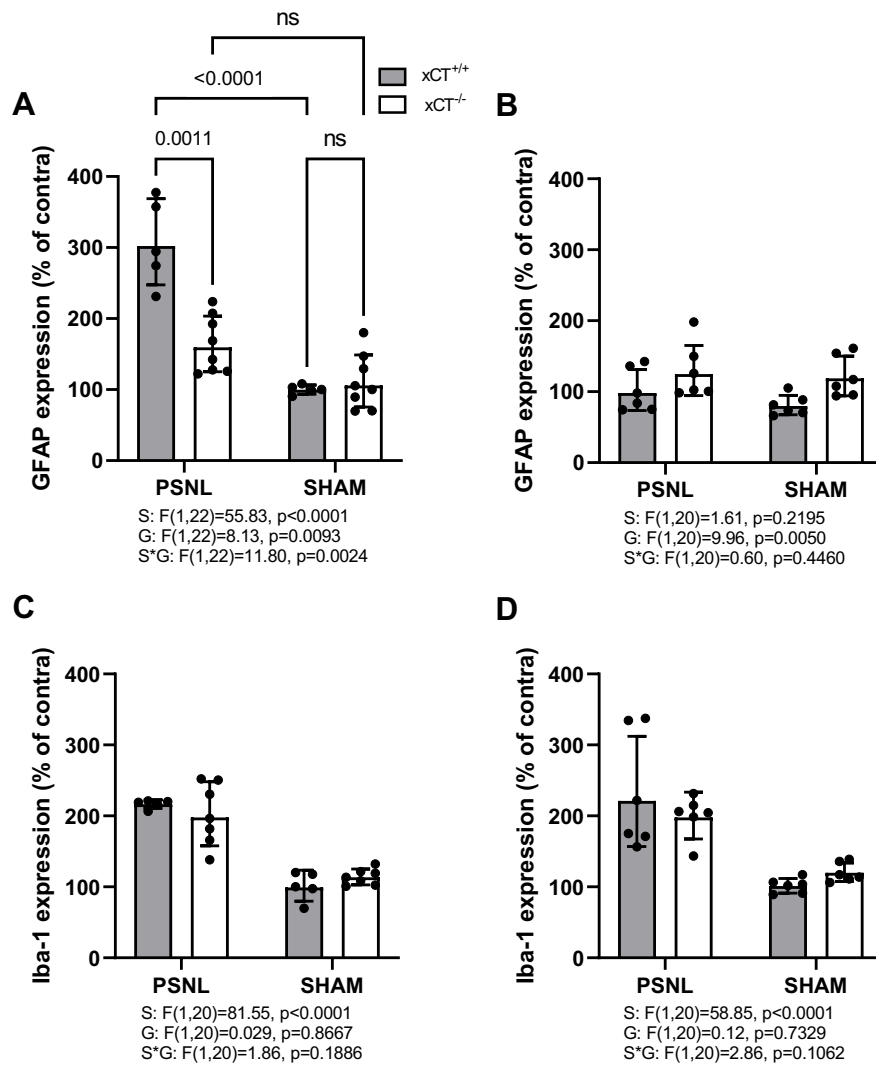


Figure 20 : Glial activation in the spinal cord following PSNL or sham surgery in $xCT^{+/+}$ and $xCT^{-/-}$ mice. Immunohistological stainings for GFAP and Iba-1 were analyzed 3 (A, B, D) and 7 (A, C, E) days after the surgery in samples from wild-type or transgenic mice lacking xCT (A). The fluorescent intensity for GFAP (B, C) and Iba-1 (D, E) in the dorsal horns was quantified. The results from the ipsilateral side are normalized and expressed as a percentage of the respective contralateral side. Images shown are representative of 5 different animals and histograms represent the geometric means with SD. Statistical analyses were performed through a two-way ANOVA model. Main and interaction effects are indicated below the graphs. When statistically significant interaction was detected, Tukey's pairwise comparisons were conducted. P-values for significant effects are reported on the graphs. *PSNL*, partial sciatic nerve ligation; *GFAP*, glial fibrillary acidic protein; *Iba-1*, ionized calcium binding adapter protein 1; *S*, surgery effect; *G*, genotype effect; *S*G*, interaction effect.



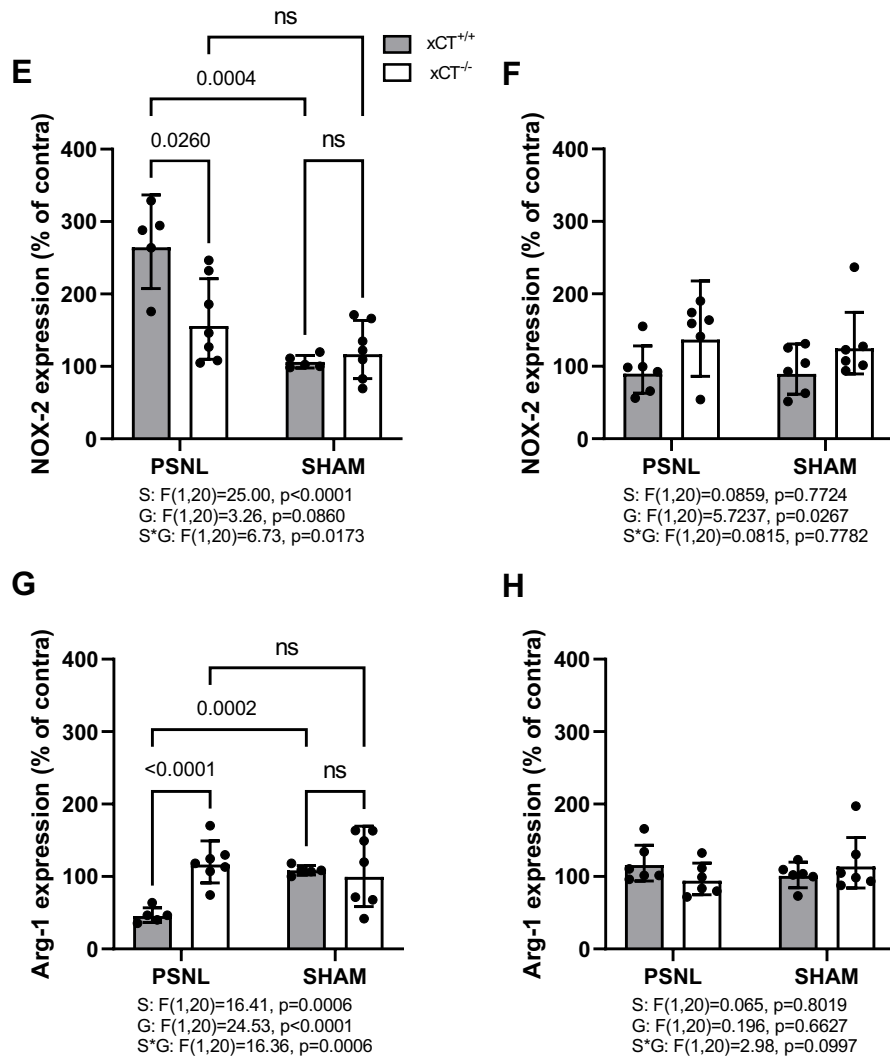


Figure 21 : Assessment of spinal mRNA of glial markers following PSNL or sham surgery in xCT^{+/+} and xCT^{-/-} mice. Three (left panels) and seven (right panels) days post-surgery, the ipsilateral and contralateral quadrants of the dorsal horn of the lumbar spinal cord were dissected and used for RT-qPCR analyses. Specific markers were used to evaluate astrocytic (A, B) and microglial (C, D) activation, GFAP and Iba-1 respectively. Other markers were used to assess microglia activation profile towards pro-inflammatory (NOX-2: panel E & F) and anti-inflammatory (Arg-1: panel G & H) phenotype. For all

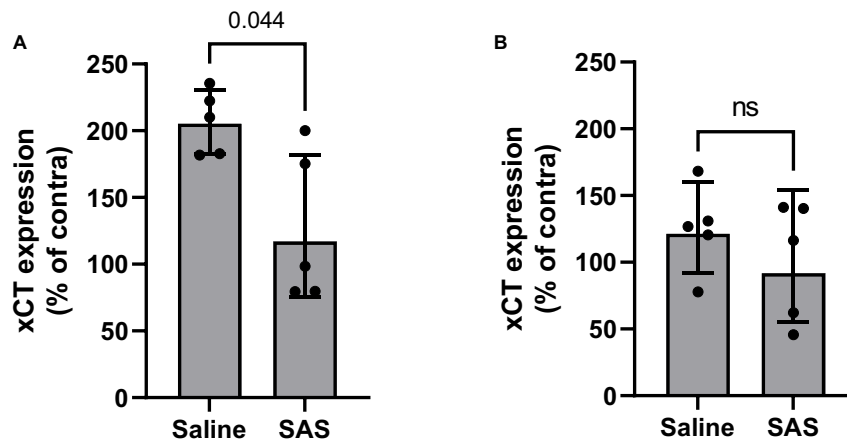
markers, the mRNA was quantified, normalized to the mean of 3 housekeeping genes (RPL-19, Ywhaz, HPRT-1) and expressed as a percentage of the respective contralateral mRNA level. As shown on the figure, data were obtained from 5-7 different animals per group and are presented as geometric mean with SD. Statistical analyses were performed through a two-way ANOVA model. Main and interaction effects are indicated below the graphs. When statistically significant interaction was detected, Tukey's pairwise comparisons were conducted. P-values for significant effects are reported on the graphs. *PSNL*, partial sciatic nerve ligation; *GFAP*, glial fibrillary acidic protein; *Iba-1*, ionized calcium binding adapter protein 1; *NOX-2*, NADPH oxidase 2; *Arg-1*, Arginase 1; *S*, surgery effect; *G*, genotype effect; *S*G*, interaction effect.

d. SAS has an anti-allodynic and anti-hyperalgesic effect

To assess the potential of targeting system x_c^- in preventing the development of neuropathic pain, a pharmacological approach was employed. Both $xCT^{+/+}$ and $xCT^{-/-}$ mice subjected to PSNL surgery were treated daily with the inhibitor of system x_c^- , SAS, or with vehicle. Treatment was initiated 2 days before PSNL and pursued until sacrifice. Mice were monitored daily for allodynia and hyperalgesia for up to 3 or 7 days after surgery, at which stage spinal cord tissue was collected for RT-qPCR analysis. As mentioned before, 3 days after PSNL, the mRNA level of xCT was significantly increased in the ipsilateral dorsal quadrant of the lumbar spinal cord (2-fold increase as compared to the contralateral quadrant). This change in xCT expression was nearly completely reversed by the administration of SAS (Figure 22A). Also, this effect appeared transient as xCT mRNA level remained unchanged 7 days post-surgery and SAS treatment was without influence at that stage (Figure 22B).

Regarding the influence of the treatment on the pain-associated behaviors, it was observed that baseline measurements of mechanical and thermal pain thresholds remained unaltered by SAS treatment in the absence of surgery (Figure 22C-H). Consistent with here-above detailed data, vehicle-treated

xCT^{+/+} mice undergoing PSNL presented a robust ipsilateral mechanical allodynia (Figure 22C-E) and thermal hyperalgesia (Figure 22F-H) that persisted for at least 7 days. Conversely, xCT^{+/+} mice receiving SAS showed a reduced hypersensitivity to the mechanical stimulus as they presented a higher 50% PWT compared to vehicle-treated mice (AUC of 11.93 ± 0.23 for SAS-treated mice and 8.18 ± 0.53 for control group, $p < 0.0001$) (Figure 22C&D). No changes were observed when testing the contralateral paw for mechanical sensitivity (Figure 22E). Besides, when challenged with thermal stimulation, SAS-treated xCT^{+/+} mice did not develop any signs of thermal hypersensitivity. Indeed, at all tested time points, the PWL was similar to the values observed at baseline (Figure 22F-H). To confirm that the observed effects were specifically driven by the inhibition of system x_c⁻, lesioned-mice lacking xCT were also subjected to the same SAS treatment. In both behavioral tests, no significant differences in the pain thresholds were observed when comparing SAS- and vehicle-treated xCT^{-/-} mice.



