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The potassium-chloride cotransporter KCC2: a new therapeutic target for spasticity and neuropathic pain

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Summary

Spasticity and neuropathic pain are common symptoms affecting patients after spinal cord injury. Spasticity is defined as an increase of the muscle tone contributing to cramps, whereas neuropathic pain is characterized by painful responses caused by a damaged nervous system. Both symptoms decrease quality of life, and currently available drugs are not uniformly useful against them. Therefore, the need to expand the range of available treatments to control these diseases is a reality in clinics.

Both symptoms arise from a reduced inhibition in the neural networks of the spinal cord. The inhibitory action depends on a low chloride ion concentration in postsynaptic neurons. This low chloride concentration is maintained by the potassium-chloride cotransporter type 2 (KCC2), which extrudes chloride ions from neurons. The expression of KCC2 is markedly decreased in motoneurons after spinal cord injury, which reduces inhibition and contributes to the development of spasticity. A similar reduction in the amount of KCC2 transporters happens in the dorsal horn of the spinal cord, causing neuropathic pain. KCC2 is an interesting therapeutic target to treat both spasticity and neuropathic pain. However, no marketed drug is able to increase KCC2 activity yet.

The present thesis aims to identify drugs capable of activating KCC2 to recover inhibition and treat spasticity and neuropathic pain.

Activation of serotoninergic 5-HT_{2A} receptors with TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide] recovers KCC2 expression in motoneurons and reduces spasticity in rats with spinal cord injury. This led us to consider if TCB-2 would increase KCC2 in the dorsal horn and alleviate neuropathic pain. We found that TCB-2 increased KCC2 in the dorsal horn of rats with a spinal cord or peripheral nerve injury. TCB-2 reduces neuropathic pain too, but only on spinal cord injury; this analgesic effect is mediated by KCC2.

In the next stage of the work, we performed a screening of drugs approved for human use from a library of compounds to identify a new KCC2 enhancer in a bioavailable and safe formulation. We identified prochlorperazine as an enhancer of KCC2 activity, recovering inhibition in motoneurons after spinal cord injury. Prochlorperazine alleviates spasticity with an efficacy that is similar to baclofen, the gold standard medication for the treatment of spasticity, and rescues the downregulation of KCC2 caused by the injury. Prochlorperazine also showed a modest reduction of mechanical hyperalgesia in animals with a spinal cord injury.

These preclinical data support prochlorperazine as a new therapy for the treatment of spasticity after spinal cord injury, having a moderate effect on neuropathic pain.

Lastly, we studied the molecular mechanisms that cause the loss of KCC2 after a spinal cord injury. Our data prove that calpain is the upstream mechanism of KCC2 downregulation and motoneuron hyperexcitability after a spinal cord injury.

This thesis validates KCC2 as a druggable target to treat spasticity and neuropathic pain after spinal cord injury.

Keywords

Spinal cord injury
Spasticity
Neuropathic pain
KCC2
5-HT_{2A}
TCB-2
Prochlorperazine
Calpain
MDL28170

Résumé

La spasticité et la douleur neuropathique sont deux symptômes qui apparaissent fréquemment après une lésion de la moelle épinière. La spasticité est définie comme une augmentation du tonus musculaire qui provoque des contractures, tandis que la douleur neuropathique se caractérise par des sensations douloureuses provoquées par une lésion du système nerveux. Les deux symptômes réduisent la qualité de vie, et les médicaments disponibles ne sont pas uniformément efficaces contre eux. Pour cette raison, le besoin d'augmenter la palette de traitements disponibles contre ces symptômes est une réalité clinique.

Ces deux symptômes résultent en partie d'une désinhibition des réseaux neuronaux de la moelle épinière. Pour être efficace, l'inhibition nécessite une faible concentration de chlorure intracellulaire dans les neurones, maintenue grâce au cotransporteur potassium-chlorure type 2 (KCC2), qui extrait les ions chlorure des neurones. L'expression de KCC2 est réduite dans les motoneurones après une lésion de la moelle épinière, ce qui entraîne une perte de l'inhibition et contribue à l'apparition de la spasticité. De la même manière, une baisse de l'expression de KCC2 dans la corne dorsale de la moelle épinière, engendre le développement d'une douleur neuropathique. KCC2 semble donc être une cible thérapeutique intéressante pour traiter la spasticité et la douleur neuropathique. Malheureusement, il n'éxiste pas encore des médicaments commercialisés capables d'augmenter l'expression et l'activité des cotransporteurs KCC2.

L'objectif de la présente thèse est d'identifier des médicaments capables d'activer KCC2 pour restaurer l'inhibition afin de traiter la spasticité et la douleur neuropathique.

L'activation de récepteurs sérotoninergiques 5-HT_{2A} avec le TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide] rétablit l'expression de KCC2 et réduit la spasticité des rats ayant subi une lésion de la moelle épinière. Ceci nous a amené à étudier l'effet du TCB-2 sur l'expression de KCC2 dans la corne dorsale et vérifier son action sur la douleur neuropathique. Nous avons découvert que TCB-2 augmente KCC2 dans la corne dorsale de rats après une lésion de la moelle épinière ou de nerf périphérique. TCB-2 réduit aussi la douleur neuropathique, mais seulement à la suite d'une lésion médullaire ; ainsi KCC2 semble intervenir dans l'effet antalgique.

Dans l'étape suivante du travail, nous avons fait un screening d'une librairie de médicaments approuvés pour un usage chez l'homme, afin de déterminer un nouveau médicament capable d'augmenter l'expression et/ou la fonctionnalité de KCC2, en une

formulation biodisponible et sûre. Nous avons identifié la prochlorperazine comme une molécule susceptible d'augmenter l'activité de KCC2. Si la prochlorperazine réduit la spasticité avec une efficacité similaire au baclofène, médicament généralement prescrit contre la spasticité chez les patients paraplégiques, et diminue la perte de KCC2 provoquée par la lésion, cette molécule a un effet plus modeste chez les animaux atteints d'allodynie mécanique suite à une lésion médullaire. Ces données précliniques montrent le potentiel pouvoir thérapeutique de la prochlorperazine dans le traitement de la spasticité après une lésion de la moelle épinière avec un effet faible sur la douleur.

En dernier lieu, nous avons étudié les mécanismes moleculaires responsables de la baisse de KCC2 à la suite d'une lésion médullaire. Nos données montrent que l'activation de la calpaïne est le mécanisme qui provoque la perte de KCC2 et l'hyperexcitabilité des motoneurones suite à une lésion de la moelle épinière.

Cette thèse valide KCC2 comme une cible thérapeutique dans le traitement de la spasticité et la douleur neuropathique suite à une lésion de la moelle épinière.

Mots clés

Lésion de la moelle épinière
Spasticité
Douleur neuropathique
KCC2
5-HT_{2A}
TCB-2
Prochlorperazine
Calpaïne
MDL28170

Resumen

La espasticidad y el dolor neuropático son síntomas que aparecen frecuentemente tras una lesión de la médula espinal. La espasticidad se define como un aumento del tono muscular que provoca contracturas, mientras que el dolor neuropático se caracteriza por sensaciones dolorosas causadas por una lesión del sistema nervioso. Ambos síntomas reducen la calidad de vida, y los fármacos disponibles no son uniformemente eficaces contra ellos. Por este motivo, la necesidad de aumentar el abanico de tratamientos disponibles contra estos síntomas es una realidad clínica.

Ambos síntomas surgen debido a una desinhibición de las redes neuronales de la médula espinal. Para ser eficaz, la inhibición necesita una baja concentración de cloruro intracelular en las neuronas, que se mantiene gracias al cotransportador potasio-cloruro de tipo 2 (KCC2), que extrae iones cloruro de las neuronas. La expresión de KCC2 se reduce en las motoneuronas tras una lesión de la médula espinal, lo que genera una pérdida de la inhibición y contribuye a la aparición de la espasticidad. De la misma manera, una reducción de KCC2 en el asta dorsal de la médula espinal contribuye a la aparición de dolor neuropático. KCC2 parece ser una diana terapéutica interesante para tratar la espasticidad y el dolor neuropático. Sin embargo, no existen aún fármacos comercializados capaces de aumentar la expresión y la actividad de los cotransportadores KCC2.

El objetivo de la presente tesis es identificar fármacos capaces de activar KCC2 para restaurar la inhibición, con el fin de tratar la espasticidad y el dolor neuropático.

La activación de receptores serotoninérgicos 5-HT_{2A} con TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl)methylamine hydrobromide] reestablece la expresión de KCC2 y reduce la espasticidad en ratas con una lesión de la médula espinal. Esto nos llevó a estudiar el efecto del TCB-2 sobre la expresión de KCC2 en el asta dorsal y a verificar su acción sobre el dolor neuropático. Descubrimos que TCB-2 aumenta KCC2 en el asta dorsal de las ratas tras una lesión de la médula espinal o de nervio periférico. TCB-2 reduce también el dolor neuropático, pero sólo tras una lesión medular; el efecto analgésico parece depender de KCC2.

En la siguiente etapa del trabajo, hicimos un screening de una librería de fármacos aprobados para uso humano, para encontrar un nuevo fármaco capaz de aumentar la expresión y/o la función de KCC2, en una formulación biodisponible y segura. Identificamos la proclorperazina como una molécula susceptible de aumentar la actividad de KCC2. La proclorperazina reduce la espasticidad con una eficacia similar al baclofeno, el medicamento que se prescribe más habitualmente a los pacientes parapléjicos para tratar la espasticidad,

y disminuye la pérdida de KCC2 provocada por la lesión. Sin embargo, la proclorperazina tiene un efecto más modesto en los animales que desarrollan alodinia mecánica como consecuencia de una lesión de la médula espinal. Estos datos preclínicos ratifican el potencial de la proclorperazina como posible indicación terapéutica en el tratamiento de la espasticidad tras una lesión de la médula espinal, con un efecto débil sobre el dolor neuropático.

Por último, investigamos los mecanismos moleculares responsables de la reducción de KCC2 como consecuencia de una lesión medular. Nuestros datos muestran que la activación de la calpaína es el mecanismo que provoca la pérdida de KCC2 y la hiperexcitabilidad de las motoneuronas tras una lesión de la médula espinal.

Esta tesis valida KCC2 como una diana terapéutica en el tratamiento de la espasticidad y el dolor neuropático tras una lesión de la médula espinal.

Palabras clave

Lesión de la médula espinal
Espasticidad
Dolor neuropático
KCC2
5-HT_{2A}
TCB-2
Proclorperazina
Calpaína
MDL28170

List of abbreviations

- **5-HT :** serotonin
- **aCSF :** artificial cerebrospinal fluid
- **ATP:** adenosine triphosphate
- **ATPase:** adenosine triphosphatase
- **BBB :** Basso-Bettie-Bresnahan test
- **BDNF :** Brain-derived neurotrophic factor
- **CaV :** voltage-activated calcium channels
- **CB1 :** cannabinoid receptors type 1
- **CB2 :** cannabinoid receptors type 2
- **CBD :** cannabidiol
- **CCC :** cation-chloride cotransporters
- **CHEPS :** contact heat-evoked potentials
- **CNRS :** Centre National de la Recherche Scientifique
- **CNQX :** 6-cyano-7-nitroquiloxaline-2,3-dione
- **DIOA:** [(dihydroindenyl)oxy] alkanoic acid
- **DL-APV :** DL-2-amino-5-phosphonovaleric acid
- **DMSO :** dimethyl sulfoxide
- **dpi :** days postinjury
- **DRG:** dorsal root ganglia
- **ECl:** chloride equilibrium potential
- **EEG :** electroencephalography; electroencephalogram
- **EGABA :** GABA equilibrium potential
- **EIPSP:** equilibrium potential of inhibitory postsynaptic potentials
- **EMEA:** European Medicines Agency
- **EMG:** electromyography; electromyogram
- **EPSP:** excitatory postsynaptic potential
- **FDA:** Food and Drug Administration
- **G:** gastrocnemius muscle
- **GABA:** γ-aminobutyric acid
- **$GABA_A$:** GABA receptors type A
- **$GABA_B$:** GABA receptors type B
- **GABAR:** GABA receptor
- **GAD:** glutamic acid decarboxylase
- **GAD65:** glutamic acid decarboxylase 65 kDa
- **GAD67:** glutamic acid decarboxylase 67 kDa
- **H reflex:** Hoffmann reflex

- **HRP:** horseradish peroxidase
- **i.p.:** intraperitoneal
- **i.v.:** intravenous
- **IgG :** immunoglobulin G
- **INaP :** persistent inward sodium currents
- **IPSC:** inhibitory postsynaptic current
- **IPSP:** inhibitory postsynaptic potential
- **IRME:** Institut pour la Recherche sur la Moelle épinière et l'Encéphale
- **KCCs :** potassium-chloride cotransporters
- **KCC2:** potassium-chloride cotransporter type 2
- **Ki :** inhibition constant
- **LTP:** long-term potentiation
- **mRNA:** messenger RNA
- **NaV:** voltage-activated sodium channels
- **NaV1.6 :** voltage-activated sodium channel 1.6
- **NEUPSIG:** neuropathic pain special interest group of the International Association for the Study of Pain
- **NKCC1 :** sodium-potassium-chloride cotransporter type 1
- **NMDA :** N-methyl-D-aspartate
- **NS neuron :** nociceptive-specific neuron
- **PAD:** Primary Afferent Depolarization
- **PBS :** phosphate-buffered saline
- **PCPZ :** prochlorperazine
- **PEST sequence :** proline (P), glutamic acid (E), serine (S) and threonine (T) sequence
- **PFA :** paraformaldehyde
- **PKC :** protein kinase C
- **PLCγ :** phospholypase Cγ
- **PNI :** peripheral nerve injury
- **PVDF :** polyvinylidene fluoride
- **RDD :** rate-dependent depression
- **S940D :** mutation consisting on substituting serine 940 from KCC2 by an aspartic acid
- **SCI :** spinal cord injury
- **SDS/PAGE:** sodium dodecyl sulphate-polyacrylamide gel electrophoresis
- **Ser940:** serine 940 of KCC2 protein sequence
- **siRNA:** small interfering RNA
- **SNI :** spared nerve injury
- **TA:** tibialis anterior muscle
- **TCB-2:** (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide
- **THC:** delta-9-tetrahydrocannabinol
- **TrkB:** tropomyosin receptor kinase B, a BDNF receptor
- **vGluT1:** vesicular glutamate transporter type 1
- **Vrest:** resting potential

- **VTA:** Ventral Tegmental Area
- **WDR neuron:** wide dynamic range neuron
- **WHO:** World Health Organisation

List of publications

Liabeuf S, Stuhl-Gourmand L, Gackiere F, Mancuso R, **Sanchez-Brualla I**, Marino P, Brocard F, Vinay L (2017) Prochlorperazine increases KCC2 function and reduces spasticity after spinal cord injury. J Neurotrauma 34(24):3397-3406.

Sanchez-Brualla I, Boulenguez P, Brocard C, Liabeuf S, Viallat-Lieutaud A, Navarro X, Udina E, Brocard F (2018) Activation of 5-HT_{2A} receptors restores KCC2 function and reduces neuropathic pain after spinal cord injury. Neuroscience 387:48-57.

Sanchez-Brualla I, Mancuso R, Boulenguez P, Brocard C, Liabeuf S, Navarro X, Udina E, Vinay L, Brocard F (in preparation) Prochlorperazine does not reduce neuropathic pain after a hemisection of the spinal cord or a spared nerve injury.

Plantier V, **Sanchez-Brualla I**, Brocard C, Gackiere F, Zanella S, Liabeuf S, Vinay L, Brocard F (in preparation) Calpain upregulates the persistent sodium current and alters the chloride homeostasis to generate spastic hyperreflexia after spinal cord injury in neonatal rats.

Sanchez-Brualla I, Brocard C, Durand J, Brocard F (in preparation) Calpains downregulate KCC2 in motoneurons after a spinal cord injury.

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1. Introduction

Spasticity and pain are common disorders affecting individuals after spinal cord injury (SCI). Spasticity is defined as a velocity-dependent increase of the muscle tone contributing to eventual spasms and contractures, whereas neuropathic pain is characterized by painful responses caused by neural damage and not by nociceptive stimuli. Both symptoms decrease quality of life, and none of the currently available drugs are uniformly effective in treating them. Therefore, the need to expand the range of available treatments to control these diseases is a clinical reality.

One trigger of spasticity and pain is a reduced inhibition in the neural networks of the spinal cord. After a spinal cord injury there is a loss of inhibition due to the interruption of the inhibitory cortical projections, but there is also a higher intrinsic excitability of the spinal networks below the lesion (Corleto et al., 2015). In normal conditions, the action of inhibitory interneurons in the spinal cord is possible thanks to a low chloride ion concentration in their target neurons, maintained by membrane proteins, the KCC2 transporters, that extrude potassium and chloride ions outside neurons (Coull et al., 2003; Vinay and Jean-Xavier, 2008). The expression of KCC2 is markedly decreased after SCI, reducing inhibition in spinal cord networks and causing spasticity (Boulenguez et al., 2010). A similar reduction in the amount of KCC2 transporters in the dorsal horn of the spinal cord is also responsible for neuropathic pain after SCI (Lu et al., 2008) or after a peripheral nerve injury (Coull et al., 2003), and preventing KCC2 downregulation avoids the development of neuropathic pain (Mòdol et al., 2014). Accordingly, KCC2 is an attractive target to restore endogenous inhibition in pathological conditions and to treat both spasticity and neuropathic pain, but the molecular mechanisms responsible for the decrease of KCC2 remain unknown and no marketed drug is able to increase KCC2 function and expression in human patients yet.

My thesis aims at completing the description of the mechanisms regulating KCC2 on the dorsal and ventral horn after spinal cord and peripheral nerve injuries and at identifying medications capable of increasing KCC2 function.

The manuscript will be composed of 4 chapters. On this, first chapter, we will expose the two symptoms that we are aiming to cure, spasticity and neuropathic pain. We will debrief current available treatments and their efficacy. We will describe in a non-exhaustive way our knowledge on the deregulations of inhibitory control in the spinal cord after a spinal cord injury, focusing particularly on the co-transporter KCC2. In a second chapter, we will summarize all the methods and techniques used during the thesis. The third chapter will present our studies that have been conducted to explore the pathways of KCC2 alteration after SCI, and the treatments we propose to boost KCC2 expression and function. Finally, in a fourth and final chapter we will discuss the data collected.

1.1. Spinal cord injury

Spinal cord injury is a complex medical issue. The spinal cord contains neural circuits that are critical for the movement of the limbs and the trunk, for the sensation of the skin, and for many autonomous activities that are essential for life. It is possible to suffer damage to only one of the networks integrating the spinal cord, producing only motor, sensory or autonomous deficits. However, the most common spinal cord injuries produce the *complex medular syndromes* in which all the functions of the spinal cord are affected (Siddall et al., 1999; Cook et al., 2007; de Miguel and Kraychete, 2009; Laso, 2010; Hubli et al., 2011). Medular section and compression are the most common forms of spinal cord injury in clinics. The spinal cord section is a good model to study spinal plasticity after a spinal cord injury and therefore it is the model that we used along this thesis.

A spinal cord section is characterized by the functional interruption of the spinal cord networks by a transverse section (Laso, 2010). It is important to consider that the diagnosis depends on a functional point of view: even if there is not a –visible- physical interruption of the spinal cord tracts, a lack of the function mediated by these tracts indicates a diagnosis of spinal cord injury. Its most usual cause is a traumatic injury –car accidents, extreme sports or gun shots being the most common factors- (Laso, 2010).

The spinal cord section has two phases: spinal shock and the phase of excessive reflex activity (Laso, 2010):

- The spinal shock phase starts immediately after the injury and it can last from days to several weeks. It is characterized by a total anesthesia, an abolition of the myotatic reflex -producing a flaccid paralysis- and the abolition of the autonomous reflexes.
- The phase of excessive reflex activity involves a recovery of the medullary reflex activity, which is exaggerated for being out of the inhibitory control of the superior centres. It is characterized by a total anesthesia, a complete “pyramidal syndrome” (paralysis with spasticity, muscular hyperreflexia, clonus, bilateral Babinski sign, phenomenon of the triple flexion -flexion of hip, knee and ankle in response to a stimulus; it implies a severe damage in the corticospinal pathway-) and reflex autonomous hyperactivity, i.e.: spastic bladder.

The section of the spinal cord can be complete -causing the interruption of all the spinal cord tracts at the affected level-, or it can be incomplete -affecting only part of the spinal cord tracts-. The consequences of the lesion in this case will depend on the spinal cord tracts affected. A classic example is the Brown-Séquard’s syndrome, which is caused by a hemisection of the spinal cord, and consists of a paralysis of the half of the body under the level of the injury that is ipsilateral to the lesion –as a result of the damage of the corticospinal

tract-, and a loss of light touch, proprioception, pain and temperature sensitivity in the skin of the contralateral half of the body under the level of the injury–as a result of the damaged dorsal columns and spinothalamic tracts-.

Appart from spinal cord section, another cause of spinal cord injury can be the **compression** of spinal cord structures. It can be extrinsic (extramedullary compression) or intrinsic (intramedullary compression, i.e.: produced by a tumor). The clinical expression of compression is similar to the one caused by a section of the compressed structures, with some differences. For example, since the injury is generally progressive in a compression, paralysis can be associated to spasticity since its onset (Laso, 2010).

In the following sections we will further describe the two signs that we studied in this thesis: spasticity and neuropathic pain. Both can appear after a medular section or a compression (Siddall et al., 1999; Cook et al., 2007; de Miguel and Kraychete, 2009; Hubli et al., 2011).

1.2. Spasticity

Spasticity is one sign of the upper motor neuron syndrome. The upper motor neuron syndrome is the set of clinical signs that appear as a consequence of the damage to upper motor neurons along the descending motor pathways (Purves et al., 2001; Laso, 2010). It is estimated that about 12 million people may be affected by spasticity worldwide, although the exact number is not known (Ertzgaard et al., 2017). The percentage of spinal cord injury patients that develop spasticity is estimated to be around 60% worldwide (Sköld et al., 1999).

1.2.1. Definition of spasticity

The medical definition of spasticity is given by Lance (1980), being "a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes ("muscle tone") with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex, as one component of the upper motor neuron syndrome".

However, Lance's definition is very restrictive for clinicians, since the clinical signs of spasticity sometimes go beyond Lance's definition, which defines spasticity considering only findings from a mobilization of the patient's limb, while spasticity can manifest also with tonic contraction of a muscle or muscle group when the patient is resting (Figure 1a), flexor and extensor spasms, and co-contraction (simultaneous contraction) of agonist and antagonist muscle pairs (Pandyan et al., 2005; Sheean and McGuire, 2009) (see Figure 1b). In recent years, several other definitions of spasticity have been proposed, which are more aligned with

clinical observations (Pandyan et al., 2005; Saulino et al., 2016). However, the definition of spasticity that is generally accepted by the whole medical community is still Lance's.

From a clinical point of view (Laso, 2010), spasticity is the hypertonus of the paralyzed limbs, and it is characterized by:

- A predominance of hypertonia on the flexor muscles of the arms, and on the extensors of the legs. The extension of the legs, if stable, may allow some patients to walk, with a characteristic scissoring gait (Furr-Stimming et al., 2014)
- Phenomenon of "the clasp-knife response": the passive mobilization of a limb meets with sudden resistance, but if enough strength is applied, the resistance decreases also suddenly, on a similar way to the functioning of a clasp-knife (Laso, 2010).

Spasticity is a condition that can be severely invalidating, and if left untreated, it can cause pain and deformities of joints (Furr-Stimming et al., 2014; see figure 1a), while also complicating the management of the daily tasks and caring that the patients need. However, at times it may not be problematic, as in the cases where it allows patients to walk. Spasticity is treated –or not- according to the needs and expectations of each patient (Ertzgaard et al., 2017).

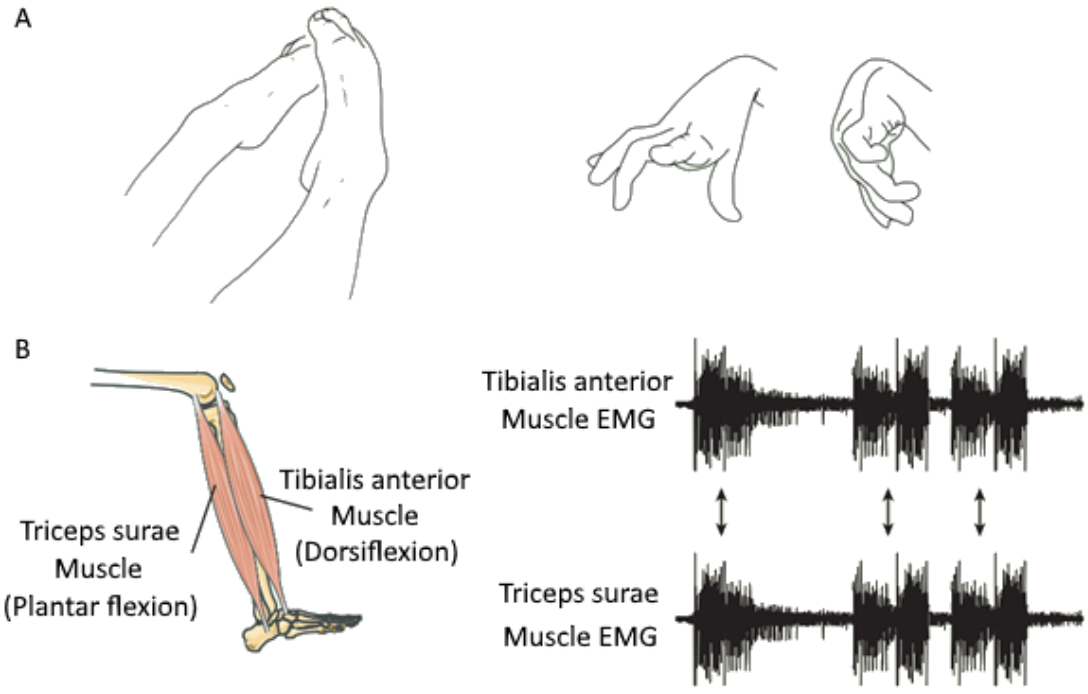

Figure 1. Clinical signs of spasticity. a) Examples of ankle and wrist of patients with chronic spasticity. The chronic, dysregulated contraction of the muscles can cause a deformation of the joints. b) Electromyograms (EMG) of hindlimb muscles (*Tibialis anterior* and *Triceps surae*, respectively flexor and extensor of the ankle), illustrating a co-contraction (double arrow) of antagonistic muscles during spasms registered on a patient with a spinal cord injury. Figure adapted from Plantier and Brocard, 2017.

1.2.2. How to evaluate spasticity in patients and animals

1.2.2.1. Things to consider when evaluating spasticity

On a clinical setting, the evaluation of spasticity is necessary to determine whether an anti-spastic treatment is needed or not, and to evaluate the efficacy of a given treatment. Something to consider before comparing clinics and laboratory techniques, is that the level of spasticity present at any given moment is affected by many external factors, including temperature, anxiety, time of the day, level of pain, body position, and whether or not the limb has been previously stretched. All these factors can confound the interpretation of repeated measurements (Sheean and McGuire, 2009). This makes the evaluation of spasticity delicate, since it should be necessary to try to minimize the external variations to be able to measure it reliably.

1.2.2.2. How to evaluate spasticity in clinics

Hypertonia can be described clinically using well-established rating scales, such as the Ashworth, or modified Ashworth scales (Ashworth, 1964; Bohannon and Smith, 1987), the Tardieu scale (Tardieu et al., 1954), and Penn scale (Penn and Kroin, 1985). These scales have been criticized for many reasons, including their inability to distinguish between the contribution of the muscle and the neuronal hyperexcitability to hypertonia. I will briefly expose these scales and compare them:

Ashworth Scale and Modified Ashworth Scale

The Ashworth Scale consists in evaluating hypertonia on a 0 to 4 range, based on the passive mobilization of a joint (Ashworth, 1964). The Modified Ashworth Scale rates spasticity from 0 to 4 using "+" in between numbers to indicate small increases in spasticity (Bohannon and Smith, 1987; Furr-Stimming et al., 2014). The modified Ashworth scale is the most widely used tool to quantify spasticity, in clinics and in scientific studies. This measure has the advantage of being easy to apply and fast to execute. It has a good intra-operator reproducibility (i.e. : the doctor tends to classify the patients' degree of spasticity correctly, according to their own standards). However, the reproducibility across different operators is weak, being a highly subjective measure. The evaluation of the Modified Ashworth Scale appears in Table 1.

0	No increase in muscle tone
1	Slight increase in muscle tone, manifested by a catch and release or by minimal resistance at the end of the range of motion when the affected part(s) is moved in flexion or extension
1+	Slight increase in muscle tone, manifested by a catch, followed by minimal resistance throughout the remainder (less than a half) of the range of motion
2	More marked increase in muscle tone through most of the range of motion, but affected part(s) easily moved
3	Considerable increase in muscle tone, passive movement difficult
4	Affected part(s) rigid in flexion or extension

Table 1. Modified Ashworth scale.

Tardieu scale

The Tardieu scale is less subject to inter-operator variability. The stretching is done at two speeds (slow and fast). The operator determines two angles: the angle (R1) at which resistance appears with a fast muscle stretch, and the final angle (R2), which reflects the maximum range of movement with a slow muscle stretch –this rules out the muscular component of hypertonia. The difference between R2 and R1 is the spasticity angle related to hyperreflexia (Haugh et al., 2006; Sheean and McGuire, 2009). Furthermore, the angle at which resistance appears, together with its intensity (calibrated between 0 and 4) are registered. This scale is the most complete measure of spasticity, but it is scarcely used, since it is complicated to perform in the context of a medical visit. The items of the Tardieu Scale appear on Table 2.

Quality of the muscle reaction	
0	No resistance through the course of the passive movement
1	Slight resistance through the course of the passive movement with no clear catch at a precise angle
2	Clear catch at a precise angle, interrupting the passive movement, followed by a release
3	Fatiguable clonus, less than 10 seconds when maintaining the pressure, appearing at a precise angle
4	Unfatiguable clonus, more than 10 seconds when maintaining the pressure, at a precise angle
Angle at which resistance appears	
	The angle is measured considering angle 0 as the position of minimum stretching for every joint. With the exception of the hip, where the angle at 0º is the anatomical resting position

Table 2. Tardieu Scale.

Penn test

It is a self-assessment of the frequency at which spasms take place (Penn and Kroin, 1985). It is less used, because it does not evaluate spasticity according to Lance's definition. For the spasticity of spinal origin, which produces spasms of the inferior limbs, it is used to evaluate the frequence and the spontaneous or induced character of spasms. This auto-evaluation scale allows a more personalized follow-up of spasticity. The questionnaire of the Penn test is represented in Table 3.

How often are muscle spasms occurring?	
0	No spasms
1	Spasms induced only by stimulation
2	Spasms occurring less than once per hour
3	Spasms occurring between 1 and 10 times per hour
4	Spasms occurring more than 10 times per hour

Table 3. Penn spasm frequency scale

Other tests

In the lower limbs, spasticity in the quadriceps can be measured by the pendulum test, in which the subject is lying on a table with the lower leg hanging. The lower leg is taken, until the knee is fully extended against gravity and then it is left, allowed to fall, oscillating until stopping at rest. The movement of the leg can be recorded with accelerometers and the pattern compared with normal subjects (Sheean and McGuire, 2009).

Electrophysiological tests

This method evaluates the excitability of the stretch reflexes on an electromyogram (Sheean and McGuire, 2009). This is usually done by obtaining the ratio between the maximum Hoffmann reflex (H wave) and the maximum M wave on an electromyogram, which is interpreted as a marker of motoneuron excitability (Angel and Hofmann, 1963).

Figure 2 shows how M wave and H reflex are obtained: when a nerve is stimulated, the electrical impulse travels orthodromically along the motor axons to stimulate the target muscle (1, green). The contraction of the muscle caused by this stimulation is the M wave. At the same time that the nerve is stimulated, the electrical impulse travels -also orthodromically- through the sensory axons, the primary afferents, to the spinal cord (2, orange), where it will stimulate motoneurons to produce another muscle contraction. This contraction is the H wave (named H because it implicates the Hoffman reflex), it is generally smaller than the M wave and it appears later on the recordings because the pathway that the

stimulation needs to follow is longer than for the M wave. The maximum H wave provides a value indicating the maximum percentage of a motoneuron pool that is recruited after a nerve stimulation (Fisher, 1992), and the maximum M wave provides a value indicating the number of motor units that are recruited on a muscle contraction, although these definitions are not exact (Pierrot-Deseilligny and Mazevet, 2000).

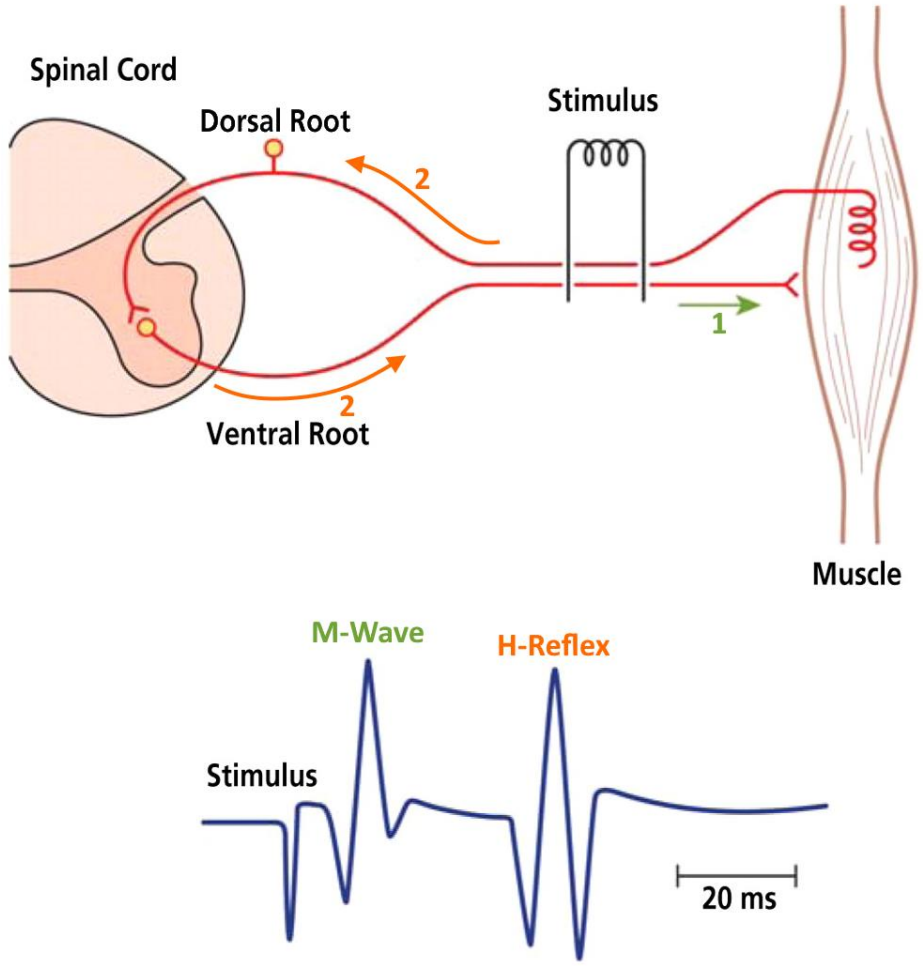

Figure 2. Anatomical pathway followed by a nerve stimulation to produce the electromyogram recording, and scheme of a recording showing the M-wave and the H-reflex. Figure adapted from Wynne et al. (2006).

The ratio Hmax/Mmax provides an easily obtained measure of motoneuron pool activation, and therefore, of motoneuron excitability (Fisher, 1992), although a strict methodology is required to be able to get valid results and interpret them (Pierrot-Deseilligny and Mazevet, 2000). The H reflex obtained by electrical stimulation cannot be compared to stretch reflexes because it does not include muscle spindle activation. Furthermore, studies in the last decades have proven that the properties of both reflexes are different beyond the contribution of the muscle spindle (Nielsen et al., 2007).

The description of the H reflex by Hoffmann, in 1918, was done on the gastrocnemius muscle, upon stimulation of the tibial nerve. This reflex could be compared to the calf reflex, also called Achilles reflex or ankle jerk reflex, and it has been the most studied since then. There is a considerable variability regarding the physiological H/M ratio of leg muscles, but the H/M ratio for calf H reflexes is normally less than 0.7 (Fisher, 1992).

The nerves that are most frequently stimulated for this test are the tibial nerve in the lower limb –as it was originally described by Hoffmann (Fisher, 1992)-, and the median in the upper limb (Marsala et al., 2005). It is important to notice that the Hmax/Mmax ratio does not always correlate with the measure of spasticity (Shemesh et al., 1977; Macdonell et al., 1989; Tekgül et al., 2013), although it correlates with the severity of spinal cord injury (Kumru et al., 2015). The H reflex is exacerbated when spasticity arises, but it is not the only component of spasticity; perhaps this is the reason why there is no direct correlation.

The strategies for the diagnosis of spasticity in clinics are depicted on Figure 3, "Diagnosis of spasticity in human patients".

1.2.2.3. How to evaluate spasticity in animals

The most widely used methods to accurately measure spasticity in animal models, are electromyographical records, mainly the Hmax/Mmax ratio, the Rate-Dependent Depression of the H reflex or the muscle spasms (Nielsen et al., 2007; Corleto et al., 2015).

Briefly, the obtention of **the Hmax/Mmax ratio** is performed in anesthetized rats, stimulation electrodes are implanted next to the sciatic nerve, and register electrodes in the plantar interosseus muscle. A slowly increasing stimulation is applied, leaving at least 1 minute in between each pulse, to obtain the maximum H wave. Afterwards, the stimulation pulse continues increasing in amplitude, to obtain the maximum M wave. The ratio Hmax/Mmax is increased in spastic animals compared to controls.

The Rate-Dependent Depression of the H reflex is obtained by stimulating the sciatic nerve and recording the M and H waves in the plantar interosseus muscle. This time, the stimulation consists of a train of pulses, at increasing frequencies. In normal rats, the H reflex shows a Rate-Dependent Depression: it becomes smaller when the sciatic nerve is stimulated at increasing frequencies. This feature is partially or completely lost on spastic rats.

The evoked muscle spams. Spasms can be evoked and analyzed in several hindlimb muscles –mainly tibialis anterior, flexor digitorum brebis, plantar interossei,…- of anesthetized or awaken spinal cord injured rodents.

To obtain the awaken spasm of the flexor digitorum brebis, the animals are introduced into a plexiglass tube and their paralyzed limbs are immobilized with tape. A pair of stainless steel stimulating needle electrodes are inserted transcutaneously into the surroundings of the tibial nerve, above the ankle. A recording electrode is inserted in the recording muscle, the reference electrode into the foot, and the ground electrode, in the base of the tail. The nerve is then stimulated with increasing stimulus intensities, to obtain H-reflex and M-wave recruitment curves, to fix a supramaximal single-pulse stimulation intensity to elicit muscle spasms (Brocard et al., 2016).

To obtain the evoked muscle spasm on anesthetized animals, the procedure is similar, just adding the anesthesia at the beginning, with no need for the plexiglass tube, and placing the animals over a heating pad to keep body temperature constant, to obtain the compound muscle action potential (M and H waves) (Valero-Cabré and Navarro, 2002).

Quantification of tail spasms. A model that deserves a particular attention is the spastic tail model, which was first developed in the cat (Ritz et al., 1992), and later developed in the rat by Bennett et al. (1999). Rats with a spinal cord transection at the level of the second sacral segment (S2), develop hypertonia, hyperreflexia, and clonus, in the tail, and these changes can be measured in the awake rat. Muscle stretch or cutaneous stimulation of the tail produce muscle spasms and increases in muscle tone, which can be measured by force recordings or electromyography.

When the tail is unconstrained, it is coiled by spontaneous or reflex-induced flexor and extensor spasms. Tail movement during spasms often triggers clonus in the end of the tail. The tail hair and skin were hyperreflexive to light touch, and sometimes light touch alone can cause clonus.

Apart from tail spasms, the rat spastic tail model presents spasticity in the tail muscles as an augmentation in the Hoffman reflexes (H-reflexes), which increase significantly 2 weeks after transection. The spasticity observed in S2 spinal cord injury rats is similar to the spasticity seen in limb muscles of humans with spinal cord injury (Bennett et al., 1999).

This model also presents long-lasting (2s) tail spasms in response to mild stimulation –like the awaken spasm- of the caudal nerve trunk (mixed nerve innervating the tail muscles) (Bennett et al., 2004). These reflexes developed gradually during the experiment, in parallel to the previously mentioned measures of spasticity. Electrical stimulation of the tip of the tail also evoked long-lasting spastic reflexes in chronically injured rats, not in acutely injured or normal rats (Bennett et al., 2004). To produce a similar reflex in acutely injured rats, a previous, conditioning C-fiber stimulation on the tip of the tail is necessary.

To conclude with this model, Harris et al. (2006) proved that spinal cord injury anesthetized rats with tail spasticity presented changes in the tail muscle phenotype: they performed EMG recordings and obtained the duration of twitchs, twitch force, peak, half-rise time, half-fall time and decay rates, to observe that the contribution of slow motoneurons increased, but it was concomitant with a lower tetanic force production, which indicates a loss of fatigue resistance.

Intracellular recordings in vivo. An advantage of animal studies is that it is also possible to examine the intrinsic excitability of the spinal motoneurons, by studying persistent inward currents and plateau potentials, in vivo (Sheean and McGuire, 2009).

Alternative approaches to get closer to clinical assessments. As we mentioned previously, the most common way to assess spasticity on a clinical examination, is by passively mobilizing the patients' limbs. This is hard to replicate in laboratory animals, since it is necessary that the limb being tested is relaxed. Nevertheless, at least one strategy to measure spasticity has been developed, that is similar to the common approaches taken on clinical settings: the computer-controlled ankle rotational system developed by Marsala et al. (2005). Briefly, rats are placed into a plastic restrainer and a hind paw attached by tape to a metal plate; this plate is connected to a computer-controlled stepping motor that can perform different angles of rotation. The paw of the rat is placed under a resistance transducer, which measures the resistance to the rotational movement at different speeds. This is not the first device created to measure spasticity: a strain-gauge system proposed to measure muscle tension had been previously proposed (Johnels and Steg, 1982), together with a technique to measure ankle torque and triceps surae EMG (Thompson et al., 1996). However, in general, the lab-designed methods to measure spasticity in awaken rodents are hard to implement due to the need of specific equipment, and the animals do not always get used to them.

The strategies to evaluate and quantify spasticity in animal models are depicted in Figure 3, "Evaluation of spasticity in animal models".

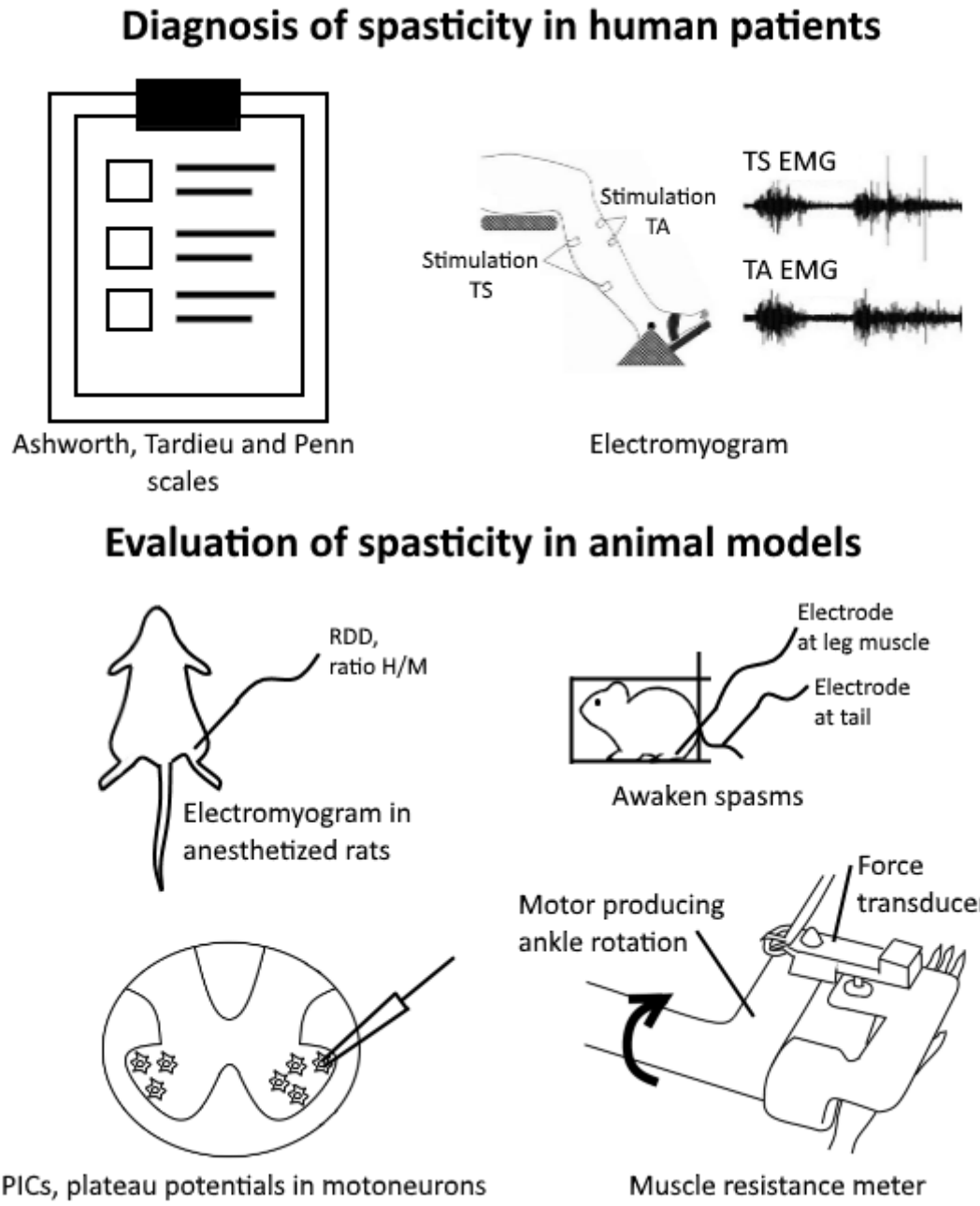

Figure 3. Comparison of the evaluation of spasticity in human patients and animal models. The tools for the diagnosis of spasticity in human patients are Ashworth, Tardieu and Penn scales, and electromyogram tests (like the ratio between the amplitude of H and M waves). The figure depicting the electromyogram tests has been adapted from Nickolls et al. (2004). The tools for evaluating spasticity in animal models are electromyogram tests in anesthetized rats, awaken spasms of leg and tail muscles, measurements of PICs and plateau potentials using intracellular electrophysiology, and devices to measure muscle tension like the muscle resistance meter created by Marsala et al. (2005) which measures the muscle resistance to an ankle rotation by using a force transducer.

1.2.3. Current pharmacological treatments for spasticity, and their limitations

There are several therapeutic interventions to manage spasticity, like physical therapy, orthoses, electro-neuromuscular stimulation, acupuncture, vibration therapy, surgical treatment and pharmacological therapy (Thibaut et al., 2013; Khan et al., 2017). Given the scope of this thesis, we will focus on pharmacological therapy. For a review of non-pharmacological interventions, see Khan et al. (2017).

The current pharmacological treatments –also called *antispasmodic agents*- for spasticity are (in order of administration –from first line, to second line, and so on-) (McIntyre et al., 2014; Ertzgaard et al., 2017):

- Oral: baclofen, benzodiazepines, tizanidine, dandrolene, cannabinoids.
- Locally injected: botulinum toxin, alcohol.

After a spinal cord injury, the first line pharmacological treatments against spasticity are baclofen, tizanidine and botulinum toxin -the last one in the case of spasticity localized to some muscles-. If these treatments are not effective, second line treatments include an intrathecal pump of baclofen, botulinum toxin injection and nerve lysis with alcohol (VIDAL). Other treatments, like dandrolene, benzodiazepines and cannabinoids are prescribed, but they have not been thoroughly tested to determine their efficacy on spinal cord injury patients (Chang et al., 2013; Ertzgaard et al., 2017). The pharmacological treatments against spasticity are represented on Figure 4 and they are more thoroughly characterized on the following paragraphs.

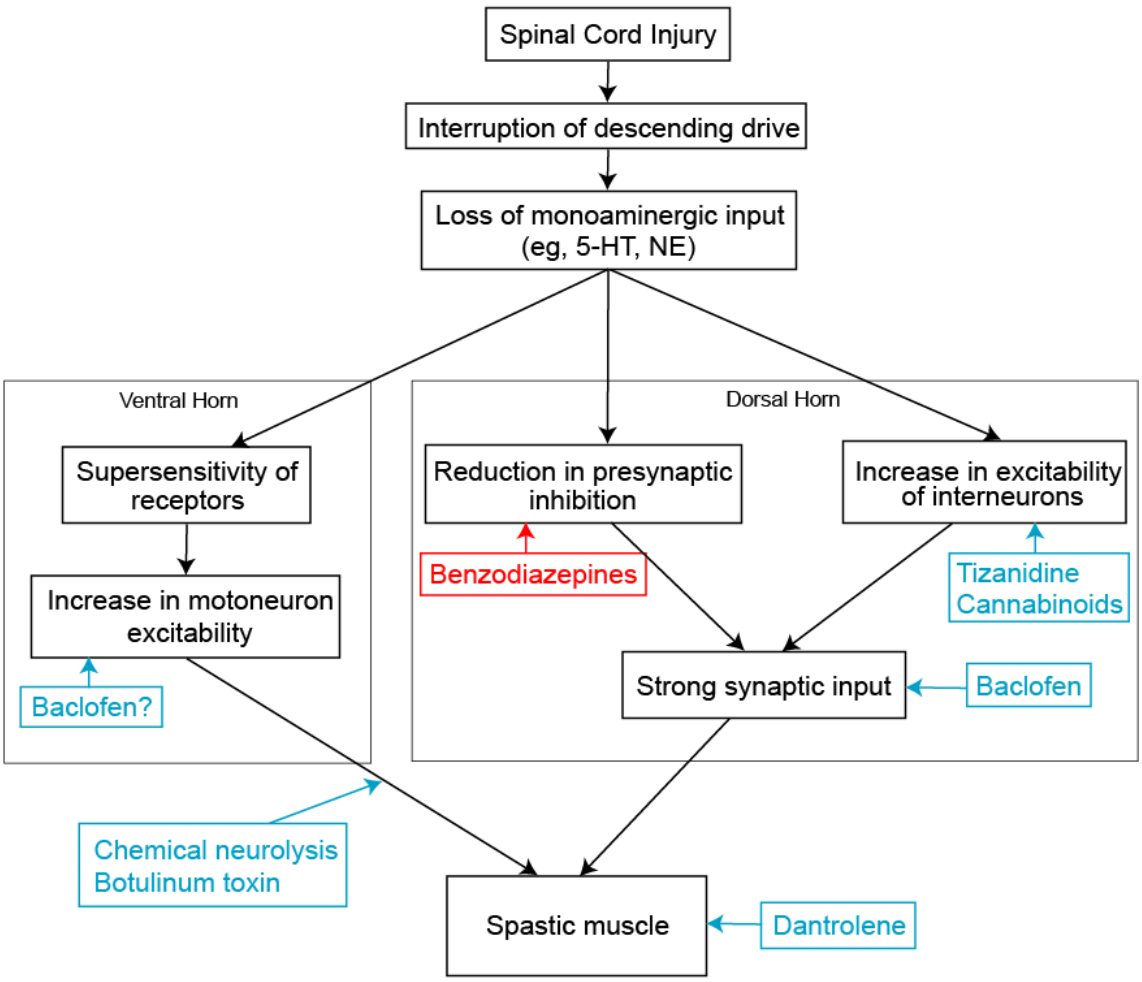

Figure 4. Pharmacological treatments against spasticity according to their mechanism of action. In blue there are the treatments that reduce the mechanism they target, and in red the ones that increase and restitute their target mechanism. Figure adapted from Elbasiouny et al. (2010).

- First line treatments:

Baclofen[a]**:** The most widely used pharmacological treatment for spasticity is oral baclofen. Baclofen is a GABA-agonist that is thought to bind and activate presynaptic GABA-B receptors selectively. Oral baclofen has proven to reduce muscle tone and frequency of spasms in several clinical trials. Baclofen hyperpolarizes spinal motoneurons, reducing their hyperexcitability and therefore reducing the hyperactivity of muscle stretch reflexes. Oral baclofen has not proven a higher efficacy against these symptoms compared to other oral

[a] The recent review by Ertzgaard et al. (2017) includes a very complete overview of the use of baclofen against spasticity. All the informations that appear in this section can be found on this review, unless we indicate it otherwise.

antispasmodic agents but it seems that generally patients prefer baclofen to the other agents. However, baclofen is water-soluble and therefore does not cross the blood-brain barrier easily. This means that patients need to take high doses of oral baclofen in order to have a concentration of the drug at the spinal cord that is sufficient to reduce their spasticity. This can cause averse side effects in 25% to 70% of patients depending on the studies, which sometimes can be intolerable. These side effects include muscle weakness, somnolence, nausea, dizziness and headache (Furr-Stimming et al., 2014).

Alternatively, baclofen can be delivered via an intrathecal pump, which reduces the dose needed at least 100 times, reducing the risk of side-effects. This technique allows direct application of baclofen into the cerebrospinal fluid at the spinal cord level; avoiding the need to cross the blood-brain barrier (Furr-Stimming et al., 2014). However, it has to be considered that the implantation of an intrathecal baclofen pump is an invasive surgical procedure that requires a highly-specialized setup, requiring a long-term commitment of patients and physicians, which restricts its use to the patients to whom the oral baclofen therapy causes severe side-effects (Furr-Stimming et al., 2014). Another aspect that is challenging from a medical point of view is that patients and caregivers need to be educated about the risks of intrathecal baclofen, especially about the risks of over- and under-dose, and how to prevent it (Boster et al., 2016). This, combined with the fact that oral baclofen is efficient in treating spasticity on many patients, makes intrathecal baclofen an interesting alternative, but with a moderate implantation compared to oral baclofen.

Tizanidine: Tizanidine is an alpha2 adrenoreceptor agonist, which reduces muscle tone by increasing presynaptic inhibition of spinal motoneurons (Simon and Yelnik, 2010). It is the drug that has been most widely compared to baclofen. They have shown to have a similar efficacy, and in a review (Dario and Tomei, 2004), tizanidine is said to be better tolerated because it causes less muscle weakness (though we need to considerate that it causes more tiredness). On a clinical trial, tizanidine has proven to reduce spasms but it did not ameliorate quality of life significantly (Taricco et al., 2006).

Botulinum toxin: The injection of botulinum toxin in spastic muscles is particularly effective in the management of spasticity. Botulinum toxin blocks the release of acetylcholine from the nerve terminal, avoiding the cycle of contractions and weakening the injected muscle (Walker et al., 2015). Botulinum toxin injection is also used to manage spastic bladder (Guillot-Tantay et al., 2017). There are seven serotypes of botulinum toxin. Only one of them is authorized in France: botulinum toxin A. It exists on the form of abobotulinumtoxinA (Dysport Ipsen Biopharm Ltd., Wrexham, UK; Medicis Pharmaceutical, Scottsdale, AZ), incobotulinumtoxinA (Xeomin; Merz Pharma, Frankfurt am Main, Germany), and onabotulinumtoxinA (Botox; Allergan, Inc., Irvine, CA). The injections of the toxin are done in the spastic muscles, under electromyographic or echographic control (Walker et al., 2015). There are no secondary effects unless the needle touches a blood vessel. If it is the case, there is a risk to develop a

botulism-like syndrome, due to the presence of the toxin in the blood (Tugnoli et al., 2002). The problem of this treatment is that the injected muscle will remain paralyzed after the treatment, which is why it is not generally recommended for children because it would interfere with their development.

- Second line treatments:

Chemical neurolysis with alcohol: Neurolysis of nerves innervating spastic muscles by the injection of alcohol has also been proposed as a treatment for spasticity (Lee and Jang, 2012). This technique can reduce spasticity and improve the range of motion during up to six months. However, this is an old technique, and it has several side effects, such as the development of pain some months after the injection, which makes its use much more restricted than for botulinum toxin –which is already much less common than baclofen-. However, it is validated as a second line treatment against spasticity in France (VIDAL).

Benzodiazepines: Benzodiazepines are positive allosteric modulators of $GABA_A$ receptor. They bind to the benzodiazepine binding site in $GABA_A$ receptors, and they increase the permeability of the pore to chloride ions when GABA binds the receptor. The benzodiazepines most commonly used in clinics are diazepam and clonazepam. According to a study (Rekand et al., 2012), benzodiazepines have an additive effect when combined with baclofen. Their use is restricted by their main side effect: sedation (Schmidt et al., 1976; Kita and Goodkin, 2000).

Dantrolene: Dantrolene acts by inhibiting calcium release from the sarcoplasmic reticulum of the muscle fiber (Abrams and Ganguly, 2015). Its advantage compared to other drugs cited in this section is that it acts directly in the muscle, having little effect on the central nervous system. However, it has the undesired side effect of reducing the muscle strength in other muscles of the body, whether or not they are affected by spasticity (Schmidt et al., 1976). This has the effect of reducing voluntary movement, which is why this drug is more indicated for patients with more severe spasticity.

Cannabinoids: Cannabinoids such as delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) bind to CB1 and CB2 receptors, and this action can modulate the effects of glutamatergic and GABAergic transmission, causing muscle relaxation and a reduction of spasticity (Taricco et al., 2006; Giacoppo et al., 2017). A spray containing a galenic preparation of THC and CBD, Sativex®, has proven to be efficient against spasticity in multiple sclerosis patients (Giacoppo et al., 2017). Cannabinoids can have anxiogenic and psychoactive side effects (Notcutt et al., 2012), and are also capable of producing dizziness, and muscle weakness (Leocani et al., 2015). Other possible side effects are somnolence, muscle weakness, diarrhea, dry mouth, blurry vision, restlessness, nausea and paranoia (Lorente Fernández et al., 2014).

To conclude this section, it is important to keep in mind that spasticity can be both invalidating and helpful for patient's life and care. Therefore, all treatments need to be adjusted to patient's therapy goals and expectations (Abrams and Ganguly, 2015; Boster et al., 2016; Ertzgaard et al., 2017). Also, given that the physiopathological mechanisms of spasticity have not been completely identified yet, the treatment for spasticity is only oriented to treat the symptoms, and not the causes of this condition.

1.3. Neuropathic pain

Recent epidemiological studies have estimated the prevalence of neuropathic pain to be 53% of the patients with spinal cord injury (Burke et al., 2017). Furthermore, beyond its own impact in quality of life, neuropathic pain presents several co-morbidities that contribute to lowering quality of life, such as depression, sleep disorders and impairments in physical function (Gilron et al., 2015). Neuropathic pain is a devastating condition, and there is great clinical interest in improving its treatment.

1.3.1. Definition

Neuropathic pain can be compared or opposed to nociceptive pain. Nociceptive pain tends to be related to tissue damage: it is very intense at the moment of the damage and reduces in intensity over time. Furthermore, general analgesic medications, such as non-steroid anti-inflammatory drugs, are effective against it. On the other hand, neuropathic pain may have a tissue injury at its onset, but it is mostly related to a dysfunction of the somatosensory system (depicted in Figure 5a): it continues even if the tissue injury has been controlled (such as after the repair of a nerve). Neuropathic pain also tends to stay or increase in intensity over time, and general analgesic medications are not effective against it, being anticonvulsants and antidepressants the most effective first-line treatments (Gilron et al., 2015).

Neuropathic pain is a sensation that needs the brain to take place. It has even been proposed, in recent years, that chronic neuropathic pain would not depend much on alterations of the somatosensory pathway, but to be more related to an affective disorder, implicating changes in the mesolimbic-cortical circuit that affect in particular the hippocampus and the ability to learn (Mansour et al., 2014). However, although the role of the brain is undeniably important, it is also well established that neuropathic pain can be caused by channelopathies affecting only the peripheral nervous system and/or the autonomous nervous system, and it is the treatment of the peripheral channelopathies which provides relief to patients (Themistocleous et al., 2014). Therefore, at least in some cases of

neuropathic pain, the pathological changes underlying its onset cannot be limited to the brain exclusively.

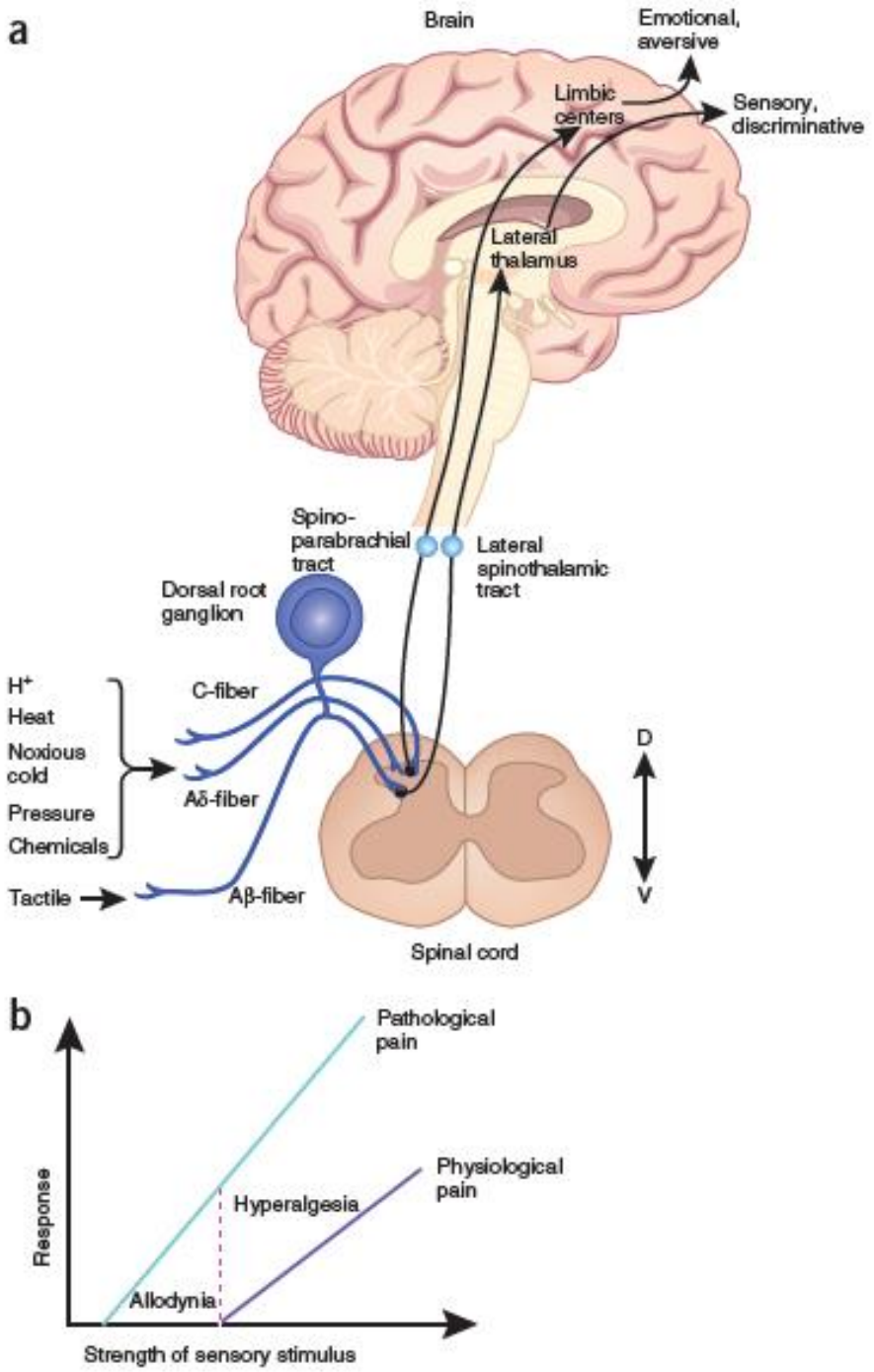

Figure 5. Sensory pathways and the development of hyperalgesia and allodynia. a) Somatosensory system: pathways that mediate the sensory and pain stimulus. b) The two categories of neuropathic pain. Allodynia is the painful response to a sensory stimulus that does not reach the nociceptive threshold. Hyperalgesia is the increase or exaggeration of the nociceptive response to a stimulus that reaches the nociceptive threshold. Figure adapted from Kuner (2010).

Two great categories of neuropathic pain are differentiated: allodynia, generally defined as pain in response to a normally non-painful stimulus, and hyperalgesia, generally defined as increased pain in response to a normally painful stimulus (Gilron et al., 2015). Figure 5b depicts this definition of allodynia and hyperalgesia. Although these definitions are still widely used and accepted, it must be noted that in 2008, the International Association for the Study of Pain task force proposed a change on these definitions, to better distinguish the contribution of the different types of fibres: hyperalgesia would be any increase in pain, including the lowering of pain threshold, while allodynia would only identify the pain that was clearly induced by low-threshold fibers (Sandkühler, 2009).

Neuropathic pain implicates a sensitization at the level of the spinal cord; this has been proposed to be due to 1) changes in the connectivity of peripheral afferents (i.e.: non-nociceptive afferents would normally activate non-nociceptive pathways, but may activate nociceptive pathways after the sensitization takes place), and 2) different sensitivity of post-synaptic receptors. These changes are facilitated by the development of inflammatory reactions mediated by immune cells and glia, and also by the loss of spinal inhibition (Garcia-Larrea and Magnin, 2008).

Given the special interest of this thesis in the area of inhibitory modulation, we have developed the consequences of the loss of spinal inhibition after a spinal cord injury on a separate section (see 1.4.2. Disinhibition of the somatosensory system).

1.3.2. Difficulty of the diagnosis in clinics

The epidemiology of neuropathic pain is particularly challenging given the diversity of diseases that can cause it. There was a thorough review on this subject by Gilron et al. (2015). Unless indicated otherwise, all the informations on this section (*1.3.2. Difficulty of the diagnosis in clinics*) are issued from this publication.

The diagnosis of neuropathic pain is generally done based on the explanations of the patient and physical examinations. The main goal is to exclude other conditions that may be treated otherwise (i.e.: a tumor that is compressing a nerve).

There are no signs that are exclusively related to neuropathic pain and do not appear in other chronic pain conditions. However, there are three words used to describe the quality of pain that are more characteristic of neuropathic pain:

- “Tingling”, also described as “pins and needles” or “prickling”
- “Burning”, also described as “hot”
- “Shooting”, also described as “electrical shocks”

Also, in recent years there has been a variety of self-report neuropathic pain assessment questionnaires, which allow to identify possible cases of neuropathic pain. These are the Michigan Neuropathy Screening Instrument, Neuropathic Pain Scale, Leeds Assessment of Neuropathic Symptoms and Signs, Neuropathic Pain Questionnaire, Neuropathic Pain Symptom Inventory, "Douleur Neuropathique en 4 questions," pain DETECT, Pain Quality Assessment Scale, and the Short-Form McGill Pain Questionnaire-2.

After the patient has been identified as being suspected of having neuropathic pain, the physical examination can verify whether they are suffering hyperalgesia, allodynia, hypoesthesia or hypoalgesia. In particular cases, other examinations may be conducted to verify the diagnosis, such as electrophysiology -microneurography-, nerve or skin biopsy, or functional brain imaging studies. For a review on this topic see Truini (2017).

1.3.3. How to evaluate pain in animals

It is crucial to understand the mechanisms at the origin of neuropathic pain in order to assess it correctly and to apply the appropriate analgesic therapies. The development of animal models of pain involving injury or disease induction on peripheral or central neurons has allowed a better understanding of the cellular and molecular mechanisms of neuropathic pain (Gilron et al., 2015).

The main problem that arises when comparing animal and clinical research in pain studies, is that the clinical diagnosis of pain is mainly done in the basis of the patient's experience of their pain. Since it is not possible to be certain of this experience in animal studies, several different ways to infer the pain experience in animals have been developed:

- Nociceptive tests: using electrical, thermal, mechanical or chemical stimuli, some of these tests quantify the latency of appearance of an avoidance behavior (tail flick test, hot- or cold-plate tests, and the radiant heat paw-withdrawal test), while others may consider the stimulus threshold that is necessary to elicit an avoidance behavior (Von Frey test, Randall-Selitto test,…); other types of tests rely on the observation and scoring of specific behaviors (like assessing cold allodynia with acetone) (Barrot, 2012):
    - Electrical thresholds: usually evaluated as a control for other experiments where electrical shocks are involved. Observed responses include flinchs, vocalizations, or an escape response, like jumping or running.
    - Tail flick test: measure of the latency for the tail flick reflex following tail exposure to a heat stimulus
    - Hot-plate test: measures the latency until paw licking takes place, when the animal is placed in a plate at 52-55ºC

- Cold-plate test: the same principle of the hot-plate test, but with a cold stimulus
- Radiant-heat paw-withdrawal test: measures the latency of the withdrawal reflex following hindlimb exposure to a heat beam
- Acetone test: a drop of acetone is applied to the hind paws. Its evaporation produces a cold stimulus, and we quantify the degree of the response, measured as the time that the rodent spends flicking the paw after the evaporation of acetone
- Von Frey test: application of plastic hairs of different thickness. The animal is placed on a grid, and the Von Frey filaments are applied to the paw. The threshold is calculated following the up-down method: starting from the first filament that produces a response, the experimenter tries the previous -thinner- filament if the response was positive and the animal retires the paw. If the response is negative, the experimenter applies the next –thicker- filament. The experimenter makes between two and 6 measures going "up" and "down" and analyzes this pattern following the method described by Chaplan et al. (1994) to obtain the nociceptive threshold. This is a measure of tactile allodynia. Since 1994, several other methods based on the same principles, have been developed to measure the threshold for tactile allodynia, using the Von Frey filaments or other devices –force transducers, etc…-.
- Randall-Selitto test: the hind paw is placed between a fixed element and a mobile blunt point which exerts a pressure that is calibrated. The measured parameter is the threshold for appearance of either a reflex withdrawal, or a vocalization
- Strain gauges fixed to blunt forceps: the measured parameter is the latency until a withdrawal response occurs.
- Nociceptive responses to chemical stimuli: the measures parameter is the behavioral scoring during a tonic pain state. The formalin test is the most commonly used, and it involves injecting a compound, formalin, on a paw of rats and mice. This compound causes a tissue injury and a continuous pain. The behavioral scoring is as follows: a rating of 1 indicates a reduction in the weight put on the injected paw, a rating of 2 indicates a complete elevation of the paw, and a rating of 3 indicates licking, biting and shaking of the paw. A mean rating is then calculated, with each rating being weighted according to the time spent in each behavior. The test lasts for about an hour. Three phases can be differentiated: 1) there is a first phase, just after the injection of formalin, of acute pain caused by direct activation of nociceptors, it corresponds to the first 10 minutes after the injection, 2) between 10 and 15 minutes after the injection there is very little nociceptive behavior, and 3) there is a late phase, of pain caused by inflammation and the sensitization of the connexions of peripheral afferents in the spinal cord (Dubuisson and Dennis, 1977; Tjølsen et al., 1992).

- Vocalizations: audible (Han et al., 2005) and ultrasound vocalizations related to neuropathic pain can be registered (Kurejova et al., 2010), but it is still debated whether vocalizations can reliably indicate the presence of pain (Yezierski, 2005).

- Facial expression of pain in rats: the measured parameter is the degree of pain the rat feels, measured in base of their facial expression (Yezierski, 2005). The advantage of this method is that it can be automatized, however it is probably limited to the detection of acute pain; it is not likely that it can be used to measure chronic pain (Yezierski, 2005).

- Operant escape responses: the animal can choose between two chambers or areas, which offer different conditions; if it feels pain in one of them it will have a tendency to prefer the other one, this behavior needs the intervention of supraspinal responses. They are considered a better measure of the animal's perception of pain. Examples are the place-preference test, the conditioned place avoidance test, and the thermal escape test (Gregory et al., 2013).

- Quality of life and function: overall assessments of activity, inactivity, grooming, eating and drinking, posture, gait, and social interactions, can indicate whether an animal is in pain (Gregory et al., 2013). Observations of excessive grooming behavior, licking, guarding or orientation can indicate the presence of pain (Yezierski, 2005).

The diagnosis and evaluation of neuropathic pain in human patients and rodents is compared in Figure 6.

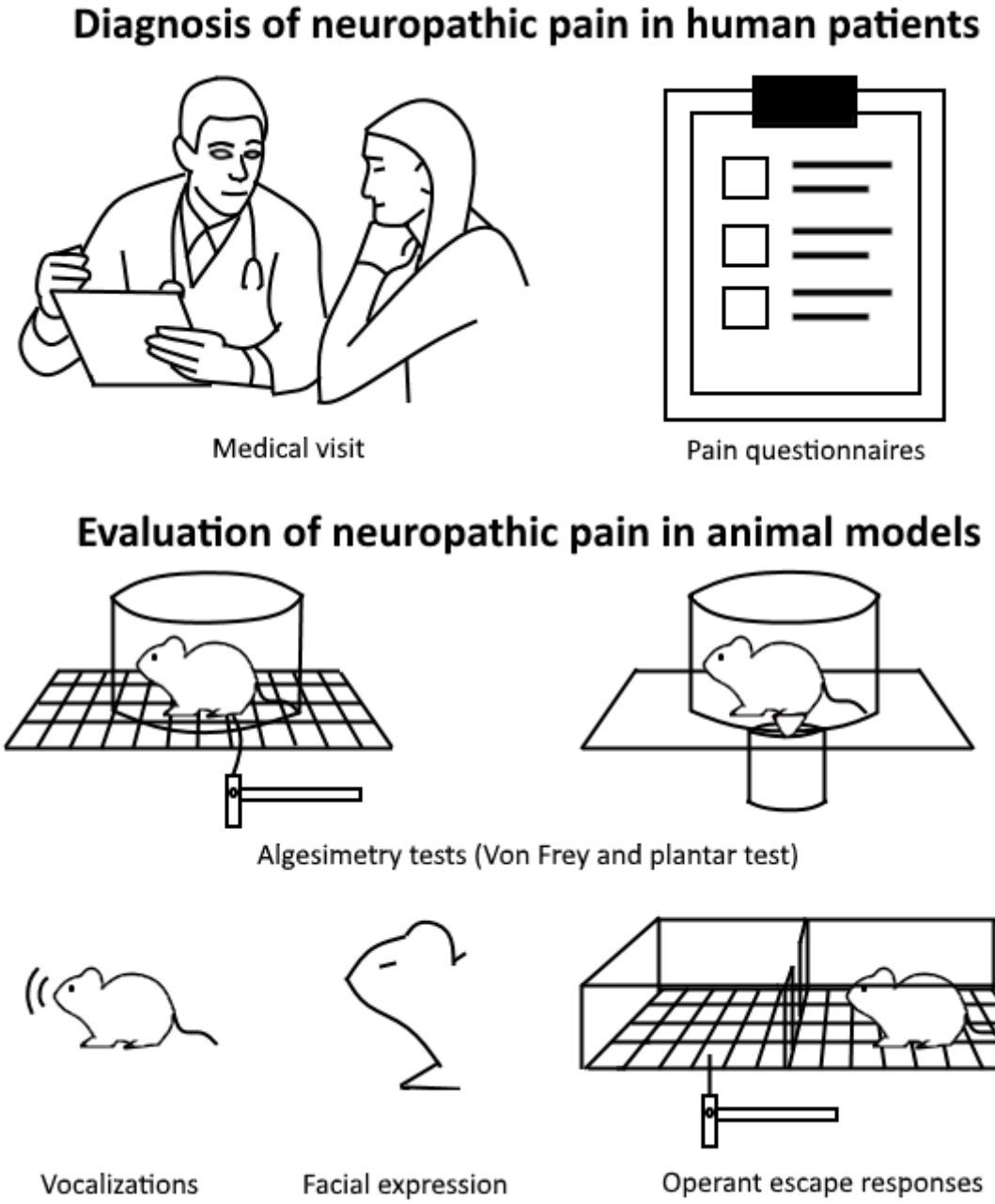

Figure 6. Methods for the diagnosis of neuropathic pain in human patients compared to the methods to evaluate neuropathic pain in rodents. The tools for the diagnosis of neuropathic pain in human patients are the medical visit and questionnaires evaluating the quality of the pain. Before making the diagnosis of neuropathic pain it is important to discard other sources of pain (injuries, arthritis, sickness, tumors,…). The tools for evaluating neuropathic pain in animal models are more indirect, and they include algesimetry tests, to measure the response of the animal to painful stimuli –only Von Frey and plantar test have been depicted, for reasons of space-, vocalizations, facial expression of the animals, and operant scape responses, which are tests where the animal will tend to avoid the area where it suffers a painful stimulation.

1.3.4. Current pharmacological treatments for neuropathic pain, and their limitations

As for spasticity, there is a variety of treatments for neuropathic pain induced by a spinal cord injury, ranging from physical therapy, to electrostimulation, surgery, psychotherapy and "soft" treatments, like acupuncture, yoga or meditation (Finnerup and Baastrup, 2012). In this section, we will focus on the pharmacological treatment for neuropathic pain.

Since not all patients respond to the treatment for neuropathic pain the same way, and for now it is hard to know whether a treatment would work before trying it on the patient, the prescription of the treatment is done following an order (from first-line medications to second-line medications if first-line are not effective, and so on). Figure 7 depicts first, second and third line medications against neuropathic pain according to the Neuropathic Pain Special Interest Group (NEUPSIG) guidelines.

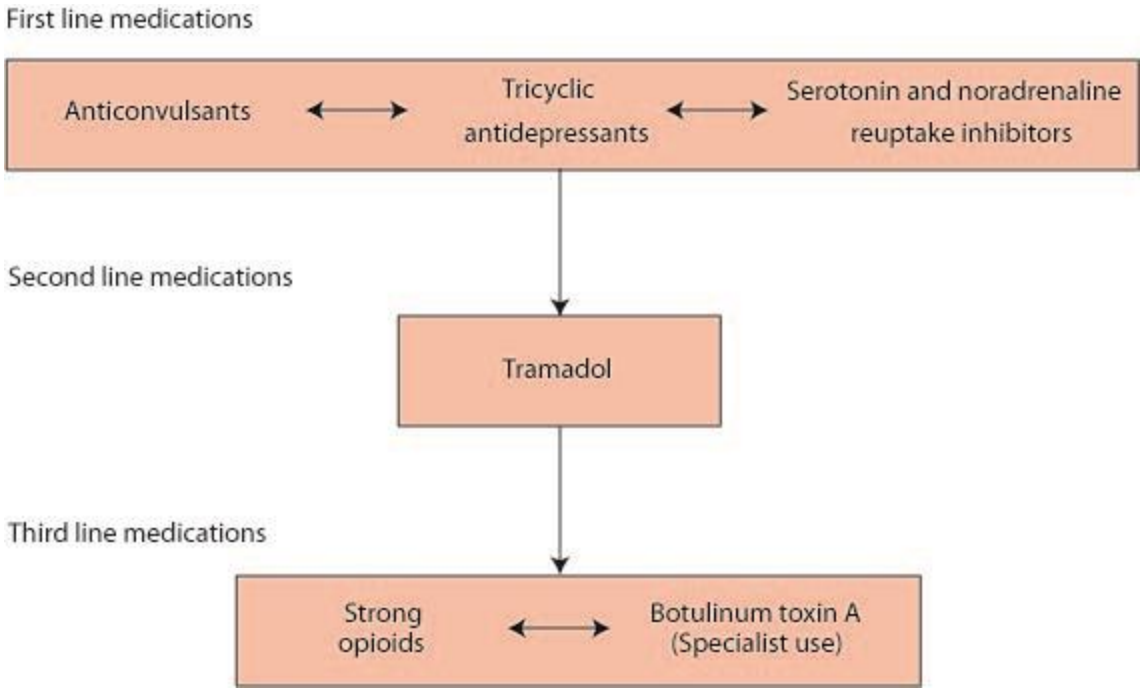

Figure 7. Algorithm for the selection of a treatment against neuropathic pain after a spinal cord injury. It is recommended to start by prescribing first line medications: anticonvulsants, tricyclic antidepressants and serotonin and noradrenaline reuptake inhibitors. If the result of the treatment with one of these medications is not satisfactory, another first line medication should be tried. If any of them works, a second line medication is proposed: tramadol. If second line medications are not effective, third line medications are proposed: strong opioids and botulinum toxin A –has only been recommended by NEUPSIG against peripheral nerve injury pain, however some studies have found it has positive results in spinal cord injury pain too-. Figure adapted from Finnerup et al. (2015), Mu et al. (2017).

- First line medications:

Anticonvulsants: gabapentin and pregabalin. Despite being derivatives of GABA, these drugs do not act on the GABA receptors but they block the α2δ subunit-containing voltage-activated calcium channels. They are overall the most effective type of drug against neuropathic pain (Mu et al., 2017). A recent study proved their effectiveness in neuropathic pain caused by spinal cord injury (Mehta et al., 2016). It has been requested by external lobbies to include gabapentin in the Model Essential Medicines List of the World Health Organisation (WHO; Smith and Raja, 2017). The Model Essential Medicines List of the WHO is a list that states the drugs considered essential to address the most important public health needs in the world (WHO website)-.

Tricyclic antidepressants: tricyclic antidepressants are among the older substances to treat depression. They contain three cycles in their structure, and they are effective against chronic pain in both depressed and non-depressed patients, although the possibility of treating comorbid depression is an important feature: many neuropathic pain patients experience depression (Gilron et al., 2015). Although tricyclic antidepressants have a proven efficacy against neuropathic pain, one meta-study showed that the most commonly prescribed tricyclic antidepressant –amytriptiline-, was not effective in reducing pain after spinal cord injury, and was only effective in patients with concomitant depression (Mehta et al., 2016). The World Health Organisation has included amytriptiline in its Model Essential Medicines List (available at the Website of the World Health Organisation)-.

Serotonin-norepinephrine reuptake inhibitors: these pharmacological agents inhibit the reuptake of monoamines from the synaptic cleft, increasing the levels of these in the synapses and therefore increasing the probability that they bind to a receptor and produce an action. Just as for amytriptiline, the most common serotonin-norepinephrine reuptake inhibitors –duloxetine, venlafaxine, trazadone-, were not effective in reducing pain after spinal cord injury, and were only effective in patients with concomitant depression (Mehta et al., 2016).

- Second line medications:

Tramadol: opioids are therapeutical agents that bind to the opioid receptors, and they have proven their efficacy against neuropathic pain (Gilron et al., 2015). They cause common adverse effects like constipation, sedation, nausea and vomiting, which are not life-threatening but are an important reason for dropout on clinical trials (Gilron et al., 2015). Consensus reports recommend opioids as a second-line or third-line therapy against neuropathic pain not only for their side-effects, but also because of well-recognized concerns regarding their great potential to cause addiction (Gilron et al., 2015).

- Third line medications:

Strong opioids: oxycodone and morphine have been proposed by the Neuropathic Pain Special Interest Group (NEUPSIG) as third line medications against neuropathic pain (Finnerup et al., 2015). They are prescribed as a third line therapy because they present the same disadvantages than tramadol, with a greater capacity to cause addiction.

Botulinum toxin injections: apart from being an antispastic therapy, botulinum toxin injections have been proposed to treat neuropathic pain (Park and Park, 2017)-. It is a third line treatment against neuropathic pain caused by a peripheral nerve injury (Finnerup et al., 2015).

- Other options:

Cannabinoids: cannabinoids are chemical agents that bind to the cannabinoid receptors CB1 and CB2. They have proved a weak efficacy against neuropathic pain following spinal cord injury -sufficiently robust studies regarding their efficacy are still lacking (Mu et al., 2017)-. However, they are among the most recommended treatments for nociceptive- or spasticity-related pain (Mehta et al., 2016). Their use is restricted in most countries due to their potential for misuse, diversion, and long-term addiction (Mu et al., 2017). Their side effects can include somnolence, “getting high”, confusion, dizziness, tachycardia and hypotension (Mu et al., 2017).

Intrathecal baclofen: beyond botulinum toxin injections, intrathecal baclofen is another antispastic therapy that has been proposed to treat neuropathic pain, as a fifth line treatment (Deer et al., 2012).

To conclude, as for spasticity, although there are drugs available to treat neuropathic pain, they may loose effectivity with time, and for some patients they are inefficient from the beginning. This is why the study of neuropathic pain mechanisms and the search for new drugs is also a medical need.

1.4. A common physiopathological mechanism involved in spasticity and neuropathic pain after SCI. A disinhibition of the spinal cord:

A spinal cord injury causes an increase in the release of glutamate, neuropeptides, adenosine triphosphate and proinflammatory cytoquines; this, together with neuroanatomical changes such as collateral sprouting, causes a central sensitization of the spinal cord (Gwak and Hulsebosch, 2011; Brown and Weaver, 2012). It is beyond the scope of this thesis to cite all the factors implicated in the development of spasticity and neuropathic pain. One of the most accepted common mechanisms implicated on the development of spasticity and neuropathic pain after spinal cord injury is a reduction of the inhibitory synaptic transmission (Hasbargen et al., 2010). Here, we will focus on mechanisms involved in the disinhibition of the spinal cord, always considering that the physiopathology of this condition is not fully characterized yet.

1.4.1. Disinhibition on the motor system

Hyperexcitability of the myotatic reflex, also named Hoffmann (H) reflex or stretch reflex, is the most classical feature characterizing spasticity. We will see the state-of-the-art about the main pathological mechanisms related to the dysregulation of the stretch reflex.

1.4.1.1. Myotatic reflex arc and its inhibitory controls

The stretch reflex is a muscle contraction of a muscle in response to stretching of the own muscle. This reflex provides continuous regulation of the muscle lenght, and it is highly developed in the antigravity muscles since it allows to keep the muscle tone that is necessary to maintain a good posture. After a stretch, the neuromuscular spindles cause a discharge of sensitive fibres Ia. The discharge is then transmitted to α-motoneurons of the concerned muscle –as an Excitatory Postsynaptic Potential-, which contracts the muscle as a response to its original stretch. The stretch reflex is depicted in Figure 8a.

On parallel to this monosynaptic circuit, there are polysynaptic circuits involving inhibitory interneurons that modulate the myotatic reflex. To avoid the exacerbation of the myotatic reflex, inhibitory GABAergic interneurons project to the synaptic terminations of Ia primary afferences (Eccles et al., 1962; Rudomin, 1999) (Figure 8b1). The release of GABA by these interneurons causes presynaptic inhibition, which reduces the firing of Ia terminations.

Presynaptic inhibition manifests itself as a reduced amplitude of the monosynaptic Excitatory Postsynaptic Potentials (EPSPs), after the repeated stimulation of Ia afferents (Curtis and Eccles, 1960). This depression is more pronounced when the stimulation frequency increases (as shown in the rate-dependent depression test).

Also, every motoneuron has a self-regulatory mechanism to stop their discharges. The axon of the motoneuron sends a collateral to a Renshaw cell, which performs a recurrent inhibition (Eccles et al., 1954; Hultborn et al., 1971) (Figure 8b2). When the motoneuron fires, it stimulates a Renshaw cell, which, as a consequence, inhibits the motoneuron on a negative feedback loop. As a consequence, all discharge from a motoneuron is followed by a stop or a reduction of its activity.

The contraction of a muscle activates Golgi tendon organs, causing a discharge of sensitive fibers Ib. Ib afferents fire in the spinal cord, activating a polysynaptic network that inhibits motoneurons, to allow the reduction of muscle tension and to protect the muscle (Laporte and Lloyd, 1952; Eccles et al., 1957; Jankowska, 1992).

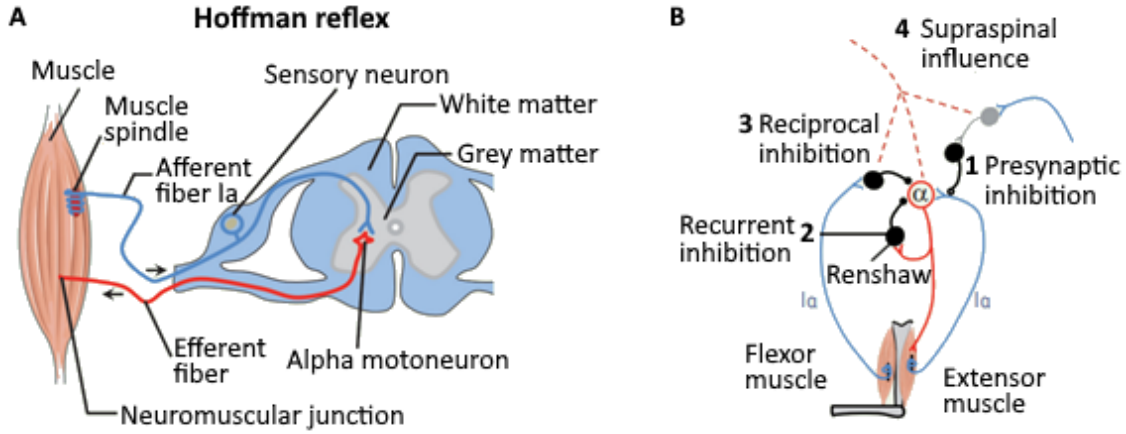

Figure 8. Hoffman reflex and motoneuron inhibitory mechanisms. a) Hoffman reflex: the stimulation of the muscle spindle by a muscle stretch stimulates motoneurons innervating that muscle on a monosynaptic circuit. The result is an action potential from the motoneuron that causes a contraction of the muscle. b) Diagram of the inhibitory mechanisms that influence a motoneuron. The diagram shows all the inhibitory mechanisms mentioned in this section -except Ib afferents and their polysynaptic circuit to inhibit motoneurons-. Figure adapted from Boulenguez et al. (2011); Plantier and Brocard (2017).

To conclude, the principle of reciprocal inhibition is applied to the fact that the contraction of a muscle causes the inhibition of its antagonist muscle (Crone et al., 1994). To achieve this, sensitive fibres Ia at the origin of the myotatic reflex also activate Ia inhibitory interneurons to inhibit the motoneurons innervating antagonist muscles (Hultborn and Udo, 1972) (Figure 8B3). Note that, beyond the segmental control of the myotatic reflex, a control is also exerted

by supraspinal centers, such as corticospinal and rubrospinal pathways, which facilitate inhibitory interneurons Ia and Ib (Lundberg and Voorhoeve, 1962; Hongo et al., 1969).

1.4.1.2. Development of spasticity: loss of inhibition on the myotatic arc

Following a spinal cord injury, the development of spasticity does not only implicate a loss of the inhibitory neurotransmission from the supraspinal centers on the myotatic arc. Spasticity does not manifest on the hours or days following a spinal cord injury, but it develops progressively, during several weeks or months, which could indicate an adaptive mechanism of the spinal inhibitory system. The inhibition in the spinal cord is downregulated due to a loss of inhibitory neurons, but most importantly, due to the plastic changes that make previous inhibitory neurons become excitatory (see Figure 9), which changes the nature of the synapses they make with motoneurons.

The reduction of the presynaptic inhibition causing the post-activation depression of Ia fibers (Figure 9, Injured), has been related to the development of spasticity. This reduction is observed on spastic patients (Calancie et al., 1993; Nielsen et al., 1993, 1995; Aymard et al., 2000; Schindler-Ivens and Shields, 2000) but also on spinalized rats (Thompson et al., 1992; Smith et al., 2017). It seems that an inhibition of presynaptic inhibition could contribute to the development of spasticity, although it has been suggested that post-activation depression is more a correlate of spasticity than a mechanism underlying it (Aymard et al., 2000).

Following a spinal cord injury, although the GABA and glycinergic terminals on motoneurons below the site of the injury are preserved, there is a loss of the number of premotor interneurons connected to these motoneurons (Khalki et al., 2018). A treatment with free moving in enriched environment, imposed locomotion in a treadmill carrousel, exercises of physiotherapy and environmentally enriched housing conditions, lead to a preservation of the interneurons premotor circuitry, with increased GABA and glycinergic terminals on motoneurons below the injury site. Rats that followed this treatment showed an improvement in their stepping and BBB score (Described in Methods: *3.2.1. BBB score*) compared to controls, and showed a locomotion that was similar to naïve rats, with some particular features, like an impaired coordination of forelimbs and hindlimbs, a higher preference for a rotate locomotor pattern than naïve rats, and a longer step cycle. The recovery of inhibitory synapses on the motoneurons seems to improve the locomotion of the rats (Khalki et al., 2018).

A reduction of reciprocal inhibition has been largely described on spinal cord injury patients (Crone et al., 2003; Knikou and Mummidisetty, 2011; Mirbagheri et al., 2014) (Figure 9, Injured), which can explain the co-contraction phenomena that are frequently observed on

spastic patients. The degree of spasticity has not shown correlation with the degree of reduction of reciprocal inhibition (Crone et al., 1994), which may be related to the difficulty to grade spasticity on a clinical exploration. Nevertheless, the alteration of reciprocal inhibition is considered to play a role on the development of spasticity (Nielsen et al., 2007).

Spastic spinal cord injury patients show a normal recurrent inhibition by Renshaw cells on a resting state (Katz and Pierrot-Deseilligny, 1982, 1999), but it is abnormally increased during voluntary movements (Katz and Pierrot-Deseilligny, 1982; Shefner et al., 1992). In contrast, a spinal cord transection on P5 rats reduced the number of motoneuron collaterals making synapses on Renshaw cells compared to uninjured rats (Smith et al., 2017), which can indicate a reduced activation of the negative feedback loop mediating recurrent inhibition (Figure 9, Injured). This difference compared to human studies may be due to the fact that the study was performed on neonatal rats. On the mature spinal network, the contribution of Renshaw cells to the myotatic reflex may be different.

Finally, if autogenic inhibition by Golgi tendon organs is always present on healthy subjects, Delwaide and Oliver (1988) have reported, instead, a facilitating effect on the spastic limbs of hemiplegic patients, which correlates with the development of hyperreflexia (Figure 9, Injured). A possible explanation for the appearence of this facilitation is an increase in the excitability of excitatory Ib pathways, similar to those facilitating extensor contraction in the spinal cord of the cat (Gossard et al., 1994; McCrea et al., 1995).

The inhibitory control of the monosynaptic reflex, and the motoneuron in general, is highly affected in neurological lesions that cause spasticity. There are alterations of the presynaptic inhibition, recurrent inhibition, reciprocal and autogenic inhibition, suggesting that a disinhibition of the spinal cord is a major mechanism in the development of spasticity.

All these changes are depicted in Figure 9.

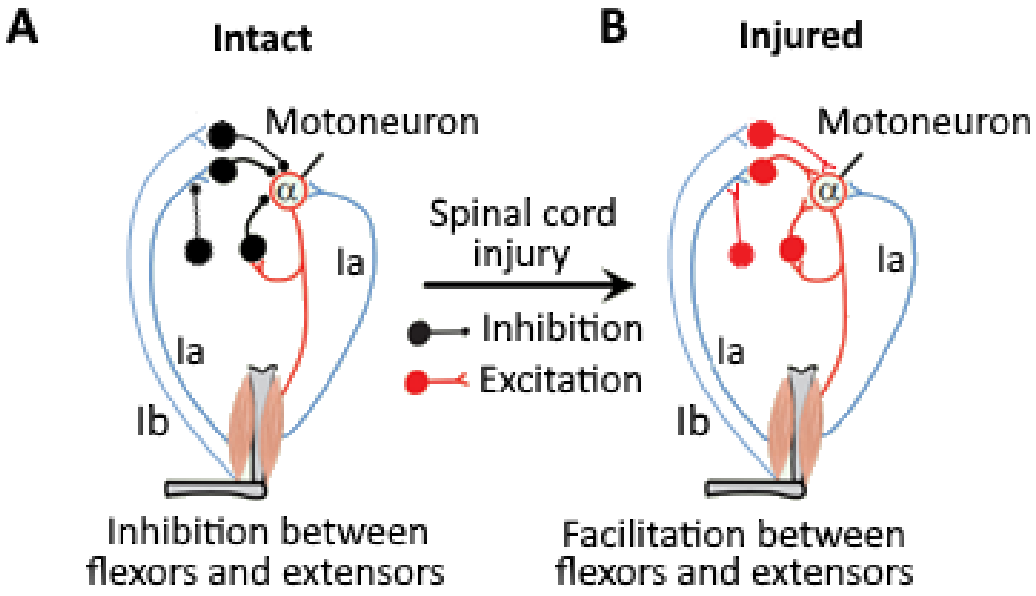

Figure 9. Disinhibition of the myotatic arc. a) Inhibitory control on intact animals. b) Inhibitory control is lost on spinal cord injured animals: inhibitory interneurons (black) become excitatory (red). Glycinergic Renshaw cells become excitatory: the release of glycine by the Renshaw cell will have an excitatory effect on the motoneuron. On a similar way, Ia afferents from antagonistic muscles, or Ib afferents from Golgi tendon organs will activate GABAergic and glycinergic interneurons, and the release of GABA or glycine from these neurons onto their synapses with the motoneuron will be excitatory. Presynaptic inhibition would be lost: the effect of releasing GABA or glycine near afferent terminals would be excitatory too. Figure adapted from Boulenguez et al. (2011); Plantier and Brocard (2017).

1.4.2. Disinhibition on the somatosensory system

1.4.2.1. The inhibitory control in the dorsal horn

Inhibition plays also a key role in the regulation of sensory networks. The gate control theory of Melzack and Wall (1965), which is the classical approach to information coding in the dorsal horn, gives a central importance to inhibition in the spinal cord, as a way to control the inputs that will reach the brain. Although this theory does not integrate all the knowledge that currently exists about the sensation of pain (Perl, 2007; Prescott et al., 2014; Zhang et al., 2015), it is still the most coherent explanation available for the transmission of pain sensation from the periphery to the brain. The gate control theory of pain is based on the following propositions (from Melzack, 1996):

1) The transmission of nerve impulses from afferent fibers to spinal cord transmission cells is modulated by a spinal gating mechanism in the dorsal horn (grey "G" neuron on Figure 10)
2) The spinal gating mechanism is influenced by the relative amount of activity in large-diameter and small-diameter fibers: activity in large fibers tends to inhibit transmission (close the gate, Figure 10, Aβ fibers in red) while small-fiber activity tends to facilitate transmission (open the gate, Figure 10, C and Aδ fibers in green)
3) The spinal gating mechanism is influenced by nerve impulses that descend from the brain (Figure 10, "central control" in blue)
4) A specialized system of large-diameter, rapidly conducting fibers (the central control trigger, Figure 10, "central control trigger" in blue) activates selective cognitive processes that then influence, by way of descending fibers, the modulating properties of the spinal gating mechanism.
5) When the output of the spinal cord transmission cells exceeds a critical level, it activates the action system –those neural areas that underlie the complex, sequential patterns of behavior and experience that are characteristic of pain- (Figure 10, in orange).

So, inhibition is present at several levels in the gate-control theory of pain: 1) the input from afferent fibers, 2) the relative proportion of large vs. small peripheral fibers' input, which determines the transmission of the impulse, 3) the descending inhibitory control from the brain, 4) all these factors prevent the activation of the brain action system that will cause a behavior characteristic of pain.

The principles of the gate-control theory of pain are applied on the development of the analgesia induced by electrical nerve stimulation (Melzack, 1996; Stanton-Hicks and Salamon, 1997).

Nociceptive fibers project mainly to laminae I and II (inner-IIi- and outer –IIo-) of the dorsal horn. Nociceptive brain projection neurons are interneurons present in lamina I and, although they have a fundamental role, they account for only the 5% of lamina I neurons (Guo and Hu, 2014). GABA immunoreactivity is present in 25% of lamina I neurons, and 30% of lamina II neurons. Most of the glycine-immunoreactive cell bodies are also GABA-immunoreactive (Polgár et al., 2003). These inhibitory cells are the substrate for the "gate" conceived by Melzack and Wall (1965) ("G" neuron in Figure 10).

$GABA_A$ receptors are present both in the primary afferent terminals and in the spinal cord neurons, and they can exert an inhibitory effect at both sites, the former being called presynaptic inhibition, while the latter is called postsynaptic inhibition. Both are regulated by spinal cord interneurons and descending afferents, both modulate physiological pain sensation (Guo and Hu, 2014) and both can be altered by an injury.

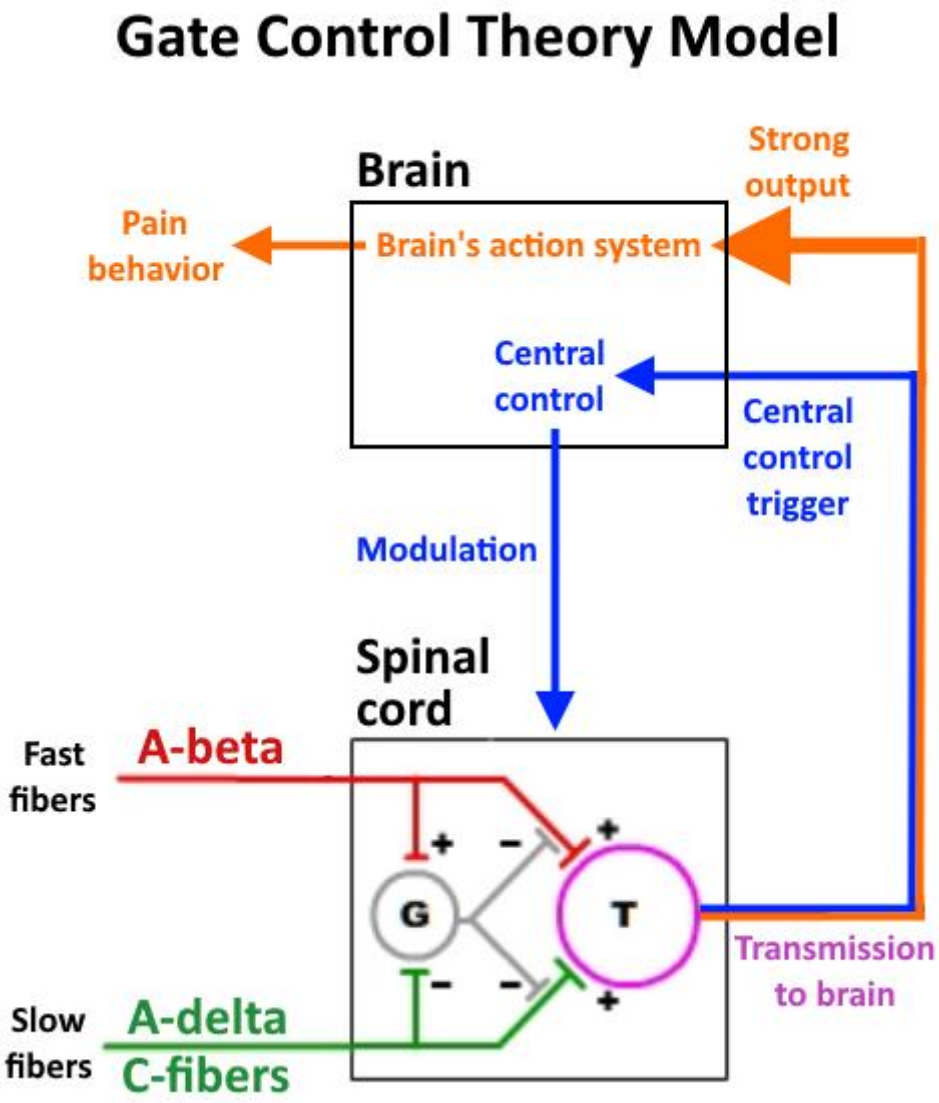

Figure 10. Gate control theory model. 1) The transmission of nociceptive impulses in the spinal cord is modulated by a spinal gating mechanism in the dorsal horn (grey "G" neuron). 2) The spinal gating mechanism is influenced by large-diameter and small-diameter fibers: large fibers inhibit transmission (Aβ fibers in red activate inhibitory "G" neuron) while small fibers facilitate transmission (C and Aδ fibers in green inhibit "G" neuron). Projection neurons mediate the transmission of nociceptive impulses to brain (T). 3) The spinal gating mechanism is influenced by the brain ("central control" in blue). 4) A specialized system of large-diameter, rapidly conducting fibers ("central control trigger" in blue) activates selective cognitive processes that then influence, by descending fibers, the spinal gating mechanism. 5) When the output of the spinal cord nociceptive transmission cells exceeds a critical level, it activates the action system –causing pain behavior- ("brain action system", in orange). Figure modified from RnCeus.com

The first stage of the sensation pathway where inhibition plays a role, is the presynaptic inhibition of the input from primary afferent terminals. Dorsal Root Ganglia (DRG) neurons contain a protein named sodium-potassium chloride cotransporter type 1 (NKCC1), which

takes chloride ions into the neuron. This allows the accumulation of a high intracellular chloride concentration. The activation of $GABA_A$ receptors on these neurons thus causes a depolarization, allowing chloride ions to leave the cell. This mechanism is known as Primary Afferent Depolarization (PAD) and, unlike the depolarization of other neurons, it reduces the probability of neurotransmitter release, producing presynaptic inhibition. Several mechanisms have been proposed to explain how a depolarization causes inhibition (such as inactivation of voltage-activated sodium –NaV- or calcium –CaV- channels, or shunting inhibition), but the exact reason why this depolarization of primary afferent terminals is inhibitory remains elusive (Guo and Hu, 2014).

The main postsynaptic inhibitory mechanism on the dorsal horn is the $GABA_A$ and glycine-mediated inhibition of interneurons and projection neurons (grey "G" and pink "T" neurons on Figure 10). Due to its importance, and because this system also has an influence in motor systems, this mechanism will be treated on a separate section (1.4.3. GABA and glycinergic Inhibition).

1.4.2.2. Development of neuropathic pain: loss of inhibition in the dorsal horn

Intrathecal administration of GABA or glycine receptor antagonists decreases nociceptive thresholds and produces behavioral indices of hypersensitivity in response to both painful and non-painful stimuli on animal models, indicating that spinal sensory systems are under tonic inhibition (Roberts et al., 1986; Sivilotti and Woolf, 1994) (Figure 11). In line with this, it is well documented that the development of neuropathic pain after nerve injury or spinal cord injury is linked to a defective inhibition (Gwak and Hulsebosch, 2011; Bonin and De Koninck, 2013; Guo and Hu, 2014).

GABAergic inhibition is disrupted in peripheral nerve injury (Moore et al., 2002; Coull et al., 2003; Scholz et al., 2005) and spinal cord injury (Drew et al., 2004).The reduction in GABAergic tone has several consequences on dorsal horn cells: 1) at least after a peripheral nerve injury (PNI), protein NKCC1 is increased in DRG neurons, increasing the intracellular chloride concentration on DRG; as a consequence, primary afferent depolarizations elicited by GABA are reduced (Kingery et al., 1988), which turns the input from primary afferents into excitatory, facilitating the activation of the pain pathway by touch inputs (Kaila et al., 2014) (Figure 11a), and 2) after a blockade of $GABA_A$ receptors, spinal lamina I and II neurons that normally only respond to high-threshold, nociceptive afferents (nociceptive-specific neurons, NS), start responding to low-threshold, non-nociceptive afferents (Aβ) (Baba et al., 2003) (Figure 11b). The neurons that respond to both nociceptive and non-nociceptive inputs are

called Wide Dynamic Range (WDR) neurons, and it is considered that an injury to the nervous system can cause a change of phenotype on neurons, from NS to WDR.

Also, the reduction of GABAergic tone in the spinal cord after injuries can cause long-term potentiation (LTP) of synapses on the nociceptive pathways (Latremoliere and Woolf, 2009; Ruscheweyh et al., 2011). This LTP of nociceptive pathways means that the downregulation of GABA can strengthen the synapses of nociceptive pathways and facilitate the transmission of nociceptive stimuli.

Since a loss of inhibition causes neuropathic pain, enhancing inhibitory neurotransmission by administering inhibitory receptor agonists or by genetic manipulation increases nociceptive thresholds and reverses indices of pain in multiple models of neuropathy (Malan et al., 2002; Hu et al., 2006).

An oral treatment with tiagabine –an inhibitor of GABA recapture- reduced neuropathic pain in patients with painful peripheral neuropathy (Novak et al., 2001). Similarly, an intrathecal treatment with baclofen applied to SCI patients –with and without neuropathic pain- reduced heat pain perception and reduced the amplitude of contact heat-evoked potentials (CHEPS), which is a measure of the transmission of C-fibers into the central nervous system (Kumru et al., 2013), another study proved that, beyond CHEPS, an intrathecal baclofen bolus reduces several types of neuropathic pain on SCI patients (Kumru et al., 2018).

To my knowledge, there are no studies in humans where the tonic inhibition of the dorsal horn has been abolished. This may be due, in part, to ethical limitations in human models: it is not acceptable to run the risk to turn a healthy volunteer into a patient with neuropathic pain (Reddy et al., 2012). Another reason may be the impossibility of measuring GABA inhibition on the dorsal horn of a human: **t**here is not an equivalent technique to local field potentials or intracellular recordings in the dorsal horn of animal models. In comparison, the characterization of the H reflex in patients to evaluate spasticity, is a correlate that is easier to obtain.

On humans, the techniques that have been used to study disinhibition of the central nervous system, are electroencephalography (EEG) and imaging biomarkers (Wydenkeller et al., 2009; Widerström-Noga, 2017). However, they are still far from being validated as having a diagnostic value for neuropathic pain, and cannot provide information about the state of inhibition of the dorsal horn.

On the next section, we will see in animal models that the spinal disinhibition due to loss of inhibitory interneurons, impaired storage and/or release of inhibitory neurotransmitters or impaired post-synaptic receptor activity has been proposed as an important pathogenic mechanism underlying disinhibition of the spinal cord.