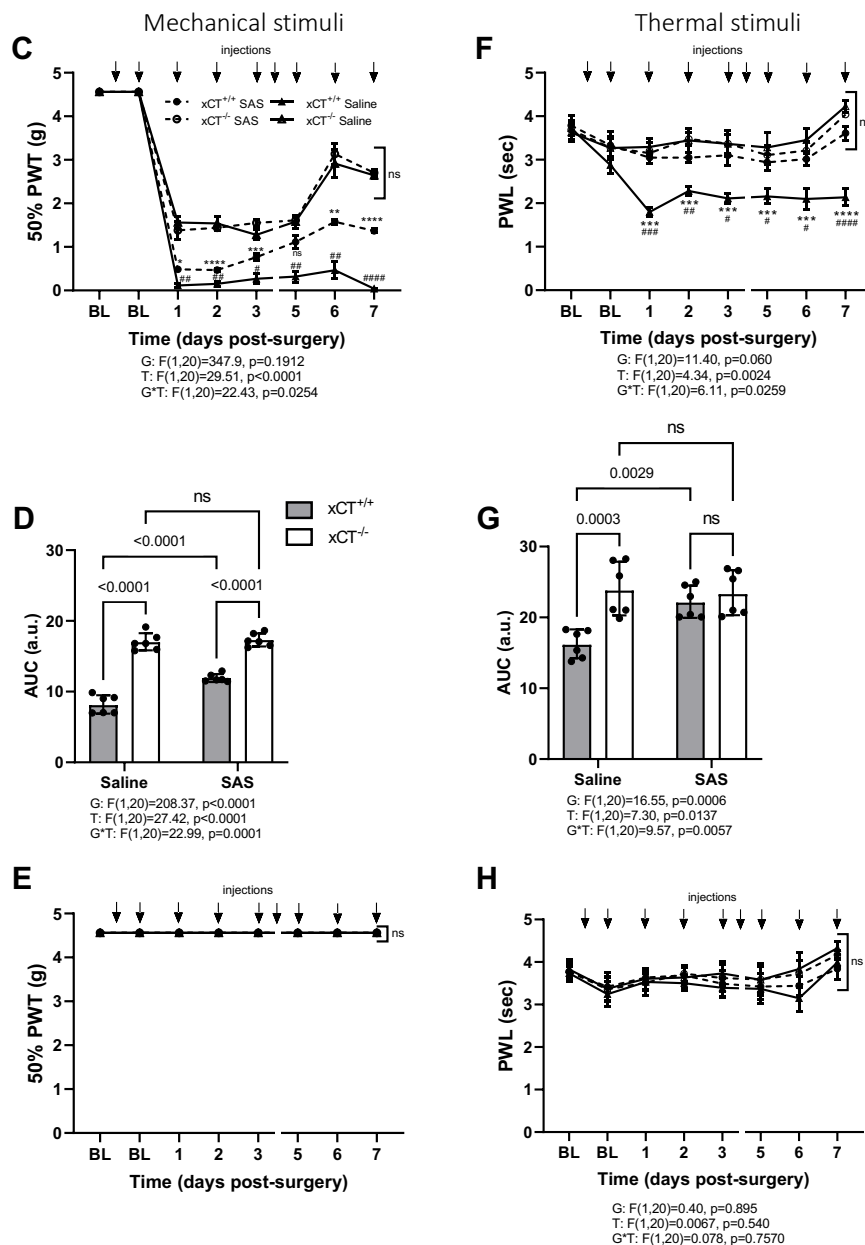


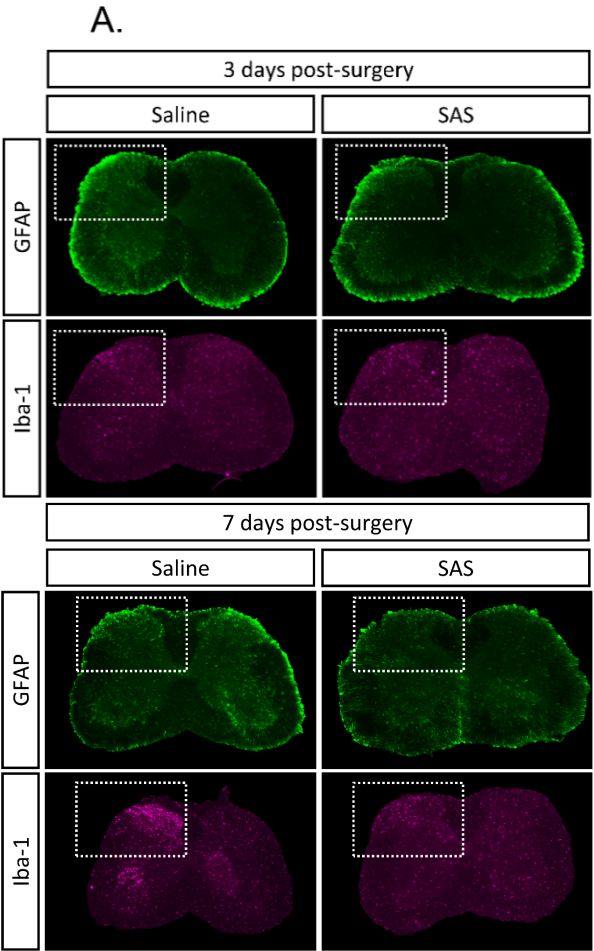
Figure 22 : Effect of SAS administration on the spinal expression of xCT and the pain hypersensitivity following PSNL surgery. Three (A) and seven (B) days post-surgery, the ipsilateral and contralateral dorsal horn quadrants of the lumbar spinal cord of lesioned- $xCT^{+/+}$ mice were dissected and used for RT-qPCR. xCT mRNA was evaluated, normalized to the mean of 3

housekeeping genes (RPL-19, Ywhaz, HPRT-1) and expressed as a percentage of the respective contralateral mRNA level. Pain hypersensitivity was assessed for the ipsilateral (C, D, F, G) and contralateral (E, H) hind paw at baseline (BL) and for 7 days following PSNL surgery. The 50% PWT to mechanical stimulation was used to determine allodynia (C-E), whereas the PWL to thermal stimulation was used as a read-out of hyperalgesia (F-H). Panel D and G depict the area under the curve for the ipsilateral 50% PWT and PWL, respectively. Data are presented as arithmetic mean with SEM (C, E, F, H) or geometric means with SD (D, G) (5 animal per group for mRNA analysis and 6 animal per group for behavioral tests). Statistical analyses were performed using a one-way ANOVA (A, B) or a two-way ANOVA (D, G) model with repeated measures (C, E, F, H). Main and interaction effects of the two-way ANOVA model are indicated below the graphs. When statistically significant interaction was detected, Tukey's pairwise comparisons were conducted. P-values for significant effects are reported on the graphs. In panel C and F, * indicates a significant difference between SAS-treated wild-type ($xCT^{+/+}$ SAS) and SAS-treated transgenic mice lacking xCT ($xCT^{-/-}$ SAS), # indicate a significant difference between SAS-treated and saline-treated animals (within the same genotype). *PSNL*, partial sciatic nerve ligation; *PWT*, paw withdrawal threshold; *PWL*, paw withdrawal latency; *AUC*, area under the curve; *I*, ipsilateral; *C*, contralateral; *SAS*, sulfasalazine; *G*, genotype effect; *T*, treatment effect; *G*T*, interaction effect.

e. SAS reduces astrocytic and microglial activation after PSNL

The effects of SAS treatment on the PSNL-induced glial activation were examined by immunohistochemistry (Figure 23) and RT-qPCR (Figure 24) for GFAP and Iba-1, in the dorsal quadrants of the lumbar spinal cord of $xCT^{+/+}$ mice daily treated with either SAS or vehicle. As previously described, lesioned-mice displayed increased ipsilateral expression of GFAP and Iba-1 (Figure 23A). Intraperitoneal treatment with SAS strongly reduced immunoreactivity of both glial markers (Figure 23B-E) particularly with respect to Iba-1 staining, 7 days after the surgery (Figure 23E). These changes were not observed by RT-qPCR (Figure 24A-D). However, pharmacological inhibition of system x_c^- leads to a significant reduction of NOX-2 expression (from 333.19 ± 51.54 % for the vehicle-treated mice to 109.86 ± 40.76 % for the SAS-treated group 3 days after the surgery, $p < 0.01$) (Figure 24E&F). No

significant difference was observed concerning the expression of Arg-1, the marker used to monitor the anti-inflammatory phenotype of microglia (Figure 24G&H).



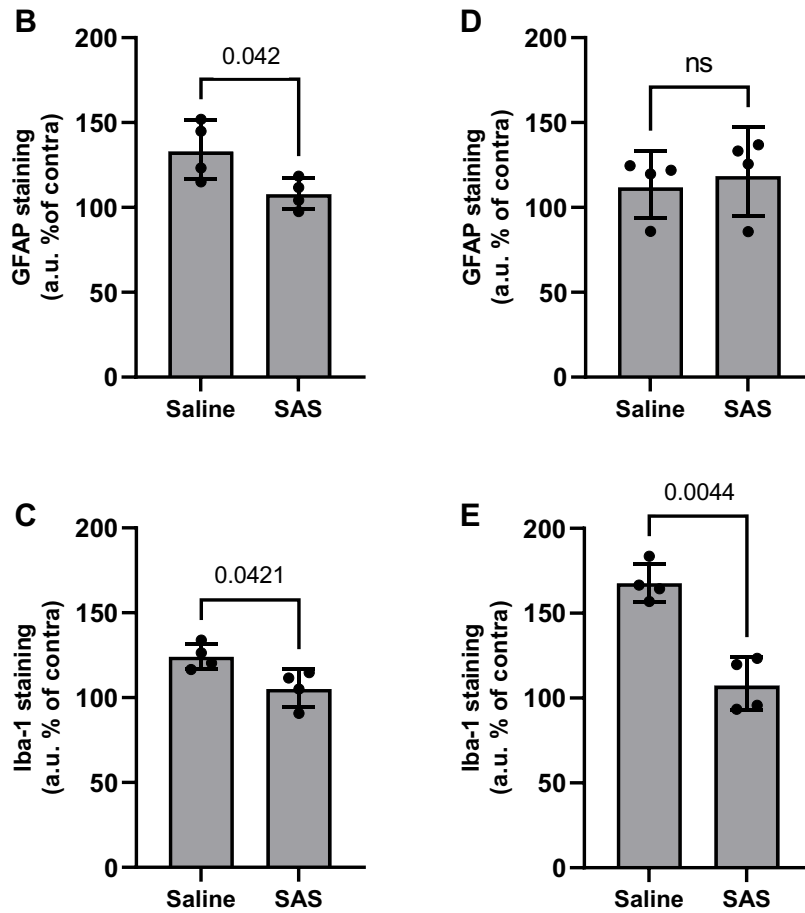


Figure 23: Effect of SAS administration on the spinal glial reactivity following PSNL surgery. Immunohistological staining of GFAP and Iba-1 was performed on lumbar spinal cord samples of $xCT^{+/+}$ mice at 3 (A, B, C) and 7 days (A, D, E) after surgery. The fluorescent intensity for GFAP (B, D) and Iba-1 (C, E) in the dorsal horns was quantified. The results from the ipsilateral side are normalized and expressed as a percentage of the respective contralateral side. Images shown are representative of 4 different animals per group and histograms represent the geometric means with SD. Statistical analyses were performed using a one-way ANOVA model. *GFAP*, glial fibrillary acidic protein; *Iba-1*, ionized calcium binding adapter protein 1; *SAS*, sulfasalazine.

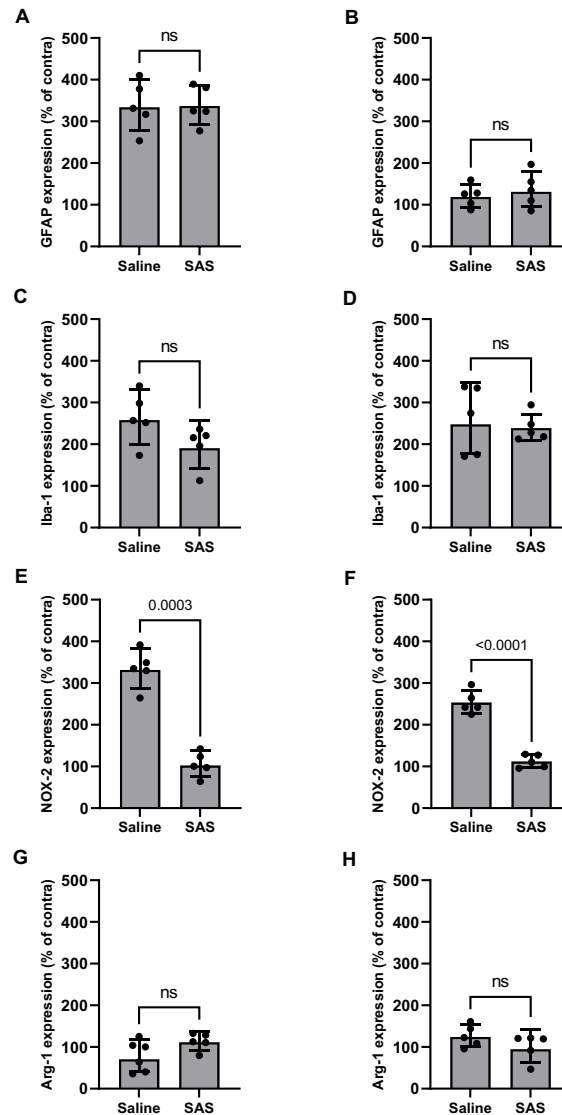


Figure 24: Effect of SAS on spinal mRNA of astrocytic and microglial markers after PSNL surgery in $xCT^{+/+}$ mice. Three (left panels) and seven (right panels) days post-surgery, the ipsilateral and contralateral quadrants of the lumbar spinal cord were dissected and used for RT-qPCR analysis. GFAP and Iba-1 were used to evaluate astrocytic (A, B) and microglial (C, D) activation, respectively. Other markers were used to assess microglia activation profile towards pro-inflammatory (NOX-2: panel E & F) and anti-

inflammatory (Arg-1: panel G & H) phenotype. For all markers, the mRNA levels were normalized to the mean of 3 housekeeping genes (RPL-19, Ywhaz, HPRT-1) and expressed as a percentage of the respective contralateral mRNA level. Data were obtained from 5 different animals per group and are presented as geometric means with SD. Statistical analyses were performed using a one-way ANOVA model. *GFAP*, glial fibrillary acidic protein; *Iba-1*, ionized calcium binding adapter protein 1; *NOX-2*, NADPH oxidase 2; *Arg-1*, Arginase 1; *SAS*, sulfasalazine.

4. Discussion

Peripheral nerve injuries often trigger maladaptive plasticity in nociceptive pathways, resulting in the pathological amplification of excitatory transmission. This is well documented in the dorsal horn of the spinal cord, which serves as the first relay center for integrating and regulating nociceptive information, contributing to the development of chronic pain (Latremoliere and Woolf 2009; Liu and Salter 2010). The reinforcement of glutamate transmission can be attributed to different mechanisms, such as an enhanced neuronal release of glutamate causing an increased activation of glutamate receptors and associated downstream signaling pathways (Liu et al. 2015; Qu et al. 2009). Besides, impaired glutamate clearance by high affinity glial transporters is also documented (Guo et al. 2019; Cavaliere et al. 2007). Modulating the molecular mechanisms that regulate glutamate transmission holds promise in preventing or alleviating chronic pain. Indeed, drugs that enhance glutamate transporter activity or antagonizing glutamate receptors were proven effective in models of neuropathic pain, but potential side effects would restrict their clinical use (Wu and Zhuo 2009; Hu et al. 2010; Simmons et al. 2002).

In addition to extensive research on glutamate transporters, accumulating evidence has recently emphasized the role of system x_c^- in modulating glutamate transmission (Baker et al. 2002). Widely distributed throughout the CNS, this cystine/glutamate exchanger responsible for the non-vesicular

glutamate release is not only implicated in the modulation of excitatory signals, but also in the defense against oxidative stress and neuroinflammation (Burdo et al. 2006; Sato et al. 2002). This dual role played by system x_c^- supports a putative involvement in diverse neurological pathologies, where both dysregulation of glutamate signaling and persistent neuroinflammation contribute to neuronal damages (Lewerenz et al. 2013). This was also evidenced in a model of bone cancer pain where increased expression of system x_c^- supports the release of glutamate, contributing to the activation of sensory neurons (Zhu, Linher-Melville, et al. 2020). In this context, the present study using a model of peripheral nerve lesion provides compelling evidence on the role played by this exchanger in the physio-pathological mechanisms of neuropathic pain.

In both rats and mice, the PSNL is a commonly used model of surgical nerve lesion to study neuropathic pain. Within a few hours after the nerve ligation, rodents exhibit signs of thermal hyperalgesia, mechanical allodynia and spontaneous pain that persist for several months (Seltzer et al. 1990). This lesion has been reported to trigger plasticity in the primary afferent fibers and sustained neuroinflammation in the dorsal lumbar spinal cord, a hallmark of central sensitization (Cavaliere et al. 2007). In the present study, the invalidation of system x_c^- appeared to influence both the amplitude and the duration of the pain sensitization after PSNL. Compared to wild-type mice, xCT knock-out mice exhibited reduced sensitivity to mechanical stimulation from the first day post-surgery, with pain sensitivity returning to baseline within just 6 days.