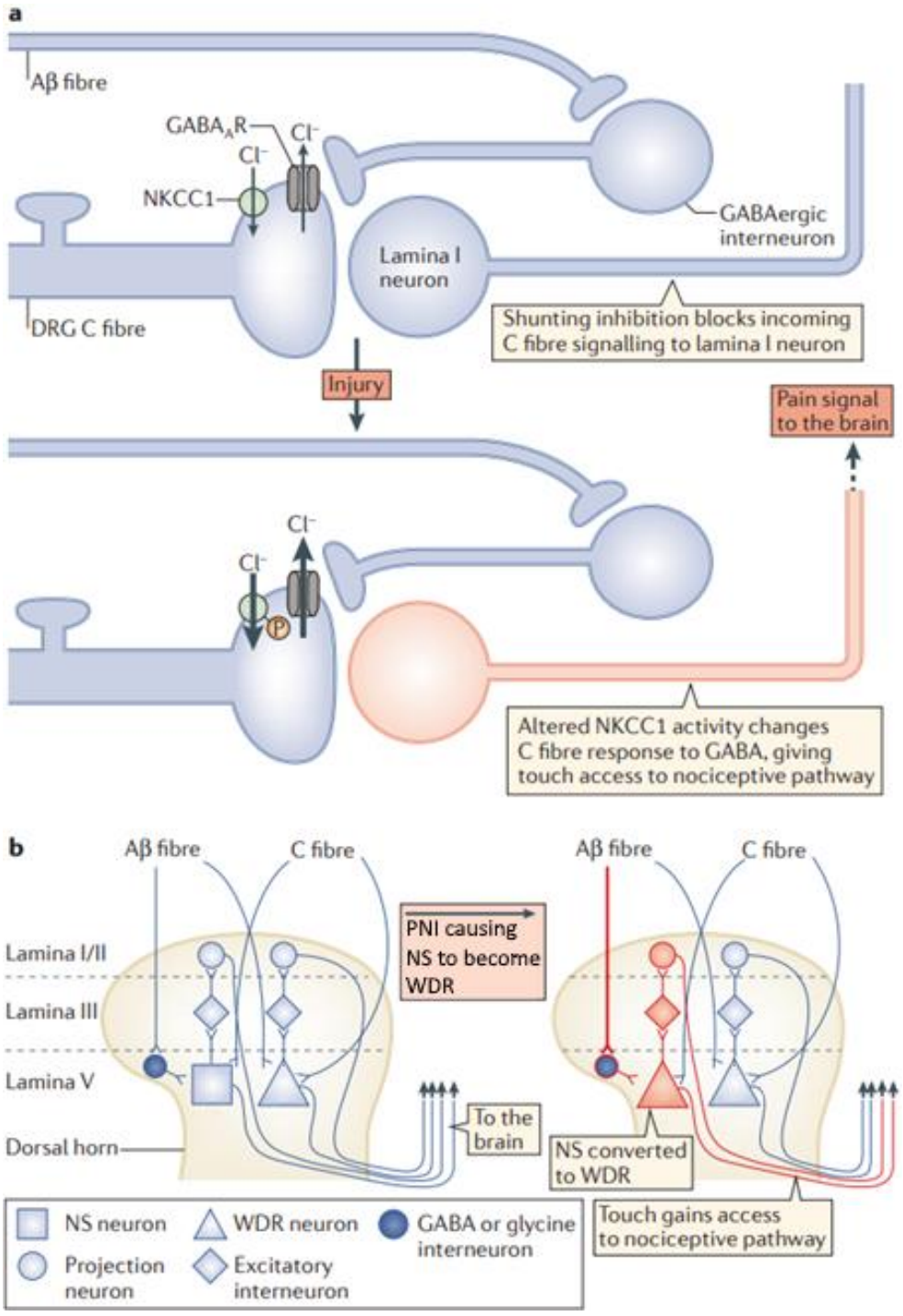

Figure 11. Cation-chloride cotransporters in pain mechanisms. a) Increased activity of nociceptors: increased NKCC1 function in primary afferent terminals after a PNI facilitates the transmission between C fibres and lamina I projection neurons. b) Aβ fibers conducting mechanical touch (not activating NS neurons) activate nociceptive pathways when NS neurons become WDR neurons: loss of KCC2 function in the dorsal horn after an injury increases the excitability of dorsal horn neurons, causing circuits that transmit a touch sensation in physiological conditions, to activate nociception-mediating circuits. Figure adapted from Kaila et al. (2014). NS neuron: nociceptive-specific neuron; WDR neuron: Wide Dynamic Range neuron; NKCC1: sodium-potassium-chloride cotransporter type 1; KCC2: potassium-chloride cotransporter type 2; PNI: peripheral nerve injury.

1.4.3. Disinhibition of the GABAergic and glycinergic system in the spinal cord

1.4.3.1. GABA/glycine receptors

At the spinal cord level, the decrease of inhibition would be caused mainly by an abnormal action of GABA and glycine neurotransmitters (Coull et al., 2003; Boulenguez et al., 2010).The GABAergic system plays and important role in motor control and sensory transmission in the spinal cord (Cramer et al., 2008; Sibilla and Ballerini, 2009). GABA receptors are present in the pre and postsynaptic sites of primary afferent terminals, at laminae I-III (Bowery et al., 1987) and at motoneurons (Delgado-Lezama et al., 2013). This system modulates the motor performance and sensory processing (Rudomin, 2009).

The fast actions of GABA and glycine are mediated by their ionotropic receptors, which are ligand-gated ion channels. Fast inhibition is mostly mediated by glycine receptors and $GABA_A$ receptors. $GABA_C$ receptors are also ionotropic, but their expression is more reduced than for $GABA_A$ receptors; since their contribution to fast inhibition is minor in this sense, it is sometimes omitted. $GABA_B$ receptors are also inhibitory, but they are metabotropic receptors, which means they are G-protein coupled receptors; they do not contribute to fast inhibitory transmission, because the consequences of their activation are slower (Purves et al., 2001).

An injury to the nervous system impairs the expression of GABA receptors in the spinal cord. After a spinal cord injury in P0 newborn rats, the expression of GABA receptors does not change between P0 and P7, while in control rats, there is a downregulation of these receptors (Sadlaoud et al., 2010). In parallel, there is an upregulation of glycine receptors that is independent of the influence from supraspinal centers, and it takes place in both conditions (Figure 12a). Another study (Khristy et al., 2009) on spinalized P5 rats looked at the changes that spinal cord injury caused in the expression of $GABA_A$ receptor subunit γ2, which is necessary for benzodiazepines' binding. The study showed that three months after the injury, there was an increase in the expression of this subunit in tibialis anterior motoneurons, and a reduction of it in soleus motoneurons, compared to naïve rats. Spinal cord transection also increased the expression of this subunit on astrocytes of both motoneuron pools, compared to naïve rats (Figure 12b).

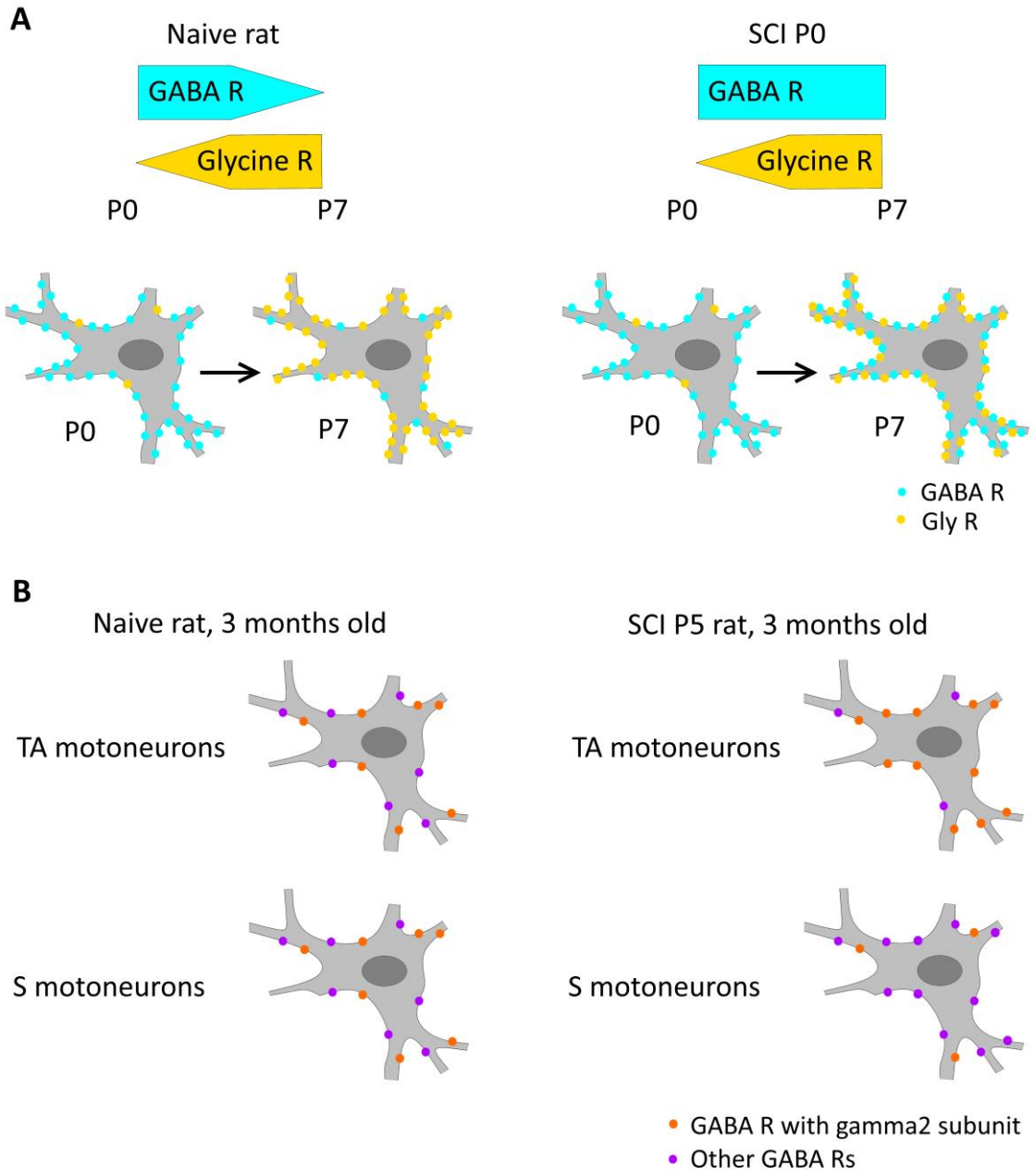

Figure 12. GABA receptors and loss of inhibition after an injury in newborn rats. a) The downregulation of the expression of GABA receptors on the first postnatal week is interrupted on rats that undergo a complete transection at P0. The upregulation of glycine receptors is independent of the spinal cord transection. b) On rats that undergo a spinal cord injury at P5, the proportion of $GABA_A$ receptors with a γ2 subunit increases on the motoeneurons of the tibialis anterior (TA) muscle, and decreases on the soleus (S) muscle, compared to naïve rats. The motoneuron shape of this figure was adapted from Betley et al. (2009).

We did not find studies showing an alteration of the total levels of GABA or glycine receptors' expression after a spinal cord injury in adult rats. However, a study from our lab (Khalki et al., 2018) showed that a complete spinal cord transection in adult rats produces an increase in the number of GABAergic axon terminals into lumbar motoneurons innervating gastrocnemius, and no difference in the number of glycinergic axon terminals innervating this muscle, compared to naïve rats, which is in line with the modifications described in newborn

rats –similar results were obtained for the tibialis anterior muscle, but the upregulation of GABA axon terminals did not reach significance-(Figure 13a). However, the number of vGluT1 afferents into motoneurons that did not have any GABAergic bouton was four times higher after the transection of the spinal cord, which could indicate an increased excitatory input into motoneurons.

A transection of the sciatic nerve without repair caused a downregulation of $GABA_B$ receptor in lamina II, 2-4 weeks postinjury (Figure 13b), possibly related to the degeneration of primary afferent endings, since this receptor in mostly localized in these afferents. The injury also caused an upregulation of $GABA_A$ receptors –on the same time postinjury- maybe because of a decrease of endogenous GABA after the injury (Castro-Lopes et al., 1995). Conversely, messenger RNA (mRNA) for $GABA_A$-receptor α2 and $GABA_A$-receptor γ2 subunits was reduced in the ipsilateral dorsal root ganglia seven days after a nerve ligation (Fukuoka et al., 1998) (Figure 13b). This may be due to the fact that mRNA and protein expression are not always correlated, but also to the fact that partial nerve injuries show different modulations of inhibitory neurotransmission than complete peripheral nerve injury (Moore et al., 2002).

In conclusion, loss of inhibition is not directly correlated with a downregulation of postsynaptic GABA and glycine receptors. Instead, it would seem that an injury to the spinal cord or the peripheral nerve increases the expression or activity of these receptors in most cases.

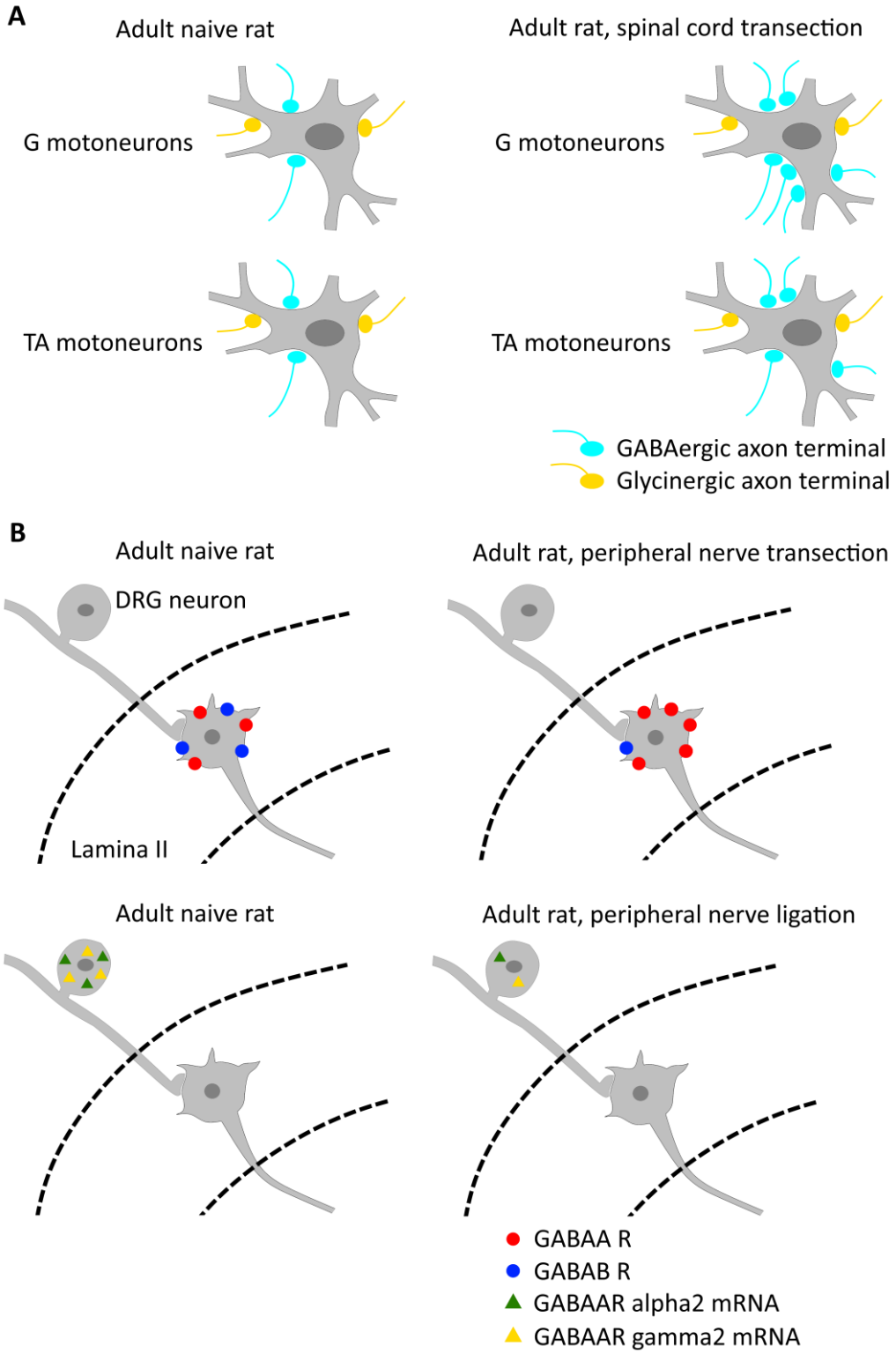

Figure 13. GABA receptors and loss of inhibition after an injury in adult rats. a) Spinal cord transection increases the number of GABAergic axon terminals on motoneurons from the gastrocnemius (G), and has a similar effect, more moderate, on tibialis anterior (TA) motoneurons, compared to naïve rats. The number of glycinergic axon terminals does not vary after the injury. b) Peripheral nerve transection on an adult rat increases the expression of $GABA_A$ receptors on lamina II interneurons and reduces the expression of $GABA_B$ receptors. Peripheral nerve ligation reduces $GABA_A$ receptor α2 and γ2 mRNA on DRG neurons. The motoneuron shape of this figure was adapted from Betley et al. (2009).

1.4.3.2. GAD expression

Glutamate decarboxylase or glutamic acid decarboxylase (GAD) is an enzyme that catalyzes the decarboxylation of glutamate to GABA. In mammals, GAD exists in two isoforms with molecular weights of 67 and 65 kDa (GAD_{67} and GAD_{65}). The function of GAD65 and GAD67 is not equivalent. In the brain, GAD65 is found mostly in axon terminals, while GAD67 is found more uniformly in all the volume of the neuron (Martin and Rimvall, 1993). GAD65 is considered to respond to short-term demands of GABA synthesis, and GAD67 to be more related with tonically-producing GABAergic neurons (Martin and Rimvall, 1993). GAD67 is expressed by most GABAergic interneurons. GAD65 is present in only a subset of them (Esclapez et al., 1994). In the spinal cord, GAD 65 is present mostly in terminals that make synapses with primary afferents (Betley et al., 2009). Considering this and the fact that GAD65 is necessary for an enhanced release of GABA at high stimulation frequencies (Tian et al., 1999), this has made authors suggest that GAD65+ neurons may be the main actors of presynaptic inhibition at sensory-motor synapses (Betley et al., 2009) (Figure 14a).

Spared nerve injury reduces GAD65 mRNA levels in the dorsal horn ipsilateral to the injury, while GAD67 mRNA levels remain stable (Bráz et al., 2012). Chronic constriction injury decreases both GAD 65 and GAD67 protein expression (Moore et al., 2002). Another study showed that the initial loss of GAD67+ cells after the injury was followed by an upregulation over their normal levels (Eaton et al., 1998). Eaton et al. (1998) proposed that the downregulation of GABA after the chronic constriction injury, could be a positive feedback signal for the increase of GAD67. Three days after the chronic constriction injury, both GAD67+ cells and GABA+ cells were downregulated. However, one week after the injury, they observed an upregulation of GAD67+ cells, which continued to be over normal levels even at 8 weeks postinjury, and it was concomitant with a downregulation of GABA+ cells, which increased progressively until 8 weeks postinjury. A cell transplant that prevents GAD67 upregulation and GABA downregulation after the injury could also alleviate neuropathic pain (Eaton et al., 1998) (Figure 14b).

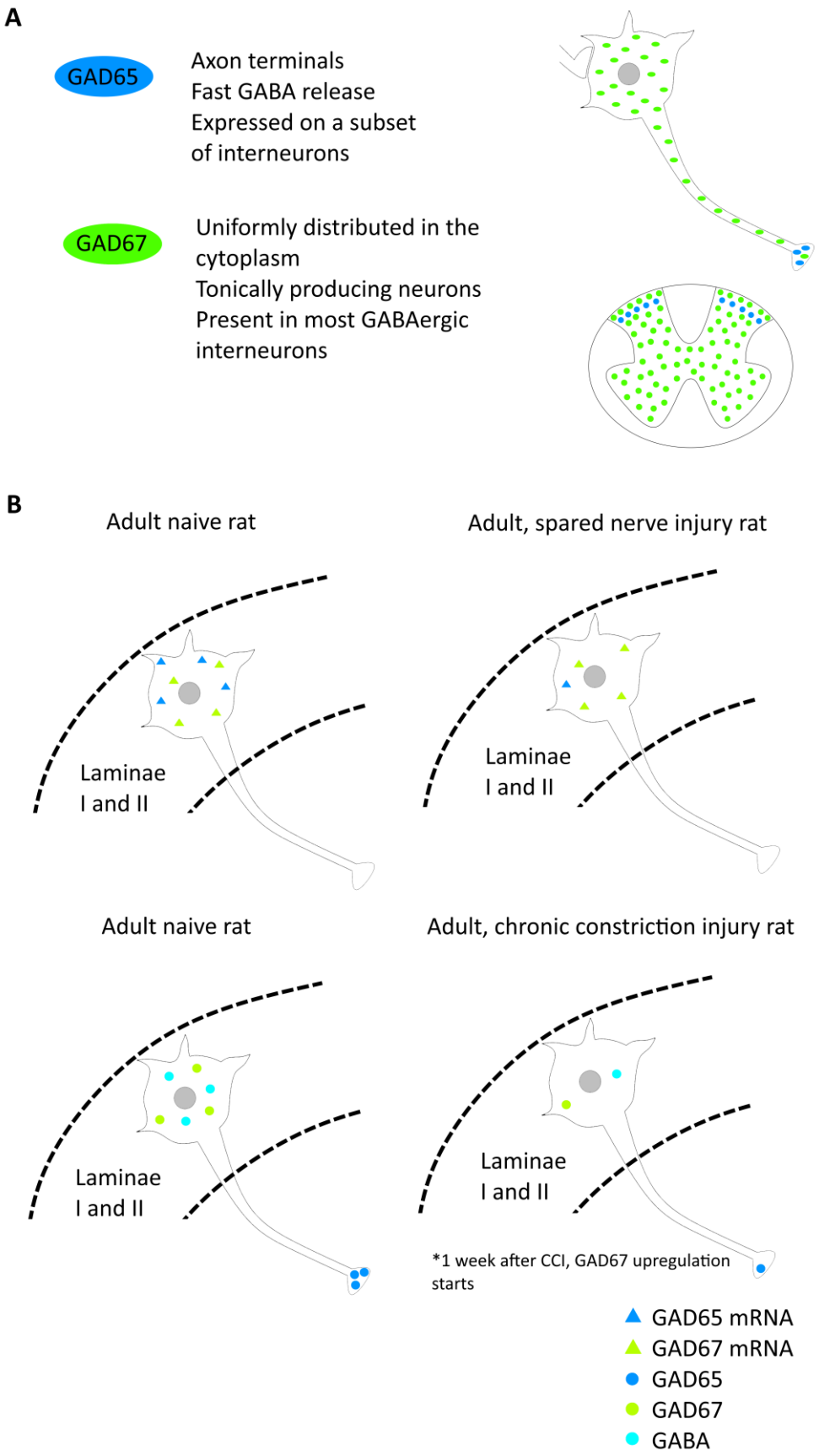

Figure 14. GAD expression after an injury. a) GAD65 and GAD67, characteristics and distribution. b) Changes in GAD65, GAD67 mRNA and protein expression after injury.

Spinal cord contusion reduces the levels of GAD65 and GAD67 in the spinal cord below the injury (Meisner et al., 2010), although in the acute phase GAD activity was significantly increased compared to naïve rats (Diaz-Ruiz et al., 2016). Spinal cord hemisection reduces the levels of GAD65 (Gwak et al., 2008), and an increase in GAD67 after hemisection by viral vectors reduced neuropathic pain (Liu et al., 2004, 2008). A complete transection, however, increases GAD67 in the dorsal and ventral horn of lumbar spinal cord in cats (Tillakaratne et al., 2002), although the activity of GAD after a spinal cord transection has reported to be altered, making the enzyme less efficient (Naftchi et al., 1979). This loss of efficiency may explain why the delivery of GAD67 by viral vectors after a complete transection reduced spastic bladder (Miyazato et al., 2009a, 2009b) (Figure 15).

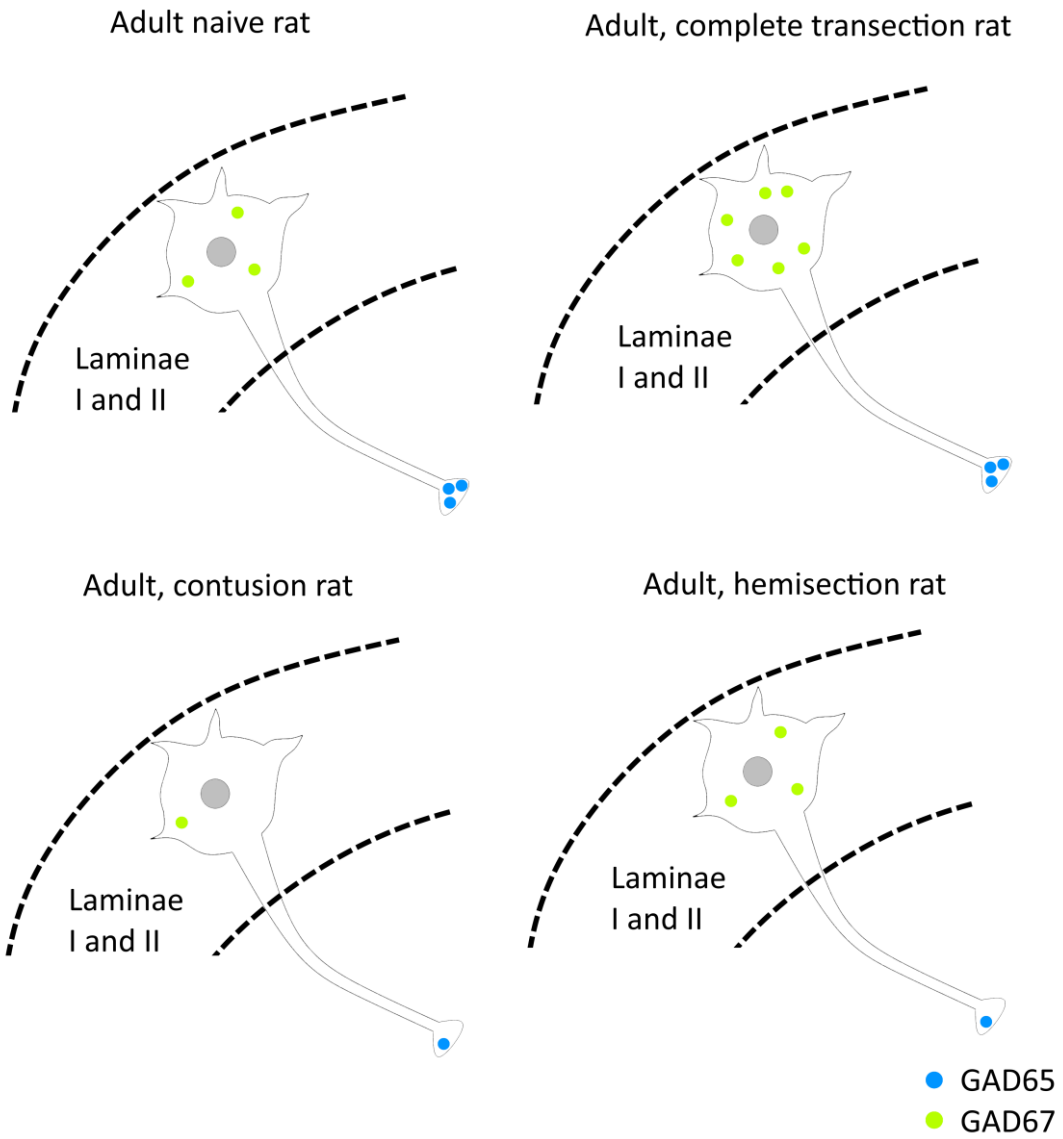

Figure 15. Changes in GAD65 and GAD67 expression on the sublesional spinal cord after different types of spinal cord injury.

1.4.3.3. Death of GABAergic neurons

Controverted results have been reported regarding GABAergic cell loss after a peripheral nerve injury, with some authors not finding a reduction on GABAergic or glycinergic cell counting (Polgár et al., 2003) while some others have described it (Castro-Lopes et al., 1993; Eaton et al., 1998; Scholz et al., 2005). Most of these authors did not consider total cell numbers, which is important to determine whether there is an apoptosis: it has been described that there is no difference between total numbers of neurons in the dorsal horn of sham rats compared with rats with a complete transection of the sciatic nerve (Coggeshall et al., 2001), chronic constriction injury (Polgár et al., 2003; Scholz et al., 2005) and spinal nerve ligation (Scholz et al., 2005). Coggeshall et al. (2001) proposed that there is not a loss of GABAergic cells after a peripheral nerve injury, but instead, that some GABA+ cells loose this marker transiently, due to the downregulation of GAD and GABA described in the previous section (1.4.3.2. GAD expression) (Figure 16a). However, after a spared nerve injury there is neuron loss, and there is also a loss of GABA+ neurons compared to sham rats (Scholz et al., 2005) (Figure 16b). In conclusion, it seems likely that there is no loss of GABA+ cells in the ipsilateral dorsal horn of the spinal cord after most types of peripheral nerve injury (Coggeshall et al., 2001; Polgár et al., 2003; Scholz et al., 2005). The only exception seems to be the spared nerve injury model, in which there is a loss of total neuron countings, and a loss of GABA+ neurons in particular (Scholz et al., 2005).

Spinal cord contusion reduced the number of GABAergic cells in lamina II of the dorsal horn in the spinal cord below the injury; some of the GABAergic neurons underwent cell death (Meisner et al., 2010) (Figure 16b). Transient spinal cord ischemia reduced the number of GABA-immunoreactive cells in the spinal cord during two weeks (Zhang et al., 1994) (Figure 16a).

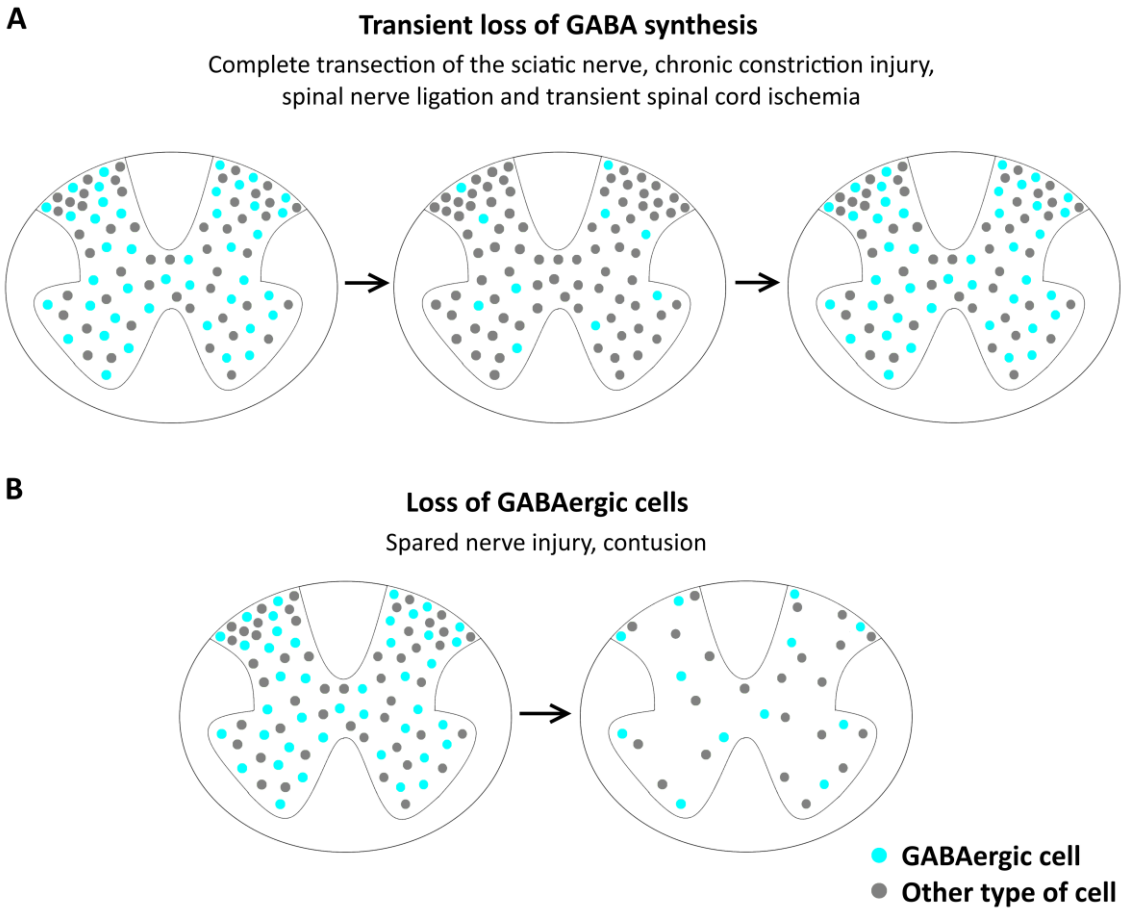

Figure 16. Loss of GABA+ cells. A) Transient loss of GABA immunoreactivity. B) Death of GABAergic neurons.

1.4.3.4. Impairment of E_{Cl}

When ligands bind to $GABA_A$, $GABA_C$ or glycine receptors, the ion channel opens, allowing the passage of chloride ions, although bicarbonate ions are also capable to pass through them, with more difficulty. The sense of the passage of chloride ions is dictated by their electrochemical gradient: if the membrane potential of the neuron is more depolarized than the reversal potential of chloride (E_{Cl}) for that neuron, the activation of glycine or $GABA_A$ receptors will cause an entrance of chloride ions (Figure 17, Intact). The entrance of negative ions in the cytosol of neurons causes a hyperpolarisation of the resting membrane potential (V_{rest}), which causes the inhibition of action potentials.

The team of John Eccles (Brock et al., 1952) described for the first time Inhibitory Postsynaptic Potentials (IPSP) at spinal motoneurons of adult cats. Several later works have allowed to characterize the dynamics of these IPSP (Lux, 1971; Llinas and Baker, 1972; Llinás et al., 1974; Allen et al., 1977). These studies have proven that IPSP are hyperpolarizing if the value of chloride equilibrium potential (E_{Cl}) is more negative than the resting potential (V_{rest}). According to Nernst equation, E_{Cl} depends on the concentration of chloride in the intracellular

and extracellular compartments. This is important to keep in mind, because for E_{Cl} to be more hyperpolarized than its membrane potential, the intracellular concentration of chloride needs to be much lower than the extracellular concentration of chloride and to keep a hyperpolarized E_{Cl}, an active transport of chloride ions out of the cell is needed.

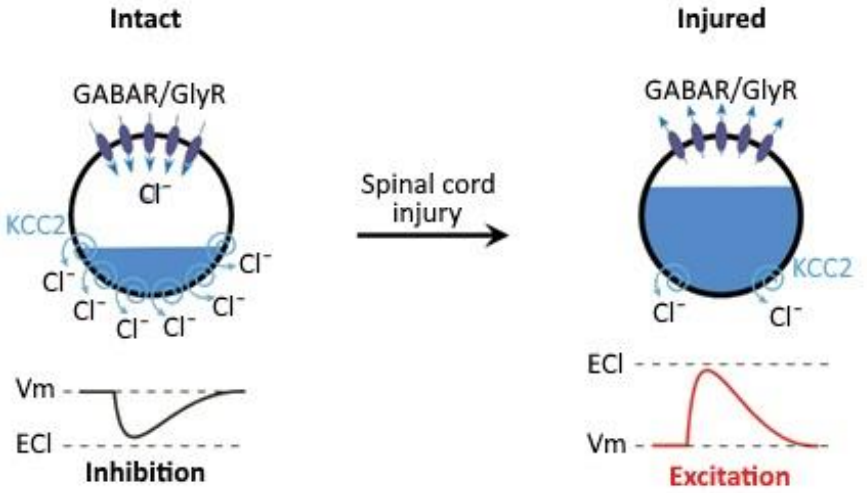

Figure 17. Consequences of E_{Cl} impairment on GABA and glycine-mediated inhibition. Intact) Inhibitory control on intact animals. KCC2 action reduces intracellular chloride concentration. Resting membrane potential of neurons (V_m) is more depolarized than chloride equilibrium potential (E_{Cl}): when GABA or glycine receptors are activated, chloride ions enter the neuron. Injured) Inhibitory control is lost on spinal cord injured animals. A downregulation of KCC2 increases intracellular chloride and this shifts E_{Cl} towards more depolarized potentials. The activation of GABA and glycine receptors lets chloride ions into the neuron; GABA and glycinergic neurotransmission becomes depolarizing. Figure adapted from Boulenguez et al. (2011); Plantier and Brocard (2017).

In the nervous system of vertebrates, there is a highly conserved mechanism dedicated to the extrusion of chloride ions from neurons, which keeps a low intracellular chloride concentration, maintaining a hyperpolarized E_{Cl}, and the protein that is implicated in it, is the potassium-chloride cotransporter KCC2 (Ben-Ari et al., 2007; Kahle et al., 2013; Kaila et al., 2014).

Beyond the actual loss of GABAergic and glycinergic receptors, cells or synthesizing enzymes, another mechanism through which an injury to the nervous system impairs inhibitory modulation is by reducing the expression and function of the potassium-chloride cotransporter KCC2 (Figure 17, Injured). We will further discuss the action of KCC2 on physiological and pathological situations in the next chapter.

1.5. KCC2

1.5.1. Nomenclature, structure, expression

KCC2 is a transmembrane protein from the solute carrier family 12 (SLC12) of electroneutral cation-chloride cotransporters (CCCs), which contains seven members:

- four potassium-chloride cotransporters, KCC1, KCC2, KCC3 and KCC4
- two sodium-potassium chloride cotransporters, NKCC1 and NKCC2
- a sodium-chloride cotransporter, NCC

These transporters associate the transport of chloride ions to the transport of sodium and potassium ions, which makes their action electronically neutral. The nine proteins have a common ancestral gene (Slc12a), and the similarity of their sequences is estimated in between 19% and 76% (Payne et al., 2003; Gamba, 2005). CCCs are constituted of 12 transmembrane segments, and an N- and C-terminal terminations that are situated on the cytoplasmic compartment.

Two members of this family, KCC2 and NKCC1, have a strong expression in the nervous system (Kaila et al., 2014) (Figure 18D); in adult mammals, KCC2 is the main chloride extruder, whereas NKCC1 is the major chloride uptake mechanism (Hartmann and Nothwang, 2014) (Figure 18B). KCC2 is present in neurons from the central nervous system, while NKCC1 is present in the central and peripheral nervous system and glia (Jayakumar and Norenberg, 2010) and has also a strong presence in kidneys (Kaila et al., 2014). KCC2 mRNA has been found in DRG neurons (Lucas et al., 2012), but whether this mRNA is translated into a functional protein is currently unknown.

NKCC1 is capable of conforming monomers or dimers, and KCC2 is capable of conforming monomers, dimers and tetramers (Hartmann and Nothwang, 2014). The C-terminal domain is thought to be implicated in the dimerization process (Hartmann and Nothwang, 2014). A question that arises is whether homo or oligomerization affects the efficacy of the CCCs transport. Whether dimerization of NKCC1 is necessary for transport activity is currently unknown; several studies indicate that KCC2 is only an active transporter as a dimer (Blaesse et al., 2006; Watanabe et al., 2009; Mahadevan et al., 2014).

The proportion of oligomerization increases along development (Figure 18A), in parallel with KCC2 action, until it becomes the principal mediator of Cl- extrusion in the adult nervous system (Blaesse et al., 2006; Uvarov et al., 2009). Some KCC2 forms have a structural role in the formation of dendritic spines (Figure 18C).

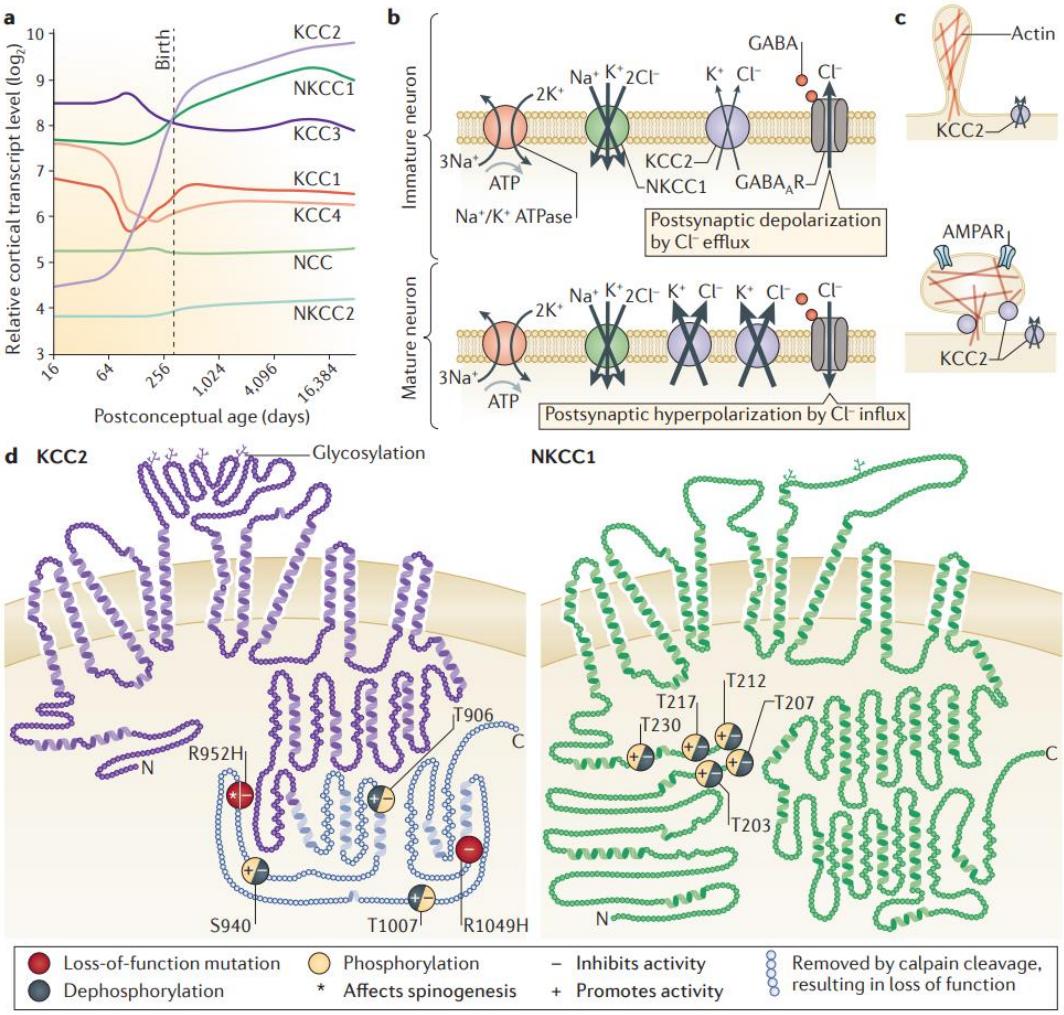

Figure 18. KCC2 and NKCC1 structure and expression along development. a) NKCC1 expression in the nervous system is quite high on the embryonic and fetal development, and it increases slightly towards birth and during postnatal development. KCC2 expression is low during embryonic development and it increases during fetal stages; its increase is slower after birth. Their development on the spinal cord happens earlier than in the brain. b) In comparison with immature neurons, mature neurons express more functional KCC2 at the cell membrane and therefore activation of $GABA_A$ receptors is hyperpolarizing. Activation of $GABA_A$ receptors at immature neurons is depolarizing due to the low KCC2 expression. c) Upregulation of KCC2 during development gives rise to forms of KCC2 that have a structural role and contribute to the formation of dendritic spines. d) Predicted secondary structures of human KCC2 and NKCC1. The main regulation sites that have been identified are indicated. Figure adapted from Kaila et al. (2014).

KCC2 seems to be only present in the somatodendritic compartment and has been described at the hippocampus (Rivera et al., 1999; Gulyás et al., 2001), cerebellum (Williams et al., 1999), cortex (Szabadics et al., 2006), brainstem (Blaesse et al., 2006), thalamus (Barthó et al., 2004), and spinal cord (Hübner et al., 2001; Stil et al., 2009). At the spinal cord level, Lu et al. (2008) shown that the vast majority of dorsal horn neurons, if not all, express KCC2; regarding the ventral horn, KCC2 is present in the membrane of motoneurons, having a strong expression as early as P10, although its maximal efficacy seems to be attained towards the

third postnatal week (Stil et al., 2009). Note that there are some neurons that do not express KCC2, like the dopaminergic neurons of the substantia nigra (Gulácsi et al., 2003) and most neurons in the thalamic reticular nucleus (Barthó et al., 2004).

1.5.2. KCC2 Function

KCC2 is a secondary active transporter: it is secondary active because its action to transport Cl^- and K^+ ions does not require ATP consumption, but it depends on the concentration gradient generated by the sodium/potassium ATPase, since KCCs use the high intracellular K+ concentration to extrude K^+ and Cl^- (Gamba, 2005). Thus, alterations of the sodium/potassium pump can affect the action of KCC2. Besides this, KCC2 is the only KCC that works constitutively in isotonic conditions (Payne, 1997; Song et al., 2002).

On physiological conditions, in adult neurons from vertebrates, KCC2 takes chloride out, driving the intracellular Cl^- concentration below its biochemical equilibrium potential, strengthening $GABA_A$ hyperpolaryzing responses and postsynaptic inhibition. KCC2 decreases the intracellular chloride concentration by extruding potassium and chloride ions on an electroneutral 1:1 ratio. However, since its action depends on the activity of sodium/potassium ATPase, its function can be inversed and it can cause a depolarization of E_{Cl} in the presence of high extracellular concentrations of potassium ions (Payne, 1997; Gamba, 2005; Vinay and Jean-Xavier, 2008). Potassium can accumulate on the extracellular compartment during long-lasting neuronal activity (Wallén et al., 1984; Marchetti et al., 2001). This can inverse the sense of KCC2 activity, making it take potassium and chloride ions into the neurons when the extracellular concentration of potassium is high, which would cause the depolarization of E_{Cl}.

The function of KCC2, its stability at the membrane or its traffic, can be modulated by phosphorylation/dephosphorylation mechanisms (Kahle et al., 2013) (Figure 19). The turnover of KCC2 is extremely fast since membrane KCC2 are partially (Rivera et al., 2004) or completely (Lee et al., 2007) endocyted in 20 minutes in basal condition. This turnover can be modified by phosphorylation/dephosphorylation processes. It is well established that certain tyrosine, serine and threonine residues from the C-terminal domain of KCC2 can be modified by kinases and phosphatases (Figure 19a). The phosphorylation of the serine 940 residue is the only known residue whose phosphorylation stabilizes KCC2 at the membrane and increases its activity (Lee et al., 2007; Medina et al., 2014). Conversely, dephosphorylation of this residue promotes the inactivation and endocytosis of the transporter. The implications of tyrosine phosphorylation have been more discussed, since different methods seemed to prove, at the same time, an increase in KCC2 activity with phosphorylation (Watanabe et al., 2009) and a decrease in KCC2 activity with phosphorylation of tyrosine residues –due to internalization

and lysosomal degradation of KCC2- (Lee et al., 2010). Finally, it is accepted that phosphorylation of tyrosines 903 and 1087 reduces KCC2 stability at the membrane and causes its degradation (Lee et al., 2007, 2010).

Several signaling pathways have been implicated in KCC2 regulation (Figure 19b).

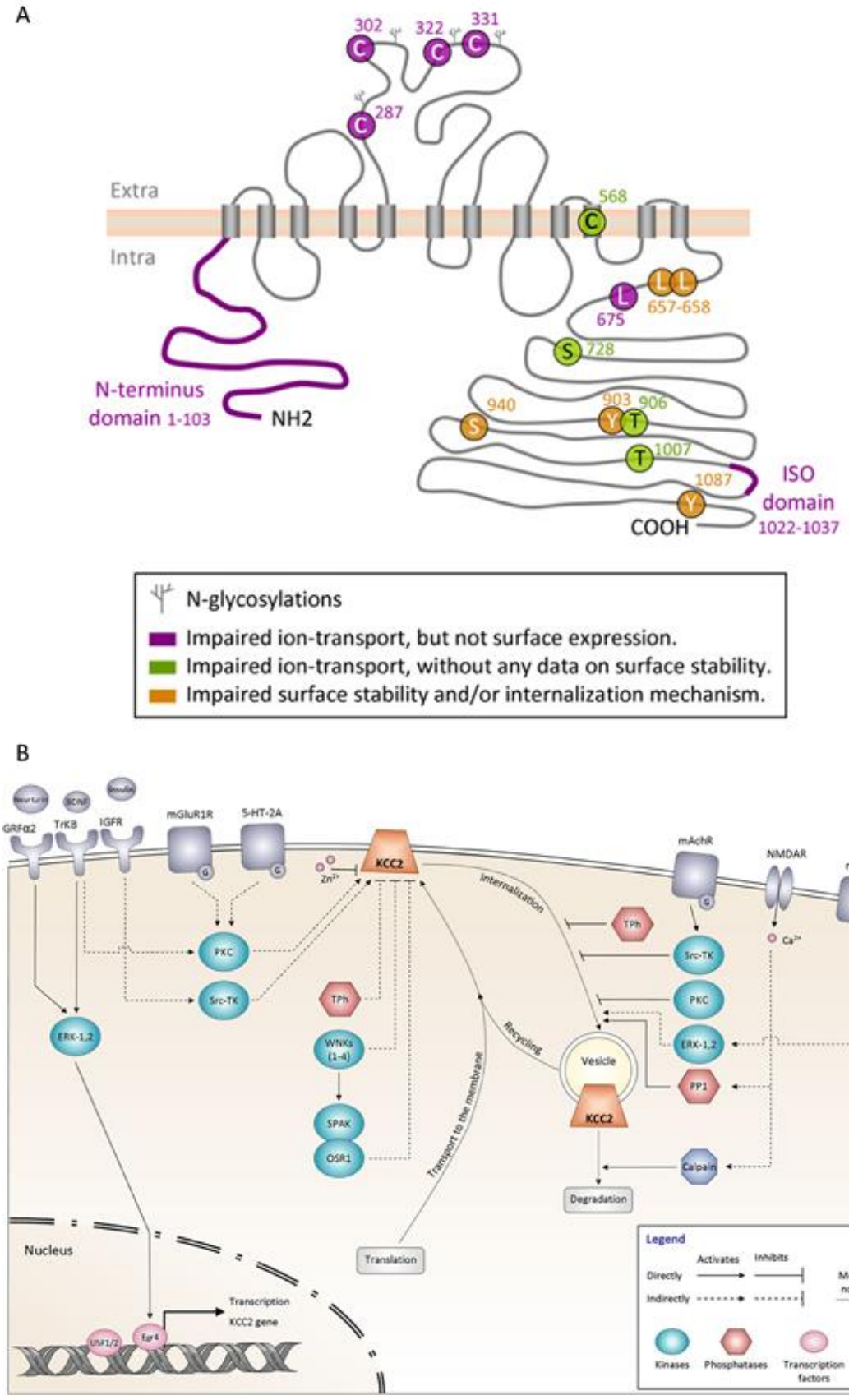

Figure 19. KCC2 C-terminal phosphorylation sites and signalling pathways. a) Residues that are necesary for KCC2 expression, based on experiments with mutant proteins. The mutation of four cysteine residues in the extracellular loop impaired ion transport through KCC2, but had no effect on

its membrane expression. Leucine 675 or ISO domain mutations had the same effect than the mutation of the 4 cysteines. Mutations on cysteine 568, serine 728 and threonines 906 and 1007 also impaired ion transport, but it is unknown whether they impair KCC2 function or its expression at the membrane. Mutations on leucine 657-658, tyrosine 903, serine 940 and tyrosine 1087 impaired KCC2 expression at the membrane and facilitated its internalization. b) Signaling pathways increasing KCC2 expression at the plasma membrane or mediating its internalization and degradation. Figure from Medina et al. (2014).

1.5.3. KCC2 in spasticity and neuropathic pain

1.5.3.1. KCC2 in spasticity

Dysregulation of KCC2 is implicated on the emergence of spasticity. Our team showed that after a spinal cord injury, the alteration of KCC2 on the sublesional region contributes to the development of spasticity (Boulenguez et al., 2010; overview in Figure 20). The immunohistochemistry showed a reduction of KCC2 expression on the membrane of lumbar motoneurons after a thoracic spinal cord injury, and it is internalized in the cytoplasm forming clusters (Figure 20a-f). These clusters are not functional: KCC2 needs to be at the membrane to be able to extrude potassium and chloride.

Following the hypothesis that this reduction on the membrane expression would cause a loss of function of the co-transporter, the team has proven that blocking KCC2 by DIOA on naïve rats induced a hyperexcitability on the spinal cord (Figure 20g) and a reduction of the rate-dependent depression (Figure 20h-i). These two signs are characteristic of spasticity. Furthermore, after a spinal cord injury, the reciprocal inhibition of motoneurons is reduced (Gackière and Vinay, 2015), which is an indicator of a reduced postsynaptic inhibition in the sublesional spinal cord. The reduction of postsynaptic inhibition is due to the dysregulation of KCC2 (Gackière and Vinay, 2015), which causes a significant depolarization of the equilibrium potential of chloride ions at the level of lumbar motoneurons and a hyperexcitability of the spinal neuronal network (Jean-Xavier et al., 2006; Bos et al., 2013). As has been mentioned in section 1.4.3.4., KCC2 is necessary for maintaining a hyperpolarized E_{Cl} that facilitates GABAergic and glycinergic inhibition.

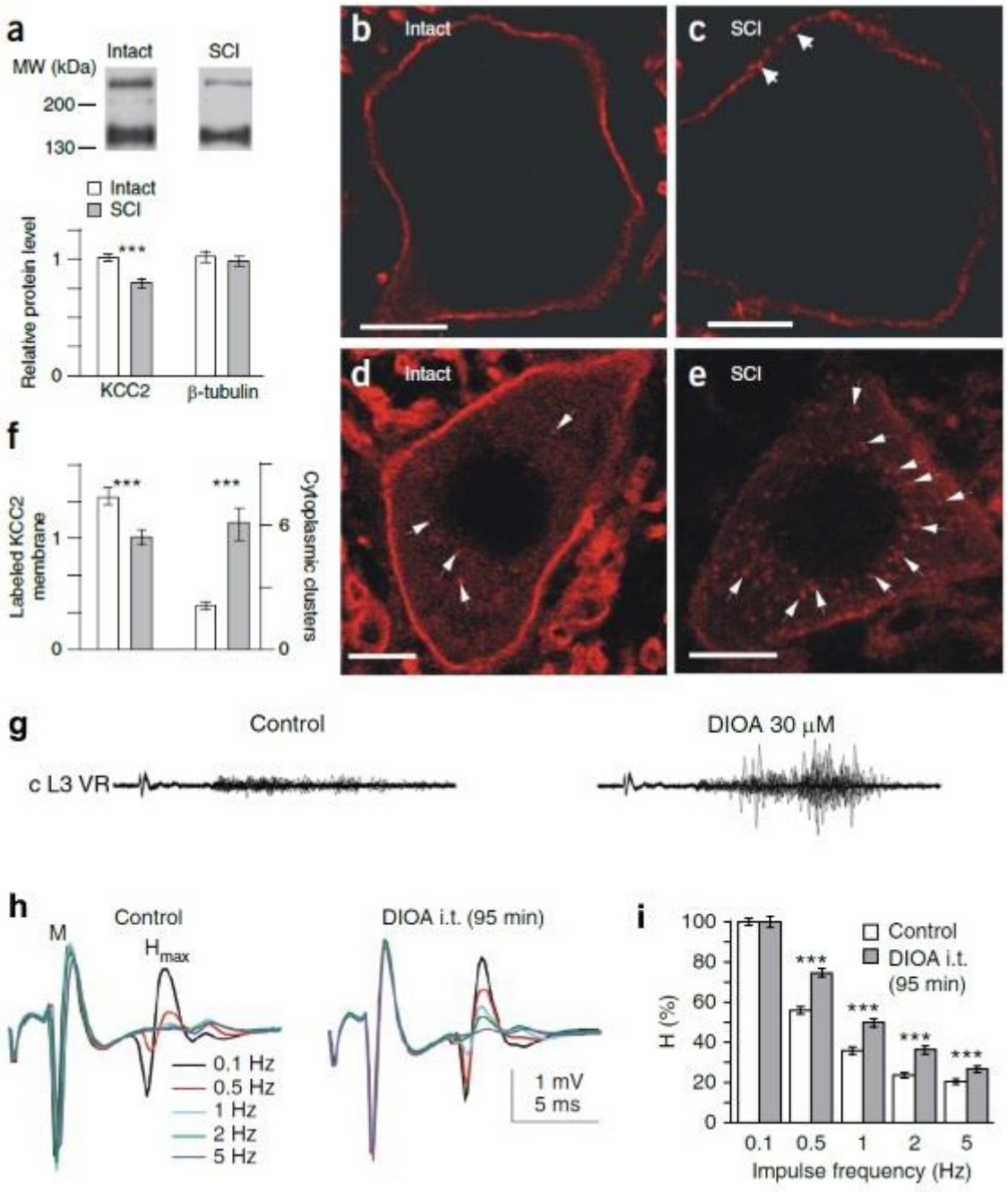

Figure 20. KCC2 downregulation on spinal motoneurons contributes to the development of spasticity after a spinal cord injury. a) Western blot studies showing a downreglation of KCC2 after a spinal cord injury. b-f) immunohistochemistry studies showing a downregulation of KCC2 at the membrane of motoneurons, together with an increase of KCC2 cytoplasmic clusters, which are not functional. g) KCC2 inhibition by DIOA on naive newborn rats causes a hyperexcitability of the monosynaotic reflex in vitro. h-i) DIOA injection on adult rats reduces rate-dependent depression in the electromyogram recordings of a hindlimb muscle. This is a sign of muscle tetanus, a kind of spasm. Data are issued from Boulenguez et al. (2010).

In physiological conditions, Brain-Derived Neurotrophic Factor (BDNF) released by microglia reduces KCC2 expression in the spinal cord. However, after a SCI, BDNF effect

reversed and it increased KCC2 expression (Boulenguez et al., 2010). Previous studies have provided evidence about how this switch on BDNF action can occur. Rivera et al. (2004) proved that BDNF activation of tropomyosin receptor kinase B (TrkB) activates two cascades, involving PLCγ and Shc, to reduce KCC2 expression on hippocampus. Using point-mutant mice, they showed that if the PLCγ cascade is not activated, the activation of the Shc cascade results in an upregulation of KCC2. Tashiro et al., (2015) proved that there is a downregulation of PLCγ after a contusion injury of the spinal cord on adult rats, whereas Shc levels were preserved. Treadmill training of these rats increased BDNF, which caused an increase on KCC2 expression, reducing spasticity and allodynia. This effect was prevented by intrathecal injection of TrkB-IgG, which inhibited the union of BDNF to TrkB receptor.

1.5.3.2. KCC2 in neuropathic pain

Several studies have proven that KCC2 dysregulation is implicated on the development of neuropathic pain. Of note, KCC2 inhibition by DIOA is pronociceptive (Austin and Delpire, 2011). However, although most studies have shown a pro-nociceptive effect of KCC2 downregulation, KCC2 reduction is not always nociceptive: there is at least one study where mice heterozygous for hypomorphic and null alleles -with about 20% of wild-type KCC2 expression-, showed a normal motor control, and a reduced sensitivity to mechanical and thermal stimulation, although they were prone to other behavioural impairments (Tornberg et al., 2005). The authors did not see a significant variation in the expression of other CCCs – although NKCC1 expression was slightly higher-, but they did not evaluate E_{Cl} or IPSPs, so a compensatory mechanism in these transgenic mice cannot be ruled out yet.

Coull et al. (2003) showed that KCC2 downregulation could cause neuropathic pain (Figure 21a) and described for the first time that a peripheral nerve injury caused a reduction of KCC2 expression at lamina I neurons of the spinal cord (Figure 21b). This had an impact on the balance of chloride ions, with a depolarization of E_{Cl} of about 20 mV on these neurons. GABAergic transmission became excitatory (Figure 21c-d), making this spinal segment more excitable. On a similar way, a spinal cord injury caused an E_{Cl} depolarisation of several mV on the same neurons of the dorsal horn. This is traduced by an alteration of KCC2 cotransporters (Lu et al., 2008). These neurons become more sensitive to the postsynaptic potentials and this contributes to the emergence of hyperalgesia.

Several groups have confirmed that KCC2 downregulation contributes to neuropathic pain after peripheral nerve injury (Coull et al., 2003, 2005; Price et al., 2005; Prescott et al., 2006; Miletic and Miletic, 2008; Janssen et al., 2012; Zhou et al., 2012; Wei et al., 2013; Mòdol et al., 2014). This downregulation is tightly linked to NMDA overactivation after a peripheral nerve injury. Zhou et al. (2012) showed that nerve injury (spinal nerve ligation) increased NMDAR

activity at the spinal cord, which was related to the downregulation of KCC2, which reduces the capacity of glycine to produce IPSPs. Reciprocally, the disrupted neuronal Cl^- homeostasis plays a critical role in potentiating synaptic NMDA activity in neuropathic pain: Li et al. (2016) proved that intrathecal delivery of KCC2 using lentiviral vectors after a peripheral nerve injury reduced neuropathic pain and restored Cl^- homeostasis, and this normalized NMDA activity.

Lu et al., (2008) proved that spinal cord hemisection caused downregulation of KCC2 below the injury level, which was related to the development of mechanical allodynia. They also showed, through patch-clamp recordings, that IPSPs were reduced after spinal cord injury, which caused that EPSPs from neurons that were normally subthreshold, were capable of causing firing. They hypothesized that partial spinal cord injury causes downregulation of KCC2, and this makes the input of some Aδ and C fibers, which is normally subthreshold, to evoke action potentials in the neurons from the dorsal horn (see Figure 10). This would be the cause of allodynia after incomplete spinal cord injury. Lu et al. (2008), showed that the depolarization of the equilibrium potential of GABA (E_{GABA}) after spinal cord injury affected most subtypes of dorsal horn neurons (vertical, radial, islet, tonic central,…).

After a spinal cord contusion, there is a decrease on KCC2, and a concomitant increase on NKCC1, which increases intracellular chloride concentration in dorsal horn neurons, causing neuropathic pain (Cramer et al., 2008; Hasbargen et al., 2010). Neuropathic pain has shown a similar pattern on a model of peripheral nerve injury: upregulation of NKCC1 expression and phosphorylation in the DRG, and downregulation of KCC2 expression and phosphorylation in the dorsal horn (Mòdol et al., 2014). But it is not the case for all types of pain: nociceptive pain caused by plantar formalin injection showed a significant decrease in KCC2 protein expression without changes in NKCC1 expression (Nomura et al., 2006). This imbalance on NKCC1-KCC2 expression seems to be characteristic of neuropathic pain, but there are some exceptions like chronic constriction injury of the trigeminal nerve (Castro et al., 2017), which did not modify KCC2 or NKCC1 expression, or chemotherapy-induced neuropathic pain (Chen et al., 2014), which showed an increase in NKCC1 without changes in KCC2.

There is a tight link between microglial activation and KCC2 function in the dorsal horn (Gwak et al., 2012) –as it was the case in the ventral horn-. A peripheral nerve injury would cause a release of ATP by the neurons, which activate P2X4 receptors on the membrane of microglial cells. This signaling pathway causes the release of BDNF by microglia, which would activate TrkB receptors from neurons, altering chloride ions homeostasis by reducing KCC2 expression and function (Coull et al., 2005; Ferrini and De Koninck, 2013). Preventing BDNF release from microglia or blocking BDNF activation of TrkB, prevented allodynia after a peripheral nerve injury. A graphic of the withdrawal threshold of rats injected with microglia, ATP and either blockers of TrkB receptors or scramble oligonucleotides is depicted in Figure 21e.

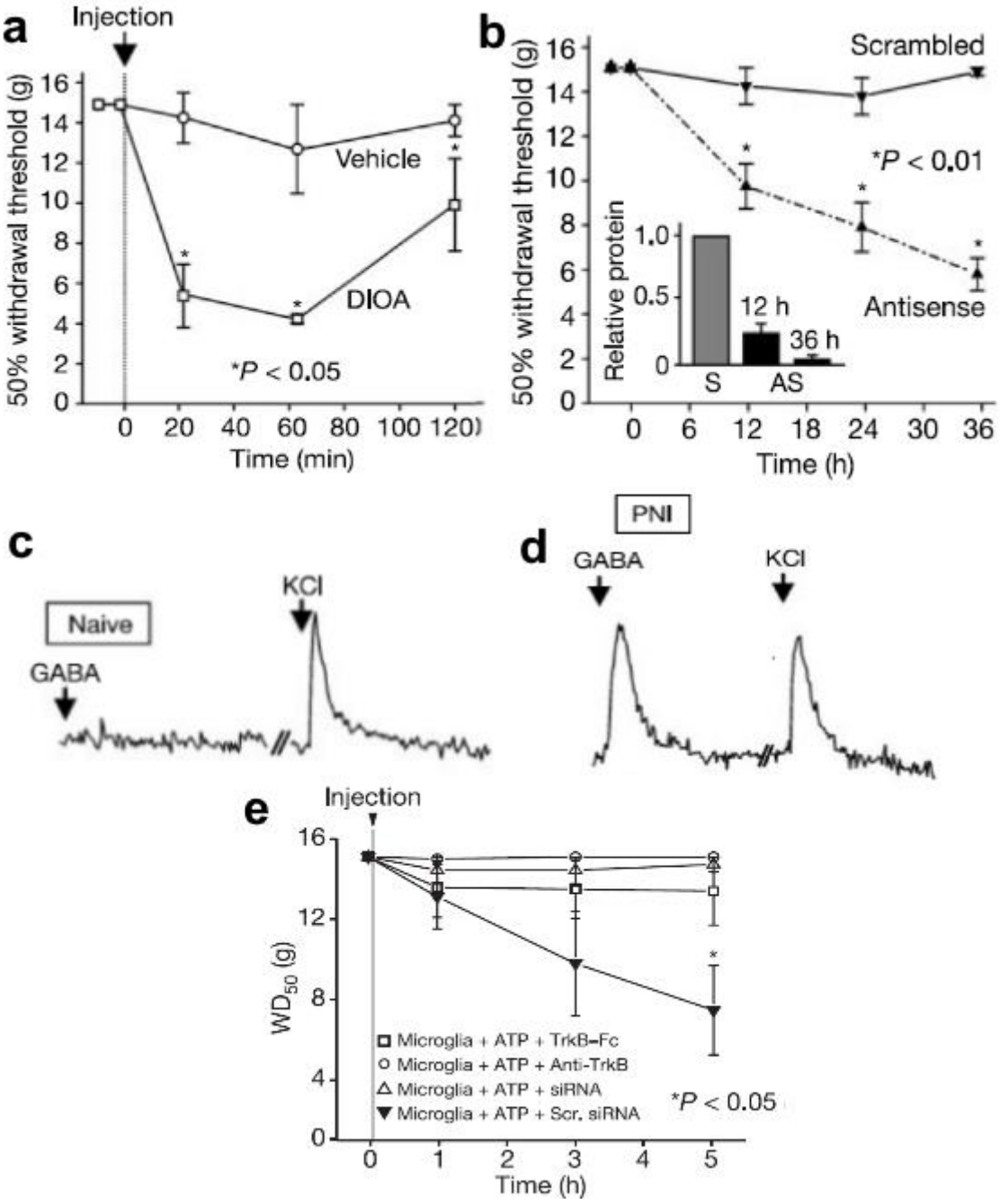

Figure 21. KCC2 impairs GABAergic transmission in the dorsal horn and facilitates pain. a) DIOA injection in naïve rats is pronociceptive. b) Administration of antisense KCC2 oligonucleotides reduces withdrawal threshold. c) Voltage-clamp of lamina I cells of naïve rats shows that GABA application does not cause any current. d) However, GABA application on lamina I cells of rats with a peripheral nerve injury (PNI) causes a depolarization of the membrane, indicating that GABA has become excitatory. e) Injection of microglia and ATP in naïve rats causes allodynia (black triangles), however, co-injection of antibodies against TrkB or small interfering RNA (siRNA) against BDNF prevented the development of allodynia (white squares, circles and triangles). Allodynia and hyperalgesia are caused by KCC2 downregulation, which is mediated by TrkB activation, dependent on BDNF release by microglia. Figure adapted from Coull et al. (2003, 2005).

It was mentioned on the previous section (1.5.3.1. KCC2 in spasticity) that treadmill training after a contusion injury in adult rats increased KCC2 at the spinal cord and reduced allodynia through a mechanism implicating BDNF (Tashiro et al., 2015), indicating that BDNF action after spinal cord injury is also analgesic.

1.5.4. Does activation of calpains relate to the reduction of KCC2 leading to neuropathic pain and spasticity?

Calpains are cysteine proteases that were discovered in rat brain preparations (Guroff, 1964). After being renamed several times, these proteases have been named CAPN for "calcium-activated neutral proteases" in 1978 (Ishiura et al., 1978). At present, 16 calpains have been discovered (Ono et al., 2016), which are divided in two subfamilies (depicted in Figure 22):

- Classical calpains (calpains 1, 2, 3, 8, 9, 11, 12, 13 and 14), which are heterodimers constituted by a calmodulin-homolog domain (EF hand domain) which allows them to interact with calcium ions, and a papain-type, cystein-proteolytic domain, which is common to all calpains
- Atypical calpains (calpains 5, 6, 7, 10, 15 and 16), which do not have the « calmodulin » domain.

Humans express 15 out of these 16 calpains (with the exception of calpain 4).

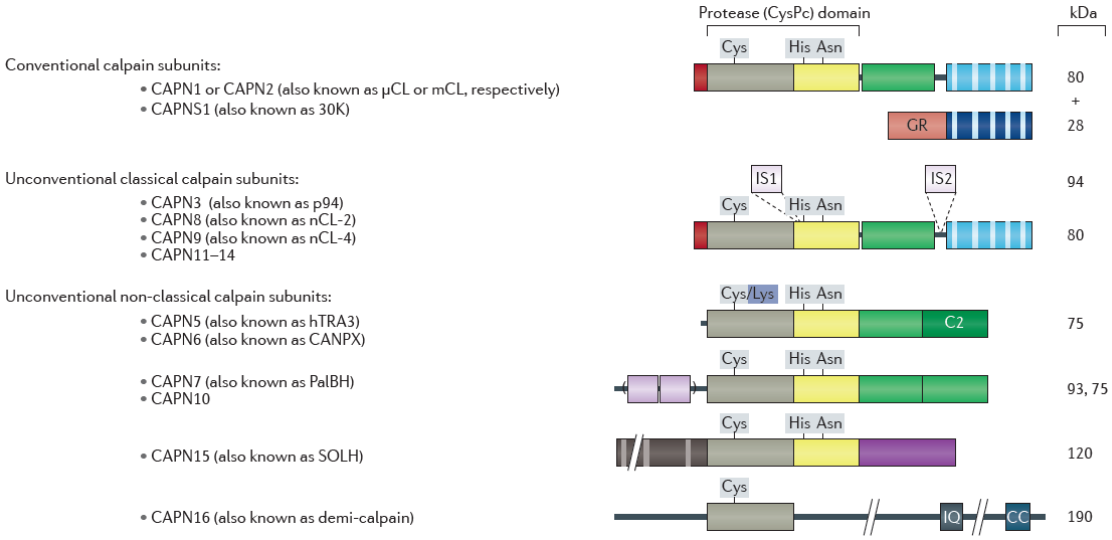

Figure 22. The fifteen calpain isoforms present in humans and their domains. Classical calpains are composed by the calmodulin-homolog domain (in blue), and a cysteine-proteolytic domain (in grey), common to all calpains. Non-classical calpains lack the calmodulin-homolog domain and express other

domains instead. Only the conventional classical calpains, calpain I and II, are heterodimers containing also a regulatory subunit of 28 kDa. Figure adapted from Ono et al. (2016)

Calpain 1 or μ-calpain and calpain 2 or m-calpain are among the most abundant calpains in the central nervous system of mammals. They have a 60% of homology in their amino acid sequence. Like all conventional calpains, they contain a catalytic subunit of 80 kDa, and a regulatory subunit of 28 kDa (Khorchid and Ikura, 2002). Calpains 1 and 2 need different calcium concentrations for their activation, and the names they received are related to this particularity. In vitro activation of μ-calpain needs calcium concentrations on the micromolar range (3 to 50 μM for half-maximal activity), while m-calpain is activated by concentrations on the millimolar range (0.4-0.8 mM for half-maximal activity) (Goll et al., 2003). Since intracellular calcium concentrations can reach micromollar concentrations at most, m-calpain could be expected to not have a high catalytic activity.They are cytosolic proteins and since their catalytic action can take place at a neutral pH (Guroff, 1964), they are capable of catalizing cytoplasmic proteins.

Several studies show an increase in the expression and activity of calpains within hours after a spinal cord injury (Li et al., 1995, 1996; Banik et al., 1997a, 1997b, 1998; Ray et al., 1999; Schumacher et al., 1999; Shields et al., 2000). This alteration of the expression of calpains seems linked to an increase in neuronal death, axon degeneration and demyelinization (Banik et al., 1997a; Shields et al., 1999; Ray et al., 2001). After the injury, there is a considerable increase in intracellular calcium, which facilitates the activation of calpains (Young, 1985; Du et al., 1999). A link has been established, during the acute phase of the injury, in the lesional region, between microglial activation and calpain activation: most of the cells with increased calpain staining were astrocytes and microglia (Li et al., 1995), and microglia and astrocytes' proliferation increased 1 to 3 days postinjury (Li et al., 1996). The beneficial effects of a calpain inhibition, on motor tasks and on the scar tissue, supports the hypothesis that there is a massive overactivation of these proteases after a spinal cord injury or a brain contusion (Banik et al., 1998; Arataki et al., 2005; Yu et al., 2013).

It has been proven that calpains cleave KCC2 (Puskarjov et al., 2012; Zhou et al., 2012; Chamma et al., 2013). The incubation of brain homogenates with calpain 2 caused a cleavage of KCC2, as shown by the reduction of protein expression in Western blot. This cleavage is blocked in the presence of MDL28170, an inhibitor of calpains (Puskarjov et al., 2012). KCC2 expression is also reduced when the activity of the preparation is increased by NMDA or a perfusion with a magnesium-free artificial cerebrospinal fluid (aCSF), which causes an increase in NMDA receptor activity, neuronal excitability and intracellular calcium, and this reduction was prevented by MDL28170. The increase in neuronal activity, which can be caused by an overactivation of NMDA receptors, increases intracellular calcium. This will activate calpains (Zhou et al., 2012), but also phosphatases which will dephosphorylate KCC2 at Serine 940 (Lee et al., 2011) this will cause KCC2 internalization and degradation by calpains (Chamma et al.,

2013). It has been proposed that this dephosphorylation causes a conformational change, exposing the cleavage region to the calpains, inducing endocytosis, recycling and degradation (Chamma et al., 2013). This degradation can be prevented by the application of a calpain inhibitor, or on a mutant where this serine is mutated into an aspartate (mutant S940D).

The amino acid sequence that is responsible of the constitutive activity of KCC2 has been identified (Mercado et al., 2006): named the ISO domain, it is on the C-terminal intracellular domain, among residues 1021 and 1035, and it is close to two predicted PEST sequences (proline, glutamine, serine and threonine) which make a privileged substrate for calpains (Shumway et al., 1999; Tompa et al., 2004; Mercado et al., 2006). Therefore, the proteolysis of these PEST sequences by calpains could make KCC2 cotransporter inoperative.

As it has been previously mentioned (section 1.5.3.2.) calpain I cleavage of KCC2 has been related to neuropathic pain after a peripheral nerve injury (Zhou et al., 2012). Inhibiting calpain I with an intrathecal injection of calpeptin reduced stablished pain hypersensitivity measured by the Von Frey test and paw pressure test. However, calpain has proven to be implicated on a wide range of processes implicating inflammatory mediators and signaling pathways related to the development of neuropathic pain (Uçeyler et al., 2007, 2010; Xie et al., 2010; Jaggi and Singh, 2012; Chen et al., 2013, 2018a, 2018b; Won et al., 2014; Zang et al., 2015; Yuan et al., 2017), so it seems that its contribution to this pathology is complex and goes beyond KCC2 downregulation.

Our team proved recently that calpains are linked to the development of spasticity after a spinal cord injury (Brocard et al., 2016). We demonstrate that calpain cleaves NaV1.6 (Figure 23a-c), causing a hyperexcitability on motoneurons (Figure 23d-e), which is related to spasticity, and a chronic treatment with MDL28170 reduces the hyperexcitability of the motoneurons and reduces also spasticity on spinal cord injured rats (Figure 23f-g). Regarding these results, the question that arises is whether calpain also cleaves KCC2 after a spinal cord injury, and whether the combination between a reduction on KCC2 and an increase on persistent inward sodium currents (INaP) could have a role in the development of spasticity.

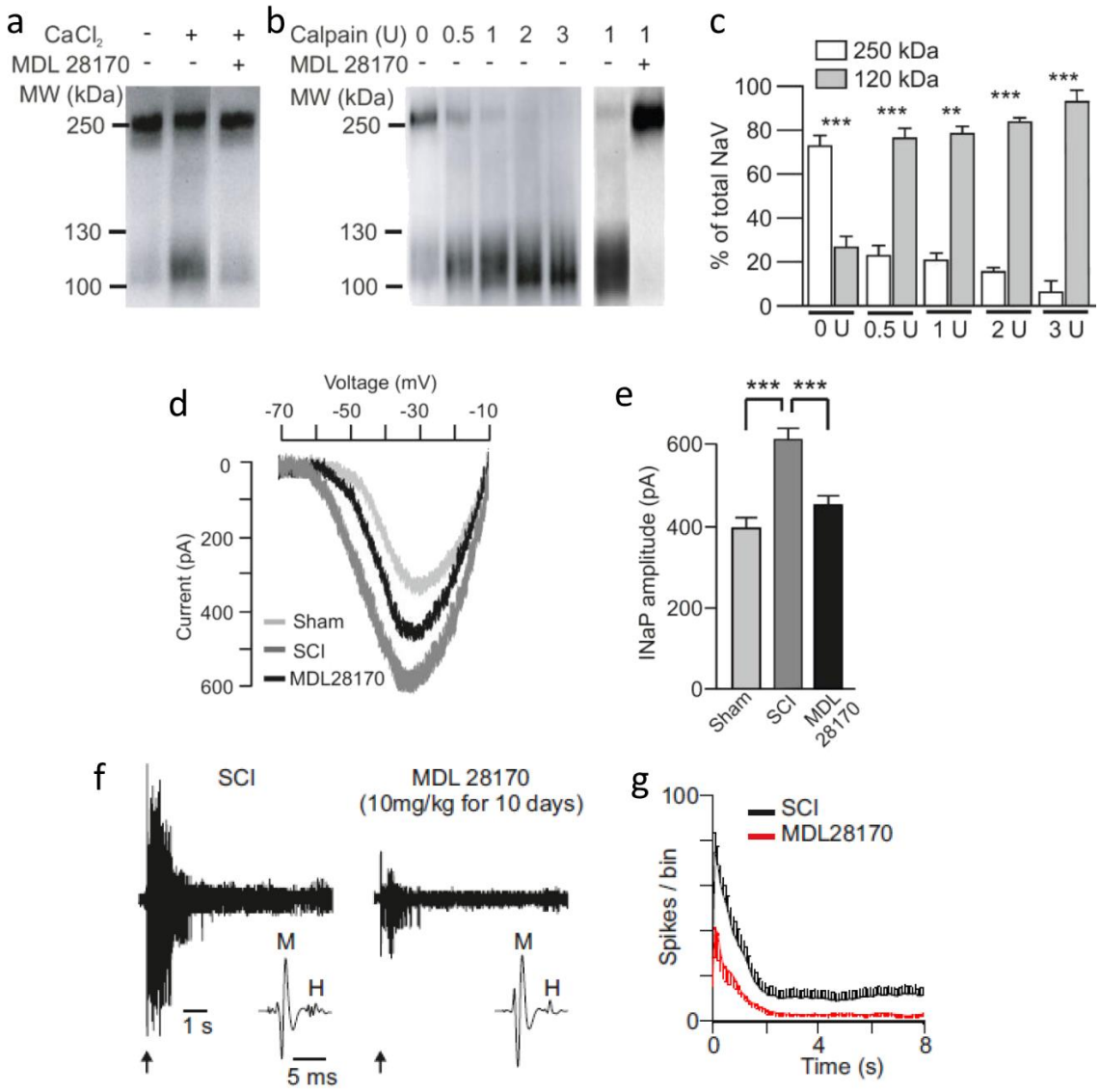

Figure 23. Calpain cleaves NaV1.6 after a spinal cord injury, causing spasticity. a) Western blot of spinal cord lysates incubated with calcium and the calpain inhibitor MDL28170. Calcium activates the proteolysis of NaV1.6 channels, which is inhibited by the addition of a calpain inhibitor. b) Increasing concentrations of calpain added to the spinal cord lysates increase the cleavage of NaV1.6 channels. c) Quantification of NaV1.6 subunits expression on the Western blot shown in B. d) The persistent inward sodium current is increased in motoneurons after a spinal cord injury, and it decreases with a chronic treatment with calpain inhibitor MDL28170. e) Quantification of persistent inward sodium current (INaP) shown in D. f) EMG of the flexor digitorum brevis muscle on SCI rats and on SCI rats after a chronic treatment with calpain inhibitor MDL28170. g) Quantification of the mean of the EMG responses shown in F for both groups of rats. MDL28170 causes a significant reduction on EMG amplitude. Figure adapted from Brocard et al. (2016).

1.6. Strategies to boost KCC2 in clinics

Given the crucial role of KCC2 on the development of neuropathic pain and spasticity after spinal cord injury, there is a compelling interest in developing drugs that can restore its normal expression. However, no drug is clinically available for this purpose yet.

On this section, we will make an overview of the strategies that have been followed to find a KCC2 enhancer, and the mechanisms we can chose to increase KCC2 expression/function to treat spasticity and neuropathic pain in clinics.

1.6.1. "Historical" KCC2 enhancers and newly synthetised compounds

KCCs were first described in red blood cells as being activated by cell swelling and N-ethylmaleimide (Garay et al., 1988); the mechanism by which N-ethylmaleimide increases KCC2 expression was recently described: it modifies KCC2 phosphorylation (Conway et al., 2017). Gagnon et al. (2013) identified two compounds, CLP257 and CLP290, which increase KCC2 and reduce neuropathic pain after a peripheral nerve ligation. Afterwards, these results have been discussed (Cardarelli et al., 2017) arguing that according to the results of the second group, CLP257 did not show an increase on KCC2 activity but only increased $GABA_A$ receptor currents.

However, the original authors and also another group, have proven the effect of CLP257 (Ostroumov et al., 2016; Gagnon et al., 2017). Gagnon et al. (2017) argued that several technical differences and some issues with the preparations could explain the divergent results from Cardarelli's group. Moreover, Oustrumov et al. (2016) found that CLP257 in slices reversed the collapse in inhibitory postsynaptic currents (IPSCs) in ventral tegmental area (VTA) neurons after stress-induced KCC2 downregulation. This confirms an enhanced Cl^- extrusion with CLP257, as this effect cannot be due to enhanced $GABA_A$ signaling, which –if KCC2 was still downregulated- would not result in increased IPSCs.

Although they have promising effects, these two drugs cannot be applied on human patients yet, since they are newly synthetized compounds that have not passed the safety tests to be commercialized as drugs for human use. The interest to find compounds that can increase KCC2 expression in human patients and are readily available has oriented us towards the repurposing of drugs that are already approved, to find an answer for this clinical need.

KCC2 enhancers are identified in Figure 24.

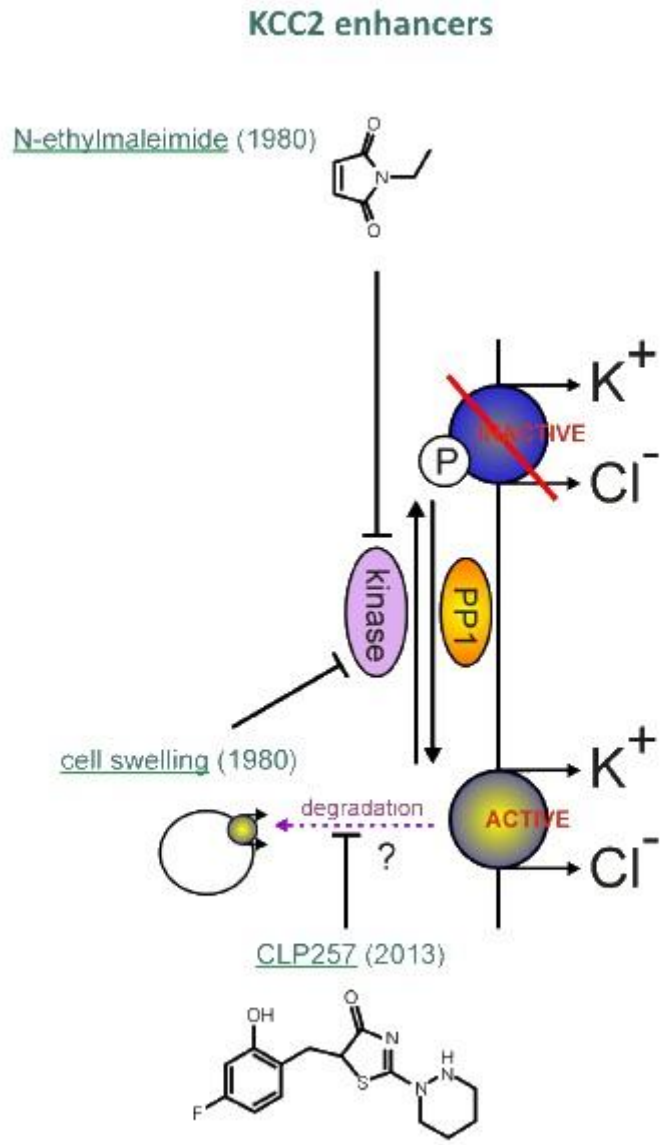

Figure 24. Enhancers of KCC2 function. Figure adapted from Delpire and Weaver (2016).

1.6.2. Serotoninergic pathways

In the healthy spinal cord, the excitability of motoneurons is set by brainstem-derived serotonin (Heckmann et al., 2005; Harvey et al., 2006). Over the last decade, studies from our group have focused on the contribution of serotoninergic projections to the maturation of spinal motoneurons and networks. The major conclusion of these studies and the leading thread of forthcoming experiments suggest that abnormalities in motor control may have serotoninergic depletion as a cause. Specifically, it appears from animals depleted from

serotonin, that a marked increase in the excitability of lumbar networks occurs (Pflieger et al., 2002), similar to that observed when spasticity emerges after SCI (Norreel et al., 2003). The lack of serotonin in the sublesional spinal cord is one of the mechanisms by which spasticity develops after SCI.

1.6.2.1. Modulation of inhibitory synaptic transmission and chloride ions homeostasis in the spinal cord by serotonin

There is an important degree of colocalization between serotoninergic and GABAergic fibers (Belin et al., 1983; Maxwell et al., 1983; Millhorn et al., 1988; Schmidt and Jordan, 2000). Also, a tight relation has been established among GABAergic inhibitory systems and the serotoninergic system (Dumoulin et al., 2000; Allain et al., 2010; Sadlaoud et al., 2010). Although the underlying mechanisms of this interaction are not completely elucidated, several observations point to a regulatory role of serotonin on the inhibitory system in the spinal cord: 1) the arrival of serotoninergic projections to the lumbar segments of the spinal cord delays the functional maturation of GABAergic neurons during the embryonic period (Allain et al., 2005) and the integrity of these projections is crucial for the depolarization-to-hyperpolarization switch (Jean-Xavier et al., 2006; Bos et al., 2013) and for the postnatal development of the GABAergic system (Sadlaoud et al., 2010); 2) The number of cells expressing mRNA of glutamate decarboxylase 67 kDa (GAD67) is strongly increased after spinal cord injury (Dumoulin et al., 2000; Tillakaratne et al., 2002) -which may have an excitatory effect (Boulenguez et al., 2010)- and transplantation of embryonic cells from raphe nucleus under the site of injury restablishes mRNA from GAD67 to the levels observed in naive animals (Dumoulin et al., 2000); 3) the activation of type 2 serotonin receptors increases the amplitude of GABAergic or glycinergic IPSPs at the dorsal horn of the spinal cord (Xu et al., 1996, 1998; Schmidt and Jordan, 2000) and reduces the monosynaptic reflex and slow ventral root potentials by activating GABA and glycinergic interneurons (Iwasaki et al., 2013), 4) activation of type 2 or type 7 serotonin receptor agonists at the spinal cord promotes the release of GABA measured by microdialisis and had an antinociceptive effect (Kawamata et al., 2002; Lin et al., 2015; Dupuis et al., 2017), and 5) a graft of serotoninergic cells after a chronic constriction injury recovered the numbers of GABA+ neurons, and this recovery of inhibition reduced neuropathic pain (Eaton et al., 1998), 5) serotonin can act as a neuromodulator of short-term depression in sensorimotor synapses; the application of serotonin on the whole spinal cord in vitro homogenized the short-term depression at different stimulation rates, while the blockade of GABAergic receptors decreased short-term depression, and converted depression to facilitation in 50% of the neurons tested (Barrière et al., 2008).

Although a close anatomo-functional relation has been found between GABAergic/glycinergic and serotoninergic systems, few studies to our knowledge have been interested in the influence of serotonin on the function of CCCs modulating chloride

homeostasis. Brustein and Drapeau (2005) proved that during the development of motor networks of the zebrafish, the exogenous application of serotonin reduces the refractory period between two spontaneous bursts, which causes an increase in excitability, and this effect was abolished by the addition of bumetanide, a blocker of NKCC1 and KCC2. According to Brustein and Drapeau (2005), serotonin would increase NKCC1 function and would take part on the maturation of the locomotor network on the zebrafish. This supports the idea of a modulation of chloride homeostasis by serotonin at early stages of development, as previously seen in fibroblasts (Mayer and Sanders-Bush, 1994). In this case, serotonin would promote an excitability, this is just due to the excitatory action of GABA during development. The conclusion of this paragraph is that serotonin can increase the function of CCCs during development.

1.6.2.2. Recovery of inhibition by recruiting 5-HT_{2A} receptors after SCI

Serotonin has proved to have a positive effect in motor recovery after a spinal cord injury (Ribotta et al., 2000; Gerasimenko et al., 2007; Ganzer et al., 2016). A previous study from our team proved that, beyond reducing spasticity, serotonin was capable of restoring the chloride homeostasis to recover the inhibitory action of GABA and glycine. Bos et al. (2013) proved that TCB-2 [(7R)-3-bromo-2, 5-dimethoxy-bicyclo[4.2.0]octa-1,3,5-trien-7-yl]methanamine], a highly specific 5-HT_{2A} receptor agonist, reduced spasticity on a rate-dependent depression test. The treatment with TCB-2 restored physiological E_{Cl} values, hyperpolarized IPSP, and restored KCC2 expression at the cell membrane. The increase on the expression of KCC2 was mediated by a calcium-independent protein kinase C (PKC), possibly PKC_{ε}, since an activator of this kinase showed a hyperpolarizing effect on the equilibrium potential of inhibitory postsynaptic potentials (E_{IPSP}) that was similar to the action of TCB-2. The main results from this work are represented in Figure 25.

5-HT_{2A} receptors are Gq protein-coupled receptors, therefore their activation initiates intracellular signaling pathways that activate PKCs. It has been proven that PKCs phosphorylate KCC2, mainly serine 940 (Ser940) (Lee et al., 2007). The phosphorylation of this residue increases the presence of KCC2 at the cell membrane and prevents internalization and degradation, probably by promoting a conformational change in the protein that increases its stability at the cell membrane. The increase in KCC2 expression and function after TCB-2 injection is probably mediated by this mechanism.

The study by Bos et al. (2013), to our knowledge, has been the only one until now, to link the activation of a particular receptor to the upregulation of KCC2 in motoneurons and the reduction of spasticity. A similar mechanism has not been described at the dorsal horn. Activation of serotonin receptors 2A at the spinal cord can facilitate inhibitory transmission in

the dorsal horn (Xie et al., 2012) and reduce neuropathic pain (Lopez-Alvarez et al., 2018). If these antinociceptive effects can be related to an increase of KCC2 expression at the membrane of dorsal horn neurons remains unknown.

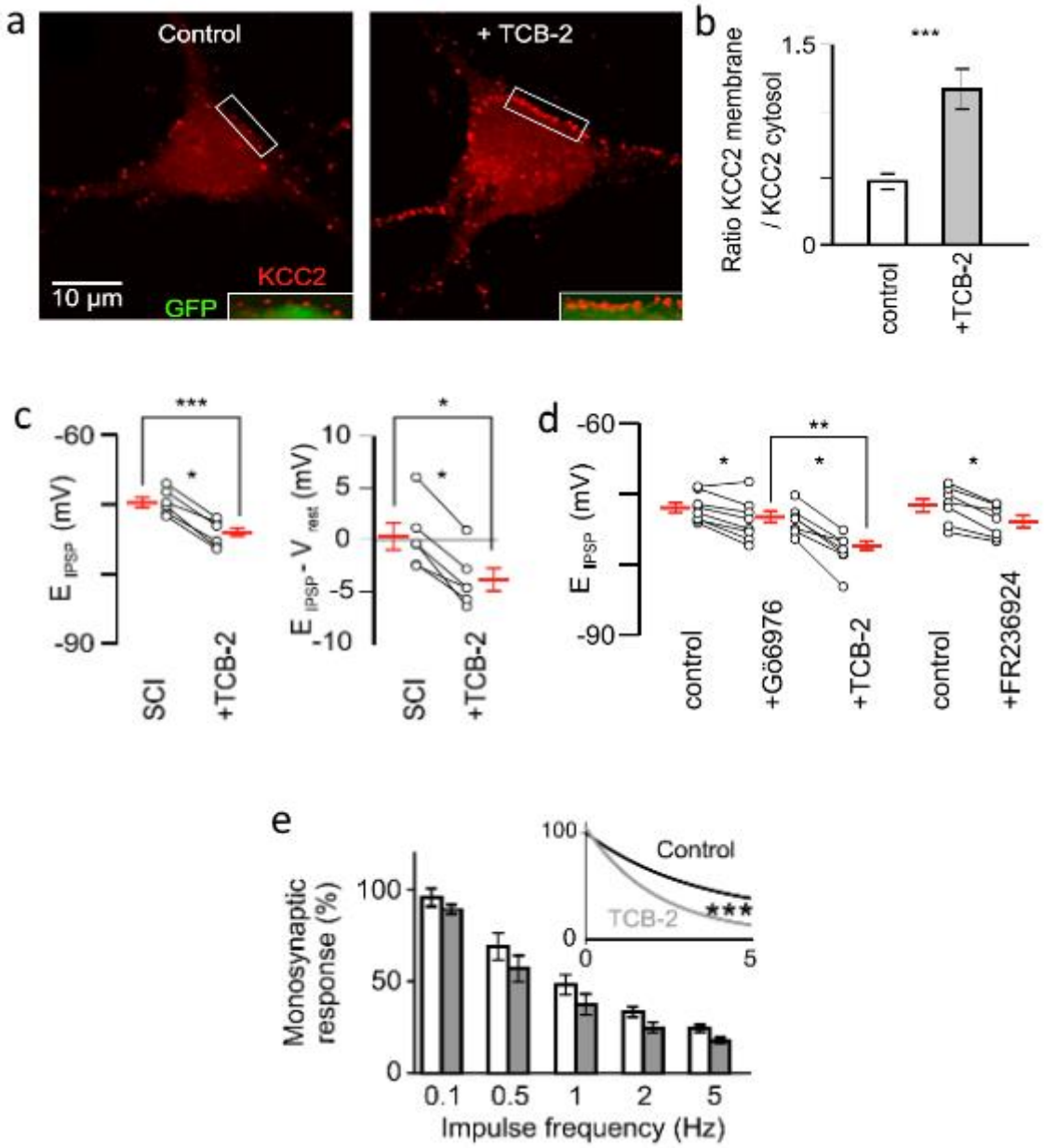

Figure 25. 5-HT$_{2A}$ receptor activation by TCB-2 increases KCC2 expression in the membrane of motoneurons and reduces spasticity after a spinal cord injury. a) KCC2 expression on motoneurons in culture, on control conditions and addition of TCB-2 on the medium. b) Quantification of KCC2 expression on cultured motoneurons. c) Intracellular recordings of E_{IPSP} on motoneurons. TCB-2 hyperpolarizes E_{IPSP} without modifying the resting potential of the motoneuron, which makes the driving force for IPSP more negative, meaning that chloride will enter the motoneurons faster after an IPSP. d) TCB-2 action on E_{IPSP} is not prevented by the addition of Gö6976, an inhibitor of calcium-dependent PKCs; however, TCB-2 action is simulated by the addition of FR236924, an activator of PKCε, a calcium-independent PKC. e) TCB-2 recovers rate-dependent depression of the H reflex on chronic spinal cord injury rats. Data from Bos et al. (2013).

1.6.3. Repositioning marketed drugs

Studying the mechanisms of regulation of KCC2 can give us valuable information to enhance KCC2 function. However, the translation of these discoveries into marketed drugs that can be given to human patients can take decades (Fugh-Berman, 2013).

The synthesis of new compounds that increase KCC2 function is a way to reach this objective. However, there is an important proportion of these compounds that do not pass the safety testing by the corresponding regulatory agency (the European Medicines Evaluation Agency –EMEA- is the organism that is responsible for safety testing in Europe). A review found that, from all drug applications submitted to the EMEA, 29% were rejected due to objections to the safety of the drugs or to their efficacy on human patients (Pignatti et al., 2002), moreover, 48% were approved after restricting their originally claimed indication. So only 23% of the applications were approved for the indications that the investors expected. This is a low percentage of success, considering that the submitted applications implicate drugs whose safety tests and clinical trials have already been conducted. All the drugs that do not pass safety tests in animals or that fail to complete clinical trials are not considered. Although some strategies have been proposed to assess the viability of this process (Wendler and Wehling, 2012), it is still impossible to predict the chances of a drug to be approved for its commercialization.

A faster and safer strategy to make a drug available for the treatment of one condition is the repositioning of drugs that are already marketed for other conditions (Ashburn and Thor, 2004). This means the drugs have already being considered safe for human use and therefore safety and clinical testing is simplified.

As a part of this thesis, we tested the efficacy of marketed drugs to increase KCC2 function on a screening. Our objective was to propose the repositioning of one or several of these drugs as KCC2 enhancers, as a treatment against spasticity and neuropathic pain caused by spinal cord injury.

1.6.3.1 Prochlorperazine

The results from the drug screening in culture cells we performed (Liabeuf et al., 2017, see Annex 1) proved that a marketed drug, prochlorperazine, is capable of increasing KCC2 expression and function.

Prochlorperazine is a phenothiazine with piperazine structure. This is a family of compounds that have antipsychotic and antiemetic properties. Beyond its effects on culture cells, prochlorperazine is also capable of hyperpolarizing chloride equilibrium potential (Figure 26a), and it recovers reciprocal inhibition and rate-dependent depression of the H reflex after a spinal cord injury (Figure 26b-c). The recovery of rate-dependent depression is correlated with a reduction of spasticity. Moreover, prochlorperazine increases KCC2 expression at the level of motoneurons after a spinal cord injury (Figure 26d-e). It is also capable of reducing spasticity.

The mechanism by which prochlorperazine modulates KCC2 function in motoneurons remains unclear (Liabeuf et al., 2017). The antipsychotic and antiemetic properties of prochlorperazine are considered to be related to its antagonist action on dopamine type 2 receptors from the limbic system and the chemoreceptor trigger zone (Smith et al., 2012). The mechanism by which prochlorperazine reduces spasticity, however, may not implicate its antidopaminergic action. After a spinal cord injury, the spinal cord is disconnected from its main source of dopamine, the A11 cell group at the diencephalon (Skagerberg et al., 1982; Sharples et al., 2014), so dopamine levels at spinal cord are already reduced. Furthermore, L-DOPA reduces spasticity by recovering rate-dependent depression of the H reflex in spinal cord injury rats (Liu et al., 2010). All these facts suggest that the reduction of spasticity by prochlorperazine is probably not mediated by its antidopaminergic action.

The increase in KCC2 expression is also unlikely to be mediated by the serotoninergic system. Prochlorperazine behaves as a partial agonist of 5-HT_{2C} receptors (Herrick-Davis et al., 2000). It is unlikely that this action can mediate its antispastic effects, since 5-HT_{2C} receptors in motoneurons become constitutively active after spinal cord injury, contributing to spasticity (Murray et al., 2010).

Phenotiazines inhibit the activity of protein kinase C (Aftab et al., 1991). The phosphorylation of KCC2 in serine residue 940 by PKC is a fast mechanism to regulate KCC2 expression and activity (Lee et al., 2007). A previously mentioned study from our team (Bos et al., 2013), proved that following spinal cord injury, a tonic activation of a calcium-independent PKC signaling pathway reduced KCC2 expression and depolarized E_{IPSP}. Therefore, the capacity of prochlorperazine to restore KCC2 function may be linked to its inhibitory action on calcium-independent PKCs.

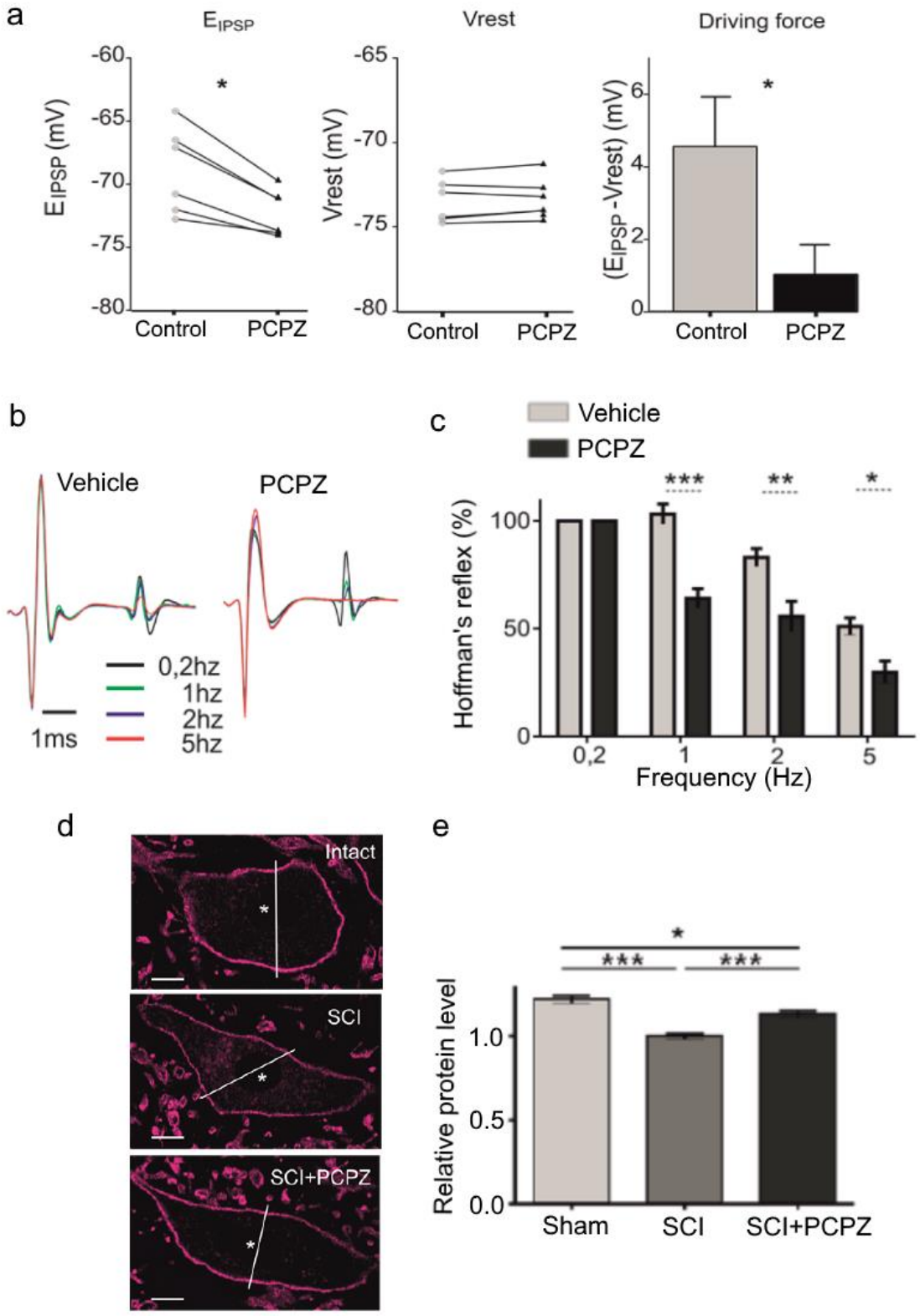

Figure 26. Prochlorperazine increases KCC2 expression and function on motoneurons after a spinal cord injury, and reduces spasticity. a) Intracellular recordings of inhibitory post-synaptic potentials (IPSPs) at different holding potentials in a motoneuron from a naïve P6 rat, before and after adding prochlorperazine to the bath. Prochlorperazine hyperpolarizes E_{IPSP}. b) Prochlorperazine recovers rate-dependent depression of the H reflex on chronic spinal cord injury rats. c) Mean amplitudes of the H reflex on spinal cord injury rats injected with vehicle or prochlorperazine. Prochlorperazine recovered

rate-dependent depression significantly. d) Immunohistochemistry of KCC2 proteins of motoneurons. e) Quantification of KCC2 protein on motoneurons. Figure adapted from Liabeuf et al. (2017).

The effects of prochlorperazine on spasticity were observed at a very low dose, compared with its daily dose for the treatment of psychosis [100 milligrams per kilogram for human oral administration, 620 milligrams per kilogram for rat intraperitoneal administration (Nair and Jacob, 2016)] which would allow to avoid the undesired secondary effects of prochlorperazine. In sum, we provided strong preclinical evidence for translation of prochlorperazine to treat spasticity in chronic spinal cord injury subjects. The translational process will be easy compared to other compounds, since prochlorperazine is a compound that has already been approved to treat human patients and has been recently patented for treating spasticity (WO 2015135947 A1).

The promising results of prochlorperazine for the treatment of spasticity through KCC2 upregulation led us to the question of whether it would also be able to reduce neuropathic pain through the same mechanism.

1.7. Objectives of the thesis

On this thesis, our goal is to produce enough molecular and electrophysiological data using our animal model (rat) to determine if any of our candidate drugs can reduce both spasticity and neuropathic pain, by reducing the decrease of KCC2 expression. Three specific aims will be developed:

Aim 1: To determine if TCB-2 is capable of activating KCC2 in the dorsal horn and of reducing neuropathic pain this way.

Aim 2: To identify new medications capable of increasing KCC2 after nervous system injuries, to treat spasticity and neuropathic pain.

Aim 3: To describe whether the downregulation of KCC2 after SCI can be related with the activation of calpains.

2. Materials and methods

The work of my thesis has been done on Wistar rats, newborn rats of both sexes (aged from P0 to P7, considering as P0 the 24 hours after birth) or adult female rats (250-300 grams).

All experimental procedures were conducted according to national regulations for animal experimentation, were approved by the local ethics committees (Comité d'Ethique en Neurosciences INT-Marseille (CEEA N°71), authorization Nb A9 01 13, and Ethics Committee of the Universitat Autònoma de Barcelona), and followed the guidelines of the European Commission (EU Directive 2010/63/EU).

I have performed all the surgeries described in this thesis, always under supervision and with help from Pascale Boulenguez, Renzo Mancuso and Philippe Marino (for the complete transection and hemisection models on adult rat) and from Cécile Brocard (complete transection of newborn rats at P0), in Marseille. I have also performed the spared nerve injury model on adult rat, after being trained by Xavier Navarro and Esther Udina, in Barcelona.

Concerning behavioural tests, I have done the algesimetry tests of adult hemisection and spared nerve injury rats, and also the BBB scores for hemisection rats. I learnt these techniques from Stefano Cobianchi and Pascale Boulenguez.

As for electrophysiology, I have done the dissections and extractions of spinal cord from newborn rats. I have done the *in vitro* recordings under supervision and with help from Laurent Vinay, Renzo Mancuso and, for most of the thesis, Frédéric Brocard. I have done the *in vivo* electromyogram recordings of adult rats to evaluate the H-reflex, or rate-dependent depression of this reflex, under supervision and with help of Philippe Marino, Esther Udina and Xavier Navarro.

The behavioural and electrophysiological data of this work are complemented by molecular and imagery techniques. I performed the immunohistochemistry analysis under supervision and with help from Cécile Brocard. Also, I performed the Western blot analysis of spinal cord samples under supervision and with help from Cécile Brocard and Sylvie Liabeuf in Marseille, and Israel Blasco and Mireia Herrando in Barcelona.

2.1 Animal models: surgeries

During the work of this thesis, we studied the motor syndromes leading to spasticity, and the sensory syndromes leading to neuropathic pain. We used three different animal models:

- To study spasticity: complete transection of the spinal cord, in female adult rats and neonate rats of both sexes
- To study neuropathic pain: hemisection of the spinal cord and spared nerve injury, in female adult rats

The animal model we used for studying spasticity was in all cases spinal cord transection. It may be argued that spinal cord contusion is a more relevant model, from a clinical point of view, but in any case, spinal cord transection reproduces the signs of a medullary section.

The animal model we used for studying neuropathic pain was spinal cord hemisection. It is neither an ideal model, from a clinical point of view, but it has been thoroughly described as a model for neuropathic pain of central origin (Christensen and Hulsebosch, 1997).

To complement the study of neuropathic pain, and to be able to avoid the interference of spasticity on our algesimetry tests, we also performed experiments on a peripheral nerve injury model, the spared nerve injury (Decosterd and Woolf, 2000).

For all surgeries in adult female rats (220–270 g), we anesthetized them with a mixture of ketamine (60 mg/kg, i.p.; Imalgene; Merial) and medetomidine (0.25 mg/kg, i.p.; Domitor; Pfizer). We also injected them with Amoxicillin (150 mg/kg, s.c.; Clamoxyl LA; Pfizer), a long-lasting antibiotic, immediately after anesthesia, and also 24 hours after the surgery.

We shaved the hair, and cleaned the skin with soap (Betadine), ethanol 70% and finally with iodine (Betadine).

After SCI, we injected 5 mL of NaCl i.p., and kept the rats warm until awakening (2h after surgery, by injection of atipamezol; 0,05ml Antisedan®). We administered Vetergesic (buprenorphin 0.05 mg/kg s-c) to the rats every 8 h for 24 h. Twice a day, we manually emptied their bladders until recovery of the urinary function, checked their temperature, and observed any clinical signs of pain or infection. We killed with a lethal dose of pentobarbital all rats presenting a more than 20% decrease in body weight, a temperature under 35 °C, or signs of autophagia.

We anesthetized neonates by hypothermia: we placed them 8-10 minutes on a -20ºC freezer, we verified that they showed no reflex responses after pinching the tail, and we put them over a cold block to perform the surgery.

2.1.1. Spinal cord transection on adult rats

Spinal cord transection was our model of choice for studying spasticity because this symptom is more frequently expressed after this type of injury than after hemisection (Kitzman, 2011) and it can be performed easily with usual surgical tools, unlike contusion injury (which needs an impactor device).