While the nociceptive behavior of mice lacking xCT was improved at both early and late stages after ligation, it is noteworthy that the upregulation of xCT mRNA in the spinal cord of wild-type animals was primarily observed at early stage after PSNL. This suggests a predominant implication of system x_c^- at the onset of the sensitization process. Hence, in the model of bone cancer-induced pain, the inhibition of system x_c^- was shown to delay the onset of

nociception (Zhu, Linher-Melville, et al. 2020). Accumulating evidence indicates that an inadequate management of acute pain can lead to its chronicisation and that early changes in the pain pathways can lead to later plastic changes responsible for persistent pain (Sinatra 2010). This putative implication of xCT at the onset of neuropathic pain is consistent with the observation that knock-out mice do not develop any sign of thermal hyperalgesia following PSNL. Indeed, if xCT^{+/+} and xCT^{-/-} mice had initially exhibited similar hypersensitivity within a few hours but showed a faster resolution in mice lacking xCT, it would suggest a later involvement of system x_c⁻. While further experiments are needed to ascertain the specific role of system x_c⁻ in the different stages of neuropathic pain. Our observations hold significant implication regarding the opportunity to target system x_c⁻ in the management or prevention of neuropathic pain.

The beneficial effect of the genetic suppression of xCT on the development and persistence of allodynia and hyperalgesia in the surgical model of neuropathic pain is consistent with the observation that such lesion triggers an increased expression of xCT in the dorsal spinal cord. Nevertheless, the use of a constitutive transgenic mice strain in which xCT expression is absent during the entire animal development raises questions related to compensatory mechanisms (El-Brolosy and Stainier 2017). To validate the findings from xCT-deficient animals, the pharmacological inhibition of system x_c⁻ in adult mice constitutes a relevant alternative approach. Indeed, considering the upregulation of xCT and the enhanced system x_c⁻ activity following sciatic nerve ligation, targeting this exchanger might help to reduce neuropathic pain symptoms. Nowadays, the most used inhibitor of system x_c⁻ is SAS, an EMA- and FDA-approved drug prescribed to treat inflammatory diseases of the intestine (Patel et al. 2019; Gout et al. 2001). Effectiveness in these disorders arises from its breakdown by the gut microbiota, generating anti-inflammatory metabolites. As only the intact parent molecule was proven effective in inhibiting system x_c⁻ (Gout et al. 2001; Chung and Sontheimer 2009; Alcoreza et al. 2019), SAS was herein administrated

intraperitoneally to reduce its metabolization. Even though some papers reported on a limited capacity of SAS to cross the BBB, several researches have demonstrated beneficial effects in nervous disorders affecting both the brain and the spinal cord such as reduced microglial activation and reduced tumor growth in mouse model of glioblastoma (Buckingham et al. 2011; Chung and Sontheimer 2009; Miladinovic and Singh). Given that system x_c^- putatively participates in the onset of neuropathic pain, SAS was administered preventively, starting 2 days before the lesion. In these conditions, a robust anti-allodynic and anti-hyperalgesic effect of SAS was observed in $xCT^{+/+}$ mice undergoing PSNL. Even though these data are consistent with the results obtained with knock-out animals, pointing out xCT as a key actor in the pain sensitization, a possible anti-inflammatory component of the treatment cannot be ruled out. Thus, some evidence indicated that SAS itself possess anti-inflammatory properties through an inhibition of the transcription factor NF- κ B involved in immune responses (Robe et al. 2004; Li and Verma 2002). Surprisingly, SAS, being a system x_c^- inhibitor, led to a downregulation of xCT mRNA. This post-translational regulation might be attributed to its anti-inflammatory properties, resulting in reduced recruitment of astrocytes and microglia which are known to upregulate xCT during inflammatory conditions. To confirm the specific role of xCT, SAS was administered to $xCT^{-/-}$ mice, but no analgesic effect was observed compared to the vehicle-treated $xCT^{-/-}$ mice. Together, these data underscore the relevance of targeting system x_c^- in neuropathic pain but call for the development of more specific inhibitors to mitigate potential side effects as previously reported with SAS (Verbruggen et al. 2021).

Knowing that system x_c^- is mainly expressed in glial cells and that over the past decades, both peripheral and central glia and immune cells have taken center stage in research on neuropathic pain, the specific implication of astrocytes and microglial cells was examined. The upregulation of xCT following PSNL surgery, was correlated with the activation of astrocytes and microglia in the dorsal spinal cord. Resident glial cells actively participate in

the strong inflammatory reaction in models of neuropathic pain by releasing mediators such as TNF- α , interleukins, chemokines, ATP. This neuroinflammatory cascade is further propagated by the recruitment of microglial cells and astrocytes (Berger et al. 2011). After both peripheral or central nerve lesions, these cells promote neuroinflammation at several levels of the pain pathway leading to the onset and progression of pain hypersensitivity. While Iba-1 is primarily recognized for its role in the initiation of neuropathic pain and GFAP for its maintenance (Basbaum et al. 2009; Campbell and Meyer 2006), results are not always straightforward as temporal dynamic changes may differ across pain models or spinal regions (Romero-Sandoval et al. 2008). At variance with several published data, our findings unveil a rapid and transient upregulation of GFAP early after the nerve lesion, prompting further investigation into the underlying chronology of this glial response. In line with the existing literature, our experimental findings regarding the persistent upregulation of Iba-1 imply that microglial activation may persist for an extended period, potentially even beyond pain resolution (Leinders et al. 2013). Emerging evidence suggests that specific subtypes of immune cells can reduce pain and contribute to the resolution of neuropathic pain (Honjoh et al. 2019; Seltzer et al. 1990). Previous studies have evidenced that activated microglia show increased system x_c^- expression and that the genetic invalidation of xCT alters their polarization profile, predominantly adopting a protective anti-inflammatory (M2) phenotype in the spinal cord of animal models of neurological diseases such as ALS (Mesci et al. 2015) or spinal cord injury (Sprimont et al. 2021). In line with this observation, our findings indicate that mice lacking xCT exhibit a different polarization profile of microglia as compared to their wild-type counterparts, suggesting that the absence of system x_c^- could beneficially balance the strong pro-inflammatory activation state commonly observed in the context of neuropathic pain. Accordingly, an altered recruitment and activation of glial cells in the spinal cord following nerve lesion was observed after pharmacological inhibition of system x_c^- with SAS. Together with a reduced

astrogliosis and microgliosis, a reduced polarization toward the pro-inflammatory microglial phenotype was observed. This evidence aligns with the results of behavioral testing indicating reduced pain sensitivity with SAS treatment as compared to vehicle-treated animals.

From an experimental standpoint, our observations highlight the need for complementary studies that would reinforce the therapeutic potential offered by blockers of system x_c^- . With a focus on the implication of system x_c^- during the onset of neuropathic pain, the present study examined animal behavior and biochemical changes during the first week after the surgical nerve lesion. Previous studies have shown that peripheral nerve lesions generate long-lasting sensitization (Echeverry et al. 2017) and the implication of system x_c^- in the persisting behavior and associated biochemical changes should also be examined at later time points. Further studies could therefore consider initiating the administration SAS after the establishment of neuropathic pain to assess the role of system x_c^- in the pain maintenance. This also underscores the opportunity of employing conditional genetic models where xCT can be silenced at later time points. Besides, it is worth mentioning that the present findings were obtained using female mice only, in order to reduce experimental variability. Hence, pain hypersensitivity in models of chronic pain, including from a neuropathic origin is sex-dependent and further studies should include both males and females (Guindon et al. 2019; Ghazisaeidi et al. 2023). Finally, previous work has revealed that xCT-deficient mice show reduced anxiety and depressive-like behaviors, supporting a role for this exchanger in several centrally controlled behaviors (Bentea, Sconce, et al. 2015). It is noteworthy that anxiety and depression are comorbidities of chronic pain and one could therefore anticipate a possible impact of inactivating or inhibiting system x_c^- (Li 2015) on brain-controlled behaviors associated with the neuropathic lesion. In a broader perspective, further studies should examine whether manipulating system x_c^- may influence the processing of pain in supra-spinal centers.

5. Conclusion

Altogether, these findings provide strong evidence for the important contribution of system x_c^- in the establishment of a persistent pathological pain state and spinal neuroinflammation after peripheral nerve lesion. This not only helps to better understand the molecular mechanisms supporting neuropathic pain but offers new perspectives for future therapies of this major clinical issue. Targeting system x_c^- appears as a unique opportunity to tackle both the altered glutamate transmission and the neuroinflammation responsible for central sensitization in patients suffering from neuropathic pain. The pharmacological manipulation of system x_c^- could therefore prevent, reduce, or alleviate neuropathic pain symptoms such as allodynia and hyperalgesia and thereby improve the quality of life of affected patients.

EXPERIMENTAL WORKPACKAGE III:

Implication of system x_c^- in Complete Freund's Adjuvant-induced peripheral inflammation and associated sensitization

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In preparation

Abstract

Background: Chronic inflammatory pain poses significant global health challenges, affecting millions of individuals worldwide and imposing a substantial burden on quality of life and healthcare systems due to inadequate relief by current clinical treatments. Macrophages orchestrate the inflammatory response upon peripheral insult, contributing to persistent inflammation and neuronal sensitization, common features of chronic inflammatory pain. Glial cells in the CNS further exacerbate hypersensitivity through the release of proalgesic mediators. It has been demonstrated that system x_c^- , expressed on both peripheral and central immune cells, significantly participates in the inflammatory response. However, its involvement in chronic inflammatory pain pathophysiology remains understudied. Considering the role of system x_c^- in modulating glutamate neurotransmission and in supporting inflammatory response, we investigated its specific implication in peripheral inflammation and the associated hypersensitivity.

Methods: Peripheral inflammation was induced by injecting Complete Freund's adjuvant (CFA) in the left hind paw of both wild-type mice and mice lacking xCT, the specific subunit of system x_c^- . We investigated the consequence of the genetic deletion of system x_c^- on the local inflammation by measuring the paw edema up to three weeks following CFA injection. During the same period, we examined the development of pain-associated behaviors accompanying peripheral inflammation as well as the level of pro-inflammatory cytokines in blood samples. Finally, our results were further confirmed by a pharmacological approach using sulfasalazine as a system x_c^- inhibitor.

Results: CFA injection was found to induce a robust and persisting increase in the paw thickness which was less pronounced in mice lacking xCT. The genetic deletion of xCT also attenuated the duration of pain hypersensitivity to mechanical and thermal stimuli after peripheral inflammation induced by CFA

injection. These effects were associated to the reduced level of systemic pro-inflammatory cytokines observed in mice lacking xCT. Pharmacological inhibition of system x_c^- by sulfasalazine also presented analgesic properties in CFA-injected wild-type mice.

Conclusion: Together, our results reveal the pivotal role of system x_c^- in the pathophysiology of chronic inflammatory pain, with xCT deficiency reducing associated pain behaviors and peripheral inflammation. Targeting system x_c^- emerges as a promising pharmacological strategy for alleviating chronic inflammatory pain, offering potential relief for affected patients.

1. Introduction

Chronic inflammatory pain represents a debilitating condition that causes significant global health challenges (Rice et al. 2016). With millions of individuals worldwide suffering from nociceptive pain of inflammatory origin, characterized by persistent inflammation and neuronal sensitization, it imposes a considerable burden on patients' quality of life and on healthcare systems (Alotaibi et al. 2023). Current clinical treatments frequently offer inadequate relief, underscoring the urgent need to develop novel effective therapeutic approaches to address chronic inflammatory pain.

Upon peripheral tissular insult, macrophages play a critical role in orchestrating the inflammatory response. Recruited at the affected site, they release pro-inflammatory cytokines and chemokines, which in turn promote local inflammation and sensitize nociceptive sensory fibers (Linley et al. 2010). This macrophage-mediated inflammatory cascade contributes to the development and maintenance of pain which is part of the five cardinal symptoms of inflammation, besides redness, heat, swelling and loss of function (Lipnik-Stangelj 2013). Peripheral inflammation is also documented to profoundly impact central processes related to pain perception, potentially leading to chronicity.

Emerging evidence highlights a crucial role for glial cells of the CNS in the pathogenesis of chronic pain with an inflammatory origin. Once activated by the peripheral trigger, microglia and astrocytes in the spinal cord or in the brainstem release a variety of proalgesic mediators that can exacerbate pain. For instance, microglial cells produce pro-inflammatory cytokines including TNF- α and IL-1 β , promoting pain hypersensitivity following inflammatory insults (Lipnik-Stangelj 2013). Additionally, it has been shown that blocking microglial activation upstream of the inflammatory input can alleviate allodynia (pain due to a stimulus that does not usually provoke pain) and hyperalgesia (increased pain from a stimulus that usually provokes pain),

common symptoms of chronic inflammatory pain (Baniasadi et al. 2020; Ledebøer et al. 2005).

Expressed on both peripheral and central immune cells, the cystine/glutamate antiporter system x_c^- , is documented to participate in inflammatory responses. Structurally, system x_c^- consists of a transmembrane Na^+ -independent amino acid transporter that is constituted of two different subunits: a heavy chain known as 4F2hc, common to several amino acid transporters, and a light chain specific subunit known as xCT (Sato et al. 1999). It transports L-cystine and L-glutamate in a 1:1 molecular ratio, preferentially importing cystine in exchange for glutamate release (Bannai and Kitamura 1980). By importing cystine which is needed for the biosynthesis of GSH, system x_c^- provides crucial protection against oxidative stress. Reports also demonstrated upregulation of xCT expression in macrophages and microglial cells during peripheral inflammation, leading the authors to assign a role in immune cell survival (Mesci et al. 2015; Nabeyama et al. 2010; Sato et al. 1995). Reinforcing the same notion, studies on mice lacking xCT showed attenuated systemic inflammatory responses in age-related priming (Verbruggen et al. 2022; Albertini et al. 2018). Moreover, the absence of system x_c^- led to reduced production of pro-inflammatory cytokines by microglia, while increasing anti-inflammatory markers (Mesci et al. 2015). Together these findings underscored that system x_c^- has a pivotal function in modulating macrophage/microglial activation and subsequent inflammatory responses, increasing the interest for its implication in inflammatory diseases such as chronic pain.

While importing cystine and regulating inflammatory response, system x_c^- also elevates extracellular glutamate concentration, reinforcing neuronal signaling (Massie et al. 2015). Alteration in excitatory neurotransmission can significantly influence synaptic activity and contribute to pathological conditions. Hence, given the critical role of glutamate transmission in pain processing, a finely tuned regulation of its extracellular levels is essential.

While most research has focused on synaptic glutamate uptake by astrocytic glutamate transporters (GLT-1 and GLAST), which accounts for over 90% of glutamate clearance in the CNS, the role of system x_c^- , responsible for a significant non-vesicular glutamate release, remains understudied in the pathophysiology of chronic inflammatory pain.

While previous study from our laboratory has shown that xCT-deficient mice exhibited reduced pain sensitivity and decreased glial activation following peripheral sciatic nerve ligation (Beckers et al. 2024), the exact role of system x_c^- in the inflammatory process leading to chronic pain remains understudied. Given this, the current study aimed at specifically examining the potential implication of system x_c^- in modulating CFA-induced behavioral hypersensitivity and the associated peripheral inflammation. Therefore, this validated chronic inflammatory pain model was combined with pharmacological and genetic manipulation of system x_c^- .