We performed spinal cord transections as previously described (Bos et al., 2013). Briefly, we anesthetized the rats as described above, we did an incision over the thoracic vertebrae T8-T13, cut the paravertebral muscles to clear vertebra T9, and performed a laminectomy of vertebra T9, removing its lamina completely. We separated the duramater from the spinal cord with fine forceps, and cut it to expose the spinal cord, then we transected the spinal cord at the thoracic level T8-T9. We dried the blood with a cotton until the spinal cord stopped bleeding. When the hemorrhage stopped, we closed the wound in layers and left the rats in their cages to recover. We injected them with a reversal agent 2 hours after the end of the surgery and also injected them the first dose of buprenorphine.

I have performed all spinal cord transections on adult rats with the help and guidance of Philippe Marino.

2.1.2. Spinal cord transection on newborn rats

Spinal cord transection in newborn rats is a model that, although it cannot be directly compared to the spinal cord transection on adult rats, it allows us to study how the changes in circuitry after the injury affect the excitability of reflexes.

Spinal cord transection in the newborn rats was performed as previously described (Norreel et al., 2003; Jean-Xavier et al., 2006; Sadlaoud et al., 2010). After anesthetizing the animals as previously described, we did a laminectomy at about T8 level (just under the scapulae), we transected the spinal cord with iridectomy scissors, and filled the lesion cavity with Surgicoll (Medical Biomaterial Products, Neustadt-Glewe, Germany). We sutured skin incisions using fine thread (PDSII 6.0, Ethicon; Johnson and Johnson, Brussels, Belgium), and let the pup recover normal temperature. When they were awaken, we cleaned any external remainings of blood, covered the scar with scotch and painted it with permanent marker to

hide the odor of the blood. We left pups together on litter with the odor of the mother for about 30 minutes after the surgery, to ensure that the mother recognized them when we bring them back to her.

Cécile Brocard taught me this surgery and helped me while I was learning how to perform it.

2.1.3. Spinal cord hemisection on adult rats

Spinal cord hemisection was our model of choice to study neuropathic pain of central origin because the interruption of unilateral pathways allows to keep the sensitivity to sensory stimulation, and the circuitry that remains or is remodelled, causes a bilateral development of chronic mechanical allodynia and thermal hyperalgesia (Christensen et al., 1996).

Spinal cord hemisection on adult rats was performed as described previously (Christensen et al., 1996): before the surgeries, we evaluated the step coordination of all rats through a BBB score (Basso et al., 1995). Also, we assessed their basal threshold for static mechanical and thermal stimulation was assessed through a Von Frey and a Ugo Basile Plantar Test, respectively (both tests are described in detail at the “Algesimetry” section).

After these tests, we performed a left hemisection of the spinal cord of the rats at the level of T9 segment under T8 vertebra: we did an incision of about 2 centimetres rostrally to T10, retired the fat, and cut the dorsal longus muscle at both sides of the spine. The muscles were separated with retractors, and the remainings of paravertebral muscles were separated from the vertebrae bodies of T7 and T8. We performed a laminectomy of T8 with the aid of a rongeur, cutting the vertebra while pulling the apophysis upwards with the aid of a forceps.

The duramater was picked and separated from the spinal cord with fine forceps, and it was cut with fine scissors to expose the spinal cord. The hemisection was performed with fine scissors (Fine Science Tools®). We introduced the open scissors between the left margin and the center of the spinal cord, with a small angle to the right, to compensate the movement of the spinal cord at introducing the scissors, and close them, sectioning the left part of the spinal cord at the level of T9 segment.

After one day, we evaluated the BBB score of the rats, to test if the lesion had been correctly performed. The rats that had lost mobility of both paws or had kept mobility of the injured paw were sacrificed.

I was taught how to perform this surgery by Pascale Boulenguez, and when operating, I was helped by her and Renzo Mancuso.

2.1.4. Spared nerve injury on adult rats

We chose the spared nerve injury as a model for peripheral nerve injury because it allows to keep the strongest, most robust level of pain (Casals-Díaz et al., 2009) for a long time (6 months, according to Decosterd and Woolf, 2000). The injury consists of ligating and sectioning the tibial and common peroneal branches of the sciatic nerve, while leaving intact the sural branch.

The neuropathic pain observed in this lesion is a mixture of different sources: the sectioned branches of the sciatic nerve are going to produce a neuroma -a tumour of nervous tissue that produces abnormal electrical signals and is painful-, whereas the intact branch that still innervates the paw, is going to produce collateral innervations of the adjacent skin territories that are denervated (Decosterd and Woolf, 2000). This pain is entirely of peripheral origin, and it is different from the neuropathic pain that is caused by nerve regeneration, which characterizes the models of chronic constriction injury or cut-and-suture. The spared nerve injury is also easy to perform, and highly reproducible.

We performed the spared nerve injury in the adult rats as previously described (Decosterd and Woolf, 2000). We did an incision on the right paw, directly through the biceps femoris muscle, exposing the sciatic nerve and its three terminal branches: the sural, common peroneal and tibial nerves. We ligated tibial and common peroneal nerves with 7-0 silk sutures and sectioned them, leaving the sural nerve intact. The section of the common peroneal and the tibial nerves was distal to the ligation, removing 2-4 mm of the distal nerve stump. Afterwards, we closed skin in layers and left animals in their cages to recover.

To perform these surgeries I was taught and assisted by Esther Udina and Xavier Navarro.

2.2. Behaviour

2.2.1. BBB score

We evaluated the motor function of the rats by means of a Basso, Bettie and Bresnahan (BBB) score (Basso et al., 1995). We left the rats in an open field, and we observed them walking around for a maximum of 4 minutes. We evaluated the BBB score of the rats on the day of the surgery (day 0), and on several days postinjury.

2.2.2. Algesimetry tests

During the week before surgery, we introduced all the rats into the devices for nociceptive threshold measurement. At each testing day, all the procedures were performed during the morning (9 a.m. – 3 p.m.). We evaluated the nociceptive threshold responses for static mechanical and thermal stimuli on both hindpaws through algesimetry tests before the surgery, and at several different days postinjury.

For each test, we tested mechanical allodynia of hemisection rats in the middle of the paw, and on spared nerve injury rats, on the lateral of the paw.

I learnt how to do algesimetry tests from Stefano Cobianchi and Pascale Boulenguez.

2.2.2.1. Von Frey test (analogic)

We used the Von Frey test to evaluate the pain response to static mechanical stimulation. We obtained the 50% paw withdrawal threshold according to the up-down method described by Chaplan et al. (1994). We placed the rats on a plastic box with a wire net floor for at least 5 minutes of habituation before testing. After this period, we stimulated the medial plant of the hindpaws in between the pads –on rats with a hemisection-, or the lateral third of the paw –on rats with a spared nerve injury-. We used Von Frey filaments (BIOSEB, Chaville, France) for stimulation, following the up-down method, departing from the 8g filament. We always tested the left paw before the right paw. We calculated the mechanical nociceptive threshold out of each of these measurements, as described by the article cited above.

2.2.2.2. Von Frey test (electronic)

We also assessed mechanical allodynia with an electronic Von Frey algesimeter (Bioseb, Chaville, France), following the protocol described by Casals-Diaz et al. (2009): we placed the rats on a plastic box over a wire mesh platform for habituation, and tested mechanical allodynia by applying to each hindpaw a 0.8 mm diameter metallic filament connected to a force sensor, increasing pressure gradually, until the rat withdrew its paw. We recorded paw withdrawal pressure three times, with 15 min interval between stimuli, for both hindpaws, at each testing day, and we used the mean of the three values to calculate the threshold value. We stimulated the rats in the same point of the hindpaws described in the last paragraph.

2.2.2.3. Hargreaves' test

We used a plantar algesimeter (Ugo Basile, Comerio, Italy) to evaluate the pain response to infrared heat stimulation. We placed the rats in the boxes at least 5 minutes before the beginning of the testing for habituation. We stimulated the rats in the same point of the hindpaws described in the last paragraphs with a hot blast from the algesimeter lamp. We set a cutoff of 20 s to avoid skin damage.

We obtained 3 values for each paw at each test, leaving at least 1 minute between one measure and the next one. We calculated the thermal nociceptive threshold as the mean of the three values obtained for each paw. In the cases where these values were too different, we took a fourth value, to improve the statistical power of this mean.

2.3. Electrophysiological tests

2.3.1. *In vitro* electrophysiology

2.3.1.1. Spinal cord preparations

We prepared spinal cords as described before (Gackière and Vinay, 2015). Briefly: we dissected spinal cords, together with spinal roots, from neonatal animals. After decapitation and evisceration, we exposed spinal cord and removed it from the duramater. The spinal cord, from spinal segments to the lesion site (T8 segment) was then removed from the vertebral column together with peripheral roots. We were especially careful to keep those from the lumbar L3 to L5 segments, which contain the motoneurons projecting to the hindlimb muscles (Nicolopoulos-Stournaras and Iles, 1983). We transferred the preparation to the recording chamber, and pinned it down in sylgard (Dow-Corning) with the ventral side up. We pinned L5 ventral and dorsal roots and placed the stimulating and recording electrodes. We pinned the roots from nearby segments (L3-L5) to stabilize the spinal cord, and cut the rest of the roots. We performed all these procedures in aCSF with a high concentration of potassium (130mM NaCl, 4mM KCl, 3.75mM $CaCl_2$, 1.3mM $MgSO_4$, 0.58mM NaH_2PO_4, 25mM $NaHCO_3$, and 10mM glucose; all compounds were from Sigma-Aldrich; oxygenated with 95% O_2/5% CO_2, pH 7.4).

2.3.1.2. *In vitro* extracellular recordings

Extracellular recordings in vitro were obtained as described elsewhere (Gackière and Vinay, 2015). Briefly, after placing the stimulating and recording electrodes near the roots of interest, we insulated them from the bath with Vaseline, and let the preparation set during 30-60 minutes before starting to record. We acquired data through an AC-coupled amplifier (bandwidth: 70–73 kHz) and a Digidata 1440A interface using the Clampex 10.2 software (Molecular Devices)."

2.3.2. *In vivo* electrophysiology: electromyographical recording of rate-dependent depression

We obtained rate-dependent depression (RDD) of the H reflex as previously described (Bos et al., 2013). Briefly: we anesthetized the rats under ketamine anesthesia (100 mg/kg i.p.) and we attached them to a metal plate placed over a heating pad. We stimulate the tibial nerve using a pair of stainless steel needle electrodes transcutaneously inserted in the vicinity of this nerve. We placed the recording electrode into the flexor digitorum muscle beneath the ankle and the reference electrode at the tip of the fourth toe of the same foot. We placed the mass electrode in the tail.

We did one stimulation to verify that electrodes were correctly placed and that we obtained an H reflex. We performed an I-V curve to determine the intensity of stimulation that elicited the highest amplitude H reflex. We stimulated at increasing stimulation rates (0.2 Hz, 1 Hz, 2 Hz, 5 Hz) to evaluate the depression of the H reflex at different stimulation rates.

Philippe Marino, Esther Udina and Xavier Navarro helped and supervised me during the electromyographical experiments.

2.4. Immunohistochemistry

Immunohistochemistry staining was conducted as previously described (Sánchez-Brualla et al., 2018). For most experiments, the protocol followed was: we perfused euthanized rats transcardially with 4% paraformaldehyde (PFA) in PBS at 4°C, ~60 min after TCB-2 or vehicle injection, ~2 h after MDL28170 or vehicle injection. We dissected out the lumbar spinal cord and post-fixed it for 8 h in 4% paraformaldehyde. We cut L5 lumbar segments of the spinal cord in 30 µm-thick sections using a cryostate. We permeated sections for 45 min (0.2% Triton X-100 in PBS-BSA 3%) and preincubated them for 30 min at 22°C in PBS containing 3% BSA and normal goat serum diluted 1/100. We incubated sections overnight at 4°C with an affinity-purified rabbit KCC2-specific polyclonal antibody (1/400, Millipore). We revealed labeling with a goat Alexa Fluor 546-conjugated rabbit-specific antibody (1/500, 1h at 22°C, Molecular Probes). We mounted coverslips in a gelatinous aqueous medium.

The details of the protocol followed at each study are mentioned on the *Methods* section of each article of the thesis.

Cécile Brocard and Esther Udina taught me, helped and supervised me during the immunostaining experiments.

2.5. Western blot

Tissue extraction and homogeneization and Western blot were performed as previously described (Brocard et al., 2016). We collected tissues from spinal cord lumbar enlargements and froze them after removing the dorsal and ventral roots. We homogenized samples in ice-cold lysis buffer (320 mM sucrose, 5 mM Tris-HCL pH 7.5, 10 µM iodoacetamide) supplemented with protease inhibitors (CompleteMini, Roche diagnostic, Basel, Switzerland). We centrifuged unsolubilized material at 14,000*g* for 70 min at 4 °C. We collected pellets and homogenized them in ice-cold lysis buffer (1% Igepal CA-630, PBS 1X, 0.1% SDS, 10 µM iodoacetamide), supplemented with protease inhibitors (CompleteMini, Roche diagnostic). We determined protein concentrations using a detergent-compatible protein assay (Bio-Rad). We loaded equal protein amounts (30 µg) from samples and separated them by size using a 6% (vol/vol) SDS/PAGE from 40% Acryl/Bisacrylamide (29/1) commercial solution. Then we transferred them to a nitrocellulose membrane and probed with an affinity-purified rabbit KCC2-specific polyclonal antibody (1/400, Millipore) at 4 °C overnight in Tris-buffered saline containing 5% fat-free milk powder and 0.05% Tween 20. We then incubated the blot for 1 h at 22 °C with an ImmunoPure goat HRP–conjugated rabbit-specific antibody (1:40,000 in blocking solution; Thermo Scientific). We blotted the proteins with the substrate HRP

immobilon Western (Merck-Millipore), and measured signal intensities with the image-analysis software Quantity-One (Bio-Rad).

The details of the protocol followed at each study are mentioned on the *Methods* section of each article of the thesis.

Cécile Brocard, Sylvie Liabeuf, Israel Blasco and Mireia Herrando taught, helped and supervised me during the Western blot experiments.

3. Results

3.1. Article I : Activation of 5-HT_{2A} receptors restores KCC2 function and reduces neuropathic pain after spinal cord injury

Activation of 5-HT_{2A} receptors restores KCC2 function and reduces neuropathic pain after spinal cord injury

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ABBREVIATIONS

- **DIOA:** [(dihydroindenyl)oxy] alkanoic acid
- **DRG:** dorsal root ganglia
- **KCC2:** potassium chloride cotransporter type 2
- **SCI :** Spinal cord injury
- **SNI :** Spared nerve injury
- **TCB-2:** (4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide

ABSTRACT

Downregulation of the potassium chloride cotransporter type 2 (KCC2) after a spinal cord injury (SCI) disinhibits motoneurons and dorsal horn interneurons causing spasticity and neuropathic pain, respectively. We showed recently (Bos et al., 2013) that specific activation of 5-HT_{2A} receptors by TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide] upregulates KCC2 function, restores motoneuronal inhibition and reduces SCI-induced spasticity. Here, we tested the potential analgesic effect of TCB-2 on central (thoracic hemisection) and peripheral [spared nerve injury (SNI)] neuropathic pain. We found mechanical and thermal hyperalgesia reduced by an acute administration of TCB-2 in rats with SCI. This analgesic effect was associated with an increase in dorsal horn membrane KCC2 signal and was prevented by pharmacological blockade of KCC2 with an intrathecal injection of DIOA [(dihydroindenyl)oxy]alkanoic acid]. In contrast, the SNI-induced neuropathic pain was not attenuated by TCB-2 although there was a slight increase of membrane KCC2 signal in the dorsal horn ipsilateral to the lesion. Up-regulation of KCC2 function by targeting 5-HT_{2A} receptors, therefore, has therapeutic potential in the treatment of neuropathic pain induced by SCI but not by SNI.

Keywords : Spinal cord injury, spared nerve injury, KCC2, neuropathic pain, 5-HT_{2A}, TCB-2.

INTRODUCTION

Spinal cord injuries (SCI) cause permanent changes in sensory functions below the lesion. The development of persistent neuropathic pain is a common side-effect following SCI which compromises patient's quality of life and impedes effective rehabilitation (Siddall and Loeser, 2001). Chronic neuropathic pain in patients with a peripheral nerve injury is also considered as the most important adverse outcome (Davis and Curtin, 2016). Our ability to relieve post-injury neuropathic pain is still limited because it is generally refractory or responds poorly to available therapies (Baastrup and Finnerup, 2008). New pharmacotherapeutic strategies are thus required to fulfill these unmet medical needs.

The neuropathic pain is often associated with a defective GABAaergic function (Gwak and Hulsebosch, 2011; Guo and Hu, 2014). Remarkably, the effectiveness of the inhibitory neurotransmitter to induce a chloride (Cl^-)-mediated hyperpolarizing current is reduced in dorsal horn neurons (Prescott, 2015). The Cl^- extruder KCC2 (potassium chloride cotransporter type 2), expressed in the spinal dorsal horn (Kanaka et al., 2001; Nomura et al., 2006), maintains low concentration of intracellular Cl^- to facilitate influx of Cl^- when $GABA_A$ receptor is activated (Boulenguez et al., 2010; Ben-Ari et al., 2012; Doyon et al., 2016). In neuropathic pain, impairment of KCC2 function causes a Cl^- gradient collapse, renders the inhibitory action of $GABA_A$ and glycine less effective and drives up the net excitability of dorsal horn neurons to induce nociception (Coull et al., 2003, 2005; Price et al., 2005; Prescott et al., 2006; Cramer et al., 2008; Lu et al., 2008; Zhou et al., 2012; Modol et al., 2014). Therefore, increasing the functionality of KCC2 to restore inhibition appears as an innovative therapeutic strategy (Gagnon et al., 2013).

Serotonin has long been linked with both central and peripheral modulation of the nociceptive signal (Viguier et al., 2013). We recently showed that the specific activation of 5-HT_{2A} receptors with TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide] corrects alterations of Cl^- homeostasis in motoneurons of rats with SCI and reduces spasticity (Bos et al., 2013). The present study aims at testing whether TCB-2 alleviates neuropathic pain in adult rats with SCI or peripheral nerve injury. We found that TCB-2 reduced mechanical allodynia and thermal hyperalgesia after SCI but not after spared nerve injury (SNI). We also provide evidence that the analgesic effect of TCB-2 is related to an increase in KCC2 function.

EXPERIMENTAL PROCEDURES

Animals

Adult female Wistar rats (230 ± 30 g) were procured from Charles River Laboratories and housed under a 12h light-dark cycle in a temperature-controlled animal care facility with *ad libitum* access to water and food. The animals were divided in 2 experimental groups (n=32 for SCI and n=16 for SNI). All experimental procedures were conducted according to national regulations for animal experimentation, were approved by the local ethics committees (Comité d'Ethique en Neurosciences INT-Marseille (CEEA N°71), authorization Nb A9 01 13, and Ethics Committee of the Universitat Autònoma de Barcelona), and followed the guidelines of the European Commission (EU Directive 2010/63/EU).

Surgical procedures

Spinal cord injury (SCI) model: Rats were anesthetized with ketamine (Imalgen, Merial, 50 mg/kg i.p.) and medetomidine (Domitor, Janssen, 0.25 mg/kg i.p.). Surgery and postoperative care were conducted as described elsewhere (Brocard et al., 2016), with the difference that the injury was a left hemisection at the thoracic T8 level and not a complete transection. In some rats, a polyethylene 5 catheter (o.d. 0.36 mm, i.d. 0.2 mm; PTFE Sub-Lite Wall, Bioseb) was intrathecally inserted. The catheter was passed through a slit cut in the spinal arachnoid of the T6–7 region, and pushed gently downward to reach the lumbar L3 level. The distal part of the tubing was firmly anchored to the T7 vertebral epiphysis tunneled under the skin and externalized at the back of the neck. After surgery, rats with a right hindlimb paralysis were discarded.

Spared nerve injury (SNI) model: Rats were anesthetized as described above. Surgery was performed on the right hindlimb as described elsewhere (Decosterd and Woolf, 2000). Briefly, the common peroneal and tibial nerves were exposed, ligated and transected below the ligations, with special care taken to avoid any damage to the sural nerve. The lesion was evaluated by pinching the 3rd and 5th toes of the injured paw, to verify that rats lost withdrawal reflex on the 3rd but not on the 5th toe.

Behavioral assessments

The rats were adapted to the testing situation for at least 15 min before stimulation was initiated. Locomotor, mechanical and heat sensitivity testing was performed prior to surgery to obtain baseline values, and then on post-operative days 7, 14 and 21 days for the SCI group and days 3, 7 and 14 for the SNI group.

BBB scoring. Hindlimb motor function was assessed based on the Basso, Beattie and Bresnahan (BBB) Locomotor Rating Scale that ranks hindlimb locomotion after SCI from complete paralysis (0) to normal and coordinated movement (Basso et al., 1995). For BBB assessment, the rats were allowed to move individually for five minutes in an open field (200 x 100 cm). Hindlimb motor function was scored based on the performance of the hindlimb ipsilateral to the spinal hemisection.

Von Frey filaments. Each rat was placed in a testing box with a wire-mesh grid floor. A series of von Frey microfilaments (starting from 8 g) were applied through the grid floor to the ventral surface of the hindpaw of the injured hindlimb of each rat. Filaments were pressed until the filament bent and held for a 3 second period or until the animal withdrew the hindpaw without ambulating. During each testing trial, the series of filaments were presented following an up-down procedure, described and validated by Chaplan et al. (1994), and the 50% response threshold was calculated for each rat.

Hargreaves's test. Thermal nociceptive threshold to radiant heat was quantified using the paw withdrawal test as described previously (Hargreaves et al., 1988). Briefly, rats were placed in a Plexiglas cubicle without restraint. After acclimation, an infrared radiant heat source was focused on the mid-plantar surface of the hindpaw (Ugo Basile model 7371, Bioseb). A cutoff stimulation time of 20 s was used in the event of no response to avoid skin damage. The paw withdrawal latency to radiant heat was defined as the time from onset of the radiant heat to the withdrawal of the rat hindpaw. The average of three estimations was taken to yield a mean paw withdrawal latency.

Drugs and experimental design

We purchased TCB-2 [(4-bromo-3,6-dimethoxybenzocyclobuten-1-yl) methylamine hydrobromide] from Tocris and DIOA [(dihydroindenyl)oxy]alkanoic acid] from Sigma Aldrich. TCB-2 was diluted in distilled water while DIOA was diluted in 3/4 NaCl 0.9% + 1/4 sodic

phosphate buffer 300 mosm, pH 7.4 and DMSO (0.1% final concentration). Three main types of experimental treatments were performed. *Treatment 1*: A single i.p. injection of TCB-2 (0.3 mg/kg) or distilled water was given on post-operative day 21 and 14 when signs of neuropathic pain were firmly established in rats with SCI and SNI, respectively. Mechanical threshold and thermal latency were then evaluated at intervals of 5, 35, 65 and 95 min and 20, 50, 80 and 110 min minutes post-TCB-2, respectively. *Treatment 2* (only conducted on SCI rats): The single i.p. injection of TCB-2 (0.3 mg/kg) and pain behavioral tests were preceded, 20 min earlier, by an intrathecal injection of DIOA (20 µg diluted in 15 µl of its vehicle + 6 µl flush with NaCl 0.9% injected over 3 min) or vehicle (3/4 NaCl 0.9% +1/4 sodium phosphate buffer 300 mosm, pH 7.4 and DMSO -0.1% final concentration-). The intrathecal DIOA injection 20 minutes before a systemic TCB-2 treatment was performed on the basis of literature (Coull et al., 2003). *Treatment 3* (only conducted on SNI rats): TCB-2 (0.3 mg/kg) or distilled water were administered i.p. daily for 7 days, starting 2 hours after the injury. Mechanical threshold was measured on postoperative days 3 and 7 immediately prior to the TCB-2 injection as well as at 1, 2 and 3 hours post-injection. The experimenter administered treatments in a blind manner.

Immunostaining of KCC2

Samples for immunohistochemical analysis were collected from animals euthanized after the last functional test [i.e. ~60 min after TCB-2 (n=6 rats; 3 with SCI, 3 with SNI) or distilled water (n=6 rats; 3 with SCI, 3 with SNI) injection]. Rats were perfused transcardially with 4% paraformaldehyde in PBS. The lumbar spinal cord was then dissected out and post-fixed for 15 h in 4% paraformaldehyde. L5 lumbar segments were cut using a vibratome or cryostat in 30 µm-thick sections. They were permeated for 45 min (0.2% Triton X-100 in PBS-BSA 3%) and preincubated for 30 min at 22°C in PBS containing 3% BSA and normal goat serum diluted 1/100. Sections were then incubated overnight at 4°C with an affinity-purified rabbit KCC2-specific polyclonal antibody (1/400, Millipore). Labeling was revealed with a goat Alexa Fluor 546-conjugated rabbit-specific antibody (1/500, 1h at 22°C, Molecular Probes). Coverslips were mounted in a gelatinous aqueous medium. All neurons of the dorsal horn (lamina I and II) were scanned using a confocal microscope (Zeiss LSM510 META) at x40 or x60 magnification

(stacks of 0.4µm-thick optical sections). Acquisition and analysis were performed blind for the different treatments.

Spinal cord samples of rats treated with TCB-2 or its vehicle were on the same slide. Confocal microscopy Z-stacks were acquired using the same parameters for all samples; all pixels from all samples were below saturated fluorescence intensity. We selected only the most superficial neurons from lamina I and II. Fluorescence signal was quantified using the Zen Software. We drew a region of interest within the plasma membrane of ipsilateral and contralateral neurons of dorsal horns and measured the mean pixel intensities from the optical section with the maximal fluorescence intensity.

For SCI rats: for each slide, mean values of fluorescence determined on the ipsilesional or the contralesional in TCB-2-treated rats were normalized by respective values measured in vehicle-treated rats.

For SNI rats: on each section, we considered the contralesional side of the spinal cord as representing normal (100%) KCC2 expression; each neuron (ipsilesional or contralesional) was normalized by the mean value of contralesional side.

Statistics

We used Student t-test and one- or two-way ANOVA with or without repeated measures as appropriate (GraphPad Software, San Diego California USA). For all statistical analyses, the data met the assumptions of the test and the variance between the statistically compared groups was similar. The significance was set at $P < 0.05$.

RESULTS

Partial motor recovery of the ipsilesional hindlimb after spinal hemisection

Motor deficits and recovery of the ipsilesional hindlimb were assessed using the BBB scale (Fig. 1A). The left spinal hemisection caused immediate paralysis of the ipsilesional hindlimb with milder effects on the contralesional hindlimb. One day after SCI, the rats dragged the left hindlimb exhibiting a BBB score of 0. At post-operative day 7, animals regained a partial left hindlimb body weight support and achieved a BBB score of 9. From the 1st till the 3rd week rats displayed a significant increase in movements of the left hindlimb and BBB scores gradually increased to 12 but remained significantly lower than preoperative values ($P < 0.01$; **Fig. 1a**). By 21 days post-SCI, rats displayed frequent-to-consistent weight-support steps and exhibited continuous plantar stepping with limited forelimb–hindlimb coordination, reaching a BBB score of 13.

Development of bilateral neuropathic pain after spinal cord hemisection

Following hemisection, rats displayed bilateral mechanical allodynia represented by a significant decrease in withdrawal threshold to Von Frey filament stimuli 7 days after the lesion in both hindlimbs ($P < 0.001$ for ipsilesional, $P < 0.01$ for contralesional; **Fig. 1b**). Bilateral thermal allodynia also developed as demonstrated by a large decrease in paw withdrawal latencies in response to thermal stimuli ($P < 0.001$, **Fig. 1c**). Afterwards, mechanical and thermal allodynia remained stable. The *pain* sensitivity to *mechanical* and *thermal* stimulation developed simultaneously in both hindlimbs, such as no differences were observed when right and left withdrawal responses were compared at each time point postoperatively ($P > 0.05$, **Fig. 1b,c**). For the next series of experiments we therefore pooled right and left paw measurements at a time (21 days post-SCI) when pharmacological intervention could be reliably tested.

TCB-2 alleviates mechanical and thermal allodynia after spinal cord hemisection

The analgesic action of 5-HT_{2A} receptors was investigated with a dose of TCB-2 (0.3 mg/kg, i.p.) previously used to alleviate spasticity in rats with SCI (Bos et al., 2013). After injection of TCB-2, paw withdrawal thresholds significantly increased in response to mechanical ($P < 0.001$, **Fig. 2a**) and thermal ($P < 0.01$, **Fig. 2b**) stimuli whereas saline injection

as a control had no effects throughout the test period ($P > 0.05$; **Fig. 2a,b**). TCB-2 reversed mechanical thresholds at the level or beyond preoperative values while thermal latencies remained below. The antinociceptive effect against mechanical stimuli was fast, lasted over a period from 5 to 65 min and peaked 35 min after the injection ($P < 0.001$, **Fig. 2a**). The behavioral sensitivity to thermal heat regained more slowly, 50 min post-injection ($P < 0.01$, **Fig. 2b**) and then slightly decreased. Altogether, it appears that the TCB-2 induced analgesia with a more pronounced effect against mechanical than thermal nociception in rats with a spinal cord hemisection.

TCB-2 alleviates SCI-induced allodynia via KCC2

The involvement of KCC2 was then investigated using an i.t. administration of DIOA, a selective KCC2 blocker (Delpire et al., 2009), injected 20 min prior the TCB-2 i.p. injection. DIOA injection prevented the TCB-2 induced antinociception against mechanical ($P > 0.05$, **Fig. 2c**) and thermal ($P > 0.05$, **Fig. 2d**) stimuli. As a control, the i.t. injection of the vehicle did not prevent the analgesic effect of TCB-2 on mechanical ($P < 0.01$, **Fig. 2c**) and thermal ($P < 0.05$, **Fig. 2d**) neuropathic pain. This indicates that TCB-2 alleviates pain mainly through a modulation of KCC2. A previous report indicated that the expression of KCC2 in dorsal horn of the lumbar enlargement was bilaterally halved in rats after hemisection (Lu et al., 2008). Spinal dorsal horn tissues were collected from rats receiving TCB-2 or vehicle treatment at peak analgesia (1 h after administration) and assayed for KCC2 protein expression in the dorsal horn. With TCB-2, KCC2 immunostaining was increased, so that a significant difference was observed compared with vehicle-treated rats (**Fig. 3**). In TCB-2-treated rats, KCC2 labeling in the membrane of lamina I and II neurons was increased by 50% in the ipsilateral side to the hemisection ($P < 0.0001$, **Fig. 3e**) and by 20% in the contralateral side ($P < 0.01$; **Fig. 3f**) compared to vehicle-treated animals.

TCB-2 does not alleviate neuropathic pain induced by SNI

To test if TCB-2 effect on neuropathic pain was not restricted to a particular type of lesion, we used the SNI, a well-standardized model of peripheral neuropathic pain. Mechanical (**Fig. 4a**) and thermal (**Fig. 4b**) pain thresholds were significantly reduced in the injured hindpaw as soon as 7 days after the lesion ($P < 0.001$) and persisted at 14 days after the injury

($P < 0.001$). No change was observed for the contralateral paw ($P > 0.05$, **Fig. 4a,b**). Therefore, compared to the contralateral side, mechanical and thermal pain thresholds were significantly lower ($P < 0.001$).

The acute administration of TCB-2 14 days after SNI did not reduce neither mechanical ($P > 0.05$, **Fig. 5a**) nor thermal allodynia ($P > 0.05$, **Fig. 5b**). Similarly, TCB-2 did not change neither thresholds nor latencies of withdrawal responses in the contralateral intact hindlimb ($P > 0.05$, **Fig. 5c,d**). Similar results were obtained when TCB-2 was daily injected as early as the day of injury for 7 days (data not shown). These results indicate that TCB-2 injection did not prevent the development of neuropathic pain after SNI.

TCB-2 restores membrane KCC2 signal in the ipsilateral dorsal horn after SNI

In vehicle-treated animals we found that KCC2 immunostaining in the membrane of neurons in the ipsilateral dorsal horn was lower ($P < 0.05$, **Fig.6e**) compared to the contralateral side in response to SNI. TCB-2 administration (0.3 mg/kg; 60 min after i.p.) restored KCC2 membrane signal in the ipsilateral dorsal horn at the level of the contralateral side ($P > 0.05$, **Fig.6e**).

DISCUSSION

We provide evidence that the activation of 5-HT_{2A} receptors by TCB-2 i) restores the cell surface KCC2 signal on dorsal horn neurons that is substantially downregulated after spinal hemisection or SNI, and ii) alleviates pain induced by a spinal hemisection, but not by SNI. The beneficial effect of TCB-2 on SCI-induced pain is likely mediated by the posttranslational modification of KCC2 as DIOA, a blocker of KCC2, abrogates the analgesic action of TCB-2. In summary, restoring KCC2 membrane expression by TCB-2 has a therapeutic potential to reduce pain developed after SCI, but it may not be enough to treat neuropathic pain after SNI.

As previously observed in behavioral studies, a spinal cord hemisection produces consistent bilateral pain-related behavior in both hindlimbs (Christensen et al., 1996; Christensen and Hulsebosch, 1997), while SNI produces a marked hypersensitivity only in the hindpaw ipsilateral to the nerve injury (Decosterd and Woolf, 2000; Rode et al., 2005; Casals-Díaz et al., 2009). These pain-related behaviors coincided with the impaired expression of KCC2 in the dorsal horns, bilaterally downregulated in hemisection animals (Lu et al., 2008) and unilaterally decreased in animals with peripheral nerve injury (Coull et al., 2003, 2005; Miletic and Miletic, 2008; Janssen et al., 2011; Zhou et al., 2012; Wei et al., 2013; Modol et al., 2014). The subsequent depolarizing shift of the Cl^- equilibrium potential caused by a decrease of KCC2 expression in the cell surface of dorsal horn neurons has been shown to attenuate inhibitory action of GABA and glycine (Coull et al., 2003, 2005; Price et al., 2005; Lu et al., 2008; Zhou et al., 2012). Therefore, impaired KCC2 activity appears to be a substrate for central sensitization of the spinal cord and consecutive neuropathic pain. In support of this, inhibition of KCC2 in intact animals led to a marked reduction in nociceptive thresholds (Coull et al., 2003; De Koninck, 2007; Austin and Delpire, 2011) while KCC2 overexpression reverses neuropathic pain in nerve-injured animals (Gagnon et al., 2013; Modol et al., 2014; Li et al., 2016).

In the present study, the acute administration of TCB-2 increased the dorsal horn membrane signal of KCC2 in rats with SCI or SNI. Interestingly, similar results have been recently reported in a rat incision pain model (Dong et al., 2016). This is in agreement with our previous findings showing that TCB-2 increases KCC2 immunolabeling on membranes of motoneurons after SCI (Bos et al., 2013). Although it may seem surprising, the fast increase on KCC2 membrane signal –which is significant as soon as 1h after TCB-2 injection- is in line

with previous reports that KCC2 expression in the membrane can be upregulated within 60 minutes (Roussa et al., 2016; Pressey et al., 2017). Even if we did not evaluate the functional consequences on chloride homeostasis, we assume that KCC2 upregulation with TCB-2 restores the Cl^- extrusion capacity in dorsal horn neurons and thereby renders the inhibitory synaptic transmission more effective to alleviate hypersensitivity. In line with this hypothesis, blockade of KCC2 by DIOA strongly reduced the analgesic action of TCB-2 on SCI-induced pain. These data are consistent with previous pharmacological studies that have indicated that 5-HT_{2A} receptor plays an antinociceptive role at the spinal cord level (Bardin et al., 2000; Seyrek et al., 2010). However, because the blockade of KCC2 by DIOA has been reported to be pro-algesic (Coull et al., 2003; Jolivalt et al., 2008), the lack of pain increasein the presence of DIOA in our study, might reflect an additional analgesic action of TCB-2 via a target different from KCC2. For example, TCB-2 can activate the release of endogenous cannabinoids (Best and Regehr, 2008), that have an analgesic effect (reviewed in Woodhams et al., 2017).

The restoration of KCC2 induced by TCB-2 in dorsal horn neurons alone is no sufficient for robust antinociception after SNI. The lack of efficacy in activating 5-HT_{2A} receptors in SNI rats could be due to a modest reduction of KCC2 membrane signal (12%). In contrast, the normalization of KCC2 levels after peripheral nerve injury induced by the blockade of NKCC1 transporters in the primary dorsal root ganglia neurons was effective in relieving hyperalgesia (Modol et al., 2014). The role and mechanisms of spinal disinhibition in neuropathic pain may be different depending on the animal model. Thus, the upregulation of NKCC1 in primary dorsal root ganglia neurons (Modol et al., 2014), or the downregulation of GAD65-67 enzymes (Moore et al., 2002) combined with decreased GABAergic cell numbers (Scholz et al., 2005) may be more determinant for the development of pain after SNI.

The 5-HT_{2A} receptor is widely distributed at all segmental levels of the spinal cord, notably in neurons of the dorsal horn with a predominant postsynaptic localization (Doly et al., 2004; Kong et al., 2010). To date, how the activation of 5-HT_{2A} receptors promotes KCC2 function in the dorsal horn neurons remains unknown. Mounting evidence [reviewed in (Mahadevan and Woodin, 2016)] suggests that the activation of a receptor coupled to a G protein, such as the 5-HT_{2A} receptor, activates a protein kinase C (PKC)-dependent phosphorylation of KCC2 which increases its stability within the plasma membrane potentially by preventing a calpain-induced proteolytic cleavage of KCC2 (Lee et al., 2007; Zhou et al.,

2012; Medina et al., 2014; Brocard et al., 2016). In keeping with this, a PKC-mediated hyperpolarization of the Cl^- equilibrium potential was observed in motoneurons below SCI under the activation of the 5-HT_{2A} receptors (Bos et al., 2013). Furthermore, nerve injury has been reported to reduce KCC2 phosphorylation in the dorsal horn that correlated well with hyperalgesia (Modol et al., 2014; Ford et al., 2015). It is thus possible that a mechanism involving a PKC signaling pathway may be responsible for the restoration of KCC2 in dorsal horn through the activation of 5-HT_{2A} receptors.

Of note, mechanical hyperalgesia induce by spinal hemisection was quickly reversed by TCB-2, but the effect on thermal hyperalgesia was more gradual and less pronounced. It is possible that TCB-2 preferentially modulates low-threshold sensory neurons rather than high-threshold nociceptive neurons, which could explain a TCB-2-induced antinociception with more emphasis on mechanical than on thermal stimuli. In line with this assumption, mechanical stimulation recruits spinal inhibitory neurons more strongly than thermal stimulation (Hammond and Drower, 1984; Furue et al., 1999; Anseloni and Gold, 2008). In this context, restoring the inhibition by enhancing KCC2 activity with TCB-2 should have more effect on mechanical hyperalgesia. A component that has not been clarified is whether TCB-2 increases KCC2 membrane signal in all types of dorsal horn neurons, or in discerning populations (either excitatory or inhibitory). KCC2 expression has been detected in the vast majority, if not all, of the dorsal horn neurons (Lu et al., 2008) but the expression of 5-HT_{2A} receptors has been reported limited in GABAergic neurons in spinal dorsal horn (Wang et al., 2009). Thus only a subset of dorsal horn interneurons might mediate the 5-HT_{2A}-mediated antinociception after SCI by potentiating the GABA/glycine-mediated Cl^- currents (Li et al., 2002). On the other hand, it is important to consider that TCB-2 could produce changes by abolishing the glial-driven mechanism of KCC2 dysfunction in neuropathic pain (Ferrini and De Koninck, 2013). The release of BDNF by glial cells increases excitability of the dorsal horn neurons by causing disinhibition via a downregulation of KCC2 (Coull et al., 2005). Taking into account the expression of 5-HT_{2A} receptors in glia (Xu et al., 2000), both indirect and direct changes in KCC2 expression can reduce central sensitization of dorsal horn neurons after SCI.

In sum, the present study provides a pharmacological rationale for the development of 5-HT_{2A} receptor agonists as a novel approach to the treatment of both chronic pain and spasticity (Bos et al., 2013) after SCI.

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DISCLOSURE

ISB performed surgeries, post-operative care, algesimetry tests, cryostate cutting, immunohistochemistry, acquisition in confocal microscopy and analysis of the spared nerve injury set of rats. **PB** and **AVL** performed surgeries, post-operative care, and algesimetry tests of the hemisection set of rats. **CB** performed perfusions. **CB and SL** performed vibratome cutting, immunohistochemistry, acquisition in confocal microscopy, and analysis of the hemisected set of rats. **ISB, FB** and **PB** wrote the paper. **XN**, **EU, LV** and **FB** designed the project and proofread the paper.

FIGURE LEGENDS

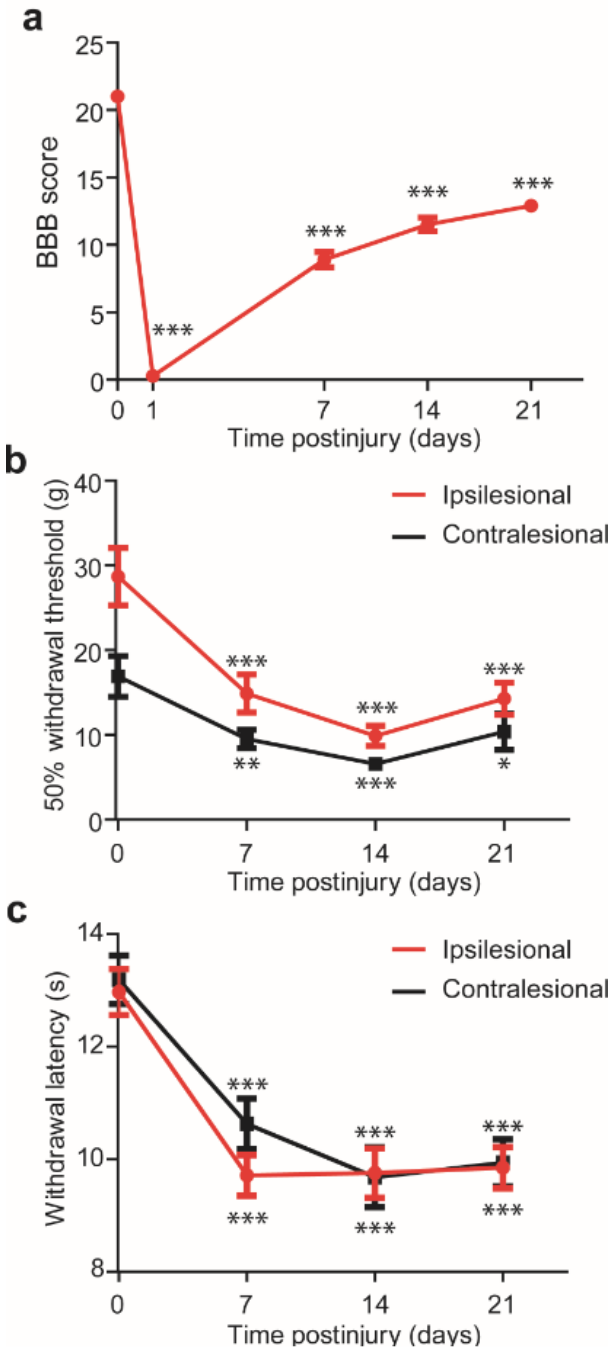

Figure 1. Spinal T8 hemisection in adult rats promotes unilateral motor deficits and bilateral allodynia. (**a**) Time course of functional recovery of the ipsilesional hindlimb as determined by Basso-Beattie-Bresnahan (BBB) locomotor score. (**b,c**) Mechanical (**b**) and thermal (**c**) paw withdrawal threshold as a function of time after the spinal cord hemisection measured in hindpaws ipsilateral (red) and contralateral (black) to the lesion. *P < 0.05, **P < 0.01, ***P < 0.001, indicate differences from the preoperative values; repeated measures one-way (**a**) and two-way ANOVA (**b,c**), Dunnett's post-test. Number of rats was 32. Data are mean ± s.e.m.

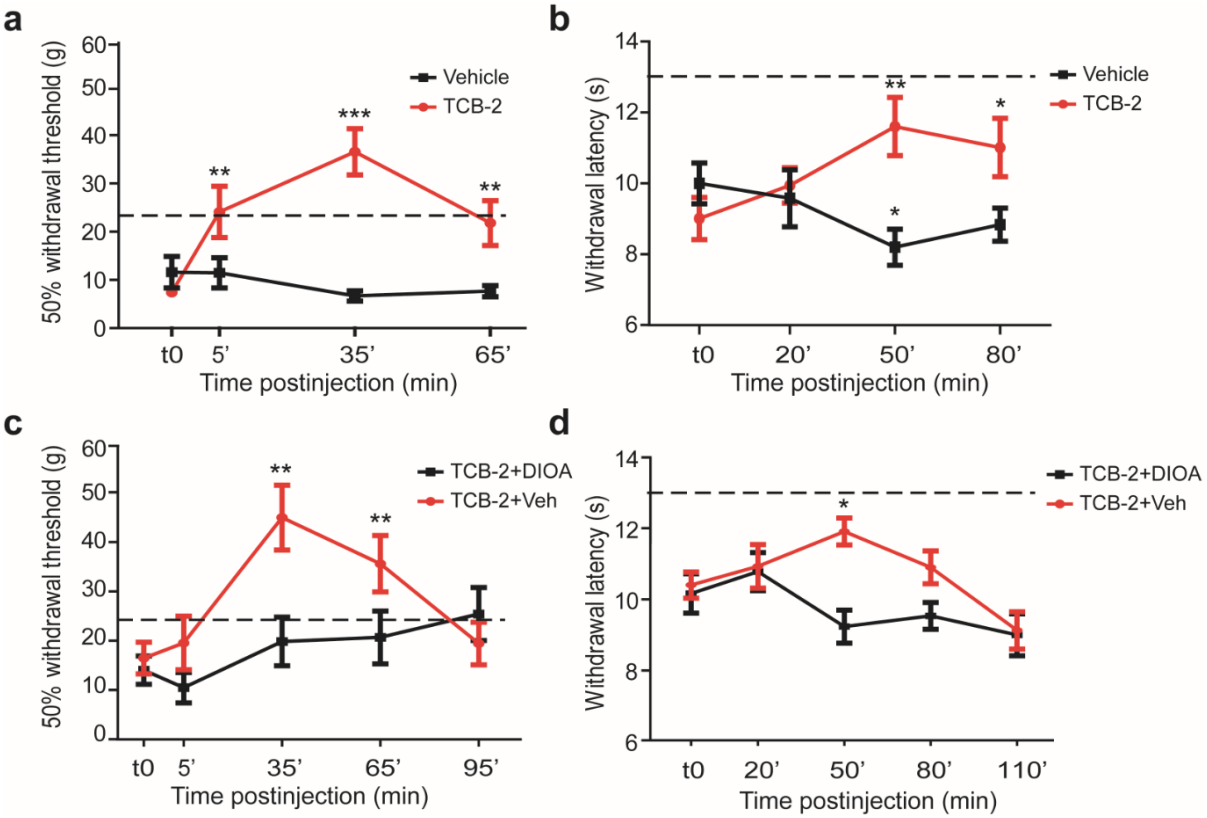

Figure 2: TCB-2 counteracts SCI-induced neuropathic pain via KCC2. (**a,b**) Mechanical (**a**) and thermal (**b**) paw withdrawal threshold as a function of time after i.p. injections of TCB-2 (red, 0.3 mg/kg, n= 8 rats) or vehicle (black, n= 8 rats) in rats with 21 days post-SCI. (**c,d**) Time course of mechanical (**c**) and thermal (**d**) paw withdrawal threshold in SCI rats (21 days post-SCI), i.t. injected with DIOA (black, 30 µg i.t., n= 6 rats) or vehicle (red, 3/4 NaCl 0.9% + 1/4 sodium phosphate buffer, pH 7.4, n= 7 rats) 20 min before an acute i.p. administration of TCB-2 (0.3 mg/kg). t0 represents the time at which TCB-2 was admistered. Plots are mean ± s.e.m. of pooled data collected from ipsilesional and contralesional hindlimbs. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, indicate differences from the pre-injection values; repeated measures one-way ANOVA, Dunnett's post-test. Dashed lines indicate the mean level of preoperative values.

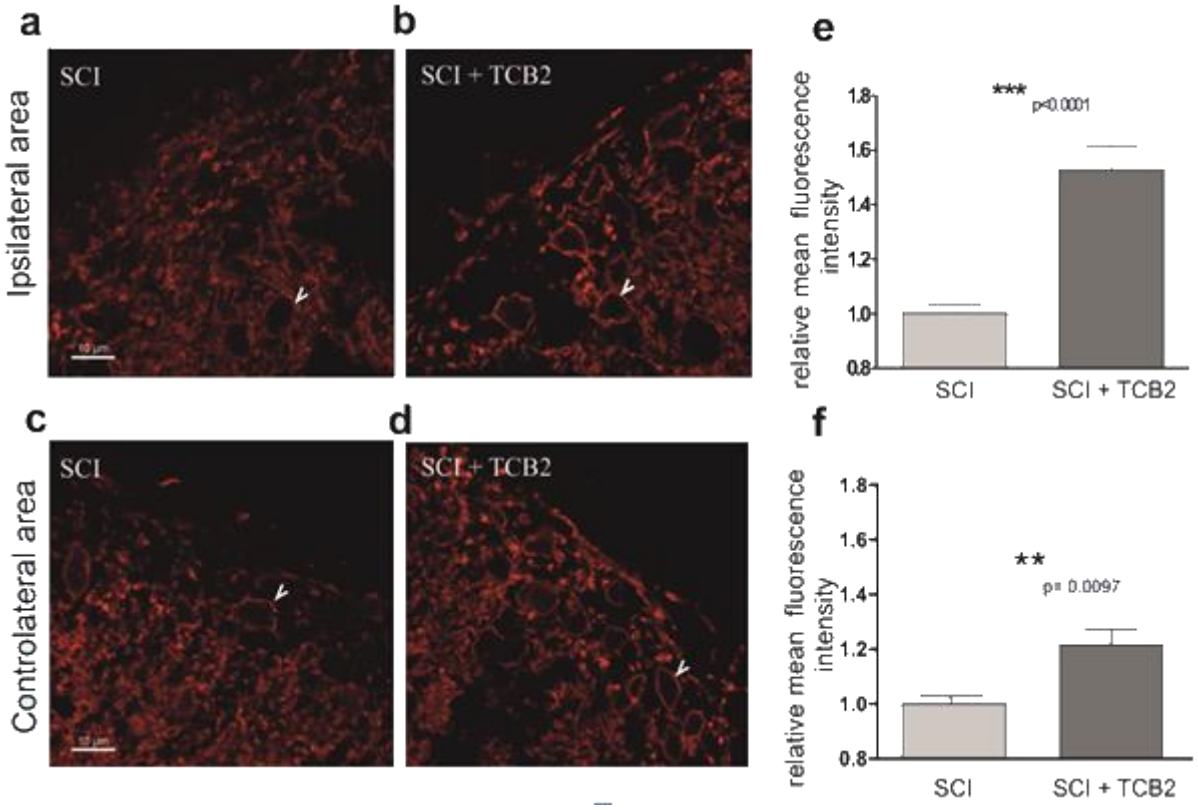

Figure 3: TCB-2 increases the lumbar (L5) dorsal horn expression of KCC2 in rats with SCI. Representative optical sections showing immunostaining of KCC2 in the superficial dorsal horn (lamina I and II) ipsilateral (top) and contralateral (bottom) to the hemisection in adult rats (30 d post-SCI) at 60 min after acute i.p. administration of vehicle (left, distilled water) or TCB-2 (right; 0.3 mg/kg). Arrowheads point to membrane-bound KCC2. Scale bar = 10 µm. Histograms represent relative immunostaining intensities obtained with KCC2 antibody in hemisection rats vehicle-treated (white) and TCB-2-treated (black) normalized to vehicle-treated controls. N = 48 cells each from 3 rats per group. **P < 0.01, ***P < 0.001, t-test. Data are mean ± s.e.m.

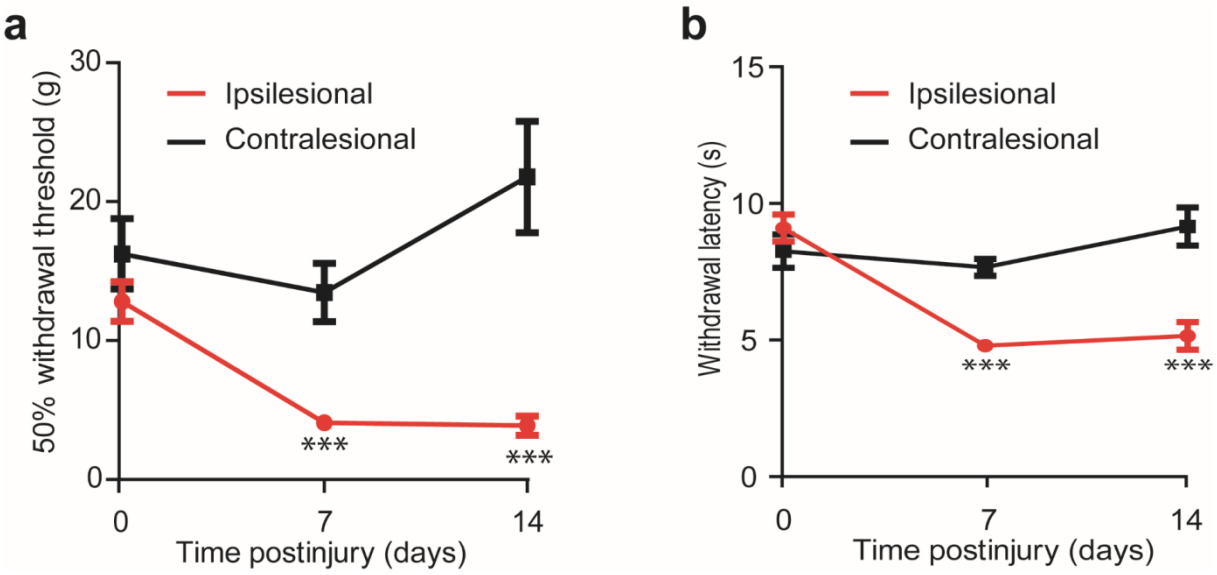

Figure 4: Unilateral spared nerve injury (SNI) in adult rats generates ipsilateral hyperalgesia. Mechanical (**a**) and thermal (**b**) paw withdrawal threshold as a function of time after injury in hindlimbs ipsilateral (red) and contralesional (black) to the lesion. ***P < 0.001, indicates differences from the preoperative values; repeated measures two-way ANOVA, Dunnett's post-test. Number of rats was 15. Data are mean ± s.e.m.

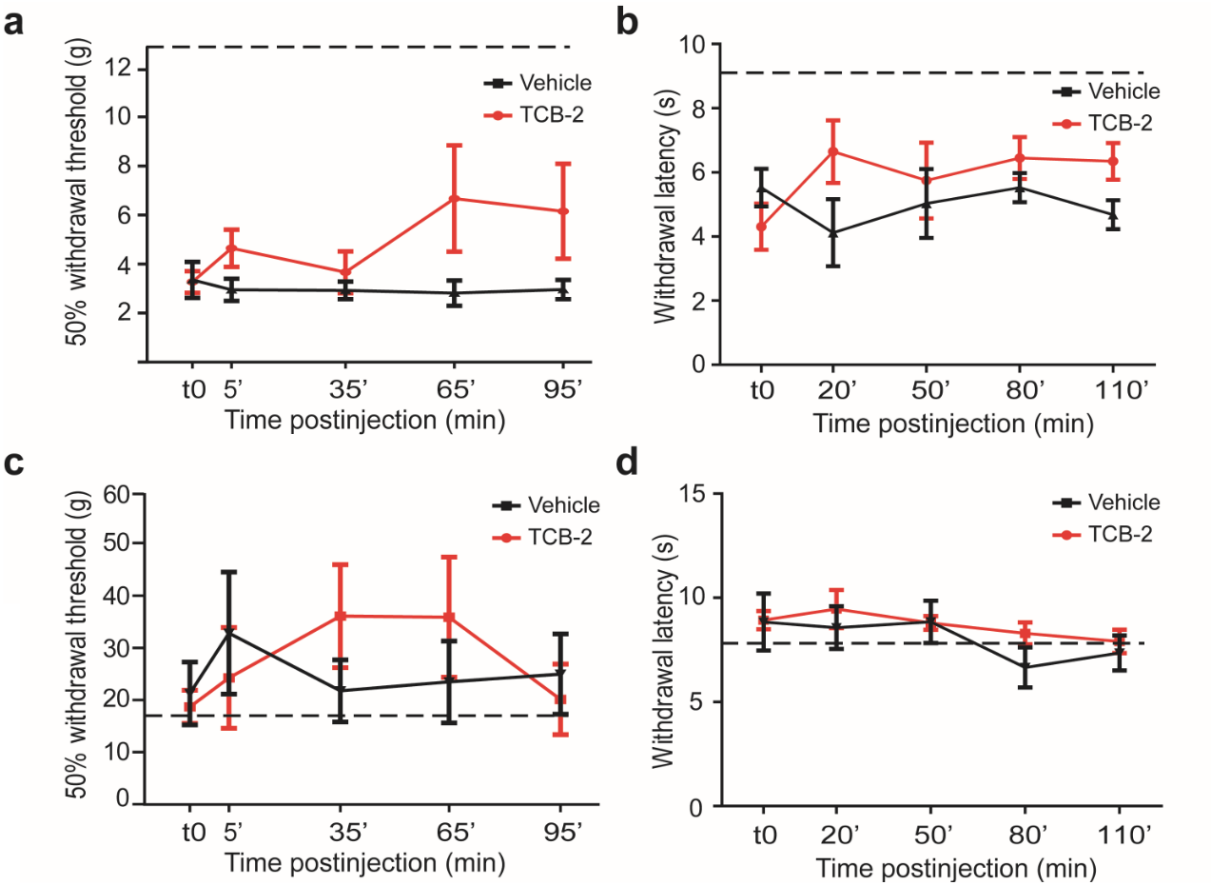

Figure 5: TCB-2 does not counteract neuropathic pain in rats with SNI. Mechanical (**a,c**) and thermal (**b,d**) paw withdrawal threshold from injured (**a,b**) and contralateral intact (**c,d**) hindlimbs as a function of time after i.p. injections of vehicle (black, distilled water) or TCB-2 (red; 0.3 mg/kg) in rats with 14 d post-SNI. Repeated measures one-way ANOVA, Dunnett's post-test. Number of rats was 8 per group. Data are mean ± s.e.m. Dashed lines indicate the mean level of preoperative values.

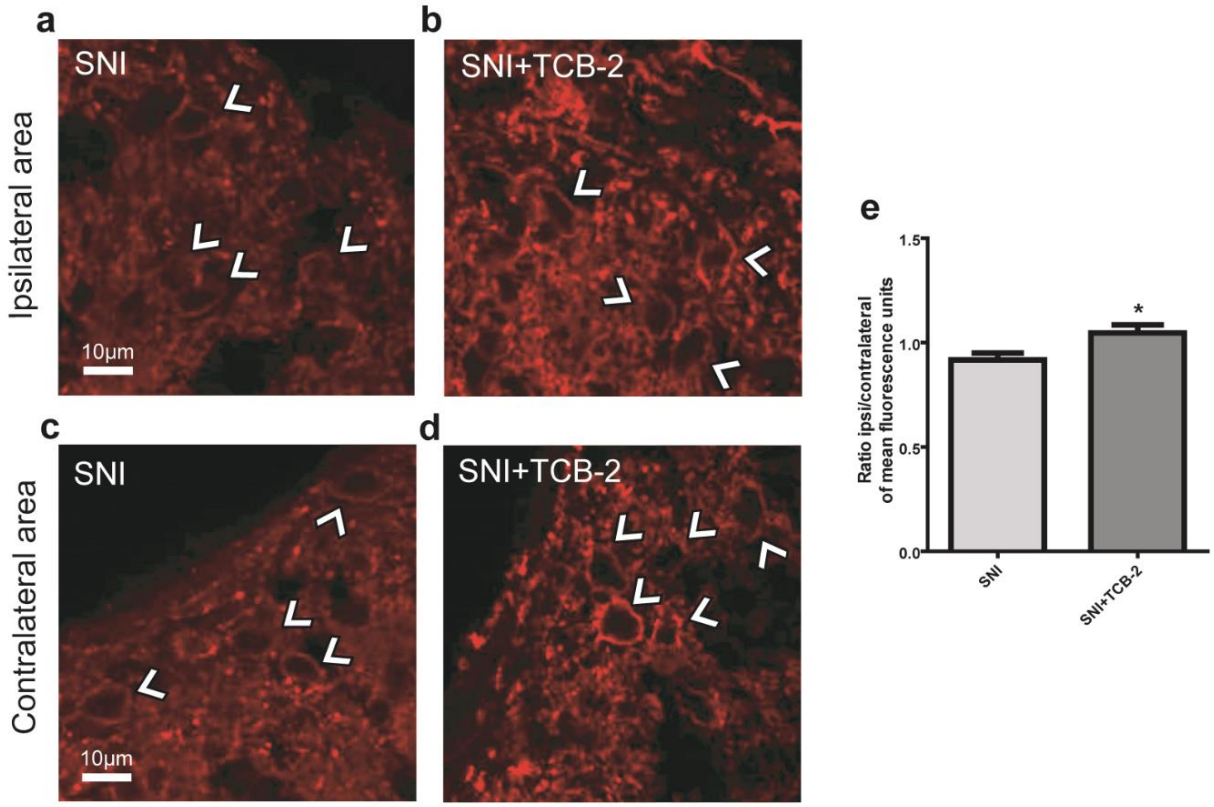

Figure 6: TCB-2 increases the lumbar (L5) dorsal horn expression of KCC2 in rats with SNI. (a-d) Representative optical sections of KCC2 immunostaining in the superficial dorsal horn (lamina I and II) ipsilateral (top) and contralateral (bottom) to the injured limb (14 d post-SNI) after acute i.p. injection of vehicle (left, distilled water) or TCB-2 (right; 0.3 mg/kg). Arrowheads point to membrane-bound KCC2. Scale bar = 10 µm. (e) Histograms representing the mean fluorescence intensity as mean fold change comparing ipsilateral/contralateral. N = 80 cells each from 3 rats per group. *P < 0.05, t-test. Data are mean ± s.e.m. in arbitrary units.

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3.2. Article II : Prochlorperazine does not reduce neuropathic pain after a hemisection of the spinal cord or a spared nerve injury

Prochlorperazine does not reduce neuropathic pain after a hemisection of the spinal cord or a spared nerve injury

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ABSTRACT

Prochlorperazine is a drug from the subfamily of piperazine phenothiazines, and it has recently been reported to increase KCC2 expression in the ventral spinal cord, reducing spasticity after a spinal cord injury. Prochlorperazine is also interesting for being available in an oral form and being overall well tolerated in acute administration. Considering all this, we wondered if prochlorperazine could also increase KCC2 and reduce neuropathic pain after a spinal cord hemisection or a peripheral nerve injury. We performed algesimetries in spinal cord hemisection rats injected with prochlorperazine at two different doses (2 mg/kg i.p.; 10 µg/kg i.v.). Prochlorperazine did not reduce thermal hyperalgesia in any case, and it reduced mechanical allodynia only transiently, when injecting the highest dose (2 mg/kg i.p.). We tested similar i.p. doses (2 mg/kg, 3 mg/kg and 10 mg/kg) on a spared nerve injury model, without any positive results. A chronic treatment with the doses 2 mg/kg i.p. and 10 mg/kg i.p. could not reduce neuropathic pain on spared nerve injury. Prochlorperazine did not prove to cause a significant increase on KCC2 protein expression on any of the two models either. Prochlorperazine does not show potential for being developed as an analgesic drug.

INTRODUCTION

There are more than 750,000 new cases of traumatic spinal cord injury every year in the world (Kumar et al., 2018). This condition has severe impact on the healthcare system and on the life of patients. About 65% of spinal cord injury patients develop neuropathic pain (Siddall et al., 2003). Neuropathic pain is also a common feature on traumatic peripheral nerve injury, a very common condition that affects between 13 and 23 out of every 100,000 people in developed countries (Li et al., 2014). Pharmacological treatment is the most widely used treatment for neuropathic pain both on spinal cord and peripheral nerve injury patients, but it is far from having satisfactory results for all patients (Finnerup et al., 2010). For this reason, there is an urgent need to find new therapeutic strategies to treat neuropathic pain.

One of the mechanisms leading to neuropathic pain after a spinal cord injury and peripheral nerve injury is spinal disregulation of chloride homeostasis, which is highly dependent on the expression of one protein: the potassium-chloride cotransporter type 2, KCC2. After an injury to the spinal cord or the peripheral nerve, KCC2 is downregulated in the membrane of spinal cord dorsal horn neurons, which increases the intracellular concentration of chloride and shifts the chloride equilibrium potential towards more positive values (Coull et al., 2003, Hasbargen et al., 2010). This reduces the capacity of chloride ions to enter the neuron and therefore the inhibitory capacity is reduced (Jean-Xavier et al., 2006, Cramer et al., 2008). We are interested in recovering KCC2 expression as a mean of reducing neuropathic pain after a spinal cord injury.

Phenothiazines are antipsychotic drugs, antagonists of D2 receptors. They are also used as antiemetics. On an acute treatment, some of them have analgesic properties against migraine pain (Becker, 2015). Migraine pain has been proposed to be neuropathic, although there is no consensus about this (Biondi, 2006). However, phenotiazines produce a number of secondary effects (Becker, 2015) that prevent them from being the drugs of choice against chronic pain, being mostly used occasionally, as a complement to other therapies (Merskey, 1997).

Prochlorperazine (PCPZ) is a drug from the subfamily of piperazine phenothiazines. It has not been reported to reduce neuropathic pain, but it reduces thermal pain in naive mice (Ghelardini et al., 2004), and on a recent publication from our team, we proved that it increases KCC2 expression in the spinal cord and reduces spasticity after a spinal cord injury (Liabeuf et al., 2017). PCPZ is also interesting for being available in an oral form and being

overall well tolerated, with secondary effects appearing rarely in acute administration (Merskey, 1997, Orr et al., 2015), although it can cause serious adverse effects on a chronic treatment (FDA, 2017). Considering all this, we wondered whether PCPZ could also reduce neuropathic pain after a spinal cord injury or peripheral nerve injury.

In this study, we determined whether the injection of PCPZ reduces neuropathic pain after a spinal cord hemisection and a spared nerve injury (SNI), using classical algesimetry tests -Von Frey and plantar test-. We complemented these results with immunohistochemistry and Western blot analyses of the expression of KCC2.

METHODS

Animals and surgery

All procedures were approved by the local Ethics Committee (Comité d'Ethique en Neurosciences-INT Marseille, License A9 01 13, and Ethics Committee of the Universitat Autònoma de Barcelona), according to French, Spanish and European regulations.

We used 37 adult female Sprague-Dawley rats for the spinal hemisection, and 20 for the spared nerve injury (SNI) experiments. The surgeries were performed as described in (Sanchez-Brualla et al., 2018). Briefly, for the spinal cord injury, we induced anesthesia in the rats with isofluorane (4%), and injected them ketamine-medetomidine (50mg/kg and 0.25 mg/kg ip, respectively). We performed an incision on the back, we cut longitudinally the dorsal longus muscle at both sides of the spine, we disengaged the muscles from vertebrae bodies T7 and T8, and performed a laminectomy of T8 vertebra using a rongeur. We cut the duramater with fine scissors to expose the spinal cord. We sectioned the left part of the spinal cord at the level of T9 segment using a pair of fine scissors. After the surgery, we left the rats in their cages to recover.

For the SNI, the surgeries were performed as described in Sanchez-Brualla et al. (2018). Briefly, we anesthetized the rats with the same anaesthetics described previously, we performed an incision through the right gluteus major and we exposed the ramification of the sciatic nerve. We ligated the tibial and common peroneal branches with 0-4 silk sutures, and we sectioned them distal to the ligatures, avoiding touching the sural branch. We sutured the muscle and the skin in layers and left the rats in their cages to recover after the surgery.

BBB score evaluation

We evaluated the motor function of the hemisection rats by means of a BBB score (Basso et al., 1995). We left the rats in an open field, and we observed them walking for a maximum of 4 minutes. We evaluated the BBB score of all rats before the surgeries, one day after the surgery -to test if the lesion was correctly performed-, and on days 7, 14 and 21 postinjury.

Algesimetry tests

We evaluated the basal threshold of the rats for static mechanical and thermal stimulation through a Von Frey and a Ugo Basile Plantar Test, respectively. On the week before surgery,

we introduced all the rats into the devices for nociceptive threshold measurement. At each testing day, all the algesimetries took place in the morning. We evaluated the nociceptive threshold responses for static mechanical and thermal stimuli on both hindpaws at different days postinjury (7, 14 and 21).

We used the Von Frey test to evaluate the pain response to static mechanical stimulation. We obtained the 50% paw withdrawal threshold of spinal cord injury rats according to the up-down method described by Chaplan et al. (1994). We placed the rats on a box with a wire net floor at least 5 minutes before testing, for habituation. After this period, we stimulated the hindpaws with Von Frey filaments (BIOSEB, Chaville, France) following the up-down method, departing from the 8g filament.

We used a plantar algesimeter (Ugo Basile, Comerio, Italy) to evaluate the pain response to infrared heat stimulation on spinal cord injury rats. We placed the rats in the boxes at least 5 minutes before testing, for habituation. We stimulated the hindpaw with a hot blast from the algesimeter lamp. The cut-off was set at 20 s to avoid tissue damage. We obtained 3 values for each paw at each test, leaving at least 1 minute between one measure and the next one. We considered the thermal nociceptive threshold as the mean of the three values obtained for each paw.

The pain withdrawal threshold for static mechanical stimulation of the SNI rats was obtained by means of a digital algesimeter. The stimulation of the paw of SNI rats was performed in the lateral side -not in the center of the paw-, since the lateral area keeps its innervation after a SNI.

Drug test

Three weeks after the surgery, hemisection rats reached the maximum level of pain (Christensen et al., 1996, Christensen and Hulsebosch, 1997). Then, we evaluated the acute effect of the injection of prochlorperazine (PCPZ, Prestwick chemical library) at different concentrations and different routes of administration, in the pain threshold. Vehicle- and PCPZ-treated rats underwent the Von Frey and Plantar Test, to evaluate the appearance of mechanical allodynia and thermal hyperalgesia with and without the drug.

For each test, we placed the rats in the Von Frey wire mesh and in the Plantar test device, we let them habituate for 5 minutes and then tested their pain threshold for 10 minutes. We performed both tests to obtain a basal measure of the pain threshold of the animals. When we finished this basal test, we injected either PCPZ or its vehicle (saline solution with 0,1% DMSO, 1ml/kg). After the injection, we placed the rats in the box for the Von Frey test, we let them habituate for 5 minutes, and then we tested their withdrawal threshold for 10 minutes. After that time, we transferred the rats to the plantar test device, where we also let them habituate for 5 minutes, then test their withdrawal latency for 10 minutes. After this test, we transferred the rats again to the Von Frey box, and we repeated this cycle 4 times. We performed each test 4 times, during a total time of 2 hours.

SNI rats develop robust pain responses as early as 7 days after the injury, and 14 days after the injury, they reach the minimum threshold for pain (Decosterd and Woolf, 2000). We determined the acute effect of PCPZ on SNI rats by an algesimetry test performed on a slightly different way: we placed the rats in the Von Frey wire mesh, we let them habituate for 5 minutes and then tested their pain threshold for 10 minutes, obtaining at least 3 measures with the electronic Von Frey test. When we finished this basal test, we injected either PCPZ or its vehicle (saline solution with 0,1% DMSO, 1ml/kg). After the injection, we placed the rats in their cages, and 35 minutes after injection we put them in the Von Frey wire mesh. We let them habituate for 5 minutes, and then we tested them for 10 minutes. After that test, we left the rats in their cages. We repeated this process 1 hour and 50 minutes after injection and 2 hours and 50 minutes after injection, to obtain the three timepoints: 45', 2 hours and 3 hours postinjection.

We determined the chronic effect of PCPZ injection on SNI rats by injecting a dose of PCPZ every day in the morning, for 14 days, starting just after the rats recovered consciousness from the surgical intervention. On test days, we performed a single Von Frey test (on days 7 and 14 postinjury), and a single plantar test (on day 5 postinjury).

Immunohistochemistry

We obtained and processed samples for immunohistochemistry as previously described (Sanchez-Brualla et al., 2018). Briefly, we collected the samples for immunohistochemical analysis from animals euthanized after the last functional test [i.e. 45 min after 10 µg/kg PCPZ

injection (n=3 SCI rats) or saline solution with 0,1% DMSO (n=3 rats; 3 with SCI, 3 with SNI) injection]. We processed equally two naïve rats. We perfused rats transcardially with 4% paraformaldehyde in PBS. We then dissected out the lumbar spinal cord and post-fixed for 15 h in 4% paraformaldehyde. We cut L5 lumbar segments in 30-mm-thick sections using a cryostat. We permeated the sections for 45 min (0.2% Triton X-100 in PBS-BSA 3%) and preincubated for 30 min at 22ºC in PBS containing 3% BSA and normal goat serum diluted 1/100. We then incubated sections overnight at 4ºC with an affinity-purified rabbit KCC2-specific polyclonal antibody (1/400, Millipore). We revealed labeling with a goat Alexa Fluor 546-conjugated rabbit-specific antibody (1/500, 1 h at room temperature, Molecular Probes). We mounted coverslips in a gelatinous aqueous medium. We scanned dorsal horn neurons (lamina I and II) using a confocal microscope (Zeiss LSM510 META) at 40X or 60X magnification (stacks of 0.4-mm-thick optical sections). We performed acquisition and analysis blind for the different treatments.

Spinal cord samples of naïve and SCI rats treated with PCPZ or its vehicle were on the same slide. We acquired confocal microscopy Z-stacks using the same parameters for all samples; all pixels from all samples were below saturated fluorescence intensity. We selected only the most superficial neurons from lamina I and II. We quantified fluorescence signal using the Zen Software. We drew a region of interest within the plasma membrane of ipsilateral and contralateral neurons of dorsal horns and measured the mean pixel intensities from the optical section with the maximal fluorescence intensity.

Western Blot

After experiments, we performed a Western blot analysis of the expression of KCC2 in the spinal cords of the SNI rats. Rats were decapitated and we retrieved the lumbar enlargement of the spinal cord and froze it on liquid nitrogen. The day of the experiment, we took the sample out of the tube where it was frozen, and cut it in four pieces, separating the ipsilateral and contralateral dorsal and ventral horn, and introducing each of these fragments in a different tube.

The protocol to extract the protein and perform the Western blot has been previously described (Brocard et al., 2016). Briefly, samples were introduced in ice-cold lysis buffer (320 mM sucrose, 5 mM Tris-HCL pH 7.5, 10 µM iodoacetamide) supplemented with protease

inhibitors (CompleteMini, Roche diagnostic, Basel, Switzerland). We used a sonicator to homogenize the samples. We centrifuged samples at 7,000g for 5 min. Then we centrifuged the supernatant at 14,000g for 70 min at 4 °C. We collected the pellets and homogenized them in ice-cold lysis buffer (1% Igepal CA-630, PBS 1X, 0.1% SDS, 10 µM iodoacetamide), supplemented with protease inhibitors (CompleteMini, Roche diagnostic). Protein concentrations were determined by using a detergent-compatible protein assay (Bio-Rad). Equal protein amounts (30 µg) from samples were size fractionated by 6% (vol/vol) SDS/PAGE from 40% Acrylamide commercial solution, transferred to a nitrocellulose membrane and probed with the rabbit KCC2-specific polyclonal antibody (1:500; Millipore) at 4 °C overnight in Tris-buffered saline containing 5% fat-free milk powder. The blot was then incubated for 1 h at room temperature with an ImmunoPure goat HRP–conjugated rabbit-specific antibody (1:80,000 in blocking solution; Vector Laboratories, Burlingame, CA, USA). The proteins were blotted with the substrate HRP immobilon Western (Merck-Millipore). Signal intensities were measured with the image-analysis software Quantity One (Bio-Rad).

Statistics

We analyzed the data from BBB and basal algesimetries by performing a D'Agostino-Pearson omnibus normality test; the groups did not show a normal distribution (data not shown); therefore, we analysed these data using the Friedmann test followed by a Dunn post hoc test.

We analysed the algesimetry tests using the Friedmann test; we performed a Dunn post hoc test, comparing the threshold at each timepoint with the threshold before injection.

We analysed the Western blot results by a Kruskal-Wallis test followed by a Dunn's multiple comparison test. To make this comparison, we separated values on: contralateral dorsal horn and ipsilateral dorsal horn.

We analysed the immunohistochemistry results using a Mann-Whitney U test.

We used GraphPad Prism 5 to perform all the statistical analyses.

RESULTS

Evolution of pain threshold and weight support in spinal cord injury

After spinal cord hemisection, rats lost the capacity to move the ipsilateral paw (Figure 1a) due to the spinal shock. However, they recovered control of the paw progressively after the injury. At 7 days postinjury, all rats were able to support their weight on all four paws, making it possible to perform the follow-up algesimetry tests on them. On parallel, the threshold for mechanical and thermal stimulation was progressively reduced for all rats at 7, 14 and 21 days postinjury (Figure 1b-c), stabilizing at 21 days postinjury, when we performed the drug tests.

Prochlorperazine shows little acute effectiveness against neuropathic pain caused by a spinal cord injury

PCPZ, as shown in Figure 2a, significantly increased pain threshold for mechanical allodynia after i.p. injection (2 mg/kg). This effect was not durable: it appeared 40' after PCPZ injection, and it was not present on the next time point, at 70' after injection. PCPZ did not show an effect on pain threshold for thermal hyperalgesia (Figure 2b).

PCPZ i.v. injection of 10 µg/kg did not have an effect on the threshold for mechanical allodynia or thermal hyperalgesia (Figure 2c-d). This dose was chosen for having previously showed to reduce spasticity (Liabeuf et al., 2017).

An acute injection of prochlorperazine does not show an upregulation of KCC2 on the dorsal horn of chronic spinal cord hemisection rats

KCC2 protein is downregulated in the superficial dorsal horn (laminae I and II) after a spinal cord hemisection, and this downregulation is bigger in the ipsilateral side to the hemisection (Lu et al., 2008).

We quantified KCC2 immunoreactivity in the dorsal horn lamina I and II neurons (Figure 3a shows our Region Of Interest) on hemisection rats treated with vehicle, compared with hemisection rats treated with PCPZ. Acute injection of 10 µg/kg on hemisection rats did not upregulate KCC2 expression on these neurons on a significant way, compared to hemisection rats (Figure 3b-c). PCPZ does not seem to mediate an upregulation of KCC2 expression in the dorsal horn.

Evolution of pain threshold on spared nerve injury

After SNI, rats keep the capacity of supporting their weight in all four paws. Mechanical allodynia is stablished as early as 3 days postinjury and it reaches its minimum at 14 days postinjury (Figure 4a, c). Rats did not show a reduced threshold for thermal hyperalgesia (Figure 4b, d), as it was published in the description of the model (Decosterd and Woolf, 2000).

Prochlorperazine does not show an acute effect on mechanical allodynia caused by spared nerve injury

Given that the only dose of PCPZ that had a significant effect on mechanical allodynia was 2 mg/kg i.p., we focused on this range of doses. We performed the test 14 days after SNI, when neuropathic pain is already present.

PCPZ, as shown in Figure 5a, did not increase pain threshold for mechanical allodynia after an i.p. injection of 2 mg/kg. The dose of 3 mg/kg i.p. showed the same absence of effect (Figure 5b), and the highest dose, 10 mg/kg i.p. showed a small, non-significant increase at 2-3 hours postinjection (Figure 5c), which is likely unrelated to the effect of PCPZ, which is maximum at 45-60 min postinjection.

The acute administration of PCPZ after SNI did not show any positive results against mechanical allodynia.

Chronic treatment with prochlorperazine does not show an effect on mechanical allodynia caused by spared nerve injury

We tried a chronic treatment with PCPZ during 14 days, starting from the day of the surgery. We tried the dose that showed some effect in SCI rats: 2 mg/kg i.p.

Chronic treatment with PCPZ did not prevent the development of mechanical allodynia: the treatment with 2 mg/kg i.p. did not increase pain threshold on this test at any of the test days -3 dpi, 7 dpi, 14 dpi- (Figure 6) compared to rats injected with vehicle. The pain threshold was even reduced, compared to vehicle-injected rats, at 3 dpi. We concluded that the chronic administration of PCPZ during the two weeks after SNI did not prevent or reduce the development of neuropathic pain.

Chronic treatment with prochlorperazine coupled with acute testing right after the injection, does not show an effect on mechanical allodynia caused by spared nerve injury

To complete this experiment, we tested whether we could find an effect on acute testing if it was coupled with a chronic treatment (Figure 7): rats received daily i.p. injections of 10 mg/kg PCPZ and on test days (3 dpi, 7 dpi), we tested their pain threshold immediately after the injection.

Chronic treatment with PCPZ did not prevent the development of mechanical allodynia in basal tests; PCPZ-injected rats showed a lower threshold that control rats at 3 dpi, and a similar threshold at 7 dpi (Figure 7a). At 3 days postinjury, PCPZ did not increase the threshold for mechanical allodynia (Figure 7b). At 7 days postinjury there was a small, non-significant increase in the threshold for mechanical allodynia on rats injected with 10 mg/kg PCPZ (Figure 7c). PCPZ did not show an effect on the withdrawal threshold for mechanical allodynia in this chronic injection-acute testing strategy.

Chronic prochlorperazine injection after a spared nerve injury does not show an increase on KCC2 or pKCC2 on Western Blot

To conclude, we performed Western Blot analyses of KCC2, on spinal cord samples from naïve and SNI rats injected with vehicle or 10 mg/kg PCPZ.

SNI shows only a slight downregulation on the expression of KCC2 on the dorsal horn in the ipsilateral side to the injury, compared to the contralateral side, while naïve animals showed an ipsilesional/contralesional ratio close to 1 (Figure 8a-b). The chronic treatment with 10 mg/kg PCPZ i.p. injection showed a tendency to increase KCC2 expression on the ipsilateral side compared to the contralateral side (Figure 8a-b). However, this tendency was not significant compared to naive or SNI rats.

DISCUSSION

The results we obtained through this work do not endorse PCPZ as being an interesting compound against neuropathic pain. PCPZ showed only a short-lasting effect on mechanical allodynia caused by spinal cord injury, at only one of the doses tested. Moreover, it did not show any effect on thermal hyperalgesia, and it did not show any significant effect on neuropathic pain induced by a SNI. PCPZ has not proven to increase KCC2 expression in the dorsal horn after an injury to the central nervous system, and it only showed a non-significant tendency towards KCC2 upregulation in the dorsal horn after a SNI.

PCPZ reduces mechanical allodynia caused by a spinal cord injury at a dose of 2 mg/kg i.p. However, this effect is temporary, it did not last for even 30 minutes, and it is not reproduced in the plantar test for thermal hyperalgesia. Considering that PCPZ can increase KCC2 in the spinal cord and recover inhibition (Liabeuf et al., 2017), it is not surprising that its effects are higher on mechanical allodynia than in thermal hyperalgesia. Mechanical stimulation recruits spinal inhibitory interneurons more effectively than thermal stimulation (Hammond and Drower, 1984, Furue et al., 1999, Anseloni and Gold, 2008) and we have previously found a similar effect with another drug that increases KCC2 expression on the dorsal horn: TCB-2 (Sanchez-Brualla et al., 2018).

On a previous study we had identified one dose of PCPZ that can reduce spasticity significantly, showing a recovery of the Rate-Dependent Depression; this dose also recovered KCC2 expression after its downregulation caused by SCI (Liabeuf et al., 2017). This dose is 10 µg/kg i.v. Therefore, we determined the effect of this dose on neuropathic pain. The dose of 10 µg/kg did not show any effect on mechanical allodynia or thermal hyperalgesia. This result was surprising.

The absence of effect of i.v. PCPZ is unlikely to be due to the change in the route of administration: in human patients, PCPZ is administered either orally –equivalent to i.p.- or intravenously (FDA, 2017), and, to our knowledge, it has not been reported to have substantially different effects. Furthermore, the dose tested has shown effects against spasticity (Liabeuf et al., 2017), as previously mentioned.

Concerning the SNI model, PCPZ did not show any antinociceptive effect on mechanical allodynia on acute administration, at the dose that showed an effect on spinal cord injury (2 mg/kg) or at higher doses (3 mg/kg, 10 mg/kg). The dose of 10 mg/kg was highly concentrated, making dilution laborious. There is not a lot of marge of maneuver beyond this dose. It appears

to us that if this dose did not reduce neuropathic pain, PCPZ is not capable of reducing neuropathic pain on an acute administration on the SNI model.

PCPZ could not avoid the establishment of neuropathic pain on a chronic treatment for one week. In fact, looking at the two graphs, our results could indicate that PCPZ injection accelerated the reduction of the nociceptive threshold for mechanical allodynia at 3 dpi, since the decrease was significant for both groups of PCPZ injections (2 and 10 mg/kg i.p.), and not for control rats. The absence of a cumulative effect of a chronic treatment was manifest even further by the experiment combining chronic injections and acute testing.

It is surprising that PCPZ has little or no effect in neuropathic pain, since it is effective against spasticity at similar doses to the ones we tested (Liabeuf et al., 2017). While it is not an established observation, many anti-spastic drugs have showed an effect against neuropathic pain too, like botulinum toxin (Finnerup et al., 2010, Park and Park, 2017), baclofen (Deer et al., 2012) and cannabinoids (Mehta et al., 2016); furthermore, neuropathic pain and spasticity have been identified as having “entangled mechanisms” that are common to both conditions (Finnerup, 2017). The H-reflex has even been proposed as an indicator of spinal disinhibition and neuropathic pain (Lee-Kubli and Calcutt, 2014, Lee-Kubli et al., 2018). This leads us to wonder how could PCPZ reduce spasticity and have no clear effect on neuropathic pain. PCPZ has previously shown to increase the threshold for thermal pain in naive mice (Ghelardini et al., 2004). In our study, using the same dose of drug, in hemisection rats, we observed no effect on thermal hyperalgesia. This may be due to a different effect of PCPZ on naive and injured nervous system. This finding may also be due to intra-species differences, either. In our experiment, we observed a significant effect only in mechanical allodynia, and it was short in time (less than 30 minutes), which is not encouraging for an analgesic drug.

A barrier that we encounter in the analysis of these data is that we do not know the mechanism by which PCPZ reduces spasticity (Liabeuf et al., 2017). PCPZ has been reported to interact with several receptors: it is an antagonist of dopamine type 2 receptors (Smith et al., 2012), a partial agonist of 5-HT_{2C} receptors (Herrick-Davis et al., 2000), an agonist of muscarinic type 1 receptor (Ghelardini et al., 2004), a blocker of α-adrenoreceptors (De B White and Richens, 1974), and it has been discussed whether it is a weak antagonist of 5-HT_3 receptors (Lehmann et al., 2013) or not (Hamik and Peroutka, 1989). However, it is not clear which of

these receptors could be responsible for the reduction of spasticity and the recovery of inhibition, or whether this depends on another action of PCPZ. Whether PCPZ acts differently in the ventral and the dorsal horn, remains to be elucidated. It is possible that differences in receptor expression in the dorsal and ventral horn can cause a different sensitivity to PCPZ.

Spinal cord hemisection downregulates KCC2 expression on the ipsilateral and contralateral dorsal horn of the spinal cord (Lu et al., 2008), and SNI downregulates KCC2 on the ipsilateral dorsal horn compared to the contralateral dorsal horn (Sanchez-Brualla et al., 2018). In this study, PCPZ showed a tendency to increase KCC2 expression on the injured dorsal horn of the spinal cord of SNI rats. This tendency was not clear on hemisection rats, however. We consider that the fact that the mean value for KCC2 immunofluorescence on the ipsilateral dorsal horn of rats treated with PCPZ compared to non-treated rats was due to fluctuations on KCC2 expression, which can be fast, and not due to a PCPZ-mediated downregulation of KCC2. The fact that this result was not significant prompts us to think that PCPZ did not have an effect on KCC2 expression after a hemisection. To conclude this paragraph, it must be noted that we perfused the rats and collected their lumbar enlargements for immunohistochemistry at a late stage after spinal cord hemisection (chronic phase, 4 months postinjury), and KCC2 expression in the dorsal horn has a tendency to recover after a hemisection (Lu et al., 2008). The absence of effect of PCPZ on KCC2 expression may be due to a partial recovery from the plastic changes induced by a hemisection.

KCC2 expression in the spinal cord is reduced after a peripheral nerve injury and a spinal cord injury (Coull et al., 2003, Lu et al., 2008), but it recovers 28 days after a peripheral nerve injury, cut and suture (Modol et al., 2014), and it shows a tendency to recover after a spinal cord hemisection (Lu et al., 2008). Our guess is that once KCC2 expression has recovered –at least partially-, it is not possible to increase it with an injection of PCPZ. To conclude this paragraph, to our knowledge, KCC2 expression on the ipsilateral dorsal horn after a SNI is downregulated compared to the contralateral dorsal horn (Sanchez-Brualla et al., 2018). It has to be taken into consideration that this is a small sample, though, where there is a tendency to change in KCC2 expression after PCPZ injection.

Altogether, these results indicate that PCPZ, although having some interesting properties for treating neuropathic pain, is not a good candidate drug for this purpose, given that 1) it has showed only a transient effect at a high dose, 2) its effects are not cumulative on a chronic

treatment: it does not prevent the establishment of neuropathic pain, neither it avoids it on an acute injection, and 3) although it shows some effect on KCC2 expression, it is not a significant effect. PCPZ has not proven potential to be developed as an analgesic drug.

FIGURES

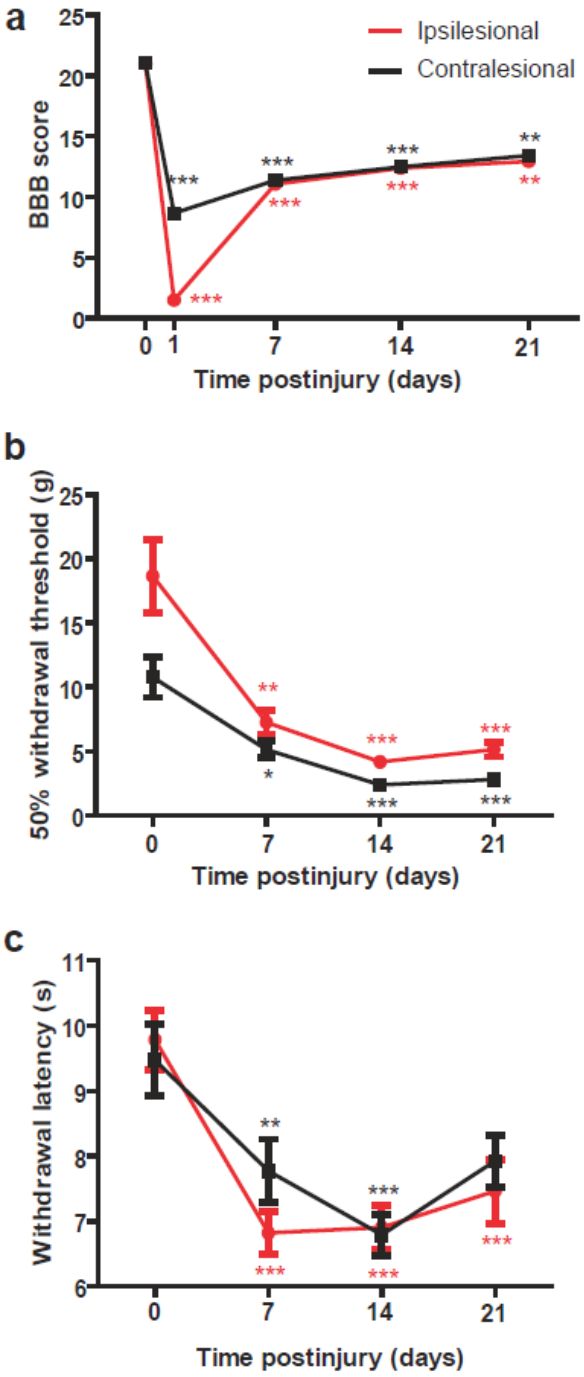

Figure 1. The effect of spinal cord hemisection on the BBB test and on the threshold of mechanical allodynia and thermal hyperalgesia. (a) BBB score of the ipsilesional and contralesional paw from the day of the surgery until 21 days postinjury. (b) Von Frey test of the ipsilesional and contralesional paw from the day of the surgery until 21 days postinjury. (c) Plantar test of the ipsilesional and contralesional paw from the day of the surgery until 21 days postinjury. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

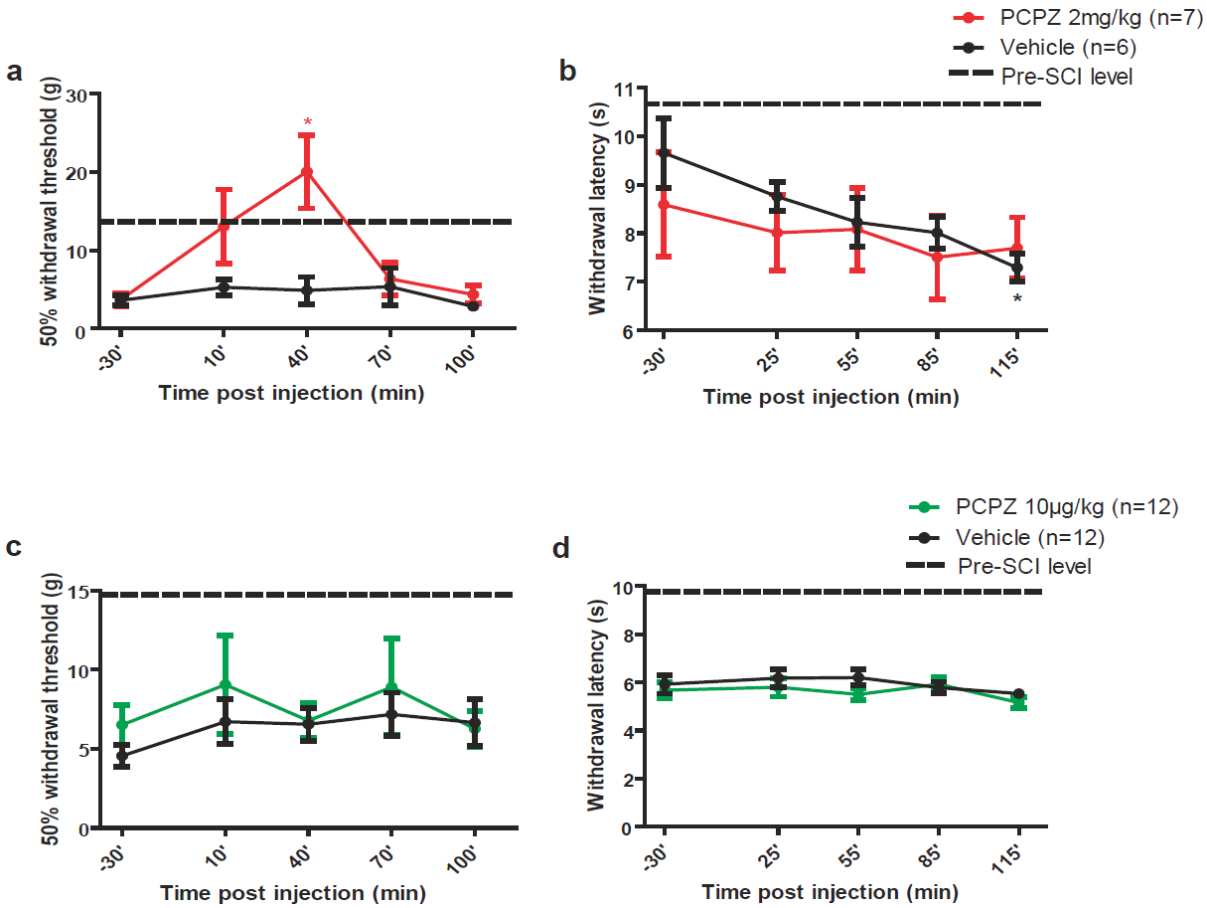

Figure 2. Prochlorperazine injection at 2 mg/kg i.p. reduces mechanical allodynia but not thermal hyperalgesia after a spinal cord hemisection. (a) Von Frey test of 2 mg/kg i.p. PCPZ and vehicle-injected rats. (b) Plantar test of 2 mg/kg i.p. PCPZ and vehicle-injected rats. (c) Von Frey test of 10 µg/kg i.v. PCPZ and vehicle-injected rats. (d) Plantar test of 10 µg/kg i.v. PCPZ and vehicle-injected rats. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

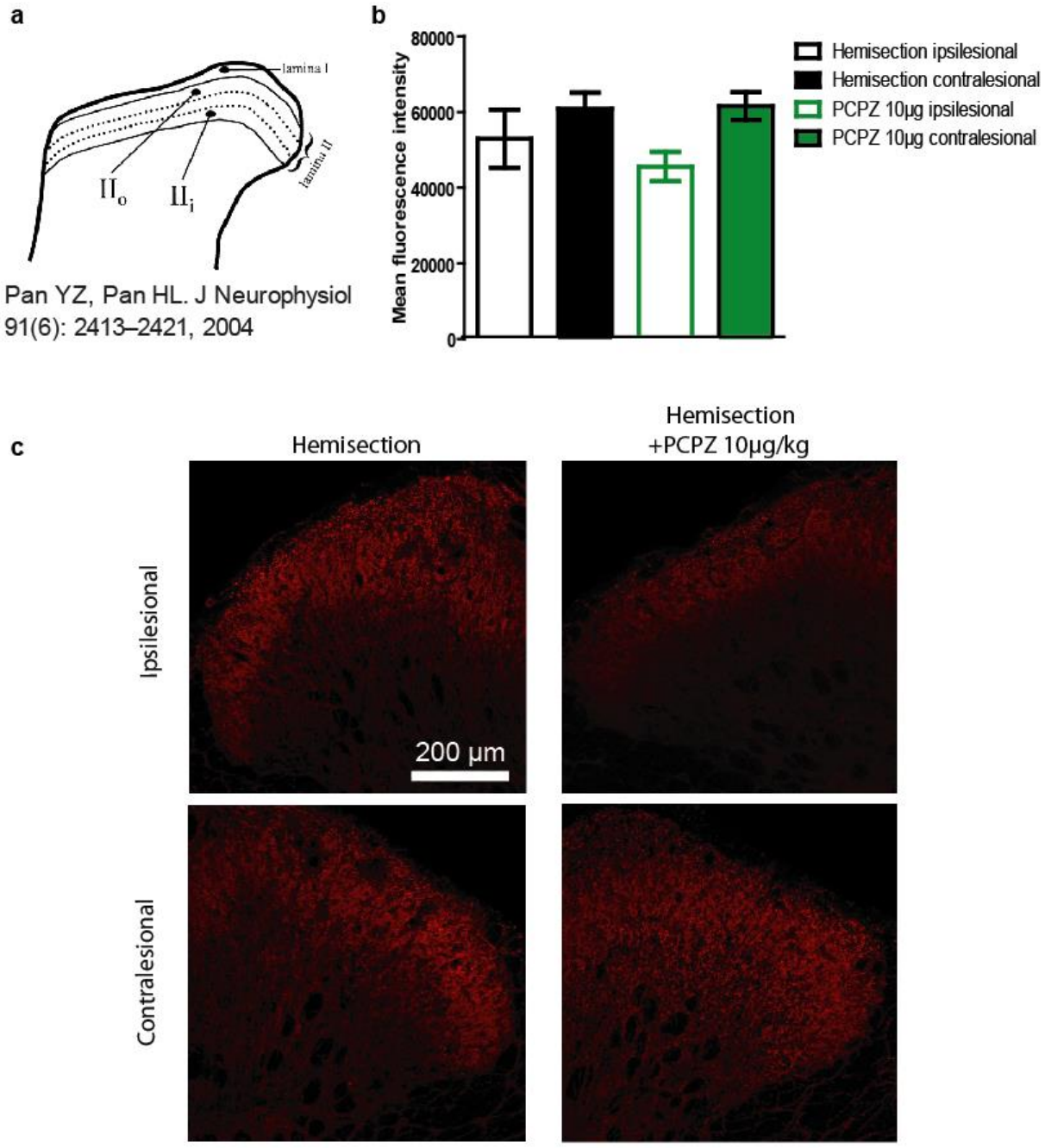

Figure 3. Chronic hemisection does not present a downregulation of KCC2 immunofluorescence on the dorsal horn. Prochlorperazine injection at 10 µg/kg i.v. does not increase KCC2 on chronic hemisection rats. (a) Mean relative intensity of fluorescence at each side of the spinal cord, on sham rats, hemisection rats injected with vehicle and hemisection rats injected with 10 µg/kg i.v. PCPZ. (b) Scheme of laminae I and II of the dorsal horn. (c) Sample photographs of KCC2 expression. Mann-Whitney U test: *=p<0.05.

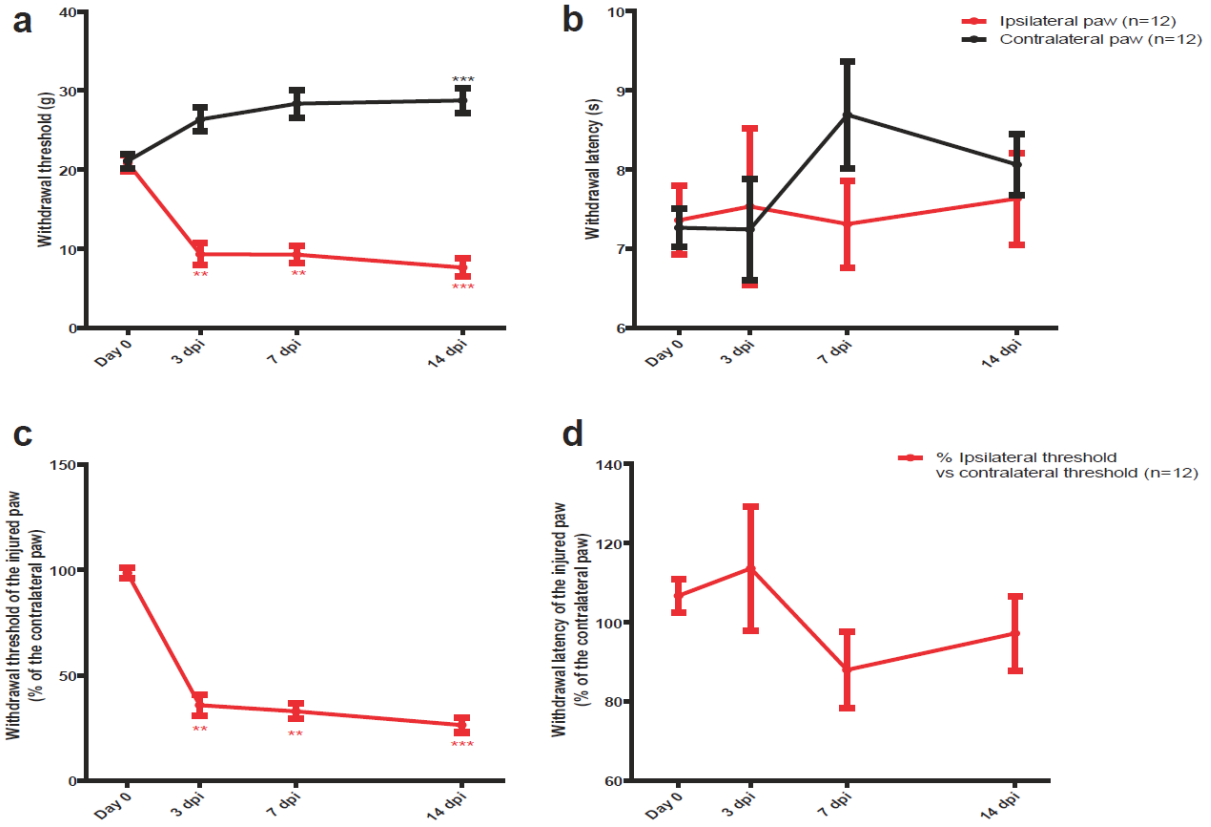

Figure 4. The effect of spared nerve injury on the threshold of mechanical allodynia and thermal hyperalgesia. (a) Von Frey test of the ipsilesional and contralesional paw from the day of the surgery until 14 days postinjury. The withdrawal threshold is presented as a percentage of the threshold for the ipsilesional paw, compared to the threshold for the contralateral paw, in (c). (b) Plantar test of the ipsilesional and contralesional paw from the day of the surgery until 14 days postinjury. The withdrawal latency is presented as a percentage of the latency for the ipsilesional paw, compared to the latency for the contralateral paw, in (d). Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

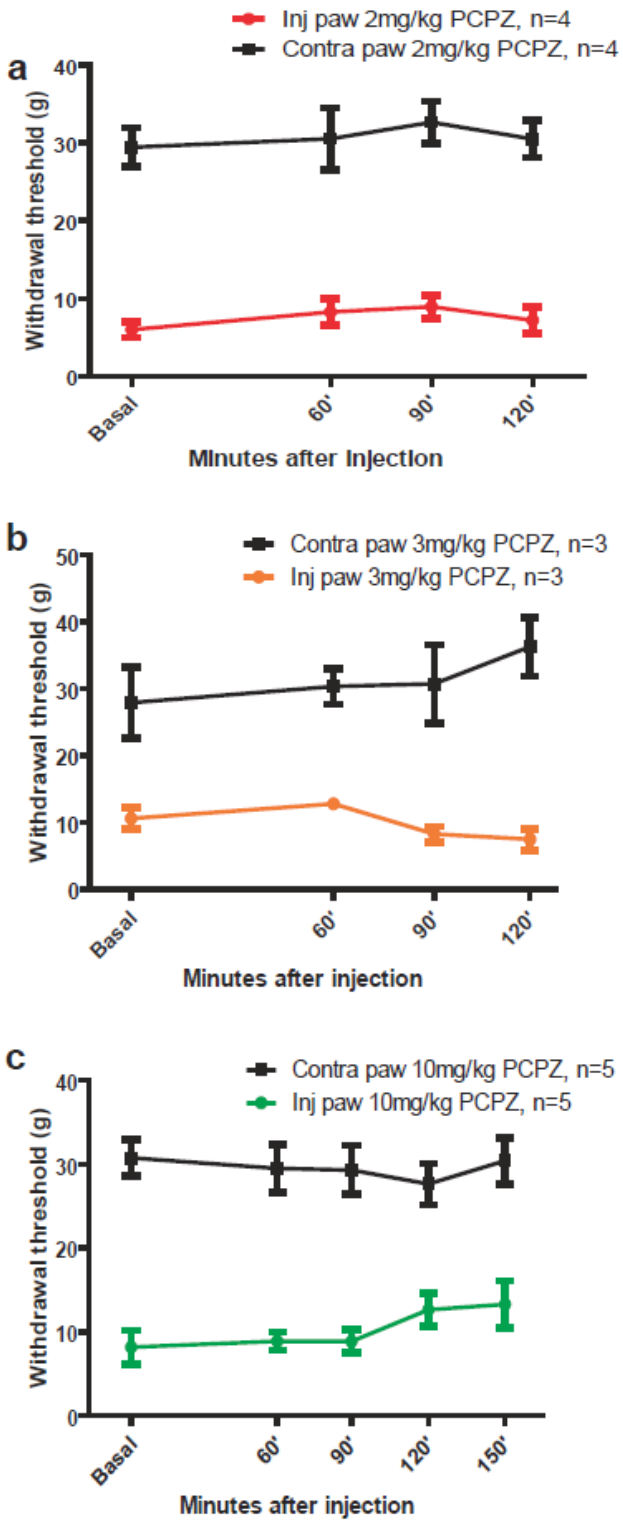

Figure 5. Prochlorperazine injection at 2 mg/kg, 3 mg/kg and 10 mg/kg i.p. does not modify the threshold for mechanical allodynia after a spared nerve injury. (a) Von Frey test of 2 mg/kg i.p. PCPZ-injected rats. (b) Von Frey test of 3 mg/kg i.p. PCPZ-injected rats. c) Von Frey test of 10 mg/kg i.p. PCPZ-injected rats. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

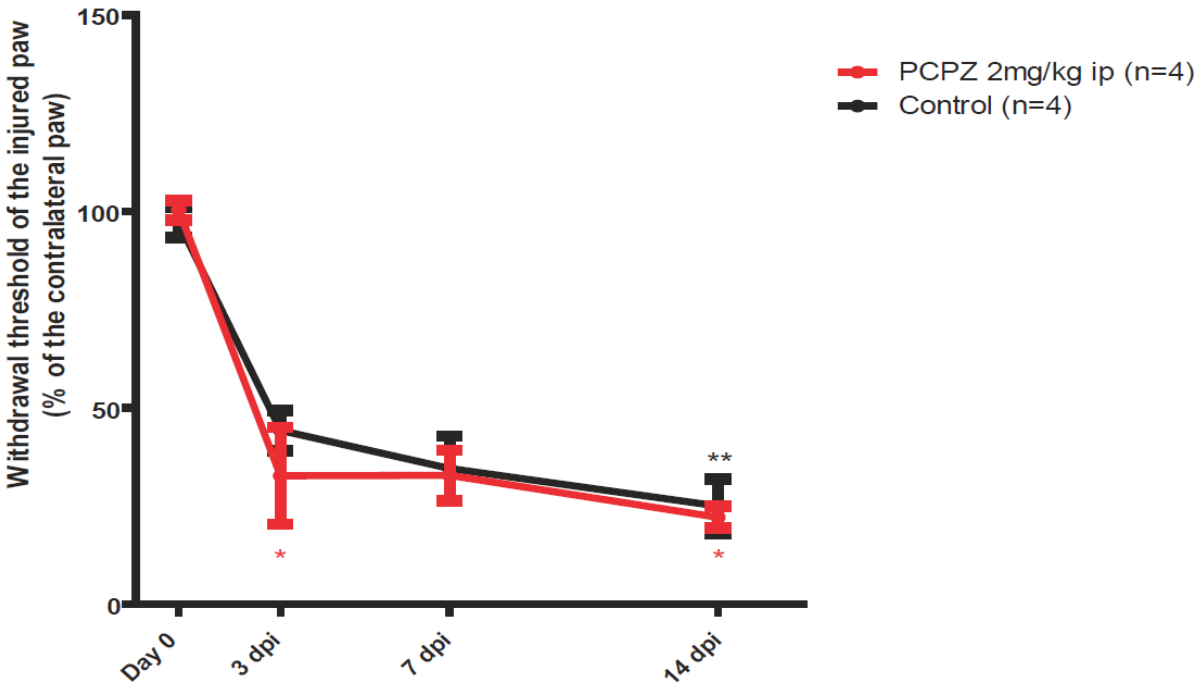

Figure 6. Chronic injection of prochlorperazine during 14 days at 2 mg/kg i.p. does not prevent the development of mechanical allodynia after a spared nerve injury. Von Frey test basal measurements at 3, 7 and 14 dpi, of 2 mg/kg i.p. PCPZ and vehicle-injected rats. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

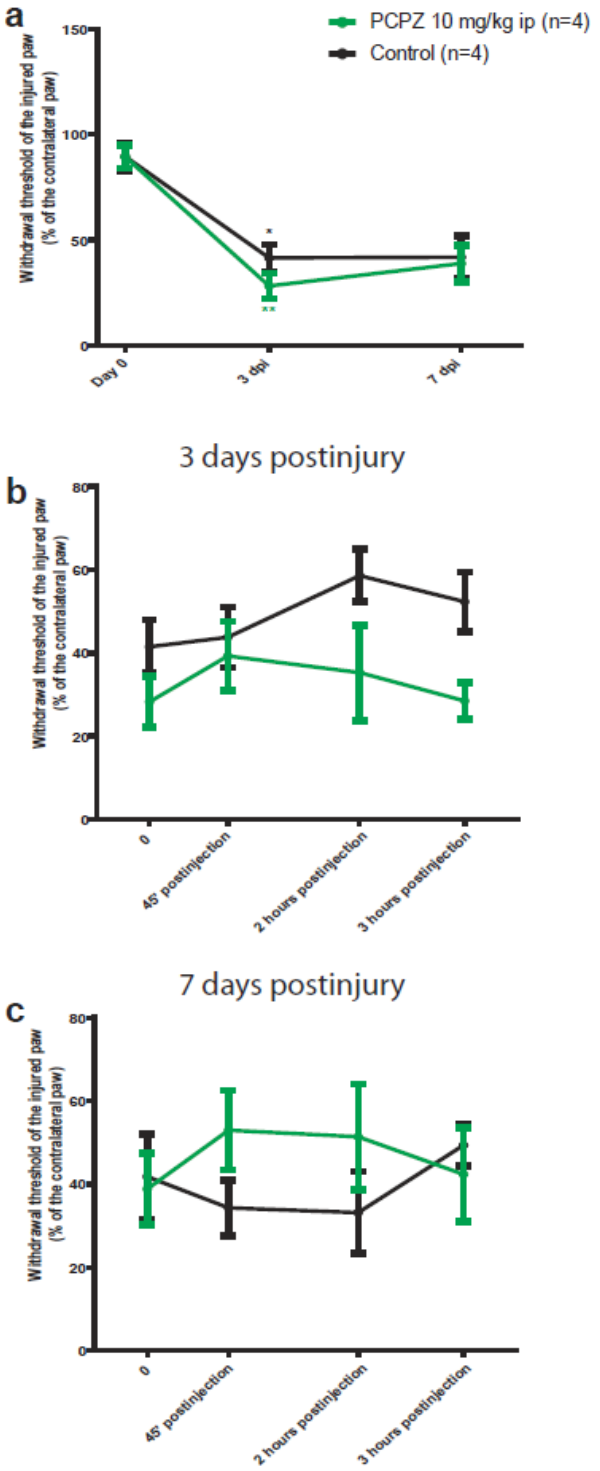

Figure 7. Chronic injection of prochlorperazine during 7 days at 10 mg/kg i.p. does not prevent the development of mechanical allodynia after a spared nerve injury. Prochlorperazine does not increase the threshold for mechanical allodynia on acute testing either. (a) Von Frey test basal measurements at 0, 3 and 7 dpi, of 10 mg/kg i.p. PCPZ and vehicle-injected rats. (b) Von Frey test of 10 mg/kg i.p. PCPZ-injected rats on the acute phase after injection, at 3 dpi. (c) Von Frey test of 10 mg/kg i.p. PCPZ-injected rats on the acute phase after injection, at 7 dpi. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

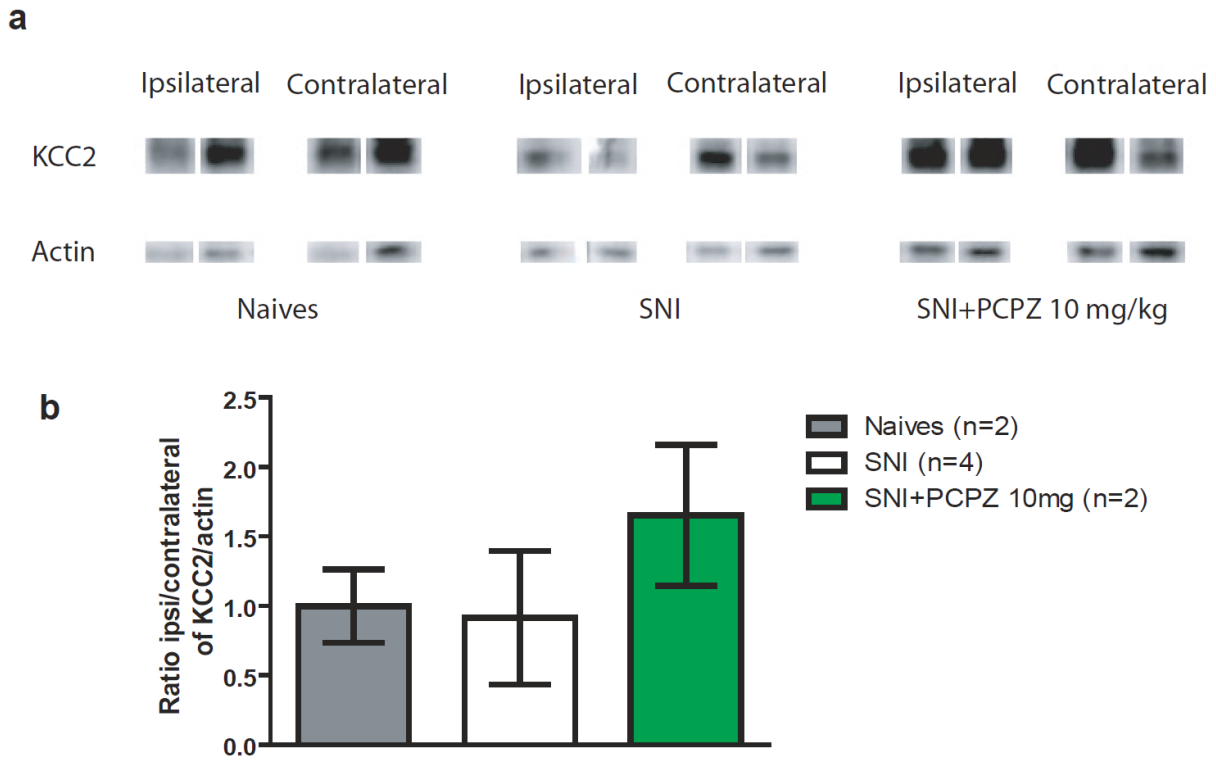

Figure 8. Spared nerve injury does not present a significant downregulation of KCC2 protein on the dorsal horn by Western Blotting. Prochlorperazine injection at 10 mg/kg i.p. shows a non-significant tendency to increase KCC2 in the ipsilesional side. (a) Western Blot membranes of the dorsal horn of vehicle- and chrochlorperazine-injected rats. (b) Quantification of total KCC2 protein on the ipsilateral and contralateral dorsal horn, on spared nerve injury rats injected with vehicle and spared nerve injury rats injected with PCPZ (10 mg/kg i.p.). KCC2 has been normalized to actin, a housekeeping protein. Kruskal-Wallis test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

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3.3. Article III : Calpains downregulate KCC2 in motoneurons after a spinal cord injury

Calpains downregulate KCC2 in motoneurons after a spinal cord injury

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Number of figures: 6
Number of pages: 21

ABSTRACT

Calpains cleave proteins when the intracellular calcium increases. They are upregulated after a spinal cord injury and they have been related to the development of spasticity after this type of lesion. The potassium-chloride cotransporter type 2 (KCC2) is downregulated after spinal cord injury, and this is related with the development of spasticity. Also, KCC2 has been previously shown to be cleaved and taken out of the cell membrane by calpain on animal models of epilepsy and peripheral nerve injury.