2. Materials and methods

a. Animals and Ethical statement

All experiments were conducted in strict accordance with the recommendations of the European commission and with the agreement of the Belgian Ministry of Agriculture (code number LA 1230618). The ethical committee of the Université catholique de Louvain for animal experiments specifically approved this study (aggregation number 2023/UCL/MD/50). Wild-type (xCT^{+/+}) and xCT knock-out (xCT^{-/-}) mice used in this study were high-generation descendants (with C57BL/6J background) of the strain previously described (Sato et al. 2005). These mice lacking xCT were generously provided by Dr. Hideyo Sato (Department of Medical Technology, Niigata University, Japan) and bred in the animal facilities of the Vrije Universiteit Brussel and the Université catholique de Louvain. All experiments combined homozygous animals obtained both from the breeding of

heterozygous animals (F1) and from a second generation using homozygous parents (F2).

All animals were accommodated under standard laboratory conditions, receiving food and water ad libitum, and were housed in the animal facility at the Université catholique de Louvain, in a 12-12h light-dark cycle, controlled temperature and humidity conditions. Mice lacking xCT were genotyped by end-point PCR on genomic DNA extracted from an ear biopsy using specific primers for the Slc7a11 gene. PCR amplification products were analyzed by agarose-gel electrophoresis to identify bands revealing the intact or truncated gene (Sato et al. 2005). Since pain hypersensitivity is sex-dependent, only female mice were used to reduce variability.

b. Experimental design

Adult female mice (wild-type and xCT-deficient mice) aged between 10 and 12 weeks were randomly subjected to subcutaneous plantar injection of CFA (1 mg/mL mycobacterium tuberculosis in 85% paraffin oil and 15% mannide monooleate, F5881, Sigma-Aldrich, MO, USA) or saline (NaCl 0.9%, Baxter). Briefly, mice were anesthetized with sevoflurane (4% for induction and 3% for maintenance with oxygen as a carrier gas). Under continuous anesthesia, mice were subcutaneously injected with 20 μ L of CFA in the plantar surface of their left hind paw, referred to as ipsilateral paw. Controls were injected with saline under the same conditions.

Baseline measurement and daily follow-up after CFA injection included assessment of body weight, spontaneous locomotion, and paw edema. Pain-related behavior (allodynia and hyperalgesia) was also evaluated on a daily basis throughout the duration of the experiment.

For the pharmacological part of the study, mice were injected for 10 consecutive days with SAS, a system x_c^- inhibitor, starting 2 days prior CFA injection.

At different time points (4, 7 or 21 days) following CFA injection, mice were sacrificed for blood sample collection.

c. Pain-related behavior testing

Mechanical hypersensitivity was assessed using the von Frey hair filament test as previously described (Bosier et al. 2015). Briefly, animals were placed in transparent chambers positioned on an elevated mesh floor. Acclimatization was allowed for 20 min. To determine the mechanical sensitivity, the “up and down” method was used (Deuis et al. 2017), with a set of 10 calibrated (0.04, 0.07, 0.16, 0.4, 0.6, 1, 1.4, 2, 4 and 6 g) von Frey hair filaments (Stoelting Co., Wood Dale, IL). The 50% paw withdrawal threshold (PWT) was then calculated using the formula previously described (Chaplan et al. 1994).

The thermal paw withdrawal latency (PWL) was used to assess hyperalgesia (Hargreaves et al. 1988) as previously described (Bosier et al. 2015). Briefly, mice were acclimated for 20 min in separated transparent, bottom-free plastic chambers placed over the glass enclosure of the Hargreaves paw thermal stimulator (University of California, San Diego, CA). A heat source was positioned underneath the plantar surface of the hind paw and the time taken to withdraw from the heat source was automatically recorded by the apparatus.

For behavioral analyses, the experimenter responsible for conducting the tests was blind to the treatment, the genotype and the injection performed on the mice.

d. Paw thickness measurement

The edema caused by inflammation was estimated by measuring the dorsal to plantar diameter of the hind paw as previously reported (Soyocak et al. 2019). Briefly, mice were held by the tail and the caliper was placed

transversely to the ipsilateral hind paw to measure its width, expressed in mm. Measurements were taken at baseline and then at different time points following CFA injection.

e. General locomotion – open field

The spontaneous locomotor activity (velocity and distance moved) of the mice was assessed by placing them in an open field (60 cm x 60 cm x 60 cm) for 15 min. This open field test was analyzed using an automated integration system (Ethovision software, Noldus), as previously described (Goursaud et al. 2015).

f. Sulfasalazine administration

Animals were randomly assigned to either the treatment or control group. An 80 mg/mL stock solution of sulfasalazine (SAS, Sigma-Aldrich, St. Louis, MO) was prepared by dissolving the powder in DMSO. The final solution was daily prepared by adding saline to reach the desired concentration of SAS. The pH was adjusted using a small volume of NaOH 0.1 M. Mice were daily injected intraperitoneally (i. p.) with 200 mg/kg of SAS (or vehicle that includes the same dilution of DMSO for the controls) (Verbruggen et al. 2021; Tsuchihashi et al. 2016) for 10 consecutive days starting 2 days prior to the CFA injection.

g. Plasma collection

Mice were euthanized 4, 7 or 21 days after CFA or saline intraplantar injection with CO₂. Blood was intracardially collected using 26G needle mounted on an EDTA-coated syringe and heparin-coated collection tubes (365966A, D. Dutscher, Bernolsheim, France). The tubes were centrifugated 3 min at 2700 g, plasma was collected and stored at -80°C before processing them in the U-PLEX assay.

h. Plasma cytokine assessment

Plasma concentrations of pro-inflammatory cytokines (TNF- α and IL-6) were determined using the Meso Scale Discovery U-PLEX customized mouse assay (MSD, Rockville, MD, USA), according to the manufacturer's instructions. Briefly, biotinylated capture antibodies were coupled to their respective linker for 30 min. Subsequently, a coating solution was prepared by combining all U-PLEX linker-coupled primary antibodies. The wells were then coated with this solution and incubated at room temperature (RT) while shaking for 1 h before rinsing the plate with PBS-Tween (0.05%). During the incubation period, a calibration curve was prepared using the provided calibrator. Samples and calibrator standards were added to each well and incubated for 1 h at RT. Following a quick washing step, detection antibodies were introduced to the wells and incubated for 1 h. After a final washing step, MSD GOLD Read Buffer was added, and the plate was analyzed using the MSD Quickplex SQ120MM.

i. Statistical analyses

Data are presented as means with standard error of the mean (SEM). Outliers were identified using the Grubbs test (α -value=0.01) and removed from the dataset when appropriate. For all analyses, a two-sided type I error of 0.05 was considered for statistical significance. Statistical analyses were performed using GraphPad Prism version 9.5.1 and R software.

For follow-up analyses, statistical comparisons were made relative to the corresponding baseline values to control for individual variability. Mixed-effects models were fitted to the data using genotype, time and treatment as the fixed effects. Repeated measurements were included in the model within individual animals. Two or three-way ANOVA were performed based on the experimental design. In case where significant interactions were detected in the model, pairwise comparisons were conducted using the Tukey post-test.

The normality of residuals was assessed using Shapiro-Wilk normality test, and equality of variances was tested using the Levene test to ensure the assumptions of the statistical tests. In case of violation, the Satterthwaite correction was used.

3. Results

a. xCT deletion attenuates edema without affecting body weight and general locomotion following intraplantar injection of CFA

The contribution of system x_c^- to chronic inflammatory pain was examined by subjecting wild-type mice and mice lacking xCT to unilateral injection of CFA into the left hind paw. Female mice were monitored for 21 days post-injection using a caliper to estimate the thickness of their ipsilateral paw. Prior to CFA injection, xCT-deficient mice exhibited similar paw thicknesses compared to their wild-type littermates. Additionally, mice receiving an injection of saline maintained paw thicknesses to pre-injection levels. As depicted in figure 25, CFA-injected mice displayed significant and prolonged paw edema, beginning as early as 4 hours post-injection, and persisting throughout the 21-day observation period. Paw thickness peaked at 5.2 ± 0.5 mm in wild-type mice 24 hours after CFA injection, compared to 2 mm in mice injected with saline. xCT-deficient mice also exhibited an increase in paw thickness over the course of the experiment, albeit less significant than that observed in wild-type mice (peaking at 4.5 ± 0.6 mm on day 1 post-injection).

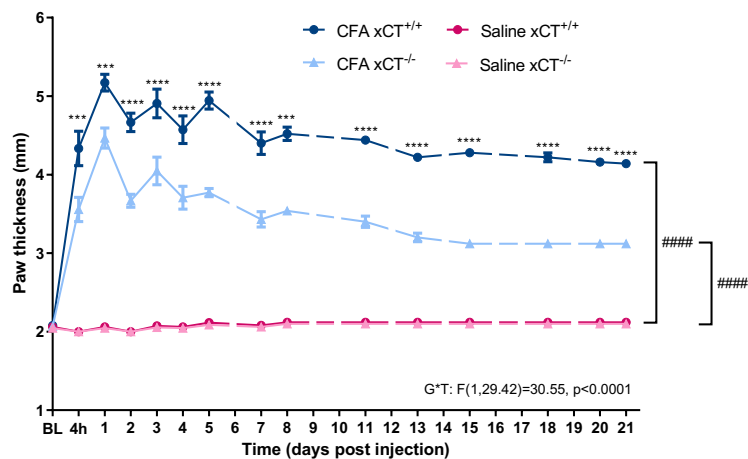


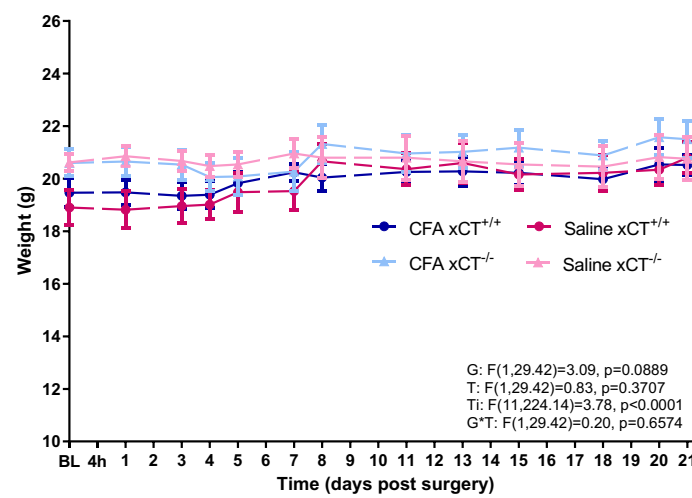
Figure 25 : Effect of CFA or saline intraplantar injection on the paw thickness at the injected site of xCT^{+/+} and xCT^{-/-} mice. Change in paw thickness (expressed in mm) as an experimental readout of local inflammation was assessed at baseline and for 3 weeks following CFA or saline injection using a caliper. Data are presented as mean $\pm$ SEM of 5 to 9 animals per group. Statistical analyses were performed using a three-way ANOVA model with repeated measures. P-values for main effects and interaction effects between treatment and genotype are indicated in the graphs. Tukey's pairwise comparisons were conducted and reported on the graphs. * indicates a significant difference between CFA-injected xCT^{+/+} mice and CFA-injected xCT^{-/-} mice, # indicates a significant difference between CFA and saline animals (within the same genotype). CFA, complete Freund's adjuvant; BL, baseline; G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment.

Daily monitored mouse body weight was unaffected by CFA injection (Figure 26A), indicating general well-being despite significant edema in the ipsilateral paw. Moreover, both xCT^{+/+} and xCT^{-/-} mice showed similar weight gain profiles during the three weeks following the injection.

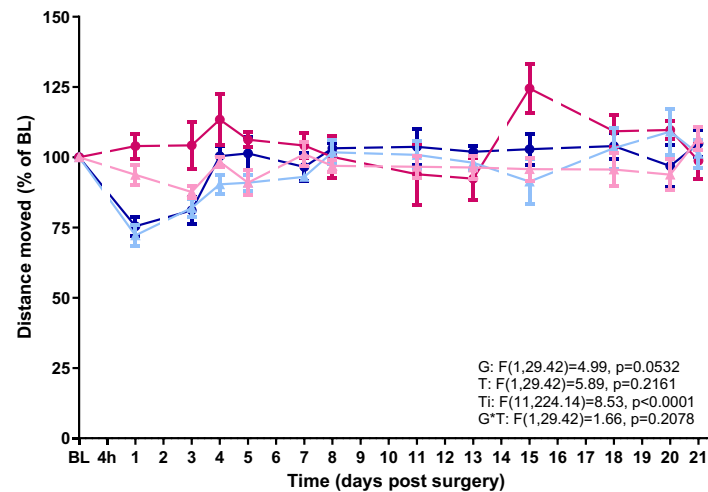
Spontaneous locomotor activity was evaluated in an open field test (Figure 26B&C). Measures of the distance traveled (Figure 26B) and the average velocity (Figure 26C) revealed no significant differences between wild-type

and xCT-deficient mice. Mice receiving CFA injection showed a transient (1 day) reduction in distance traveled and average velocity compared to baseline measurements, but the observed effects were similar in both genotypes. Together, these results allow us to exclude a potential locomotor bias in the assessment of other parameters.

A



B



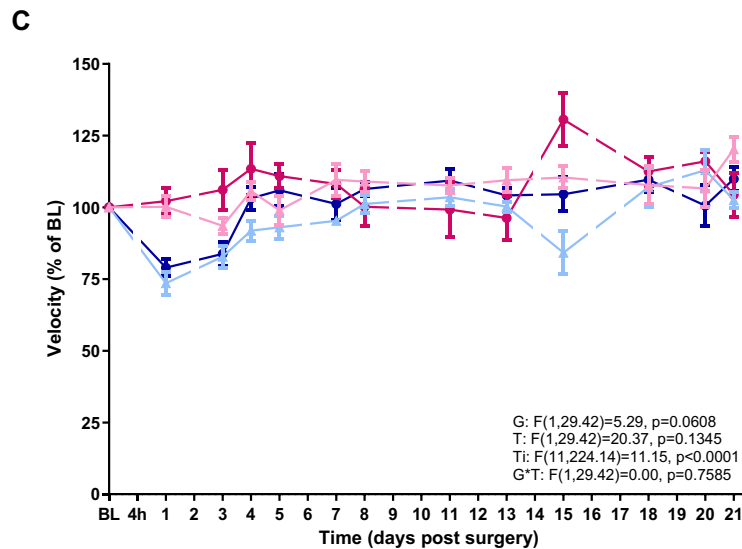


Figure 26: Effect of CFA or saline intraplantar injection on weight and locomotion in $xCT^{+/+}$ and $xCT^{-/-}$ mice. Weight (A) was assessed at baseline (BL) and regularly for up to 3 weeks after CFA or saline injection. In the open field test, the distance traveled (B) and the average velocity (C) were recorded. Data are presented as mean $\pm$ SEM of 5 to 9 animals per group. For panel B and C, data are expressed as a percentage of baseline. Statistical analyses were performed through a three-way ANOVA model with repeated measures. P-values for main effects and interaction effects between treatment and genotype are indicated in the graphs. *CFA*, complete Freund's adjuvant; *BL*, baseline; *G*, genotype effect; *T*, treatment effect; *Ti*, time effect; *G*T*, interaction effect between genotype and treatment.

b. Mice lacking xCT present a reduced hypersensitivity to mechanical and thermal stimuli following intraplantar injection of CFA

Peripheral inflammation is known to induce sensitization characterized by the development of enhanced sensitivity to mechanical and thermal stimulations. Therefore, female $xCT^{+/+}$ and $xCT^{-/-}$ mice were monitored for 21 days post-injection using a von Frey (Figure 27A&B) and a Hargraeves test

(Figure 27C&D) to respectively evaluate CFA-induced allodynia and hyperalgesia. As illustrated in figure 27A, prior to CFA injection, xCT-deficient mice exhibited an equivalent mechanical sensitivity as compared to wild-type mice. Additionally, control mice (receiving saline injection) displayed transient hypersensitivity, as from the 3rd day post-injection, they regained a PWT similar to that measured before the injection and this transient hypersensitivity appeared comparable between both genotypes. Besides, xCT^{+/+} animals administered with CFA displayed significant and prolonged mechanical allodynia, evident as early as 4 hours after CFA injection. The 50% PWT reached a minimum value of 0.0015 ± 0.0052 g on day 1 and persists until day 18 (with a 50% PWT value of 0.052 ± 0.022 g), with some modest recovery on days 20 and 21 (0.16 ± 0.08 g and 0.20 ± 0.09 g, respectively) after injection. In xCT-deficient mice, following the initial decrease, the 50% PWT to mechanical stimulation tended to progressively recover as from day 15 post-injection (reaching 1.86 ± 0.50 g on day 21 post-injection), indicating that the hypersensitivity observed in xCT^{-/-} animals is transient compared to that observed in their xCT^{+/+} littermates. When testing the contralateral paw, a temporary reduction in the 50% PWT was noted during the first 4 hours for the saline-injected mice and within the first 2 days for the CFA-injected mice (Figure 27B). However, by day 4 after injection, all mice demonstrated sensitivity to mechanical stimulation comparable to the baseline level.

When testing the response to thermal stimulation using the Hargraeves test, xCT^{+/+} mice receiving CFA injection developed a persistent hyperalgesia as indicated by the decreased latency for the ipsilateral paw withdrawal reaching 40% of the baseline value after only 4 hours (Figure 27C). This hypersensitivity to thermal stimuli persisted for the entire duration of the experiment. While mice lacking xCT also developed ipsilateral hyperalgesia after CFA administration, as soon as 3 days post-injection, this decrease was less pronounced compared to that observed in wild-type animals. No significant changes were observed when testing the contralateral paw for thermal sensitivity (Figure 27D).

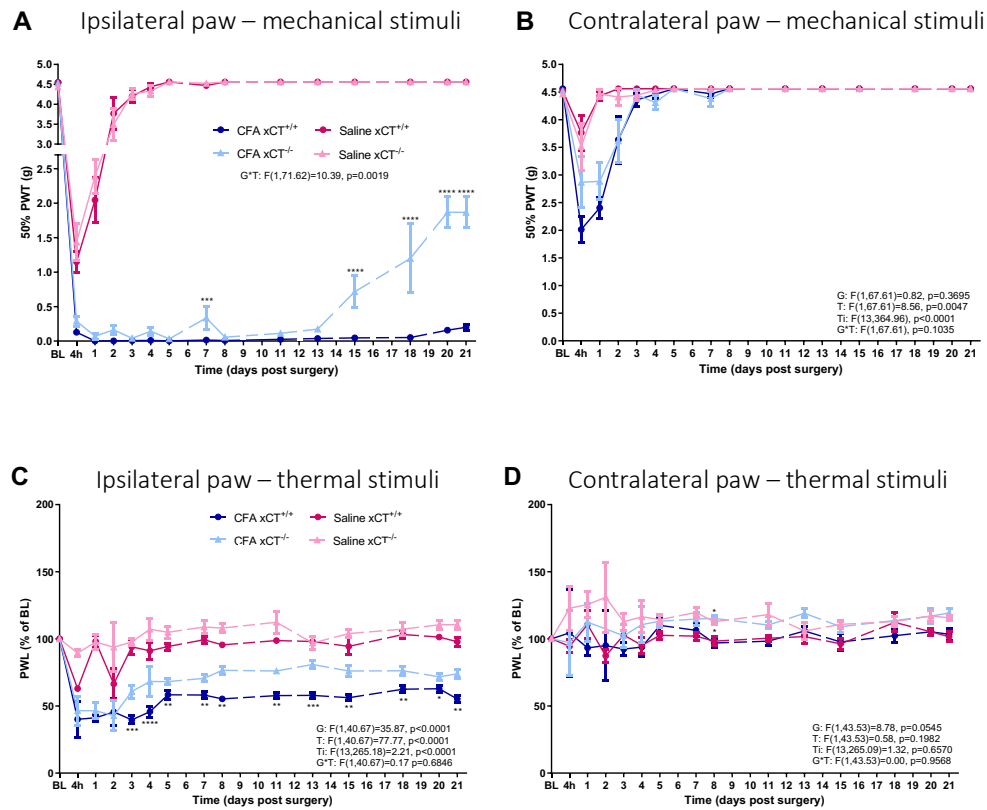


Figure 27: Allodynia and hyperalgesia in xCT^{+/+} and xCT^{-/-} mice following CFA or saline intraplant injection. Pain hypersensitivity was assessed upon stimulation of the ipsilateral (A, C) and contralateral (B, D) hind paws at baseline (BL) and for up to 21 days after CFA or saline injection. The 50% PWT to mechanical stimulation was used to determine allodynia (A, B), whereas the PWL to thermal stimulation was used as a read-out of hyperalgesia (C, D). Data are presented as mean $\pm$ SEM of 5 to 9 animals per group. Statistical analyses were performed through a three-way ANOVA model with repeated measures. P-values for main effects and interaction effects between genotype and treatment are indicated in the graphs. Tukey's pairwise comparisons were conducted and reported on the graphs. * indicates a significant difference between CFA-injected xCT^{+/+} mice and CFA-injected xCT^{-/-} mice. *CFA*, complete Freund's adjuvant; *PWT*, paw withdrawal threshold; *PWL*, paw withdrawal latency; *BL*, baseline; *G*, genotype effect; *T*, treatment effect; *Ti*, time effect; *G*T*, interaction effect between genotype and treatment.

c. xCT deletion results in attenuated peripheral inflammation following intraplantar injection of CFA

After observing reduced CFA-induced pain behaviors in mice lacking xCT along with a decrease in peripheral edema as compared to their wild-type littermates, we decided to evaluate the effect of system x_c^- on inflammation induced by peripheral injection of CFA. Therefore, blood samples were collected at different time points following CFA or saline injection, and the concentrations of IL-6 and TNF- α were determined. Considering that the absence of xCT had a predominant influence on the recovery of pain sensitivity, we focused our analysis on the evolution of cytokine levels in the three weeks following the initial inflammatory insult. Thus, in mice receiving a saline injection in the paw, plasma levels of IL-6 remained relatively unaffected over time, both in xCT^{+/+} and xCT^{-/-} mice (Figure 28A). However, in animals receiving the CFA injection, the plasma IL-6 level increased over time in xCT^{+/+} mice, while conversely, it tended to decrease in xCT^{-/-} animals. A significant difference was indeed observed between both genotypes at 21 days post-injection (Figure 28B).

When considering plasma concentrations of TNF- α , xCT^{+/+} mice receiving saline injection, presented a tendency for an increase in concentration over time. However, TNF- α levels remained stable in transgenic mice lacking xCT (Figure 28C). Additionally, overall TNF- α levels were lower in xCT^{-/-} compared to xCT^{+/+} mice. A similar pattern was observed in CFA-injected mice, with a time-dependent increase in xCT^{+/+} mice and relatively stable levels in xCT^{-/-} mice. After statistical analysis, this translated into a significant difference between the two genotypes at 7 and 21 days after CFA injection (Figure 28D).

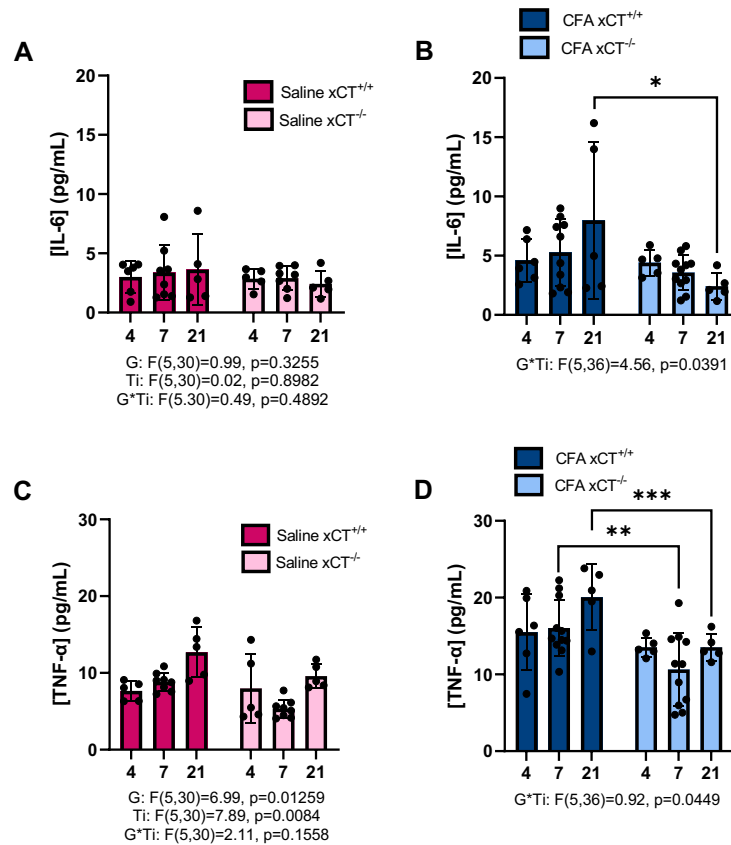


Figure 28 : Plasma pro-inflammatory cytokine level at 4, 7 and 21 days following CFA or saline intraplantar injection of xCT^{+/+} and xCT^{-/-} mice. Plasma IL-6 and TNF-α concentration were measured to assess systemic inflammation induced by CFA or saline administration at different time point following the injection. Data are presented as mean ± SEM of 5 to 10 animals per group. Statistical analyses were performed through a two-way ANOVA model. P-values for main and interaction are indicated in the graphs. Tukey's pairwise comparisons were conducted and reported on the graphs (*p<0.05, **p<0.01, ***p<0.001). *G*, genotype effect; *Ti*, time effect; *G*Ti*, interaction effect between genotype and time.

d. SAS possesses anti-allodynic and anti-hyperalgesic properties driven by system x_c^- inhibition

To further evaluate the implication of system x_c^- on the development of chronic inflammatory pain, we examined the consequence of its pharmacological inhibition. Both wild-type and xCT-deficient animals were subjected to CFA injection and treated for 10 consecutive days with either saline or SAS (200 mg/kg once per day, i.p.). The mice were regularly monitored over 3 weeks following the CFA administration. Baseline measurements revealed that SAS injection had no influence on paw thickness (Figure 29A) or sensitivity to mechanical (Figure 29B) and thermal (Figure 29C) stimuli, as thresholds remained unchanged following SAS treatment prior to CFA injection.

When examining the influence of SAS treatment on CFA-induced paw edema, it was observed that CFA injection induced an increase in paw thickness in vehicle-treated mice, albeit less pronounced in xCT^{-/-} as compared to xCT^{+/+} mice, as mentioned before. However, wild-type mice receiving SAS exhibited reduced paw edema as early as 2 days after CFA injection, reaching levels comparable to those observed in vehicle-treated xCT^{-/-} mice (Figure 29A).

Regarding pain-associated behaviors, mechanical hypersensitivity was relatively similar between vehicle-treated and SAS-treated xCT^{+/+} mice, at least until day 13 post-injection. From that time point, a progressive recovery was observed in SAS-treated xCT^{+/+} animals as indicated by the increase in the 50% PWT. Therefore, SAS-treated xCT^{+/+} mice displayed a profile similar to mice lacking xCT (Figure 29B).

As detailed before, xCT^{+/+} mice injected with CFA developed significant hypersensitivity when tested for thermal stimulation, evidenced by a decreased latency before paw removal. Again, within few days after CFA injection, xCT^{+/+} mice receiving SAS exhibited reduced PWL compared to vehicle-treated counterparts, an effect maintained throughout the

experimental period (Figure 29C). Finally, to confirm that the effects observed using SAS were related to system x_c^- inhibition, $xCT^{-/-}$ mice received the same treatment. No differences were observed in paw thickness or in thermal and mechanical sensitivity between vehicle-treated and SAS-treated mice.