On this study, we studied spontaneous activity at the lumbar level; spinal cord injury increased bursts of spontaneous activity on lumbar segments, and these were significantly reduced by the addition of MDL28170 to the bath. On a similar way, we observed animals at P5-P8 to determine that spinal cord injury increases the number of spontaneous movements, and acute injection with MDL28170 could reduce the frequency of these movements. We also found that KCC2 is upregulated by the acute injection of MDL28170 after a spinal cord injury.

We propose that the increase on spontaneous activity is a physiological correlate of downregulation of KCC2 by calpain. We determined whether calpain downregulates KCC2 after a spinal cord injury using immunohistochemistry and Western blot. We found for the first time that, both in newborn and adult spinal cord injury rats, spinal cord injury downregulates KCC2, and a chronic treatment with calpain inhibitor MDL2810 after spinal cord injury prevented this downregulation. Spinal cord injury also depolarized chloride equilibrium potential, and chronic treatment with MDL28170, an inhibitor of calpains, prevented this depolarization.

INTRODUCTION

There are 1200 new cases of spinal cord injury (SCI) every year in France (Institut pour la Recherche sur la Moelle épinière et l'Encéphale [IRME], 2017). This condition has severe impact on the healthcare system and the life of patients. About 60% of SCI patients develop spasticity (Skold et al., 1999). Spasticity has received many definitions, but the one proposed by Pandyan et al. (2005), based on a previous definition given by Tardieu, has been advocated on the last years (Ertzgaard et al., 2017): "disordered sensori-motor control, resulting from an upper motor neurone lesion, presenting as intermittent or sustained involuntary activation of muscles". Spasticity symptoms include hyperreflexia, spasms, muscle cramps, rigidity, and muscular hyperactivity from a neurological origin in general.

The first line treatments against spasticity are physiotherapy, occupational therapy, rehabilitation, orthopedics and neurosurgery. These treatments are frequently coupled with pharmacological therapy when spasticity is problematic (VIDAL). However, since the mechanisms that cause spasticity are not well known, the treatment is still only symptomatic, not directed at curing the causes of the spasticity. The pharmacological treatment for spasticity is far from having satisfactory results for all patients. For this reason, there is an urgent need to find new therapeutic strategies to treat spasticity.

Calpains are a family of proteins with enzymatic properties: they are cysteine proteases that are present at the cytoplasm of cells and they can be active at a neutral pH. This makes them capable of cleaving cytosolic proteins when they are active (Guroff, 1964). Two of the most abundant calpains in the nervous system of mammals are calpains I and II (Geddes and Saatman, 2010, Singh et al., 2014). Calpains have been identified as being important after SCI: preventing their activation improves motor recovery (Yu et al., 2013). Calpains have recently been identified as cleaving voltage-activated sodium channel 1.6 (NaV) after a SCI; after its cleavage, NaV1.6 becomes overactivated, contributing to the development of spasticity (Brocard et al., 2016).

Considering the wide range of activity of calpains and the fact that after a SCI there is an increase in intracellular calcium on spinal cord neurons (Young, 1985) and more particularly, on motoneurons (Li et al., 1996), it is possible to assume that calpains may modify other proteins that may also contribute to the development of spasticity. One of the proteins that are cleaved by calpain is the potassium-chloride cotransporter type 2 (KCC2) (Puskarjov et al., 2012, Zhou et al., 2012).

KCC2 has been related to a variety of health issues concerning a hyperexcitability of central nervous system neurons. A dysregulation of KCC2 has been implicated in the pathophysiology of several disorders like epilepsia (Hekmat-Scafe et al., 2006, Sivakumaran et al., 2015), amyotrophic lateral sclerosis (Fuchs et al., 2010), neuropathic pain (Coull et al., 2003), and it has also been implicated in the development of spasticity after a SCI (Boulenguez et al., 2010). KCC2 downregulation in the membrane of spinal cord neurons increases the intracellular concentration of chloride and shifts the chloride equilibrium potential towards more positive values (Coull et al., 2003, Hasbargen et al., 2010). This reduces the capacity of chloride ions to enter the neuron and therefore the inhibitory capacity at the spinal cord is reduced (Jean-Xavier et al., 2006, Cramer et al., 2008).

We hypothesized that calpain cleaves KCC2 after a SCI, which would contribute to spasticity, altogether with the cleavage of NaV1.6 channels, and this would increase the spontaneous activity of the network. In this work, we prove that calpains cleave KCC2 after a SCI and this cleavage is necessary for the downregulation of KCC2 that is observed in the chronic phase after the lesion.

We performed electrophysiological tests and measured spontaneous activities, to determine the effect that calpain has in network activity at the spinal cord. Then, we characterized the expression of KCC2 after SCI and with a chronic treatment of MDL28170 injections, on newborn and adult rats, by Western blot, immunohistochemistry and sharp electrode technique.

METHODS

Animals and surgery

All procedures were approved by the local Ethics Committee (Comité d'Ethique en Neurosciences-INT Marseille, License A9 01 13, according to French and European regulations.

We used 20 adult female Sprague-Dawley rats for the complete transections of the spinal cord. The surgeries were performed as described in Brocard et al. (2016). Briefly, for the SCI, we induced anesthesia in the rats with isofluorane (4%), and injected them ketamine-medetomidine (50mg/kg and 0.25 mg/kg ip, respectively). We performed an incision on the back, we cut longitudinally the dorsal longus muscle at both sides of the spine, we disengaged the muscles from vertebrae bodies T7 and T8, and performed a laminectomy of T8 vertebra. We cut the duramater with fine scissors to expose the spinal cord. We sectioned the spinal cord completely at the level of T9 segment using a pair of fine scissors. We sutured the wound in layers. After the surgery, we left the rats in their cages to recover.

For the SCI on newborn rats at P0, the surgeries were performed as described at Norreel et al. (2003). Briefly, we anesthetized the rats by hypothermia, we made an incision on the back, and moved the brown fat upwards, out of the surgical space. We identified the scapulae muscles, traced an imaginary line underneath them, and performed a laminectomy of the vertebra just above this line. We cut the spinal cord with small scissors and put an absorbable hemostyptic agent (Surgicoll®) between the two pieces of the spinal cord to prevent descending axons from reaching the sublesional spinal cord. When the bleeding stopped, we put the brown fat back into its place, and sutured the skin of the rats. We performed the aftercare the same way for spinal cord injured and naïve newborn rats -to prevent their mother to recognize the injured ones-, and we brought them back to their mother.

Extracellular recordings on isolated spinal cord

We performed the dissection of the spinal cord as previously described for intracellular recordings. We pinned down ventral L5 roots and put en passant electrodes in contact with them to record electrical activity. We isolated the electrodes with Vaseline and left the preparation to recover for 1 hour under continuous perfusion with artificial cerebrospinal fluid (aCSF) with a concentration of 4 mM KCl.

After this time, we recorded spontaneous activity on the ventral root of the spinal segment L5.

Some of the recordings of spontaneous activity on P5 animals were done at a concentration of 30 µM MDL28170 diluted on the aCSF.

Video recordings of spontaneous activity on newborn rats

We recorded SCI newborn rats (P5-P8) on video to analyze their spontaneous activity. We placed the rats over a hotplate at 33ºC and recorded their activity before injections of MDL28170 (40, 60 and 120 mg/kg i.p.) or its vehicle (DMSO) and after, for up to 9 hours in the same day, with a follow-up the day after.

We quantified spontaneous activity by counting the number of twitches that rats performed during 10-minute intervals, until 9 hours postinjection. We compared the numbers of twitches on animals injected with MDL28170, DMSO and naïve animals. We also considered the number of twitches on naïve animals injected with MDL28170 at its highest dose (120 mg/kg).

Chronic treatment with MDL28170 injections

We injected 10 mg/kg MDL28170, an inhibitor of calpains per day for ten days, on adult and newborn rats. MDL28170 was diluted on DMSO.

Due to technical constraints, the treatment of SCI newborn rats used for intracellular recordings of E_{IPSP} in vitro lasted only 7 days. It is not possible to record from rats beyond P7 in our setup.

Immunohistochemistry

We obtained and processed samples for immunohistochemistry as previously described (Sanchez-Brualla et al., 2018). Briefly, we collected the samples for immunohistochemical analysis from animals euthanized after the last injection (i.e. 1-2 hours after MDL28170 [n=3 adult SCI rats, n=3 newborn SCI rats] or saline solution with 0,1% DMSO injection [n=3 adult SCI rats, n=3 newborn SCI rats]).

We perfused adult rats transcardially with 4% paraformaldehyde in PBS. We then dissected out the lumbar spinal cord and post-fixed for 15 h in 4% paraformaldehyde. We then

introduced samples in PBS-sucrose (20%) at 4ºC and the day after we included them in OCT medium (Tissue-Tek®) to freeze them. We dissected the spinal cord from newborn rats on aCSF (120 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 20 mM D-glucose). We then post-fixed the sample in 4% paraformaldehyde for 7 hours, then in PBS-sucrose (20%) at 4ºC and the day after we included samples in OCT medium (Tissue-Tek®) to freeze them.

We cut L5 lumbar segments in 30-mm-thick sections using a cryostat. We permeated the sections for 45 min (0.2% Triton X-100 in PBS-BSA 3%) and preincubated for 30 min at 22ºC in PBS containing 3% BSA and normal goat serum diluted 1/100. We then incubated sections overnight at 4ºC with an affinity-purified rabbit KCC2-specific polyclonal antibody (1/400, Millipore). We revealed labeling with a goat Alexa Fluor 546-conjugated rabbit-specific antibody (1/500, 1 h at room temperature, Molecular Probes). We mounted coverslips in a gelatinous aqueous medium. We scanned ventral horn using a confocal microscope (Zeiss LSM510 META) at 40X or 60X magnification (stacks of 0.4-mm-thick optical sections). We performed acquisition and analysis blind for the different treatments.

Spinal cord samples of rats treated with MDL28170 or its vehicle were on the same slide. We acquired confocal microscopy Z-stacks using the same parameters for all samples; all pixels from all samples were below saturated fluorescence intensity. We selected only motoneurons. We quantified fluorescence signal using the Zen Software. We drew a region of interest within the plasma membrane of motoneurons and measured the mean pixel intensities from the optical section with the maximal fluorescence intensity.

Western Blot

We performed a Western blot analysis of the expression of KCC2 in the spinal cords of the SCI rats. We followed the protocol described on Brocard et al. (2016). Briefly, one month after SCI in adult rats, and at P7 for newborns, rats were decapitated and we retrieved the lumbar enlargement of the spinal cord and froze it immediately using a metal block at -80ºC. Samples were introduced in sucrose buffer (sucrose 0.32M, tris HCl pH7.5 5mM, sodium vanadate 10mM, sodium fluoride 10mM, sodium pyrophosphate 10mM, iodoacetamide 10µM), supplemented with protease inhibitors (CompleteMini, Roche diagnostic, Basel, Switzerland). We homogenized the samples.

To obtain the total fraction of proteins, we centrifuged samples at 14000g, for 30 minutes, at 4ºC, and collected the supernatant.

To obtain the membrane fraction of proteins, we centrifuged samples for a first time at 7,000*g* for 5 min on sucrose buffer. Then we centrifuged the supernatant at 14,000*g* for 70 min at 4 °C. To obtain the membrane fraction of proteins, we resuspended the pellets in ice-cold lysis buffer (1% Igepal CA-630, PBS 1X, 0.1% SDS, 10 µM iodoacetamide), supplemented with protease inhibitors (CompleteMini, Roche diagnostic, Basel, Switzerland).

Protein concentrations were determined by using a detergent-compatible protein assay (Bio-Rad). Equal protein amounts (30 µg) from samples were size fractionated by 6% (vol/vol) SDS/PAGE from 40% Acrylamide commercial solution, transferred to a nitrocellulose membrane and probed with the rabbit KCC2-specific polyclonal antibody (1:500; Millipore) at 4 °C overnight in Tris-buffered saline containing 5% fat-free milk powder. The blot was then incubated for 1 h at room temperature with an ImmunoPure goat HRP–conjugated rabbit-specific antibody (1:80,000 in blocking solution; Vector Laboratories, Burlingame, CA, USA). The proteins were blotted with the substrate HRP Immobilon Western (Merck-Millipore). Signal intensities were measured with the image-analysis software Quantity One (Bio-Rad).

Statistics

Western Blot results, In vitro analysis of E_{IPSP} and extracellular recordings of spontaneous activity were compared using a Mann-Whitney U test. Immunohistochemistry results were compared using a Student t test.

In vivo analysis of video recordings of the spasms of newborn rats were compared using a Friedman ANOVA followed by a Dunn post-hoc test that compared all values of spasms at different timepoints to the value of the number of spasms before injection.

We used GraphPad Prism 5 to perform all the statistical analyses.

RESULTS

Spontaneous activity on naïve newborn rats decreases during the first postnatal week

Newborn rats present bursts of spontaneous activity that decrease along their first postnatal week. We recorded the spinal cord of P1-P6 rats and noted a high activity at P1-P2, with a high number of bursts, amplitude, duration, area under the curve and frequency (Figure 1, a-f). This activity was reduced progressively along the first postnatal week, being minimal at P5-P6. For all the parameters considered, there was a significant reduction of activity during the first postnatal week (Figure 1, a-f) and the values at P3-P4 were intermediate (Figure 1, a-f).

SCI on newborn rats prevents the decrease of spontaneous activity along the first postnatal week

SCI on newborn rats at P0 showed a tendency to increase spontaneous activity compared to age-matched naïve rats as early as one day postinjury (P1, Figure 2a). We put the values for naïve rats next to the SCI bars for comparison (Figure 2b-f).

The evolution of the five parameters we used to describe the bursts of spontaneous activity along the first postnatal week was not homogeneous on SCI rats. Amplitude and area under the curve showed an increased excitability compared to age-matched naïve rats but, similarly to the development of naives, they showed a tendency to decrease progressively between P1-P2 and P5-P6 (Figure 2c, e). Number and frequency of bursts decreased between P1-P2 and P3-P4 and increased again at P5-P6 (Figure 2b, f). In contrast, duration of bursts followed the opposite pattern, it increased slightly between P1-P2 and P3-P4, and decreased between P3-P4 and P5-P6 (Figure 2d).

Application of MDL28170 on the bath reduces spontaneous activity on SCI newborn rats at P5

The application of MDL28170 (30 µM) reduced spontaneous activity on SCI newborn rats at P5 (Figure 3a). MDL28170 application reduced the number of bursts, duration, amplitude and area under the curve of the bursts (Figure 3b-e). MDL28170 increased the instantaneous frequency of the bursts by clustering them, making bursts happen more close to each other (Figure 3f).

The effect of MDL28170 was not a cause of degradation of the spinal cord due to a long time post dissection; we found a partial wash of its effect on almost all the parameters we studied (Figure 3b-e) except on the frequency (Figure 3f). Wash recordings also showed more clustered bursts compared to recordings under MDL28170 and recordings without drug.

Acute injection of MDL28170 on P5-P8 SCI newborn rats reduces spontaneous activity

We recorded SCI newborn rats on bouts of 10 minutes, when kept at 33ºC and counted the number of spontaneous movements or spasms they made before and after an acute injection of MDL28170 (40 mg/kg, 60 mg/kg or 120 mg/kg) or its vehicle (DMSO). Figure 4a is a picture of the setup.

Injection of 40-60 mg/kg MDL28170 reduced significantly the number of bursts and this reduced activity was maintained during 4 hours. The effect lost its significance at 5 hours postinjection and returned to baseline at 7 hours postinjection (Figure 4b). The effect of MDL28170 is dose-dependent since injection of 120 mg/kg MDL28170 reduction in the number of bursts was even more steep, to the point of reaching values similar to naïve animals (about 15 bursts on 10 minutes) between 3 and 5 hours postinjection (Figure 4b). The effect was still significant 9 hours postinjection when we brought them back to the mother, and we needed to repeat the test the day after to register a return to the baseline (Figure 4b).

Injection of the highest dose of MDL28170 (120 mg/kg) did not alter significantly the number of twitches of naïve rats (Figure 4c). Injection seems to have the effect of reducing the number of bursts for a short time (Figure 4c).

Prevention of calpain activation after SCI prevents KCC2 downregulation on SCI newborn rats

After a SCI on newborn rats, there is a downregulation of KCC2 on its monomeric (Figure 5a, b) and oligomeric (Figure 5a, c) forms in the total fraction of protein. Chronic treatment with MDL28170 injections prevented KCC2 downregulation, keeping KCC2 oligomer expression a ≈60% higher than SCI rats (Figure 5d, f), while monomer expression was not significantly increased (Fig 5d, e). Quantification of KCC2 immunofluorescence on the cell membrane of P7 newborn SCI rats showed that chronic treatment with MDL28170 increased KCC2 immunofluorescence ≈50% compared to vehicle-injected rats (Fig 5g-i).

Prevention of calpain activation after SCI prevents KCC2 downregulation on SCI adult rats

After a SCI, there is a downregulation in the expression of KCC2, which becomes about 84% of KCC2 expression in control rats before SCI on Western blot, and about 66% on immunohistochemistry (Boulenguez et al., 2010). We analyzed the expression of KCC2 by Western blot after SCI in adult rats. Analysis of KCC2 expression on the total fraction did not show any effect of the chronic treatment with MDL28170 (Figure 6a-c). The analysis of the membrane fraction showed no change on the expression of KCC2 monomers (Figure 6d,e), but the chronic treatment with MDL28170 increased the presence of KCC2 oligomers at the membrane by a ≈40% (Figure 6d,f). This result is confirmed when we analyzed the KCC2 expression at the membrane of motoneurons by immunohistochemistry. The chronic treatment with MDL28170 injections also increased KCC2 fluorescence at the membrane by a ≈40% (Figure 6g-i).

DISCUSSION

The results we obtained through this work support our hypothesis that calpain is an important mediator of the downregulation of KCC2 on the spinal cord after SCI in adult and newborn rats. Furthermore, calpain inhibition reduces the upregulation of spontaneous activity on SCI newborn rats.

Since their discovery (Guroff, 1964), calpains have been implicated on a wide range of diseases and have been proposed as therapeutical targets for many of them. On the nervous system, calpains have been implicated in neurodegenerative diseases like Alzheimer, Parkinson's and ALS, and they play a role in spinocerebellar ataxia, lissencephaly and brain ischemia (Ono et al., 2016). Previous work from our lab showed that calpains are overactivated after a SCI (Brocard et al., 2016), cleaving voltage-activated sodium channels NaV1.6. Calpains are implicated on the downregulation of KCC2 on other diseases of the nervous system (Puskarjov et al., 2012, Zhou et al., 2012). In this study, we found that the same cleavage of KCC2 by calpain seems to be taking place after SCI, and it affects spontaneous activity on the neural network of the spinal cord.

Spontaneous activity is an electrical activity generated and transmitted in all immature neural networks. It has been proposed to have a role in the network's "maturation" to a functional state (Blankenship and Feller, 2010). In motor systems, it translates on spontaneous movements that embryos of different species perform (Landmesser and O'Donovan, 1984), these movements continue to take place in newborn babies (Hadders-Algra, 2018) and its study is critical for predicting the development of abnormalities or diseases (Kwong et al., 2018). KCC2 is an important contributor to spontaneous activity: KCC2 KO mice show an increased spontaneous neuronal network activity at hippocampus, this activity has been considered epileptiform and it develops as early as E18.5, when KCC2 is not even considered to be functional (Khalilov et al., 2011). In our study, we showed that SCI exacerbated spontaneous activity and that MDL28170 reduced this spontaneous activity in vitro. In vivo, we showed that the number of spontaneous movements was increased after a SCI, and it could decrease, almost to the level of naïve animals, after MDL28170 injection.

We have no proof that MDL28170 reduced the exacerbated spontaneous activity thanks to KCC2 upregulation, but it has been previously described that KCC2 is crucial for spontaneous

activity in newborn rodents (Stil et al., 2011), we can argue that there is a correlation between MDL28170 upregulation of KCC2 expression and the decrease on spontaneous activity.

Calpain has proven to play a role on network activity too: after a mild mechanical injury of a dissociated cortical neural network, calpain inhibition restored the normal activity of the network (Patel et al., 2012). Another study showed that tetrodotoxin inhibited spontaneous activity on neural networks, and inhibition of calpain activity was protective on these neural networks, not by restoring normal levels of spontaneous activity but by delaying neuronal death (Schonfeld-Dado et al., 2009). It is possible that the action of calpain on spontaneous activity was not direct but mediated by other signaling cascades.

Beyond the incidence of spasticity, restless leg syndrome is more frequent on SCI patients than on healthy population (Kumru et al., 2015); furthermore, periodic leg movements during sleep may be increased on human SCI patients (Ferri et al., 2015), although this study did not make a comparison with healthy controls, and we are not sure whether these movements can be compared with the twitches we registered. Twitching in newborn rats has been studied during their sleep, and it is considered to be important for the development of sensorimotor networks (Blumberg et al., 2013, Blumberg and Dooley, 2017). The same group has found that a thoracic transection of the spinal cord reduced hindlimb twitching on P8 rats during sleep by 35-50% compared to control rats (Blumberg and Lucas, 1994), but these results have been contradicted by Esteves et al. (2004), who found that SCI increased limb movements during sleep, while sham rats showed no limb movements. Our results are in the direction of Esteves et al.: SCI increases limb movements. In this context, a treatment with calpain inhibitor MDL28170 reduces hindlimb movements, to the levels present on naïve rats.

After a SCI in adult rats, there is a downregulation of KCC2 in the lumbar segments of the spinal cord (Boulenguez et al., 2010). In this study, we showed that this downregulation of KCC2 takes place also after a SCI on newborn rats. By Western Blot, we showed a preservation of KCC2 in the lumbar spinal cord mediated by calpain inhibition in both adult and newborn rats, and by immunohistochemistry we showed that KCC2 expression was preserved in the membrane of motoneurons on rats injected with MDL28170, compared to DMSO. The results from Western Blot, moreover, indicate a preservation of KCC2 oligomeric form, which is the only active form of KCC2, according to recent studies (Blaesse et al., 2006, Watanabe et al.,

2009, Mahadevan et al., 2014). This indicates that not only the expression of KCC2 but also its function can be preserved after SCI by a calpain inhibitor.

The agent used to prevent calpain activation was MDL28170. MDL28170 is an inhibitor of calpains I and II (datasheet from Merck®). These are two of the most highly expressed calpains in the central nervous system, together with calpain V (Geddes and Saatman, 2010, Singh et al., 2014). These two calpains have been implicated in several diseases of the nervous system (for a review see Ono et al. [2016]). More particularly, calpain I is activated after SCI (Springer et al., 1997, Du et al., 1999, Yu et al., 2013). One caveat to the specificity of MDL 28170 is its activity against cathepsin B. It has been difficult to separate activities of cathepsin B and calpain, although MDL 28170 provides a 2.5-fold separation for these enzymes (Mehdi, 1991)(Ki=25 and 10 nmol/l for cathepsin B and calpain, respectively). However, after SCI, no lysosomal cathepsin B activity has been established outside the injury epicenter (Ellis et al., 2005). Furthermore, inhibitors targeting cathepsin B do not prevent the proteolytic cleavage of sodium channels (von Reyn et al., 2009). Furthermore, MDL28170 has previously proven to prevent KCC2 downregulation by inhibiting calpain (Puskarjov et al., 2012), while no relationship between cathepsin B-mediated lysosomal degradation and KCC2 has been previously established, to our knowledge. In fact, leupeptin, a blocker of serine proteases (Wang, 1990) does not alter total KCC2 protein levels (Puskarjov et al., 2012).

We chose to study calpain activation on a chronic condition, although most studies have focused on its activity on the acute phase of SCI (Banik et al., 1997, Ray et al., 1999, Schumacher et al., 1999). This model was more aligned with our research interests, since spasticity appears in the chronic phase, weeks or months after SCI takes place, and once developed, often becomes chronic (Finnerup, 2017). A treatment of spasticity needs to be effective on a chronic administration; the first hours after the injury are generally dedicated to stabilization of the patient and therefore it is not common to administer drugs or other substances in this period. Furthermore, spasticity is sometimes beneficial for patients, so the treatment for each patient tends to be tailored to their particular needs and expectations (Ertzgaard et al., 2017).

We did not find significant differences between KCC2 downregulation after SCI on adult and newborn rats in function of the age of the animals, neither we found different effects of the calpain inhibitor, MDL28170. There are differences on the neuroplasticity after a SCI between newborn and adult rats, the recovery of descending input being easier on newborn rats (Bregman et al., 1993) but, to our knowledge, the differences on this recovery have not

been linked with calpain function. Calpain activity increases with aging (for a review on this topic see Nixon [2003]) but whether there are significant changes between birth and adulthood, or whether the proportion of calpain isoforms present on the spinal cord changes with aging, has not been completely elucidated. In any case, our results show that the result of SCI and calpain inhibition is similar in both situations, regarding KCC2 expression.

EIPSP is hyperpolarized after a chronic treatment with MDL28170, proving that the increase on KCC2 that can be seen on immunohistochemistry and Western Blot has a functional correlation.

Considering the present work, it is possible to say that calpain is responsible for KCC2 downregulation after a SCI, since calpain inhibition shows a clear preservation of KCC2 protein from cleavage. But it is not possible to discard that other proteins may contribute to KCC2 downregulation after SCI. BDNF is an example of a protein released by spontaneous activity (Kuczewski et al., 2008) which regulates KCC2 expression (Rivera et al., 2004, Tashiro et al., 2015). It must be taken into consideration, however, that BDNF and calpain have been shown to participate in similar signaling pathways, such as inhibiting synaptic vesicle endocytosis (Wang and Zhang, 2017), so it is not impossible that the pathways activated by BDNF are mediated downstream by calpain. On a similar way, we can say that calpain inhibition reduced the exacerbated spontaneous activity caused by a SCI, and it depended on KCC2, but we cannot be sure about the possible implication of other mechanisms.

To our knowledge, we are the first to describe a clear effect of calpain on KCC2 downregulation after a SCI and to link it to a functional outcome like spontaneous activity. Our results indicate an interesting venue on reducing calpain activation after a SCI to preserve KCC2 expression.

FIGURE LEGENDS

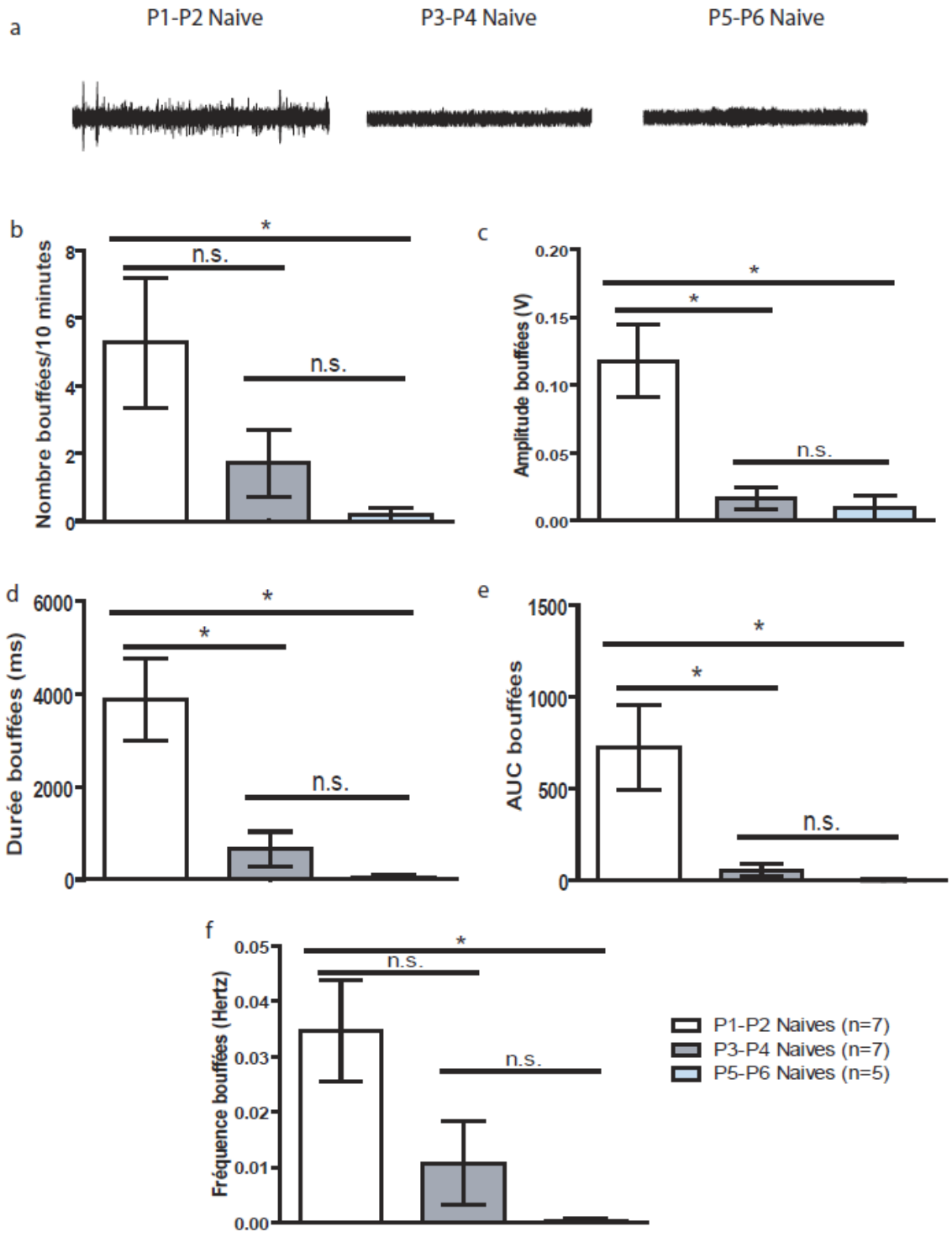

Figure 1. Electrophysiological recordings of spontaneous activity on naïve P1-P6 rats. a) Ten-minute recordings of spontaneous activity on naïve P1-P6 rats. b) Number of bursts during 10-minute recordings. c) Amplitude of bursts. d) Duration of bursts. e) Area under the curve of bursts. f) Frequency of bursts. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

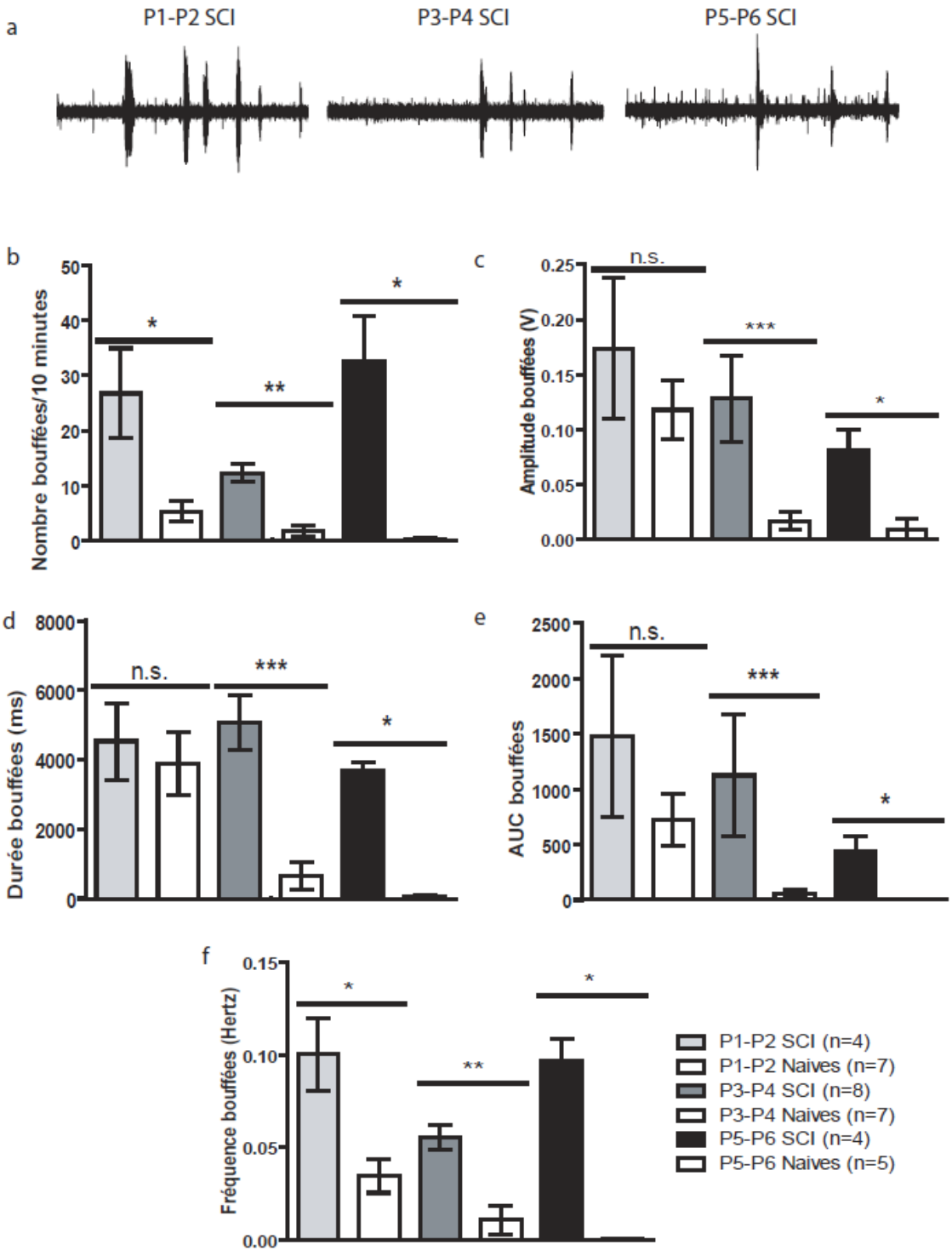

Figure 2. Electrophysiological recordings of spontaneous activity on SCI P1-P6 rats. a) Ten-minute recordings of spontaneous activity on SCI P1-P6 rats. b) Number of bursts during 10-minute recordings. c) Amplitude of bursts. d) Duration of bursts. e) Area under the curve of bursts. f) Frequency of bursts. Results from age-matched naïve animals are at the right of every SCI column, for comparison. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

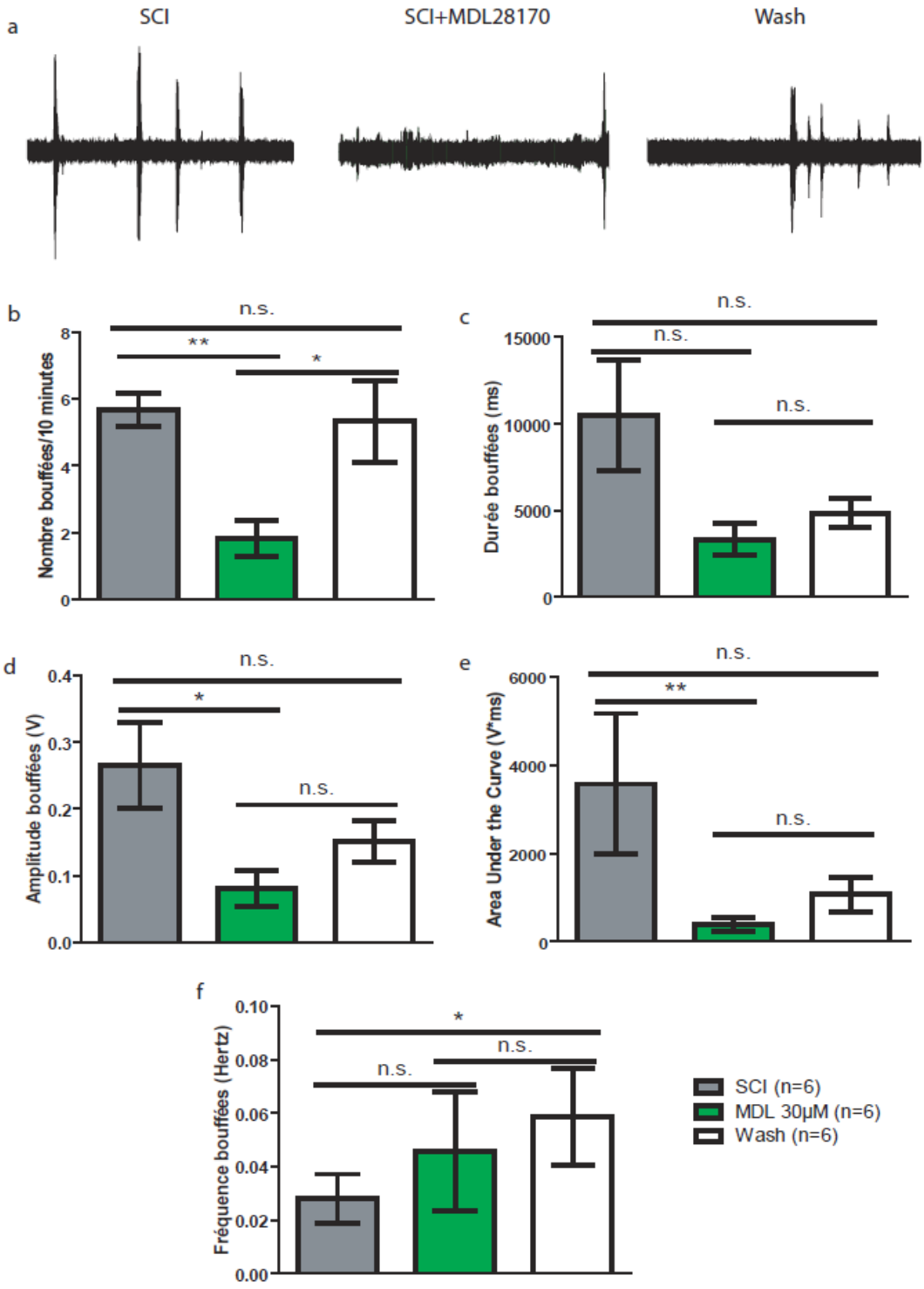

Figure 3. Electrophysiological recordings of spontaneous activity on SCI P5 rats on the presence of MDL28170. a) Ten-minute recordings of spontaneous activity on SCI P5 rats on aCSF, on the presence of MDL28170 and during the wash. b) Number of bursts during 10-minute recordings for the three conditions. c) Amplitude of bursts for the three conditions. d) Duration of bursts for the three conditions. e) Area under the curve of bursts for the three conditions. f) Frequency of bursts for the three conditions. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

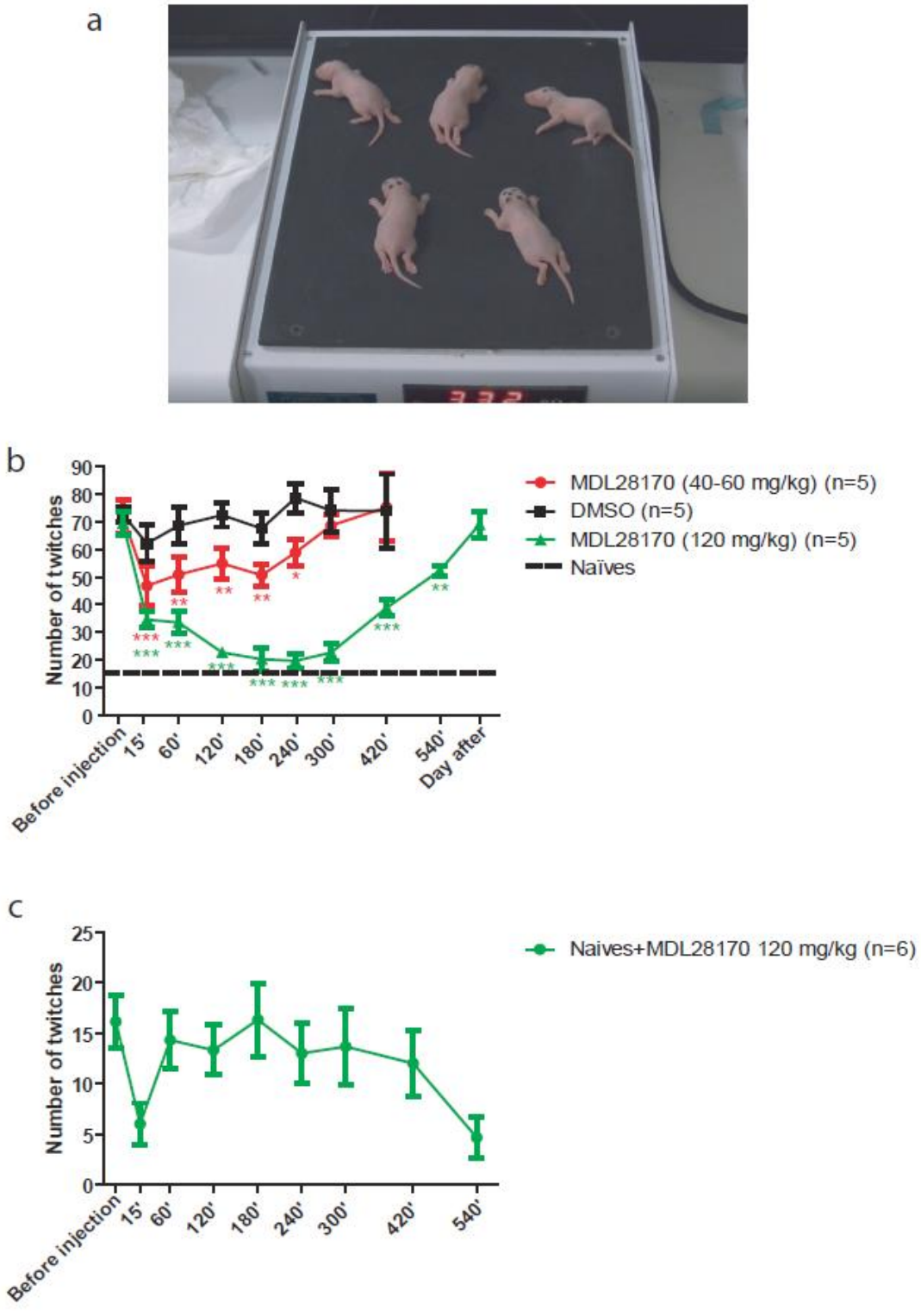

Figure 4. Video recordings of spontaneous activity on P5-P8 rats. a) Image of the setup for recording spontaneous activity. b) Number of twitches of SCI P5-P8 animals, after injection of MDL28170 (40-60 mg/kg or 120 mg/kg) or its vehicle (DMSO). The dashed line indicates the number of twitches on naïve animals. c) Number of twitches on naïve animals with an injection of MDL (120 mg/kg), its vehicle (DMSO) or with no injection. Friedman test and Dunn's multiple comparison test: *=p<0.05, **=p<0.01, ***=p<0.001.

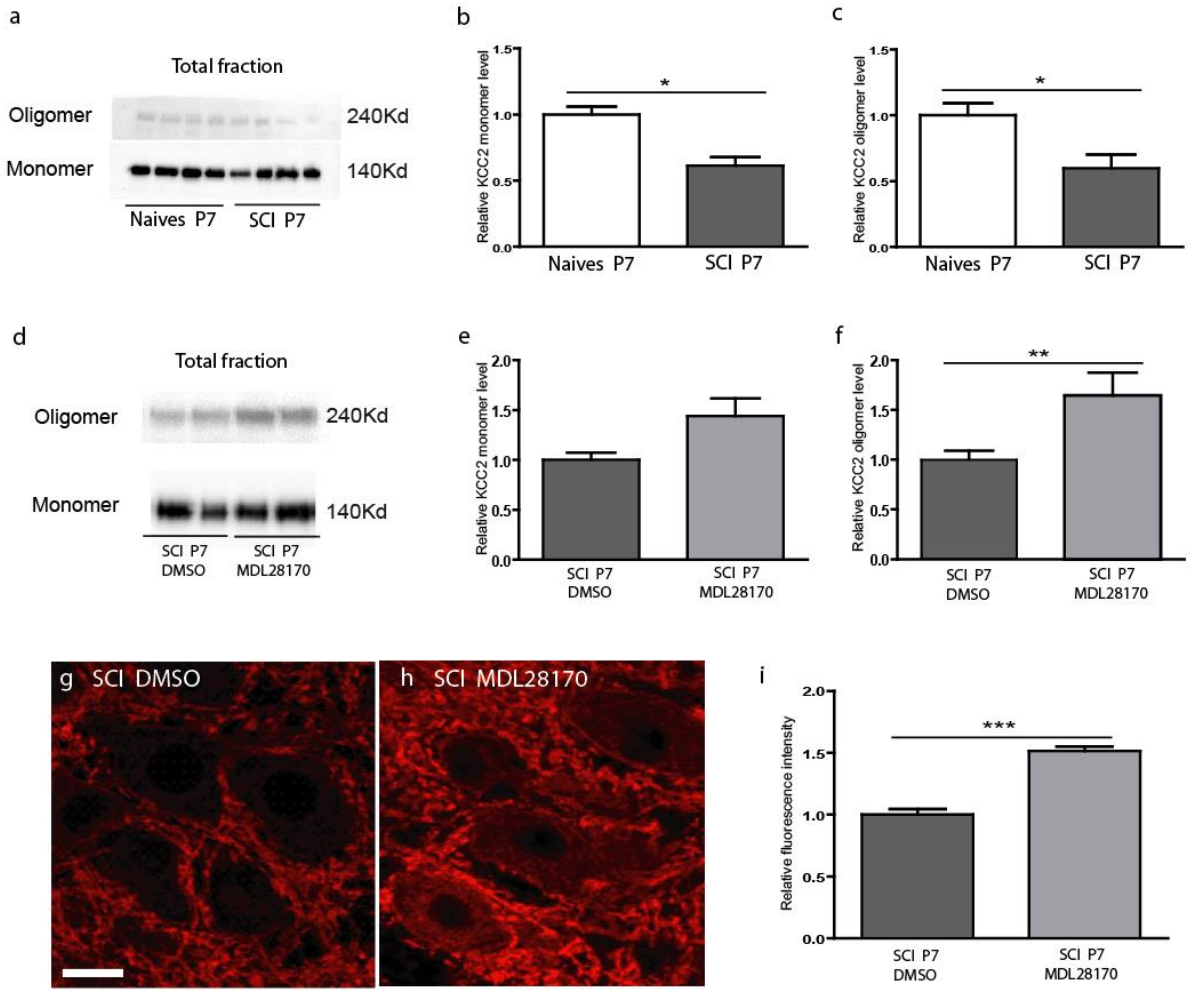

Figure 5. Results of immunostaining KCC2 in lumbar L4-L5 motoneurons of SCI P7 rat treated by intraperitoneal injection with vehicle or MDL28170 (10mg/kg for 7 days). Western blot (a), and quantification of KCC2 on its monomeric (~140kDa) (b) or oligomeric form (~240kDa) (c) on the total fraction of protein from the lumbar spinal cord of naïve and transected P7 rats. Western blot (d), and quantification of KCC2 on its monomeric (~140kDa) (e) or oligomeric form (~240kDa) (f) on the total fraction of protein from the lumbar spinal cord of transected rats at P7 treated chronically with MDL28170 injection or DMSO injection. Mann-Whitney test: *=p<0.05, **=p<0.01. Images of immunofluorescence (40x oil immersion objective) of transected rats treated chronically with DMSO injection (g) or MDL28170 injection (h). Mean relative intensity of fluorescence of motoneurons in the spinal cord, on transected rats treated chronically with MDL28170 injection or DMSO injection (i). Student t test: *=p<0.05, **=p<0.01, ***=p<0.001.