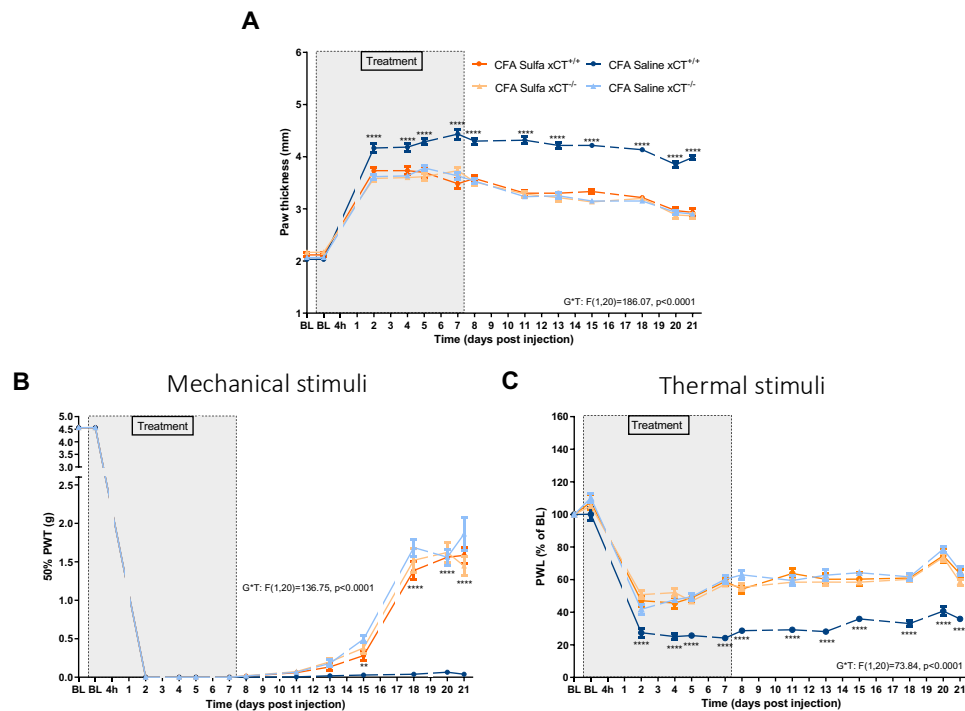


Figure 29: Effect of SAS treatment on the paw thickness and the pain hypersensitivity following CFA injection. Paw volume (expressed in millimeter) was assessed at baseline and for 3 weeks following CFA injection using a caliper (A). The paw thickness was used to determine local inflammation. Pain hypersensitivity was assessed for the ipsilateral hind paw at baseline (BL) and for up to 21 days after CFA injection. The 50% PWT to mechanical stimulation was used to determine allodynia (B), whereas the PWL to thermal stimulation was used as a read-out of hyperalgesia (C). Data are presented as mean $\pm$ SEM of 6 animals per group. Statistical analyses were performed through a three-way ANOVA model with repeated measures. P-values for main effects and interaction effects between genotype and treatment are indicated in the graphs. *CFA*, complete Freund's adjuvant; *PWT*,

*paw withdrawal threshold; PWL, paw withdrawal latency; BL, baseline; SAS, sulfasalazine; G, genotype effect; T, treatment effect; Ti, time effect; G*T, interaction effect between genotype and treatment.*

4. Discussion

Inflammatory pain is a multifaceted biological response that engages the somatosensory, immune, neuronal, and vascular systems in reaction to tissue damage. Initially, acute inflammation, which results in pain perception, serves as a protective function by initiating the healing process and restoring tissue integrity. However, in some cases and for reasons that are still under investigation, inflammation and the accompanying pain may persist, evolving into maladaptive and chronic pain. The persistence of inflammation is associated with neuronal sensitization in the pain pathways primarily induced by immune mediators such as pro-inflammatory cytokines including TNF- α , IL-1 β and IL-6 (Clark and Vissel 2016; Zhang and An 2007; Littlejohn 2015; Scholz and Woolf 2007). These mediators directly bind their receptors expressed on neurons and glial cells within the central and peripheral nervous systems (Scholz and Woolf 2007; Szelényi 2001; Cook et al. 2018; Guo et al. 2007). By doing so, pro-inflammatory cytokines modulate the expression and activity of ion channels and receptors crucial for pain transmission (Linley et al. 2010; Zhang, Bi, and Gan 2018; Kawasaki et al. 2008; Carlton and Coggeshall 1999). Also, the inflammatory cytokine TNF- α was shown to modulate the expression of astrocytic high-affinity glutamate transporters (GLT-1 and GLAST), in charge of maintaining low glutamate concentrations in the synaptic cleft and avoid hyperexcitability (Sitcheran et al. 2005). Chronic pain is indeed characterized by disrupted glutamatergic neurotransmission, partially attributed to altered GLT-1 and GLAST expression and functionality (Guo et al. 2019). In a mouse model of inflammatory pain induced by peripheral injection of CFA or formalin, a GLT-1 enhancer was found effective in reducing allodynia (Alotaibi et al. 2023; Alotaibi and Rahman 2019).

Alike glutamate transporters, emerging evidence suggests that system x_c^- could also be regulated by the presence of inflammatory mediators such as LPS, TNF- α and IL-1 β (Sato et al. 1995; Jackman et al. 2010). Since system x_c^- is responsible for glutamate release from activated astrocytes, microglial cells and macrophages, it is considered as an indirect contributor to synaptic transmission (Massie et al. 2015; Kigerl et al. 2012). By increasing glutamate release and the consequent activation of glutamate receptors and their downstream pathways, enhanced activity of system x_c^- could participate in the dysregulated glutamate transmission, supporting the development of chronic pain. Expressed by immune and glial cells, system x_c^- is also a key player in modulating immune and inflammatory responses. Notably, the genetic deletion of xCT was shown to attenuate inflammation in various *in vivo* and *in vitro* models of nervous disorders (Mesci et al. 2015; Albertini et al. 2018; Verbruggen et al. 2022).

In both rats and mice, the intraplantar injection of CFA is a commonly used experimental model for persistent inflammatory pain (Fehrenbacher et al. 2012). Within a few hours after CFA injection, rodents exhibit signs of peripheral inflammation with an important local edema associated with severe mechanical allodynia and thermal hyperalgesia that persist for several weeks (Stein et al. 1988; Fehrenbacher et al. 2012). This model has also been reported to trigger central plasticity with sustained neuroinflammation in the dorsal spinal cord (Raghavendra et al. 2004; Li et al. 2005). The present study aimed at elucidating the role of system x_c^- in the peripheral inflammatory response and the associated persistent pain. Before evaluating the impact of xCT deletion on pain associated behaviors, the spontaneous locomotion was monitored using the open field test to exclude potential biases associated with CFA injection that could lead to a reduced mobility. Such biases might otherwise influence paw withdrawal responses during the Von Frey or the Hargreaves tests. Our results demonstrated that, on average, throughout the duration of the experiment, neither wild-type nor transgenic mice lacking xCT exhibited significant changes in spontaneous locomotion following injection

with CFA or saline. These results are in accordance with the meta-analysis of Burek and colleagues highlighting that unilateral intraplantar injection of CFA does not impact exploratory behavior tested in an open field up to 6 weeks post-injection (Burek et al. 2021).

We here report that the invalidation of system x_c^- significantly influences the peripheral inflammation induced by CFA, as evidenced by the local edema at the injected paw and the level of inflammatory cytokines. Compared to wild-type mice, xCT-deficient mice exhibited a noticeable reduced paw edema both in the acute stage and during the entire extend of the experiment (3 weeks). As described by Mesci and colleagues when studying microglial response in a model of ALS, this attenuated local inflammation in xCT^{-/-} mice may be explained by the reduced release of pro-inflammatory mediators by activated macrophages recruited at the injection site (Mesci et al. 2015). Using a model of peripheral inflammation, the present observation strengthens the already established evidence underscoring the significant role of system x_c^- in regulating the immune responses.

Besides reduced edema, hypersensitivity to mechanical and thermal stimulation was attenuated in xCT^{-/-} mice injected with CFA compared to xCT^{+/+} mice. While the behavioral tests revealed similar sensitization in the initial days following CFA injection in both genotypes, xCT^{-/-} mice showed partial recovery within the first weeks following the injection whereas strong allodynia and hyperalgesia persisted for up to three weeks in wild-type animals. Since the nociceptive behavior of mice lacking xCT only improved at later stages after the lesion, this suggest that system x_c^- activity participates in the maintenance of pain rather than in its onset. Nevertheless, it is noteworthy that the genetic suppression of xCT in mice also impacts on the early immune responses, as shown by the reduced maximal paw thickness measured after the CFA injection. This influence of xCT in the initiation of the tissue response leading to sensitization is consistent with our previous observations in a model of neuropathic nerve lesion (Beckers et al. 2024). It

is demonstrated that certain cytokines are not only involved in the initiation but also the persistence of pathological pain through the continuous activation of nociceptive sensory neurons in the periphery (Zhang and An 2007). Also, accumulating evidence indicates that early biochemical modifications along nociceptive transduction pathways may have delayed consequences in pain pathophysiology (Victoria and Murphy 2016). To fully understand the role of system x_c^- , the use of pharmacological or genetic approaches targeting system x_c^- a few days after triggering the inflammatory insult, would help to determine whether xCT deletion can reverse established inflammatory pain¹.

Following CFA injection in the hind paw of mice, systemic inflammation is observed within a few days and persists for several weeks. We here provide evidence that the kinetic profile of this inflammatory response differs between xCT^{+/+} and xCT^{-/-} mice. Specifically, the expression levels of pro-inflammatory cytokines tend to increase over time in xCT^{+/+} mice, whereas they stabilize or even decrease in mice lacking xCT. This could explain the impact of xCT deficiency on pain perception, as increasing evidence revealed that pro-inflammatory cytokines recruit immune cells and influence sensory transduction and synaptic plasticity along the entire nociceptive axis (Ozaktay et al. 2002; Zhang and An 2007). Genetic knockout or neutralizing agents targeting these cytokines were proven efficient in supporting analgesia (Taves et al. 2013; Hess et al. 2011; Clark and Vissel 2016). Hence, plasma levels of selected cytokine have been correlated with both neuroinflammation and pain and may serve as biomarkers for chronic pain (Xu, Yang, et al. 2018). Therefore, the altered profile of peripheral pro-inflammatory cytokines in xCT-deficient mice observed in our study appears as a putative mechanism explaining the early recovery observed when

¹ These experiments are currently ongoing and will be included in the manuscript before submission for publication.

measuring allodynia and hyperalgesia in mice lacking xCT or in mice treated with SAS.

Finally, the results obtained using the genetic model of constitutive xCT deletion were corroborated using a pharmacological approach. We demonstrated that inhibiting system x_c^- by systemic treatment of mice with SAS was effective in reducing allodynia and hyperalgesia. Indeed, pre-emptively treated xCT^{+/+} mice exhibited a faster resolution of pain-related behavior when assessed with a mechanical stimulus. Similarly, SAS treatment reduced not only the duration but also the amplitude of thermal hyperalgesia and inflammation-induced paw edema. While the intact SAS behaves as a blocker of system x_c^- , the metabolites of SAS generated in the gut have anti-inflammatory properties via NF- κ B inhibition (Wahl et al. 1998; Gout et al. 2001). Nevertheless, it appears that the reduced edema of the inflamed paw and pain-related behaviors observed in xCT^{+/+} mice treated with SAS is not linked to its action on NF- κ B but rather to its inhibition of system x_c^- , as no difference in edema and pain sensitivity are observed in xCT^{-/-} mice. Although these data highlight the opportunity of targeting system x_c^- in chronic inflammatory pain, it would be pertinent to develop and test specific pharmacological inhibitors to confirm that the observed effect are indeed specifically driven by system x_c^- inhibition.

Alongside its impact on the inflammatory and immune response, system x_c^- plays a predominant role in the modulation of glutamate level in the brain parenchyma. In a mouse model of cancer-induced pain where system x_c^- expression is increased, enhanced glutamate release is observed which importantly contributes to the activation of sensory neurons (Zhu, Linher-Melville, et al. 2020). Furthermore, neuroinflammation was also shown to regulate both glutamate receptors and transporters (Tilleux and Hermans 2007; Haroon et al. 2017). Therefore, it would be essential to assess central and peripheral glutamate sensitization in xCT-deficient mice following CFA injection to determine whether the observed effects are only related to a

reduced inflammation or also rely on the modulation of excitatory transmission.

5. Conclusion

In conclusion, we here report for the first time that system x_c^- plays a role in the pathophysiology of chronic inflammatory pain. Our findings demonstrate that the absence of xCT moderates painful behaviors associated with CFA-induced peripheral inflammation. Specifically, xCT-deficient mice exhibit diminished pain hypersensitivity, which correlates with reduced systemic inflammation and edema at the injection site. With consistent data obtained using SAS as a pharmacological inhibitor of system x_c^- , this study paves the way for the future development of specific drugs targeting this exchanger to alleviate chronic inflammatory pain in patients.

GENERAL DISCUSSION

Central sensitization stands as one of the prevailing mechanisms to explain the presence of neuropathic pain. It refers to an adaptation of the CNS to persistent inflammation and an increase in glutamatergic transmission, leading to enhanced synaptic excitability in the pain pathways. Supporting this altered glutamatergic homeostasis, numerous studies have shown dysregulation of the astrocytic EAATs in models of neuropathic pain. However, in this context, little is known regarding the implication of another glial actor strongly involved in the regulation of glutamatergic transmission, namely system x_c^- . Additionally, it is now well-recognized that this exchanger also participates in the control of the inflammatory response, another crucial element in the pathological context of chronic pain. **Therefore, this led us to hypothesize that system x_c^- might be of importance in the pathophysiology of neuropathic pain and therefore, targeting system x_c^- could be beneficial in two ways. First, by reducing the release of glutamate and thus decreasing excitatory neurotransmission in the pain pathways. Second, by shifting the microglial activation towards the neuroprotective phenotype to reduce persistent inflammation during the progression of neuropathic pain and avoid plastic adaptation in the CNS.**

The main objective of this thesis was therefore to provide insights into the role of system x_c^- in the pathogenesis of chronic pain and to propose potential therapeutic strategies for alleviating chronic pain symptoms. To address this goal, we formulated several questions that were answered throughout this thesis. Specifically, we employed two validated models of chronic pain: one involving neuropathic properties induced by partial ligation of the sciatic nerve, primarily affecting central pathways, and the other one is an inflammatory pain model induced by the peripheral injection of CFA into the hind paw. We took advantage of a genetically modified mouse model lacking xCT, the specific subunit of system x_c^- . Besides, we validated our findings using a pharmacological approach to assess the potential clinical relevance.

1. Summary of the findings

In an initial workpackage, we optimized and validated a novel methodological approach to assess the functionality of system x_c^- , which is essential for understanding the biological implications of the altered xCT expression seen in several nervous disorders. This method involved the use of tritiated glutamate as a substrate for the reversed transport mediated by system x_c^- . Validation of the uptake assay was conducted in primary astrocyte cultures, in transfected cells that show constitutive overexpression or silencing of xCT as well as in crude synaptosomes from nervous tissue samples. By running the assays in Na^+ -free buffers, we were able to specifically measure the functionality of system x_c^- while avoiding potential interference from glutamate uptake by EAATs. The specificity of the assay was further confirmed using selective pharmacological inhibitors or by experiments conducted in tissue samples from transgenic mice lacking xCT. This methodological approach provides a reliable tool for screening and characterizing new system x_c^- inhibitors which have been frequently cited as valuable drugs for nervous disorders and cancer.

We used this assay in our second workpackage. Using a standardized mouse model of neuropathic pain which consists in a unilateral PNL, we evidenced that xCT was specifically up-regulated in the ipsilateral lumbar dorsal spinal cord of lesioned animals at early stage. Besides, behavioral responses to mechanical and thermal stimuli revealed that animals lacking xCT did not show constitutive hypersensitivity but show significantly lower allodynia and hyperalgesia after sciatic nerve lesion compared to their wild-type littermates or following SAS treatment. Focusing on the well documented early glial responses to the lesion, we evidenced an increased expression of GFAP and microglial M1 markers in the dorsal horn of the spinal cord in wild-type animals. These biomarkers for astrogliosis and inflammation after PNL were found to be significantly lower in animals lacking xCT or following the pharmacological SAS treatment.

We extended our research in the third workpackage by analyzing the implication of system x_c^- in the inflammatory component of chronic pain. We reported that the deletion of xCT resulted in a reduced hypersensitivity to mechanical and thermal stimuli in a mouse model of inflammatory pain which consists of subcutaneous injection of CFA in the hind paw. Mice lacking xCT also exhibited a reduced peripheral inflammation characterized by lower systemic levels of pro-inflammatory cytokines as well as a reduced paw edema following CFA injection.

2. Opportunities and limitations of the experimental models

Before moving to the interpretation of our findings, it is essential to acknowledge both the opportunities and limitations inherent in the experimental models employed. While these models offer valuable insights, they also possess inherent constraints that warrant consideration. By critically examining these factors, we can ensure a more nuanced understanding of the research outcomes.

a. Rodent versus human studies

Findings presented in this thesis are exclusively derived from studies conducted in rodents rather than in humans. Studies on humans are undoubtedly more clinically relevant, but also present several constraints or limitations.

Working with human volunteers or patients offers significant advantages, primarily because observing and analyzing chronic pain in human subjects allows researchers to witness firsthand the complexity of the condition as it manifests in real-world scenarios. Human studies also provide insights into the intricate interactions between biological, psychological, and sociocultural factors, offering a comprehensive understanding of chronic pain development and management.

However, human studies also present certain drawbacks. The inherent variability among human populations can complicate data interpretation and generalization, as individuals may exhibit diverse pain experiences due to intrinsic (e.g. genetic, metabolic status, comorbidities) or extrinsic (environment) factors. Moreover, tracking the molecular or cellular mechanisms underlying chronic pain in humans can be challenging due to the poor availability of biological samples. Ethical considerations further restrict the types of studies and interventions that can be conducted on human subjects.

In contrast, rodent models offer a controlled experimental setting with standardized experimental design. These models allow for the manipulation of the abovementioned intrinsic and extrinsic variables to address specific mechanisms underlying chronic pain. Genetic modification techniques enable the generation of transgenic rodents with targeted alterations in pain-related gene candidates, facilitating the study of disease mechanisms. Furthermore, rodent models allow for strictly defined experimental protocols and conditions, thereby reducing variability and increasing reproducibility across studies (Sadler et al. 2022).

While rodent models provide valuable insights into the underlying mechanisms of chronic pain, they may not fully capture the complexity and heterogeneity of the human condition. Besides, some physiological functions may also differ between species as it is the case for glial cells (Degl'Innocenti and Dell'Anno 2023; Smith and Dragunow 2014). This discrepancy in experimental models can pose challenges when attempting to translate findings from rodent studies to clinical applications. Moreover, the subjective nature of pain perception in humans adds another layer of complexity, making it difficult to directly correlate rodent behavioral responses with human pain experiences. Especially since rodent studies essentially focus on the measure of experimentally induced pain, we therefore lose the possibility to evaluate spontaneous pain, which is more clinically relevant,

especially when considering the efficacy of new drugs. Collaborative efforts between fundamental scientists and clinicians are needed to bridge the gap between preclinical and clinical research and ultimately develop more effective treatments for chronic pain.

b. Neuropathic pain model

The first animal model to study neuropathic pain appeared a few decades ago. It consisted in a complete transection of the sciatic nerve, often accompanied with a lesion of the saphenous nerve (Wall et al. 1979). A few years later, Bennett and Xie developed a new model which consisted in the placement of a loose chronic ligature on the sciatic nerve of rodents, triggering behaviors similar to those observed in humans suffering from neuropathic pain (Bennett and Xie 1988). Since then, several models have been developed, to better understand the pathophysiological mechanisms of neuropathic pain as well as the efficacy of different treatments (Challa 2015). In this thesis we decided to exploit the PSNL mouse model characterized by gliosis, disrupted glutamate homeostasis and neuroinflammation, all correlating with the persistence of pain behaviors such as allodynia and hyperalgesia, making this model ideal to study neuropathic pain preclinically (Seltzer et al. 1990; Korah et al. 2022).

This choice was first motivated by the strong expertise of our laboratory in investigating the central mechanisms underlying neuropathic pain using this model. While most of the studies were conducted on rats, we had to adapt and validate the procedure to mice since we wanted to include transgenic mice lacking xCT (Malmberg and Basbaum 1998).

A second advantage of this model is that it consists in a *partial* lesion of the nerve that preserves some innervation of the paw allowing for the testing of hypersensitivity behaviors. The partial ligation indeed leaves a fraction of the nerve fibers unaffected, thereby reducing the risk of motor deficits. A discrete area of the paw is therefore propitious for stimulation during behavioral tests

to evaluate hypersensitivity development (Swett and Woolf 1985). Also, PSNL closely mimics certain aspects of human neuropathic pain conditions, making it more clinically relevant for studying mechanisms and testing potential treatments (Decosterd and Woolf 2000). It reflects the focal nature of many neuropathic pain syndromes observed in patients. While the procedure is well-characterized and relatively straightforward to perform, the partial nature of the model inevitably introduces challenges to precisely define the extent of the damage to the nerve fibers, leading to some variability in experimental outcomes (Decosterd and Woolf 2000).

It is worth mentioning that rodent models may not be ideal in all cases. Therefore, it would be preferable to validate the implication of system x_c^- in neuropathic pain across multiple models simultaneously, or to explore its implication on a model such as the modified SNI (mSNI), which encompasses both pronounced and attenuated pain in the animals (Taccola et al. 2016). This would allow for the establishment of more robust correlations between system x_c^- , biological markers and pain sensitivity.

c. Inflammatory pain model

As for neuropathic pain, inflammatory pain can be modeled by a variety of procedures. Most of them consist in the injection of inflammatory agents into the rat or mouse hind paw (Hargreaves et al. 1988; Iadarola et al. 1988; Millan et al. 1988; Larson et al. 1986). Among the injected components, formalin, carrageenan, and CFA are the most frequently used. While formalin injection is mainly used for acute inflammatory studies, carrageenan and CFA are specifically employed when considering persistent pain (Hargreaves et al. 1988; Iadarola et al. 1988). While the injection of CFA solution induces a strong hypersensitivity and a cutaneous inflammation characterized by redness and edema which appear within 2 hours and persist for more than 2 weeks, the injection of carrageenan also produces hypersensitivity, but its duration is usually limited to maximum 1 week (Ren and Dubner 1999). For

this reason, we preferentially opted for the CFA model, as our main objective was to reproduce chronic inflammatory pain. This model offers an extended duration for pain-related behavior assessment, providing a wider experimental window to discern differences between wild-type and mice lacking xCT.

Furthermore, the inflammatory response triggered by CFA involves complex interactions between immune cells, glia, and neurons, offering insights into the intricate pathophysiology of chronic pain (Raghavendra et al. 2004; Lu et al. 2022). However, it is important to acknowledge that while CFA injection effectively reproduces certain aspects of chronic inflammatory pain, it may not fully replicate the multifaceted nature of clinical pain conditions, necessitating complementary models for comprehensive preclinical investigations.

3. Sex-difference in pain

In recent years, there has been growing interest in exploring sex differences in pain. As previously discussed, epidemiological and clinical evidence indicates that women are more susceptible to chronic pain, and some studies suggest that they may experience more severe clinical pain (Fillingim et al. 2009; Fillingim et al. 2003; Tang et al. 2012). This pattern is further corroborated by findings from animal models of chronic pain, consistently demonstrating greater pain sensitivity and lower response to analgesic treatments in female mice compared to males (Lee et al. 2023; Liu, Karagoz, et al. 2020; Nicotra et al. 2014). Despite the higher prevalence of chronic pain in women, female animals are greatly underrepresented in preclinical pain research (Beery and Zucker 2011). The absence of female subjects means that fundamental differences between the sexes in pain pathophysiology have been overlooked. This sex bias also reduces the translational relevance of preclinical research because of inadequate representation of the clinical population.

While the precise etiological factors underlying these sex differences remain unknown, it is likely that various biological and psychosocial (e.g. stress exposure or catastrophizing) processes are contributing factors. For instance, differences in sex hormone levels and endogenous opioid functioning between the sexes may influence pain sensitivity (Zubieta et al. 2002; Craft 2007; Craft et al. 2004). Smith and colleagues described that women experiencing high levels of estradiol and low levels of progesterone tend to display reduced pain sensitivity and increased binding of brain μ -opioid receptors compared to those with lower estradiol levels. Conversely, low endogenous opioid neurotransmission was linked to low estradiol levels (Smith et al. 2006). Mogil and coworkers have demonstrated that female mice subjected to ovariectomy displayed similar patterns of analgesia modulation as males in response to swim stress-induced analgesia (Mogil et al. 1993). The same group also evidenced that male C57BL/6J mice displayed greater sensitivity to morphine than did their female counterparts (Kest et al. 1999). However, it seems that this difference is also dependent on the genotype of the mice, which could explain inconsistencies in the literature (Kest et al. 1999; Mogil et al. 2000).

Furthermore, as previously mentioned, the immune system plays a critical role in mediating chronic pain by directly interacting with neurons through inflammatory signals. The first evidence for the sexually dimorphic role of microglia contributing to pain hypersensitivity arose from the discovery that intrathecal administration of LPS induced mechanical allodynia in male mice and was without influence in females (Sorge et al. 2011). A few years later, they demonstrated that targeting microglial activation could alleviate pain symptoms in male mice when delivered to the spinal cord, whereas this treatment failed to produce similar results in females (Sorge et al. 2015). This dimorphic role of microglia in the pathophysiology of neuropathic pain has been deeply investigated revealing sex-specific transcriptome in spinal microglia (Fiore et al. 2022). Despite observing comparable levels of microglial proliferation in the dorsal horn of both sexes, female mice appear

to employ an alternative, microglia-independent pathway to modulate pain hypersensitivity. Instead, adaptative immune cells, potentially T cells, may participate in mediating pain hypersensitivity in female mice (Mapplebeck et al. 2016; Sorge et al. 2015). Indeed, it is well characterized that after peripheral nerve injury, T cells infiltrate the spinal cord and are involved in pain hypersensitivity (Cao and DeLeo 2008; Costigan et al. 2009). Importantly, female mice lacking T cells are able to switch to the microglia-dependent pathway to mediate hypersensitivity (Sorge et al. 2015).

Due to the abovementioned-related sex differences in pain processing or perception, we decided to enroll only animals from one sex in our study, to reduce variability. Since male mice from our animal facilities were already dedicated to other projects, we deliberately decided to characterize the pain response in female mice. In our neuropathic pain mouse model, we reported differences in microglial activation between $xCT^{+/+}$ and $xCT^{-/-}$ mice. Indeed, transgenic mice lacking xCT showed reduced levels of Iba-1 in the spinal dorsal horn following PSNL as compared to their wild-type littermates. This was in accordance with pain hypersensitivity, as $xCT^{-/-}$ mice exhibited lower pain as well. Given that pain hypersensitivity appears to be also mediated by activation of other immune cell types, in particular T cells, it would be of interest to investigate how these cells are activated in our model. Additionally, considering the observed differences in microglial activation between $xCT^{+/+}$ and $xCT^{-/-}$ female mice, it would be certainly worth to repeat these experiments in males. Since the difference is already evident in female, we can expect even more pronounced effects in males.

4. Sulfasalazine specificity

SAS has received considerable attention as inhibitor of system x_c^- . There exists an abundance of evidence supporting its efficacy in inhibiting this transporter. It has indeed been widely employed in numerous preclinical

studies involving system x_c^- . This is the main reason why we decided to use it as inhibitor of system x_c^- in our study.

However, it has been established that SAS possesses peripheral anti-inflammatory properties, primarily attributed to its metabolites generated in the intestine, inhibiting the NF- κ B pathway (Wahl et al. 1998). To reduce the potential bias arising from this aspect, we administered the SAS solution intraperitoneally, in order to bypass its metabolization. Additionally, as a control, we administered SAS to mice lacking xCT and detected no additive effect. Thus, although the xCT deficient animals already exhibited reduced pain due to the absence of xCT itself, no additional effect was observed further suggesting that the observed analgesic effect is attributable to its inhibition of system x_c^- .

The chemical structure of SAS suggests a pharmacokinetic profile that restricts its CNS penetration. Despite the absence of studies determining the precise amount of compound reaching the CNS, some studies have demonstrated SAS effects on brain tumors, suggesting its ability to cross the BBB. This may however rely on an increased BBB permeability under certain pathological conditions. Another possible explanation could be the utilization of a transporter expressed in the BBB (Oatp2b1), as described by Kusuhara and colleagues (Kusuhara et al. 2012).

We acknowledge that relying solely on the use of this inhibitor may not be sufficient to definitively establish the role of system x_c^- in neuropathic pain. While in our study SAS primarily served to pharmacologically validate findings from the transgenic animal model, exploring other inhibitors would offer additional confirmation of our results. Given the promising implications of this transporter in various cancers and neurological pathologies, ongoing efforts are aimed at developing novel, more specific, and potent compounds targeting the system x_c^- (Hang et al. 2022).

5. Glial activation kinetics

Pathological pain has traditionally been attributed to dysfunctional neuronal activity. While neuronal functioning is indeed altered, there is compelling evidence highlighting the pivotal role of glial cells, particularly astrocytes and microglia, in the pathophysiology of chronic pain (Wieseler-Frank et al. 2004; Scholz and Woolf 2007; Milligan and Watkins 2009). Nevertheless, understanding the kinetics of glial cell activation in the context of pathological pain remains a challenge, often complicated by inconsistencies in the literature. Discrepancies may arise from variations in experimental techniques (RT-qPCR, immunohistochemistry) and animal models employed, as well as the selection of the markers (e.g. GFAP, S100b, Aldh1l1 for astrocytes and CD11b, Iba-1, OX-42 for microglia). Additionally, the heterogeneous nature of glial activation across different CNS regions adds another layer of complexity to understand their temporal dynamics (Lawson et al. 1990; Gwak et al. 2012). Also, glial cells operate within a feedback loop, where the release of mediators perpetuates their own activation. As a result, the kinetics of astrocyte and microglial activation are intricately intertwined and not easily delineated.

Following peripheral nerve injury, it is commonly believed that microglia activation precedes astrocyte activation (Cavaliere et al. 2007). Indeed, microglial activation tends to be limited in time, reverting to quiescent state within few days or weeks following the peripheral insult. In some cases, it may persist at reduced levels during later stages (Kohno et al. 2018). It has been shown that inhibiting microglial activation is sufficient to attenuate pain behaviors only at early stages following nerve injury (Raghavendra et al. 2003). Indeed, microglial inhibitors show low efficacy in reducing pain when administered at later stages after nerve injury (Raghavendra et al. 2003). Conversely, robust astrocyte activation appears relatively early after nerve injury but generally extends beyond the duration of the injury, which supports its role in chronic pain. It may even persist for 9 months depending

on the experimental model (Gwak et al. 2012). Thus, astrocyte activation appears to cover both the onset and maintenance of chronic pain. While some studies indicated that mediators derived from activated microglia initiate the astrocytic activation, other highlighted that astrogliosis may occur in the absence of microgliosis (Robinson et al. 2014; Hald et al. 2009). This suggests that injured afferent neurons may trigger astrocyte activation independently of microglial cells.

Our findings, however, deviate from some published results, since a rapid but transient upregulation of GFAP – an astrocytic marker - is observed within 3 days after sciatic nerve injury. Concomitantly, microglial cells are activated at early stages and persist for the entire duration of the experiment. When considering microglial activation, our findings are consistent with the literature since it is commonly suggested that microgliosis can persist for few days or weeks after nerve injury. Although we only followed the animals for 7 days, activation of microglia appeared preserved throughout this time frame. It would be of interest to envisage a longer follow-up to examine whether the observed effect at short time point remains or induces changes at later stages. With respect to astroglial activation, we unexpectedly observed that astrogliosis was transient, in discrepancy with studies proposing that astrocytes play a role not only in the acute-to-chronic pain transition but also in its maintenance (Gao 2010b; Ji et al. 2016). This underscores the complex glial activation kinetics and the importance of investigating multiple models and markers simultaneously to gain a comprehensive understanding of the dynamic activation of glial cells in pain pathophysiology. Unfortunately, even if glial cell modulators such as minocycline have shown promise in animal models of neuropathic pain, clinical trials using oral minocycline have not been as successful as expected (Arout et al. 2019). This divergence with preclinical models may be due to interspecies differences in the pharmacokinetic properties of minocycline or its interactions with non-microglial targets, which could diminish its pain-relieving effects. Developing new compounds specifically targeting microglia

or astrocytes would facilitate studies on their involvement in pathological conditions.

6. Targeting system x_c^- as a new strategy to reduce chronic pain

An increasing body of research is highlighting the critical role of system x_c^- in neurodegenerative pathologies, which frequently involve (neuro)inflammation and disruption of glutamatergic transmission. Consistently, most of the studies have reported an enhancement or upregulation of system x_c^- in the disease models that were used, and this was confirmed in our study on chronic pain (Pampliega et al. 2011; Mesci et al. 2015; Massie et al. 2008; Beckers et al. 2024). These findings suggest that targeting this transporter could hold promise as a therapeutic strategy in future treatments of nervous disorders. Given the potential significance of inhibiting system x_c^- , we prospect that concerted efforts will be mobilized towards the development of effective pharmacological inhibitors, which could offer novel avenues for patients suffering from several diseases and in particular chronic pain.

a. Is system x_c^- playing a predominant role in the onset or maintenance of chronic pain?

As for most studies evidencing new mechanism or molecular actors in an experimental model of nervous diseases, a key question to be answered is to determine the implication of system x_c^- at the onset or during the maintenance of the pathology. In the present study, all data provide evidence for a role of system x_c^- in chronic pain, but it remains challenging to ascertain its implication at the different stages of the disease. Deciphering this distinction holds significance in guiding the timing and approach of interventions targeting this exchanger, determining whether drugs should be administered preventively, curatively, or both. While our findings provide some insights into this question, additional investigations are necessary to

fully understand the extent of the implication of system x_c^- in chronic pain. The question is further complicated by the fact that early processes may impact late stages of the disease.

In the context of neuropathic pain, our study suggests that system x_c^- likely plays a role in the initiation of pain, as evidenced by the early upregulation of xCT mRNA in the spinal cord of wild-type animals after PSNL. This is consistent with the observation that xCT deficient mice do not develop any sign of thermal hyperalgesia following nerve injury. Hence, in the model of bone cancer-induced pain, the inhibition of system x_c^- was shown to delay the onset of nociception (Zhu, Linher-Melville, et al. 2020). Accumulating evidence indicates that early changes in the pain pathways can lead to plastic adaptations responsible for delayed consequences such as persisting pain (Sinatra 2010). Within the scope of this project, our investigations have been primarily focused on short-term studies, limiting the extrapolation of our findings to the maintenance phase of chronic pain.

In the model of inflammatory pain, we extended our research for up to 3 weeks after the administration of CFA, providing valuable additional insights. The constitutive deletion of xCT or the preventive inhibition of system x_c^- using SAS consistently resulted in a significant reduction in both pain hypersensitivity and peripheral inflammation. However, it is noteworthy that the temporal dynamics of these effects differ from those observed in the neuropathic pain model. Specifically, in the early stages following CFA injection, xCT^{-/-} animals displayed comparable pain sensitivity to their wild-type counterparts, suggesting a delayed onset of discernible differences. This temporal shift in response dynamics prompts further investigation into the underlying mechanisms governing the role of xCT in the initiation versus maintenance phases of chronic pain. Therefore, while our findings contribute significantly to understanding the acute effects of xCT modulation in pain models, comprehensive longitudinal studies are warranted to elucidate its long-term implications in chronic pain states.

To provide more precise understanding of the temporal implication of system x_c^- in chronic pain, we are currently conducting a study involving the administration of SAS once pain is established. Concretely, SAS will be administered 7 days after the initial insult and the impact of this treatment on the mouse behavior will be followed for two weeks. Additionally, we currently develop new mouse models allowing for conditional xCT deletion upon administration of a pharmacological agent (inducible Cre-ERT2 recombinase technology with the administration of tamoxifen). Pending optimization of the induction protocol, this approach will allow to silence system x_c^- at a defined time point during the onset or maintenance of the neuropathic pain process (Kim et al. 2018). Moreover, this approach would also ensure the absence of compensatory glutamatergic or oxidative stress-related adaptation that may have developed when using constitutively xCT deleted models (El-Brolosy and Stainier 2017). Indeed, to date no particular deleterious effects have been observed following constitutive and systemic xCT deletion in terms of oxidative stress, which may be explained by the potential upregulation of other transporters responsible for cystine uptake to allow for sufficient GSH synthesis. Nevertheless, unpublished findings from Bentea, Verbruggen and colleagues, including RNAseq and metabolomics data, show no compensation at the transcriptomic and metabolic levels in the hippocampus.

b. Is system x_c^- playing a predominant influence on neuroinflammation or glutamatergic sensitization in the context of chronic pain?

Another important question that remains only partially addressed is the mechanism by which system x_c^- is participating in chronic pain. Research conducted in our laboratory primarily focused on investigating the altered control of glutamate homeostasis by glial cells in chronic pain. However, several studies indicate that the influence of system x_c^- in the context of

nervous disorders extends beyond the modulation of glutamate transmission. Hence, the majority of the studies on system x_c^- examine its role on inflammation and immune responses whereas the impact on glutamate transmission remains secondarily addressed.

Nevertheless, given the compelling behavioral data accumulated during the present research program and the documented influence of xCT deletion on reducing glial activation and peripheral inflammation, we remain convinced that both mechanisms could be contributive. Indeed, considering the physiological function of system x_c^- , its enhanced activity in a neuropathic pain context should inevitably increase glutamate release, supporting enhanced excitatory transmission and synaptic efficacy (Figure 30).

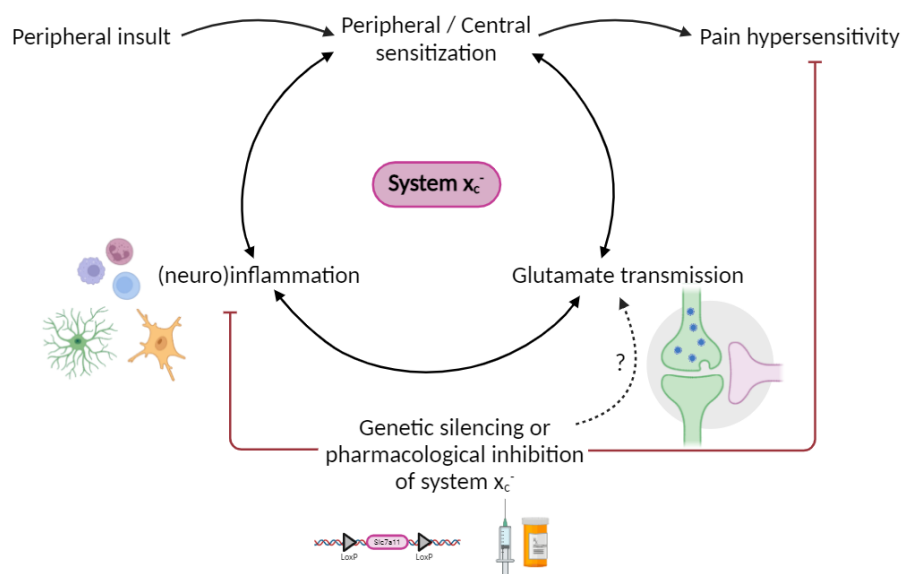


Figure 30: Interpretation of the results of this PhD thesis. For more details, see the description in the text above. Created with BioRender®.

This is consistent with findings in the context of cancer-induced pain, where tumors are found to exhibit overexpression of system x_c^- , leading to increased glutamate release that subsequently activates nociceptors triggering pain (Zhu, Linher-Melville, et al. 2020). Hence, to specifically address the question

of a putative impact of system x_c^- on glutamate sensitization in the context of chronic pain, additional research is needed. Such studies could consider the phosphorylation of specific NMDA receptor subunits or the activation of genes such as *cfos* or ERK in the spinal cord or supraspinal structures, as indirect readouts of glutamate sensitization (Qu et al. 2009; Guo et al. 2002; Gao and Ji 2009; Liu and Salter 2010).

c. System x_c^- and the modulation of oxidative stress in neuropathic pain

It is well established that oxidative stress is an important component in the pathophysiology of chronic pain (Teixeira-Santos et al. 2020; Carrasco et al. 2018). Given that system x_c^- plays a role in the transport of cystine, and thereby actively participates in the synthesis of GSH, an important ROS scavenger in the CNS, it appears paradoxical to consider targeting the system x_c^- as a promising new therapeutic strategy for chronic pain. However, although cancer cells or primary astrocyte cultures survive without xCT only when supplemented with antioxidant substances, no sign of increased oxidative stress in the absence of functional system x_c^- is observed in *in vivo* models (Massie et al. 2011). Indeed, xCT deficient mice appear to live longer and show better health status as compared to their wild-type littermates (Frare et al. 2023; Verbruggen et al. 2022). This suggests that alternative pathways to face oxidative stress compensate the absence of cystine import by system x_c^- to support GSH synthesis.

Although we did not specifically address oxidative stress in this study, since the inhibition of system x_c^- essentially has beneficial effects in this pathological context, we believe that any potential detrimental effect related to the reduced import of cystine is compensated by other mechanisms.

d. Importance of system x_c^- and glial cells in comorbidities of chronic pain

Finally, system x_c^- and glial cells have emerged as important players in stress and depressive-like behavior (Sild et al. 2017). Since depression and pain are often associated with an elevated level of inflammation, increasing studies investigate the role of glial and immune cells in this dynamic interaction. Activated microglial cells after peripheral nerve injury are detected in contralateral brain structures. Importantly, this is not restricted to the somatosensory cortex related to pain discrimination, but is also seen in brain structures associated with affect (Taylor et al. 2017). Moreover, targeting inflammatory mediators or their downstream cascades has been shown to improve depressive-like behavior and pain (Campos et al. 2020).

Concerning system x_c^- , Bentea and colleagues have demonstrated that transgenic mice lacking xCT exhibit decreased anxiety and depressive-like behaviors (Bentea, Demuyser, et al. 2015). Albertini and co-workers also evidenced that $xCT^{-/-}$ mice presented a reduced LPS-induced depressive like behaviors as compared to their $xCT^{+/+}$ littermates (Albertini et al. 2018). It is worth emphasizing that in both human and rodent models, the development and persistence of chronic pain has been extensively associated with anxiety and stress (Blackburn-Munro and Blackburn-Munro 2001; Li 2015). Indeed, altered mood behaviors, such as anxiety and depression, are frequently seen as comorbidities of chronic pain. Furthermore, anxiety has also been proposed as a predisposing factor for pain chronification (Doan and Blitz 2020). Thus, pain-driven anxiety could operate as a trigger for pain reinforcement. Indeed, individuals experiencing chronic pain are three times more likely to develop anxiety or depression while conversely, patients suffering from depression face a threefold higher risk of developing chronic pain (Li 2015). It would therefore be interesting to evaluate whether the blunted response to pain stimuli in mice lacking xCT is linked to their resilience to anxiety. Indeed, targeting system x_c^- could potentially be

beneficial not only for alleviating pain through peripheral and spinal mechanisms but also for addressing the psychological comorbidities, thereby promoting the overall well-being of patients (Nicholson and Verma 2004; Palazzo et al. 2015). This could potentially act synergistically to further enhance the quality of life for patients experiencing chronic pain.

7. General conclusion and perspectives on the role of system x_c^- in neuropathic and inflammatory chronic pain

Currently, there remains a significant shortage of therapeutic solutions for patients suffering from chronic pain. Thus, there is an important need to explore novel treatment strategies to alleviate the burden on individuals affected by this debilitating condition. Indeed, existing treatments often lack sufficient efficacy or favorable side effect profiles to efficiently relieve patients, particularly over extended periods.

The project of this PhD thesis examines the implication of system x_c^- and the consequences of its genetic silencing or pharmacological modulations in the pathophysiological context of neuropathic and inflammatory chronic pain. Despite certain limitations inherent to many preclinical studies, the importance of system x_c^- in the mechanisms underlying chronic pain should not be overlooked. Our research provides additional insights primarily linked to neuroinflammation and glial activation. While it would be important to also explore the involvement of system x_c^- in dysregulated glutamatergic transmission, we remain convinced that this protein plays a significant role in chronic pain pathophysiology. To our knowledge, we herein provide the first evidence for the implication of this exchanger in chronic pain contexts, necessitating further research to confirm or expand our findings before considering clinical translation.

Moreover, in this PhD thesis, we primarily focused on peripheral and spinal mechanisms that are often considered in preclinical chronic pain studies. However, since nociception and pain perception need to be considered as

different entities, it would be relevant and necessary to evaluate supraspinal outcomes to determine the impact of system x_c^- modulation on pain perception rather than nociception. Since pain is known to modulate brain cortical activity in humans and animals, we envisage to assess electroencephalographic signals of the somatosensory cortex as a biomarker of pain (LeBlanc et al. 2016). This would also help us to obtain information regarding spontaneous pain state rather than evoked pain as electroencephalogram recordings can be realized on awake, freely behaving animals (LeBlanc et al. 2016). In addition, further studies should also consider the activity in subcortical structures to appreciate the importance of system x_c^- in the multi-dimensional experience of pain, including affective and emotional aspects.

Concerning the clinical opportunity of targeting system x_c^- , we have so far only tested SAS, which unfortunately does not exhibit specificity for this exchanger. This work calls for the development of novel and more specific inhibitors that would be essential to further confirm our interpretations. If we can replicate the same beneficial effects on pain hypersensitivity, this would support the drugability of system x_c^- . Worth mentioning, as SAS is already FDA- and EMA-approved, the transition to clinical phases could be facilitated. Considering the complex mechanism supporting chronic pain, it seems unlikely that targeting system x_c^- alone will fully relieve patients suffering from chronic pain. More probably, treatment targeting system x_c^- should be considered in combination with other already developed therapies to potentiate their efficacy and potentially reduce the dosages of active compounds, reducing the risk of side effects.

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