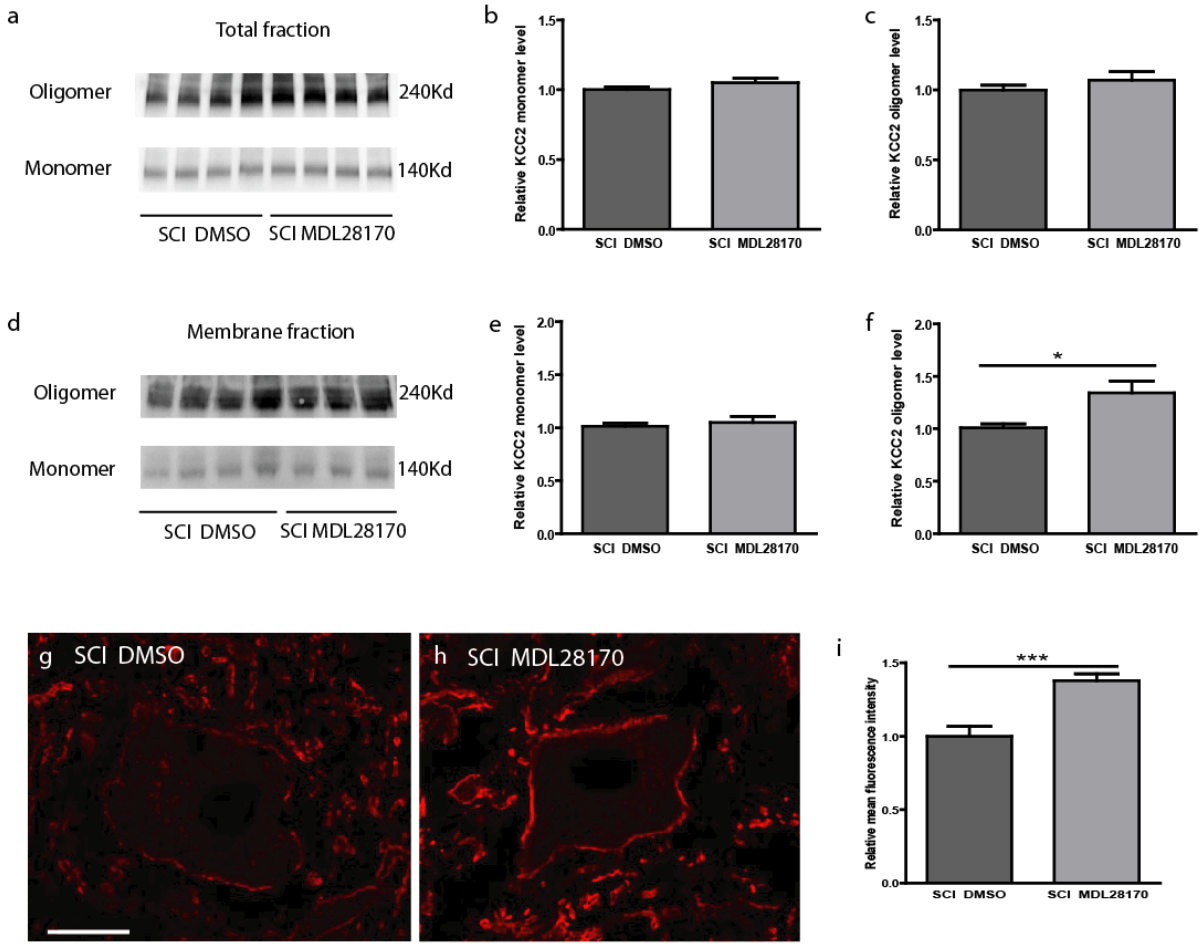

Figure 6. Results of Western Blot and immunostaining of KCC2 in lumbar L4-L5 motoneurons of SCI adult rat treated by intraperitoneal injection with vehicule or MDL28170 (10mg/kg for 10 days). Western blot (a), and quantification of KCC2 on its monomeric (~140kDa) (b) or oligomeric form (~240kDa) (c) on the total fraction of protein from the lumbar spinal cord of transected rats treated chronically with MDL28170 injection or DMSO injection. Western blot (d), and quantification of KCC2 on its monomeric (~140kDa) (e) or oligomeric form (~240kDa) (f) on the membrane fraction of protein from the lumbar spinal cord of transected rats treated chronically with MDL28170 injection or DMSO injection. Mann-Whitney test: *=p<0.05. Images of immunofluorescence (40x oil immersion objective) of transected rats treated chronically with DMSO injection (g) or MDL28170 injection (h). Mean relative intensity of fluorescence of motoneurons in the spinal cord, on transected rats treated chronically with MDL28170 injection or DMSO injection (i). Student t test: *=p<0.05, **=p<0.01, ***=p<0.001.

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4. General discussion and perspectives

On this last section of the thesis, we are going to discuss the relevance of the main findings derived from this work, some general observations we may get from them, and the studies that would be needed to complete this knowledge. Four points stand out to us as important discussion points:

- Mechanisms behind KCC2 downregulation after a spinal cord injury: completing the puzzle
- Different effects of medications on the ventral and dorsal horn of the spinal cord
- Are there differences between neuropathic pain of central and peripheral origin?
- New venues for developing treatments against spasticity and neuropathic pain

4.1. Mechanisms behind KCC2 downregulation after a spinal cord injury: completing the puzzle

The work of my thesis has served to: 1) identify TCB-2 as capable of increasing KCC2 and reducing neuropathic pain of central origin, 2) determine that prochlorperazine does not have a strong effect on neuropathic pain, and 3) specify the role of calpain on KCC2 downregulation after a spinal cord injury (SCI). The discoveries made along the thesis are depicted in Figure 27.

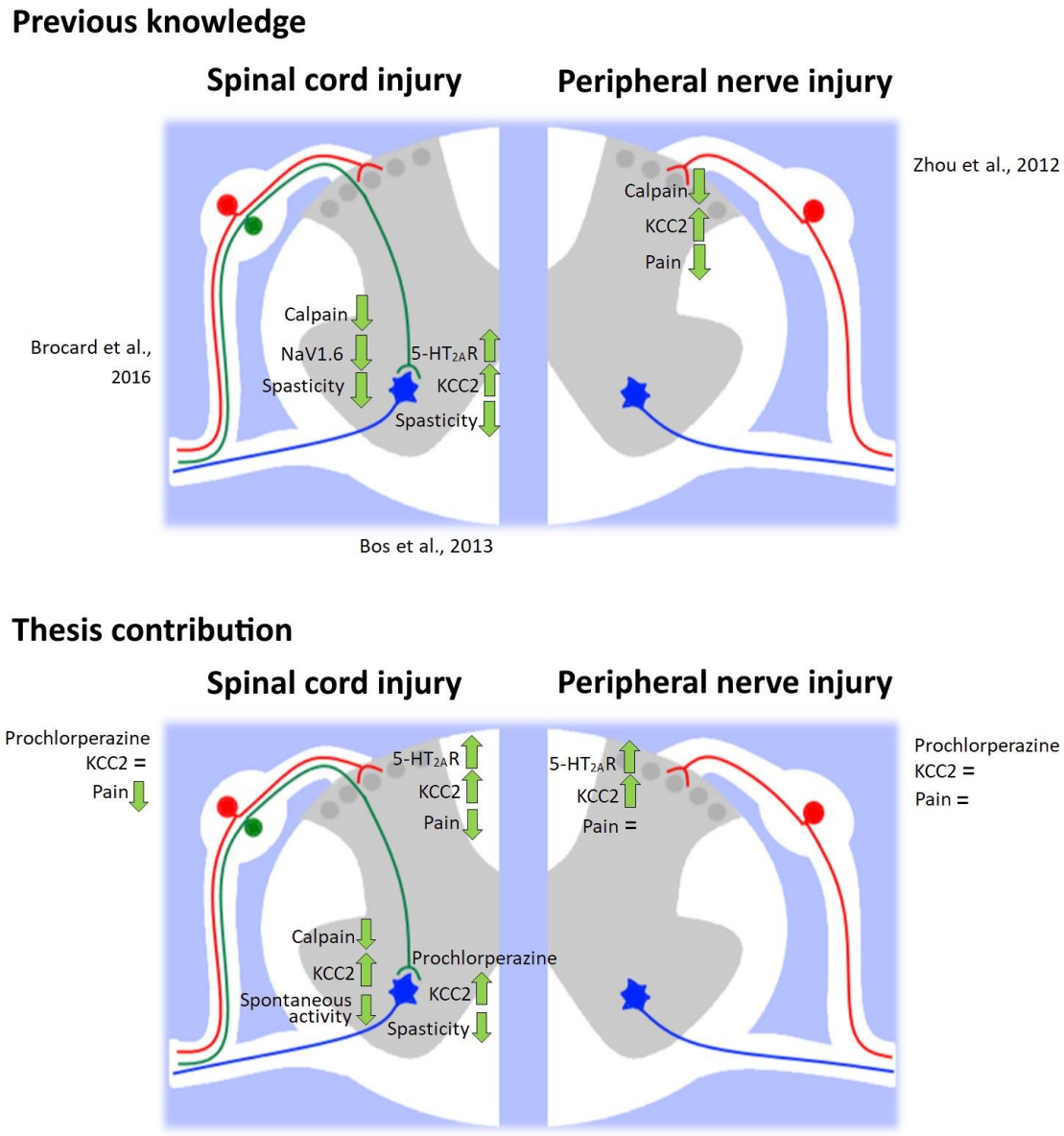

Figure 27. The contribution of this thesis to completing the map of mechanisms regulating KCC2 expression after a spinal cord injury. Previous studies had proven that activation of 5-HT_{2A} receptors can increase KCC2 in motoneurons and reduce spasticity (Bos et al., 2013), that calpain inhibition causes an upregulation of KCC2 in the dorsal horn and a downregulation of neuropathi pain (Zhou et al., 2012), and a downregulation of NaV1.6 which mediates an alleviation of spasticity (Brocard et al., 2016). This thesis has contributed to complete this information: activation of 5-HT_{2A} receptors can increase KCC2 in the dorsal horn and reduce neuropathic pain after a spinal cord injury, calpain inhibition can upregulate KCC2 in the ventral horn, reducing spontaneous activity, and finally, we identified a drug, prochlorperazine, which is capable of increasing KCC2 expression in motoneurons and reducing spasticity, and has a moderate effect on neuropathic pain after a spinal cord injury, although it does not show an upregulation of KCC2 in the dorsal horn after any type of injury.

4.1.1. TCB-2 increases KCC2 in the ventral horn after a spinal cord injury: the work of this thesis shows that it also increases KCC2 in the dorsal horn after a spinal cord or peripheral nerve injury

KCC2 expression and function on the cell membrane depends on its phosphorylation on certain residues, which is mediated by PKCs (Lee et al., 2007). Since this was described, there have been efforts to find ways of increasing this phosphorylation in order to boost the activity of KCC2. Bos et al. (2013) found that an agonist of 5-HT_{2A} receptors can increase KCC2 in motoneurons after a spinal cord transection and reduce spasticity.

We found that agonist of 5-HT_{2A} receptors TCB-2, can increase KCC2 in the dorsal horn laminae I and II, after a spinal cord and peripheral nerve injury (Sanchez-Brualla et al., 2018).

There is a well-known link between serotonin neurotransmission and neuropathic pain, specially regarding fibromyalgia or neuropathic pain with a psychological compound (Gilron et al., 2013; Sagheddu et al., 2015), and diabetic neuropathy (Sindrup et al., 1990)-. However, there have not been many studies about a possible link between serotonin and KCC2 expression. To our knowledge, in fact, the aforementioned study by Bos et al. (2013) is the only one that has been published.

An interesting venue to investigate after our study would be whether the intracellular signaling pathways mediating KCC2 upregulation after the activation of 5-HT_{2A} receptors are the same on both the ventral and dorsal horn of the spinal cord: the study by Bos et al. (2013) revealed that KCC2 upregulation by activation of 5-HT_{2A} receptors (5-HT_{2A}Rs) was mediated by a calcium-independent PKC, activation of PKC_{ε} hyperpolarized E_{IPSP}, and prevention of calcium-dependent PKC activation hyperpolarized E_{IPSP}, which is directly related to an upregulation of KCC2. To completely understand the mechanisms regulating KCC2 expression on the dorsal horn, it would be helpful to determine whether KCC2 upregulation on the dorsal horn is also PKC dependent, and if that was the case, whether the PKC-mediated signaling pathways are similar to the ones mediating this effect in the ventral horn.

PKC phosphorylates serine 940 on KCC2, which 1) stabilizes KCC2 on the neuronal membrane reducing its turnover rate (Lee et al., 2007), and 2) causes a conformational change that protects KCC2 from calpain hydrolysis (Lee et al., 2011). Mahadevan and Woodin (2016) have proposed that the main element upstream of KCC2 regulation by PKC signaling are G protein-coupled receptors (GPCRs), and they reviewed the knowledge that exists on this area. G_q-protein coupled receptors activate PKC and they have been related to KCC2 upregulation in several structures from the nervous system. In particular, apart from 5-HT_{2A} receptors, type

I metabotropic glutamate receptors (mGluR1 and mGluR5) (Banke and Gegelashvili, 2008), metabotropic zinc receptors (mZnRs) (Chorin et al., 2011; Saadi et al., 2012; Gilad et al., 2015), A3A-type adenosine receptors (Ford et al., 2015) and oxytocin (Leonzino et al., 2016) have proven to be able to increase KCC2 presence on the cell membrane and its function. Furthermore, activation of Gq-GPCR opioid receptors increases PKC activity in the dorsal horn (Mao et al., 1995; Mayer et al., 1999) and it has been proposed that the recovery of inhibition in the dorsal horn mediated by opioid drugs may be done through a KCC2 upregulation (Mahadevan and Woodin, 2016).

However, not all G_q-protein coupled receptors increase KCC2 function: activation of muscarinic acetylcholine receptors (mAChRs) on hippocampal neurons downregulates KCC2 function (Lee et al., 2010), although this needs to be interpreted carefully: muscarinic receptors were activated with carbachol, which is an agonist of all muscarinic receptors; M1, M3 and M5 are G_q-PCRs, but M2 and M4 are $G_{i/o}$-PCRs (Haga, 2013). This distinct effect has been related to the intracellular calcium concentration, and a significant release of calcium from intracellular stores could have a role on KCC2 downregulation: a robust activation of α1-adrenergic receptors (Hewitt et al., 2009), or relatively high concentrations of caffeine (Fiumelli et al., 2005) downregulates KCC2 function.

KCC2 inhibitors sometimes block G_q-GPCRs. VU0240551 may inhibit A1- and A3-type adenosine receptors (Delpire et al., 2009), and its analogue VU0463271 may bind to α1-adrenergic receptors (Sivakumaran et al., 2015). Whether the action of KCC2 inhibitory compounds depends on GPCR-mediated mechanisms or not, remains to be determined.

Considering this evidence, is there proof for or against the hypothesis that PKC activation by 5-HT_{2A} receptors may have the same effect on KCC2 expression on the ventral and dorsal horn? Data are not conclusive regarding that: 1) PKC is present in both the substantia gelatinosa and in the ventral horn on healthy human subjects, being more abundant in the dorsal horn (Krieger et al., 1995), 2) a chronic intrathecal injection of morphine that could induce morphine tolerance increased PKC on the DRG and the dorsal horn but not in the ventral horn of the spinal cord (Jin and Yu, 2010), 3) PKC has been shown to contribute significantly to the development of persistent pain in animal models (Coderre, 1992), 4) mGluR1/5-mediated activation of PKC on the dorsal horn seems to be specifically tied to regulation of neuropathic pain: it is antinociceptive against mechanical and cold hypersensitivity caused by a peripheral nerve injury, but not on the formalin test (Yashpal et al., 2001), 5) the subtypes of PKC are not homogeneously expressed in the spinal cord, for example, PKC_γ is mainly expressed in the dorsal horn and has been related to tactile allodynia (Polgár et al., 1999; Peirs et al., 2014).

An astonishing finding is that, in contrast to Bos et al. (2013) results, where activation of PKC_ε reduced excitability of motoneurons, Dutra et al. (2015) showed that PKC_ε was instead

necessary for the maintenance of chronic neuropathic pain. Another study showed PKC_{ε} is involved in the maintenance of the sensitization of TRPV1 receptor on neuropathic pain (Malek et al., 2015). Although these mediators activate a huge variety of intracellular signals, it is surprising that they show the opposite effects when comparing the ventral and the dorsal horn.

Another result from Bos et al. (2013) was that inhibition of calcium-dependent PKCs hyperpolarized E_{Cl}, facilitating inhibition. PKCγ is a calcium-dependent PKC tightly related to the development and maintenance of neuropathic pain (Basbaum, 1999). PKC_{γ} KO mice were shown to have a significantly reduced mechanical and thermal allodynia following chronic constriction injury of the nerve (Malmberg et al., 1997), which could indicate that, as in the ventral horn, an inhibition of calcium-dependent PKCs would recover inhibition.

TCB-2 is a potent 5-HT_{2A} ligand (Di Giovanni and De Deurwaerdère, 2017): it is unlikely that its effect is mediated by another receptor (like 5-HT_{2C}). Although there are several pathways that can activate PKC and therefore increase KCC2 function, regarding our results, we can only conclude that 5-HT_{2A}R should be implicated (Schulte et al., 2018).

To conclude with this part, beyond the molecular mechanisms behind KCC2 regulation after a spinal cord injury, it would be interesting to investigate the modification of the electrical properties of dorsal horn neurons by serotonin. For that reason, I would suggest to start by determining which type of neurons from the dorsal horn are affected by serotonin. Recent efforts have been directed to identifying and describing neuronal populations in the dorsal horn (Yasaka et al., 2010). It is possible that TCB-2 is only exerting its action on a particular population of neurons from the dorsal horn.

4.1.2. Prochlorperazine increases KCC2 in the ventral horn after a spinal cord injury, but it is not capable of increasing KCC2 in the dorsal horn on the same condition, and has a non-significant effect after a peripheral nerve injury

Prochlorperazine, an antagonist of dopamine receptors type II with wide actions over other type of receptors, has proven to increase KCC2 in the ventral horn and to reduce spasticity after a spinal cord transection (Liabeuf et al., 2017).

We found that prochlorperazine does not show to increase KCC2 on the dorsal horn after a spinal cord hemisection, and shows only a light, non-significant trend after a peripheral nerve injury. Furthermore, prochlorperazine does not show a clear effect against neuropathic

pain on any of the two models we used, it only increased the pain threshold significantly during 30 minutes at a relatively high dose: 2 mg/kg i.p.

The fact of not knowing the mechanism of action of prochlorperazine makes it difficult to determine why it does not show an effect on neuropathic pain. It has been reported by Ghelardini et al. (2004) that prochlorperazine exerted an antinociceptive effect against thermal and visceral pain, which was mediated by muscarinic receptors. However, activation of muscarinic acetylcholine receptors can downregulate KCC2 function (Lee et al., 2010). It is possible that a mechanism that would downregulate KCC2 expression on a naïve animal could be antinociceptive, if the downregulation of KCC2 was somehow compensated, rescued. However, on an animal affected by neuropathic pain, the loss of inhibition after the spinal cord injury may make any modification of KCC2 expression enough to avoid an analgesic effect of prochlorperazine.

However, as it was suggested on the previous section regarding 5-HT_{2A}, this drug may have different actions on different types of neurons: prochlorperazine has proven to increase KCC2 on motoneurons (Liabeuf et al., 2017) but in our results it did not prove any difference on KCC2 expression on dorsal horn neurons, compared to vehicle injection, which might exclude the possibility of causing a downregulation of KCC2. It seems clear that prochlorperazine has a different effect on cultured hippocampal neurons, spinal motoneurons and dorsal horn neurons. With our present data, we cannot know whether prochlorperazine has a different effect on different types of dorsal horn neurons.

In any case, we consider the data obtained about prochlorperazine as being still preliminary: for these results to be published we still need to increase the number of samples we tested, and to add some experiments, especially regarding the quantification of KCC2 expression after different doses of prochlorperazine. For example: we do not know the effect on KCC2 of 2 mg/kg i.p., the only dose that proved an increase on the pain threshold after a spinal cord injury. On a similar way, we need to increase the number of samples on the Western blot because the absence of significance when comparing prochlorperazine 10 mg/kg i.p. to vehicle-injected SNI animals, is most probably due to the size of the sample.

4.1.3. Besides downregulating KCC2 on the dorsal horn after a peripheral nerve injury, calpain downregulates KCC2 on the spinal cord after a spinal cord injury

After a spinal cord injury, there is an immediate increase in the expression and activity of calpains (Li et al., 1995, 1996; Banik et al., 1997a, 1997b, 1998; Ray et al., 1999; Schumacher et al., 1999; Shields et al., 2000). The injury causes a considerable increase in intracellular

calcium, which facilitates the activation of calpains (Young, 1985; Du et al., 1999) on neurons and glia (Li et al., 1995, 1996).

It has been described that calpains cleave KCC2 (Puskarjov et al., 2012; Zhou et al., 2012; Chamma et al., 2013). The incubation of brain homogenates with calpain 2 caused a cleavage of KCC2, shown by Western blot, which is blocked in the presence of MDL28170, the calpain inhibitor (Puskarjov et al., 2012). KCC2 expression is also downregulated when there is an increase in neuronal activity, which can be caused by an overactivation of NMDA receptors, increasing intracellular calcium. NMDA overactivation will activate calpains (Zhou et al., 2012), but also phosphatases which will dephosphorylate KCC2 at Serine 940 (Lee et al., 2011) this will cause KCC2 internalization and degradation by calpains (Chamma et al., 2013). It has been proposed that this dephosphorylation causes a conformational change, exposing the cleavage region to the calpains, inducing endocytosis, recycling and degradation of KCC2 (Chamma et al., 2013).

As it has been previously mentioned in the introduction (section 1.5.3.2. and 1.5.4.) calpain I cleavage of KCC2 has been related to neuropathic pain after a peripheral nerve injury (Zhou et al., 2012). Inhibiting calpain I with an intrathecal injection of calpeptin reduced stablished pain hypersensitivity measured by the Von Frey test and paw pressure test. However, calpain has proven to be implicated on a wide range of processes implicating inflammatory mediators and signaling pathways related to the development of neuropathic pain (Uçeyler et al., 2007, 2010; Xie et al., 2010; Jaggi and Singh, 2012; Chen et al., 2013, 2018a, 2018b; Won et al., 2014; Zang et al., 2015; Yuan et al., 2017), so it seems that its contribution to this pathology is complex and goes beyond KCC2 downregulation.

Our team has recently proved that calpains are linked to the development of spasticity after a spinal cord injury (Brocard et al., 2016): calpain cleaves NaV1.6, causing a hyperexcitability on motoneurons, which is related to spasticity, and a chronic treatment with MDL28170 reduces the hyperexcitability of the motoneurons and reduces also spasticity on spinal cord injured rats. On this thesis, we showed that the increase on spontaneous activity on the newborn rat spinal cord that is upregulated by the spinal cord injury can be reduced by MDL28170, and this treatment also prevented the downregulation of KCC2. Finally, a chronic MDL28170 treatment, prevented the downregulation of KCC2 in both newborn and adult spinal cord injury rats.

Spontaneous activity is an electrical activity generated and transmitted in all immature neural networks. It is thought to have a role in the network's "maturation" before it is functional (Blankenship and Feller, 2010). KCC2 is an important contributor to spontaneous activity: a downregulation or absence of KCC2 has been related to pathologically increased spontaneous activity. KCC2 KO mice show an increased spontaneous neuronal network activity at hippocampus, which has been considered epileptiform (Khalilov et al., 2011), and

an increased spontaneous activity at spinal motor networks, which however does not affect flexor-extensor alternating pattern in fictive locomotion (Stil et al., 2011). In our study, we showed that SCI exacerbated spontaneous activity and that MDL28170 reduced this spontaneous activity in vitro. In vivo, we showed that the number of spontaneous movements was increased after a SCI, and it could decrease, almost to the level of naïve animals, after MDL28170 injection. The link between calpain itself and spontaneous activity is more vague, though after a mild mechanical injury of a dissociated cortical neural network, calpain inhibition restored the normal activity of the network (Patel et al., 2012). These two studies are, to our knowledge, the only ones linking calpain and spontaneous or network activity before the work of this thesis: they indicate that calpain can reduce exacerbated spontaneous activity, but they do not specify the mechanism by which this happens, and they are exclusively studies in cell cultures. We think the data supporting a role for KCC2 on spontaneous activity is more conclusive, and therefore we think the development of exaggerated spontaneous activity after a SCI depends on KCC2 downregulation, mediated by calpain.

With this study, we complete the information that exists about the influence of calpain activation on KCC2 expression after traumatic injuries to the nervous system. We establish that calpain upregulation after a spinal cord injury downregulates KCC2 expression, and we correlate it to the increase in spontaneous activity on newborn rats, which can be related to the increase on network activity on adult rats.

4.2. Different effects of medications on the ventral and dorsal horn of the spinal cord

Following spinal cord injury, neuroplasticity, which is essential for the recovery of neurological functions, can cause spasticity and neuropathic pain. These two conditions share several features: have a late onset after the spinal cord injury, once they develop, they tend to become chronic, and some of the drugs against neuropathic pain have been suggested to be useful against spasticity, like gabapentin and pregabalin (Finnerup, 2017). However, pharmacological drugs that are useful against one of these conditions are not always effective against the other one: amytriptiline, a tricyclic antidepressant used against neuropathic pain, has been reported to increase spasticity, and baclofen, the gold standard against spasticity, has not proven an effect against neuropathic pain (Finnerup et al., 2015), although some clinical trials have reported so (Kumru et al., 2013). Cannabinoids have proven efficacy on spasticity caused by ALS (Mehta et al., 2016), but the evidence available about their efficacy against neuropathic pain is inconclusive (Finnerup et al., 2015).

Among the drugs that we tested along this thesis, TCB-2 proved to reduce both spasticity (Bos et al., 2013) and neuropathic pain (Sanchez-Brualla et al., 2018), while prochlorperazine can reduce spasticity (Liabeuf et al., 2017) but not neuropathic pain. We did not test the effect of an inhibition of calpain on neuropathic pain. Unfortunately, since we do not know the mechanism of action of prochlorperazine, we cannot make assumptions about it.

Molecular mechanisms behind spasticity and neuropathic pain may differ; besides, motoneurons and dorsal horn neurons are not similar cells, although they may share many common features and signaling pathways. Drug development from a researching point of view tends to take place as 1) discovering the pathological mechanism of one disease or syndrome, and 2) developing a drug that would act on this mechanism. However, from a clinical point of view, the treatments that are applied are the ones that work, although sometimes we may not know exactly how they function. The advantage of working in the whole animal allows to see the effect of the drugs we tested on measures of spasticity and neuropathic pain, and it will be interesting to use that information to direct future research.

Neuropathic pain and spasticity are not caused by a single alteration or a linear process: they involve complex neuroplastic changes that take place in the neural network (Finnerup, 2017). The reason why some drugs are useful against both spasticity and neuropathic pain and others are not, may be another clue in the direction of the mechanisms underlying these conditions. Many receptors, ionic channels and signaling pathways are affected and modified by neuroplasticity after the spinal cord injury. The fact that some medication can have an effect or not on these conditions, may indicate that some of these changes are more relevant than others. And, recovering the cited examples, that voltage-activated calcium channels are essential for the symptoms of both spasticity and neuropathic pain (pregabalin and gabapentin), that monoamines' signaling is more relevant for neuropathic pain than it is for spasticity (tricyclic antidepressants), and that activation of $GABA_B$ receptors can prevent spasticity, but not neuropathic pain.

4.3. Are there differences between neuropathic pain of central and peripheral origin?

Two of the drugs we tested, TCB-2 and prochlorperazine, showed more clear effects on neuropathic pain after a spinal cord hemisection than after a peripheral nerve injury. This situation poses a question: is it logical that the treatment against neuropathic pain had a different outcome on neuropathic pain of central origin, compared with the neuropathic pain of peripheral origin? Why could this happen?

Regarding human patients, it is expensive to conduct randomized controlled trials of marketed drugs, and it is hard to find data including enough patients and similar evaluation of pain medications to be able to conclude whether or not they are effective in human patients. Meta-analyses are extremely important and in the last years, several have taken place (Attal et al., 2010; Attal and Bouhassira, 2015; Finnerup et al., 2015; Aviram and Samuelly-Leichtag, 2017; Derry et al., 2017; Duehmke et al., 2017; Mücke et al., 2018). Given the scarcity of studies, evidence-based guidelines are generally oriented to treat neuropathic pain in general, without making a distinction between different conditions (Finnerup et al., 2015). This makes it hard to determine whether neuropathic pain of central or peripheral origin have different responses to treatments.

Nevertheless, there is one exception: it has been described that trigeminal neuralgia has a distinct response to drug treatment than other conditions causing neuropathic pain (Cruccu et al., 2008; Attal et al., 2010). To give an example, the first line pharmacological treatments that have been recommended in this condition are carbamazepine or ox-carbazepine (Cruccu et al., 2008; Attal et al., 2010), which are not among the first line treatments for general neuropathic pain (tricyclic antidepressants, serotonin-noradrenaline reuptake inhibitors, pregabalin and gabapentin). Although trigeminal nerve is considered part of the central nervous system for being a cranial nerve, it can be argued that its pathology (normally due to an entrapment and compression of the nerve) is more close to a peripheral nerve injury, so it may reinforce the idea that neuropathic pain caused by a peripheral or a central condition, may need different treatment.

There are some data available that allow to predict therapeutical options for treating neuropathic pain of central or peripheral origin, for example tricyclic antidepressants have proven to be efficient against neuropathic pain of central and peripheral origin, gabapentin and pregabalin have proven to be efficient against neuropathic pain of central but not peripheral origin, and serotonin and noradrenalin recaptation inhibitors and opioids have proven efficacy against neuropathic pain of peripheral, but not central origin (Baastrup and Finnerup, 2008). The evidence for other pharmacological treatments is very limited.

The fact that we find a higher effect of a 5-HT_{2A} agonist on central neuropathic pain, although inhibitors of monoamine recaptation are more effective against peripheral neuropathic pain, may have two possible explanations: 1) accumulation of serotonin in the spinal cord may activate 5-HT_{2A} receptors, but it may activate other types of serotonin receptors, which could be the ones mediating the effect we see, since serotonin can have varied effects in function of the receptor it binds to (Bos et al., 2013), and 2) the effect of monoamine recaptation inhibitors may be due to the accumulation of noradrenalin instead of serotonin. The first option does not seem probable because, precisely, with TCB-2 we are selectively activating a receptor that increases KCC2 expression, and when inhibiting the recaptation of serotonin, all serotonin receptors may be activated, while some downregulate

KCC2 (Bos et al., 2013); the only possibility is that actually 5-HT_{2A} agonism after a peripheral nerve injury was increasing neuropathic pain, which has been previously described by Aira et al. (2010) the same group also described that 5-HT_{2A} activation reduced the spinal effect of mu–opioids (Aira et al., 2012). So we would suggest that the presence of noradrenaline may act synergistically with the activation of 5-HT_{2A} receptors, or have an additive effect itself, which would increase the response to peripheral neuropathic pain. Probably the higher effect of serotonin and noradrenaline recaptation inhibitors is due to the preservation of the monoamine pathways after a peripheral nerve injury, which does not happen after a spinal cord hemisection.

It is not completely surprising that neuropathic pain of central or peripheral origin had different responses to treatments: the ion channels that are affected on peripheral afferents after a peripheral nerve injury are not the same affected by a spinal cord injury (Hains et al., 2003; Waxman and Hains, 2006; Todorovic and Jevtovic-Todorovic, 2013; Waxman and Zamponi, 2014). But the subject of peripheral vs. central sensitization is intrincated, since the mechanisms that mediate both processes are different, but both processes seem to take place after both a peripheral nerve injury and a spinal cord injury (Vranken, 2012). After a peripheral nerve injury (cut and suture) there is a modification on CCCs that "climbs" up the pain pathway, from the DRG until reaching the brain (Mòdol et al., 2014). After SCI, there is a persistent upregulation of voltage-activated sodium channel NaV1.8, expressed almost exclusively in primary afferent neurons. Selectively knocking down this gene, reduced hypersensitivity of pain withdrawal reflexes: this lead the authors to determine that persistent pain after spinal cord injury is maintained by primary afferent activity (Yang et al., 2014).

Since we cannot be sure of at which structure do the pharmacological treatments that we have tried have their action (central or peripheral), it is hard to say why some treatments act on both, and some do not. Further investigation in this subject may lead to a better understanding of the pathophysiology of pain and the action of analgesics. A preparation like the one used by Evans et al. (1993), in which the nerve is microdissected but left in situ, combined with the analysis of spinal local field potentials, could allow to determine whether the drugs tested have a peripheral effect or not: since the drugs could only act on the peripheral nerve. If they have an effect with this preparation, then, their action is –at least partially- peripheral.

4.4. New venues for developing treatments against spasticity and neuropathic pain

We developed the existing knowledge about three previously identified drugs that are capable of increasing KCC2: MDL-28170, TCB-2 and prochlorperazine. We found that KCC2 is downregulated by calpain after a spinal cord injury, we discovered that TCB-2 can increase KCC2 in the dorsal horn and decrease neuropathic pain of central origin (Sanchez-Brualla et al., 2018), and we found that prochlorperazine, despite being effective against spasticity (Liabeuf et al., 2017), is not worthy of being developed as a drug against neuropathic pain. Our experiments with these three drugs offer different opportunities for further drug development.

Prochlorperazine is the candidate that may be closer to preclinical development: its use is approved in human patients, its side effects are relatively safe (Merskey, 1997; Orr et al., 2015), a previous study from our laboratory identified it as a promising drug against spasticity (Liabeuf et al., 2017) and it has been patented for this purpose (WO 2015135947 A1). Unfortunately this drug seems not suitable for treating neuropathic pain.

TCB-2 is a hallucinogenic drug (Di Giovanni and De Deurwaerdère, 2017) and is not approved for a pharmacological use in humans, therefore it is unlikely that it will go into the market as a drug against spasticity or neuropathic pain. Since the papers about this drug have already been published, it is not possible to make a patent out of it. However, we contributed to the existing knowledge about serotonin receptors in neuropathic pain and maybe in the future, some good agonist of 5-HT_{2A}Rs that can undergo further preclinical development may be found or synthetized.

Finally, the target that is probably less developed for its translation into clinics is calpain: 1) MDL28170 is not a compound approved for its use on human patients, and even if it was, since the publication about its role on spasticity has already been made, it cannot be patented; 2) calpain is not an ideal pharmacological target, since it is not directly regulated by a surface receptor or protein, which is the case for the promising drugs targeting the pain system (Manning, 2004); 3) furthermore, if calpain was to be targeted, a very selective and subtle inhibitor needs to be found or synthetized: calpain cannot be completely inhibited because it is necessary for cellular processes; finding a specific inhibitor of calpain I or calpain II may be interesting too (Ono et al., 2016). If calpain is to be inhibited, it would be necessary to restrain and target the therapy as much as possible. A possible approach in the future, may be gene therapy with interference RNA against calpain mRNA.

All the pharmacological therapies that we tested are still far from being translated into clinics, but the work of this thesis helps increasing the knowledge about them, to facilitate that maybe within some years, one of them may be tested on a clinical assay. The interest of basic research on the treatment against spasticity and neuropathic pain is to provide more options of treatments for the medical doctors, so the probability of finding some therapy for every patient will increase. The aim of this thesis has been to contribute to this goal.

5. Conclusions

The three studies that are part of this thesis have produced the following main conclusions:

1) Activation of 5-HT_{2A} receptors increases KCC2 expression after a spinal cord injury and a peripheral nerve injury. This effect reduces neuropathic pain of a central origin, but does not affect neuropathic pain of peripheral origin.

2) Prochlorperazine does not increase KCC2 expression in the dorsal horn after a spinal cord or peripheral nerve injury. This drug has proven a short effect on neuropathic pain from central origin, only at a high dose, and no effect on neuropathic pain from peripheral origin.

3) Spinal cord injury increases spontaneous activity in the spinal cord of neonatal rats *in vitro*, and it also increases twitches recorded *in vivo*. Calpain inhibition reduces both spontaneous activity *in vitro* and twitches *in vivo*. These effects are related to an increase on KCC2 on the spinal cord, as shown on Western Blot and immunohistochemistry. The same effect on KCC2 takes place on adult rats. These results indicate that calpain cleavage of KCC2 is a major cause of KCC2 downregulation after a spinal cord injury.

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Annex I : Prochlorperazine induces KCC2 function and reduces spasticity after spinal cord injury

PROCHLORPERAZINE INCREASES KCC2 FUNCTION AND REDUCES SPASTICITY AFTER SPINAL CORD INJURY

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ABSTRACT

In mature neurons, low intracellular chloride level required for inhibition is maintained by the potassium-chloride co-transporter KCC2. Impairment of Cl^- extrusion following KCC2 dysfunction has been involved in many CNS disorders such as seizures, neuropathic pain or spasticity after a spinal cord injury (SCI). This makes KCC2 an appealing drug target for restoring Cl^-homeostasis and inhibition in pathological conditions. In the present study, we screen the Prestwick Chemical Library® and identify conventional antipsychotics phenothiazine derivatives as enhancers of KCC2 activity. Among them, prochlorperazine hyperpolarizes the Cl^- equilibrium potential in motoneurons of neonatal rats and restores the reciprocal inhibition after SCI. The compound alleviates spasticity in chronic adult SCI rats with an efficacy equivalent to the anti-spastic agent baclofen, and rescues the SCI-induced downregulation of KCC2 in motoneurons below the lesion. These preclinical data support prochlorperazine for a new therapeutic indication in the treatment of spasticity after SCI and neurological disorders involving a KCC2 dysfunction.

INTRODUCTION

Glycine and GABA are the major inhibitory transmitters in the CNS. The strength, as well as the polarity, of $GABA_A$ and glycine receptor-mediated chloride synaptic inputs depends on the regulation of the intracellular concentration of chloride ions ($[Cl^-]_i$). [1,2]. A low $[Cl^-]_i$ is needed for inhibition to occur and is primarily maintained in healthy mature neurons by the potassium-chloride co-transporters KCC2 in the plasma membrane, which extrude Cl^- [3,4]. In many neurological disorders associated with enhanced excitation, the global expression of KCC2 is downregulated thereby depolarizing the Cl^- equilibrium potential and reducing the strength of postsynaptic inhibition [5-8,8-13].We have previously reported that the disinhibition of motoneurons linked to a dysfunction of KCC2 after spinal cord injury (SCI) predisposes to spasticity [14,15], a motor disorder clinically characterized by spasms, clonus and hyperreflexia [16-18]. Spasticity negatively influences quality of life, but none of the currently available drugs (baclofen, tizanidine, botulinum toxin A…) are uniformly useful in reducing all symptoms associated to spasticity [19]. Therefore, efforts which aim at finding chemical activators of KCC2 to restore inhibition by lowering $[Cl^-]_I$ might provide a new effective therapy for spasticity and, more broadly, for a wide range of neurological disorders.

A recent high-throughput screening assay identified CLP257 as a positive modulator of KCC2 able to restore impaired Cl^- homeostasis in dorsal horn neurons derived from a rat model of neuropathic pain [20]. Likewise, activation of $5\text{-}HT_{2A}$ receptors with TCB-2 repairs alterations of Cl^- homeostasis in motoneurons of spastic rats following SCI [21]. Although promising, transfer of CLP257 or TCB-2 from research discovery to patients is a costly and arduous undertaking. The strategy of repurposing approved drugs for another indication reduces time and effort in bringing a new chemical entity from bench to bedside. To this end, we have screened molecules from the Prestwick Chemical Library® consisting of offpatent approved drugs to identify a new KCC2 enhancer in a bioavailable and safe formulation. We identified piperazine phenothiazine derivatives as pharmacologically active agents for activating KCC2. We focused on prochlorperazine and found that in addition to activate KCC2 function, it reduces spasticity in rats with SCI.

MATERIAL AND METHODS

Animals

Wistar rats were housed under a 12 h light/dark cycle in a temperature-controlled area with *ad libitum* access to water and food. All animal care and use conformed to the French regulations (Décret 2010-118) and were approved by the local ethics committee CEEA 71 - Comité d'éthique en neurosciences - INT Marseille (authorization Nb A9 01 13).

Surgery

The surgery and postoperative care were described previously. [18] Briefly, we anesthetized adult female Wistar Rats (225/250g Charles River, Burlington MA USA) with a mixture of ketamine (Imalgen®, Merial, Duluth, Georgia, USA, 50 mg/kg ip) and medetomidine (Domitor®, Janssen Pharmaceutica, Beerse, Belgium, 0.25 mg/kg ip) and neonates by hypothermia. Amoxicillin was administered after anesthesia (150 mg/kg, s.c.; Duphamox LA® ; Pfizer Inc, NY, USA). After laminectomy, the spinal cord was transected at T8 thoracic level (for neonates at the day of birth). In some adult rats, a catheter was inserted with the distal end in proximity to the lumbar enlargement. Sham-operated animals were subjected to all procedures except the spinal cord transection.

Drugs

We purchased: perphenazine, prochlorperazine dimaleate (PCPZ), fluphenazine, trifluoperazine dihydrochloride, thioproperazine dimesylate, thiethylperazine from Prestwick Chemical® (Illkirch, France) or Tocris (Bristol, UK); ouabaine, bumetanide, acetophenazine, baclofen, DIOA [(dihydroindenyl)oxy]alkanoic acid] and NEM (NEthylmaleimide) from Sigma Aldrich (St Louis, MO, USA) ; perazine from Santa Cruz Biotechnology (Dallas, TX, USA); 2-amino-5-phosphonovaleric acid (AP5) and 6-cyano-7-nitroquin-oxaline-2,3-dione (CNQX) from Abcam (Cambridge, UK). AP5 and CNQX were prepared in aCSF, ouabaine in H_2O, and bumetanide, phenothiazines piperazine, baclofen and DIOA in DMSO.

Drug administration and assessment of spasticity in adult spinal rats.

We used the rate-dependent depression (RDD) of the H-reflex as a measurement of spastic symptoms. [15] A pair of stainless steel stimulating needle electrodes was inserted transcutaneously into the surroundings of the tibial nerve, a recording electrode into the flexor digitorum brevis muscle (FDB, flexor of the lateral four toes) and the reference electrode was placed s.c. into the foot. We stimulated the tibial nerve for 0.2 ms at 0.2 Hz with increasing current intensities and determined the intensity necessary to get a maximal H response. Then, we used this intensity for trains of 12 stimulations at 0.2, 1, 2 and 5 Hz. Normalization of the amplitude of responses (the first three discarded) to the controls evoked at 30-s intervals (mean of five pulses) determined the level of the RDD.

Chronic spastic rats were treated with a single i.v. administration of PCPZ (10µg/kg in saline + 0.1% DMSO), baclofen (2mg/kg; in saline + 0.1% DMSO) or vehicle (saline + 0.1% DMSO). DIOA was i.t. delivered (40 µg in DPBS, calcium, magnesium + 10% DMSO). The RDD was evaluated twice before drug injection and at intervals of 20, 40, 60 and 80 min after the drug injection.

In vitro electrophysiological recordings.

The spinal cord below T8 was isolated from neonatal rats [postnatal day (P)4–P6] as previously described (Vinay et al., 1999) and transferred to the recording chamber perfused with an oxygenated (95% O_2/5% CO_2) aCSF composed of the following (in mM): 130 NaCl, 4 KCl, 3.75 $CaCl_2$, 1.3 $MgSO_4$, 0.58 NaH_2PO_4, 25 $NaHCO_3$ and 10 glucose, pH 7.4 (24-26°C). Extracellular recording/stimulation were made from lumbar L3-L5 ventral (VR) and dorsal (DR) roots by contact stainless steel electrodes insulated with Vaseline. A glass suction electrode was also used to stimulate the ipsilateral ventral funiculus at the L2–L3 level. AC recordings from VR were amplified (×2,000) and band-pass filtered from 70 Hz to 3 kHz. Current clamp recordings from L4–L5 motoneurons were performed in DCC mode with an Axoclamp 2B amplifier (Molecular Devices, Sunnyvale, CA, USA). Motoneurons were identified by an antidromic response to VR stimulation. Microelectrodes were filled with 2 M K-acetate (90–150 MΩ). Intracellular signals were sampled at 10 kHz (Digidata 1440a; Clampex 10 software; Molecular Devices).

To estimate the ionic gradient of Cl^- efflux in motoneurons, we measured the reversal potential of the inhibitory postsynaptic potential (E_{IPSP}) evoked by ipsilateral ventral funiculus stimuli and pharmacologically isolated with CNQX (10 µM) and AP5 (50-100 µM). The amplitudes of IPSPs measured at various holding potentials (500-ms-long current pulses) were plotted to obtain E_{IPSP} from the regression line.

The reciprocal inhibition was assessed by using a variation of the monosynaptic model technique [22]. We evoked monosynaptic reflexes in VR L5 by a supramaximal stimulation of the DR L5 (0.3 ms duration). When this stimulation was preceded by a stimulation of the DR L3 (2 x times the threshold (T) for the monosynaptic reflex) with a delay ranging from 0 to 40 ms, the amplitude of the L5 monosynaptic reflex was reduced, indicative of the reciprocal inhibition. Normalization of the amplitude of the L5 monosynaptic reflex recorded before and after the conditioning DR L3 stimulation were used to assess the reciprocal inhibition.

Stable mammalian cell line expressing KCC2

The HEK-293 cells stably expressing KCC2 were grown to confluence at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium/Nutrient mixture F-12 supplemented with foetal bovine serum (10%), penicillin (50 U/m) and streptomycin (50 pg/ml; Life technologies, Carlsbad, CA, USA). Puromycin (20µg/ml, Life technologies) was added to select KCC2-expressing cells.

Thallium (Tl_+) influx assay

The FluxOR™ Thallium detection kit from Life Technologie was used. Briefly, HEK-293 cells were loaded with the Tl_+-sensitive fluorescent dye FluxOR for 90 min. Once the unloaded dye was removed, cells were supplemented with assay buffer containing ouabain (200 µM) and bumetanide (10 µM). Cells were then plated (100.000 cells/75 µl/well) in clear-bottomed, black-walled 96-well plates (Greiner Bio-One, Monroe, NC, USA). Compounds to be assayed from the Prestwick library collection were added (5 µl/well) by using the Biomek® NX Laboratory automation workstation (Beckman Coulter, Villepinte, France) and incubated for 15 min before measuring an initial baseline fluorescence (490 nm excitation and 520 nm emission) onto the screening system POLARstar omega (BMG Labtech, Ortenberg, Allemagne). The fluorescence signal was initiated by adding 20 µl of a 5×Tl_2SO_4 solution (final concentration of 2 mM Tl_2SO_4) and recorded 30 minutes after. The test was conducted for each plate using 2 columns on which cells on half were treated with 33 µM NEM, an accepted activator of KCC2, [23] as positive control and

the other half with no compounds as negative control. After subtracting each well's fluorescence obtained after compound addition (baseline fluorescence), activity was expressed as percentage change of Tl_+ influx in compoundtreated cells versus influx in vehicle-treated cells. Hits were selected as compounds that cause an increase >20% without apparent effect on parental untransfected HEK cells. Hits were retested in triplicate. Positive compounds were further evaluated by testing them at varying concentrations. To evaluate the quality of the Tl_+ flux assay, the value of Z' factor was calculated using the following equation: Z'=1-[3 SD of sample + 3 SD of control]/[mean of sample-mean of control]. At the end of the fluorescent assay, cell viability was checked by the PrestoBlue® Cell Viability Reagent (Life technologies) protocol. Briefly, cells viability in 96-well plates was expressed as the 570-nm absorbance ratio of drug-treated cells compared with cells incubated in the absence of drug (medium). The result was expressed as percentage of the control (defined as 100%). The initial screen of 880 compounds was performed at 2µg/ml (3 to 10µM), 12µg/ml (18 to 60µm) and 20µg/ml (30 to 100µM).

Immunostaining KCC2

Adult rats were perfused transcardially with 4% paraformaldehyde (PFA, Sigma Aldrich) in PBS (bioMérieux SA, Marcy l'Etoile, France) at 4°C. Tissues were post-fixed overnight (16 hours) in 4% PFA and washed 3 times with PBS. Lumbar spinal cords (L4-L5) were then embedded in 4% low melting agarose and sectioned (30 µm) with a vibratome. Sections from all samples were mounted on the same poly-lysine coated slides and processed for immunohistochemistry. Slices were thus (i) dried overnight, (ii) permeated (45 min) in PBS with 3% Bovin Serum Albumin (BSA, Sigma Aldrich) and 0.2% Triton x-100 (iii) washed in PBS (2 x 5 min), (iv) preincubated (1 h) in PBS with normal goat serum (3% BSA, 1/100 normal Goat serum), (v) washed and incubated overnight at room temperature in the affinity-purified rabbit anti-KCC2 polyclonal antibody (diluted 1:500; Merck-Millipore Billerica, Massachusetts, USA; Cat# 07-432, RRID:AB_310611), (vi) washed in PBS (3 x 5 min) (vii) incubated (1 h) with a goat anti-rabbit IgG conjugated to Alexa-546 (diluted 1:500; Life Technologies), (viii) washed in PBS 3x5 min, and finally (ix) coverslipped with a gelatinous aqueous medium. In control experiments, the primary antiserum was replaced with rabbit immunoglobulin fraction during the staining protocol. Images were taken using the confocal microscope Zeiss, LSM500 at X60 magnification, digitized in stacks of 0.5 µmthick optical sections and processed with the Zen (Zeiss) software.
We quantified KCC2 immunolabeling of motoneuron (identified as the biggest cells in the ventral horn). Line scans show peaks of intensity at the periphery of the cell body (Fig. 3D), likely plasma membrane. We drew a region of interest within the presumed plasma membrane and measured the mean pixel intensities in this delimited area [15,18,21]. Values from Sham, SCI and PCPZ treated-rats were then normalized to the mean value measured from sections of SCI rats on the same slide.

Statistical analysis

Group measurements were expressed as means ± s.e.m. We used Mann-Whitney tests, Kruskal-Wallis tests and one- or two-way ANOVAs with or without repeated measures as appropriate (Graphpad Prism 5 software). For all statistical analyses, the data met the assumptions of the test and the variance between the statistically compared groups was similar. The significance was set at $P < 0.05$.

RESULTS

Phenothiazine piperazine derivatives upregulate KCC2 function

To identify KCC2 activators from the Prestwick Chemical Library®, we used a standard thallium (Tl^+) flux assay reported as suitable for high throughput screening in HEK-293 cell line expressing KCC2 (Delpire et al., 2009;Zhang et al., 2010; Medina et al., 2013). In principle, upon the activation of KCC2 in reverse mode, the uptake of Tl^+ into cells releases a fluorescent signal from the pre-loaded FluxOR™ dye which reports the activity of KCC2. The initial screen of 880 compounds performed at the concentration of 12 µg/ml (18 to 60 µM), yielded 121 hits with more than 20% increase in the fluorescent signal (**Fig.1a**). The Z factor with an average of 0.83 ± 0.004 (range 0.75 to 0.92) conformed to the assay quality requirement (**Fig. 1b**). We then selected 27 compounds among these 121 hits and that were ineffective on naïve HEK293-cells. A secondary screen tested each compound in triplicate and confirmed the activity of approximately 60% of them (17/27). A classification of positive hits revealed 4 molecules (perphenazine, prochlorperazine dimaleate, trifluoperazine dihydrochloride and thioproperazine dimesylate) that belong to antipsychotic piperazine phenothiazine derivatives characterized by a three-ring structure and a piperazine substituent on nitrogen (**Fig. 1c**). Based on their commercial availability, 8 piperazine phenothiazines were purchased for further evaluation (chemical structure of each compound is illustrated in **Fig. 1c**). To determine their potency, dose-response curves (range: 0.1 to 50 µM; example for prochlorperazine in **Fig. 1d**) were performed in parallel to a counter screen to evaluate their cytotoxicity. All compounds were effective with the minimal effective concentration ranging from 3 to 12 µM (see **Fig. 1c**). Prochlorperazine, thiethylperazine, fluphenazine and perazine were the most potent activators (~140% of basal activity). Among these molecules, prochlorperazine dimaleate (termed henceforth as PCPZ) exhibited the most potent effect with the lowest minimal effective concentration (3 µM) without apparent cytotoxic effect at concentration below 200 µM (see **Fig. 1c**).

Therefore, from a drug repurposing point of view, PCPZ was selected for further experimental investigations of the molecule.

PCPZ enhances Cl^- extrusion capacity in motoneurons

By means of intracellular recordings, we investigated whether PCPZ could enhance Cl^- extrusion capacity of lumbar motoneurons recorded in *in vitro isolated whole spinal cord preparation* from intact neonatal rats [postnatal day (P)4–P6]. The effect of PCPZ was examined by determining the reversal potential of inhibitory post-synaptic potentials (E_{IPSP}) as a measure of the KCC2 function [24]. We found that PCPZ (10 µM) hyperpolarized E_{IPSP} within 20–25 min by -3.3 ± 0.66 mV (**Fig. 2a**; n=6; *p<0.05 Wilcoxon test). Given that there was no change of the resting membrane potential (V_{rest}; n=6; **Fig. 2a**; p>0.05, Wilcoxon test), the net driving force for Cl^- (E_{IPSP}-V_{rest}) increased significantly (n=6; **Fig. 2a**; *p<0.05, Wilcoxon test), potentially strengthening the inhibitory synaptic transmission.

PCPZ strengthens inhibition and decreases spasticity in rats with SCI.

To test the ability of PCPZ to enhance the post-synaptic inhibition, we took advantage of the recent *in vitro* demonstration that the reciprocal inhibition in the spinal cord is markedly reduced after SCI [25]. Typically, flexor-related Ia interneurons [recruited by stimulation of the dorsal root (DR) L3] inhibits L5 extensor-related motoneurons [26] such as when the DR L5 stimulation was preceded by DR L3 stimulation, there was a reduction of the DR L5-evoked monosynaptic response (**Fig. 2b**). This reduction almost disappeared in spinal cord isolated from neonatal rats that underwent SCI

at birth (**Fig. 2b**). In those rats, the bath application of PCPZ at a concentration as low as 10 µM significantly reduced the amplitude of the L5 monosynaptic response to values seen in intact animals (**Fig. 2b**, n=6; ###, p < 0.001; two-way repeated measures ANOVA). Altogether, these results indicate that PCPZ is able to restore the reciprocal inhibition after neonatal SCI.

The Hoffmann reflex (H-reflex), resulting from the monosynaptic activation of motoneurons by Ia afferents, declines in amplitude over repetitive stimulation and becomes more depressed as the frequency of stimulation increases [27,28]. The lower rate dependent depression (RDD) of the H-reflex in spastic *patients* and *animals* identifies RDD as a reliable assessment of spasticity [15,29,30]. As the low RDD is indicative of a spinal disinhibition and a KCC2 dysfunction (Boulenguez et al., 2010), we tested whether the hyperpolarizing shift of E_{IPSP} in motoneurons by PCPZ would restore the RDD in adult rats with chronic SCI (21 d post-SCI). The i.v administration of PCPZ (10 µg/kg) or vehicle (DMSO) did not affect the maximal amplitude of H-reflex and M-wave (**Table 1**) but significantly increased the RDD to values seen in SCI animals (**Fig. 3a**). At frequencies of 1 Hz and higher, the amplitude of the H wave over repetitive stimulation was smaller than after vehicle injection, with a maximal effect 80 min after PCPZ administration (*n* = 9 vehicle, n=8 PCPZ; **Fig. 3a**; *2-way ANOVA test with bonferroni post-test; ns p > 0,5; *p < 0.5; ** p < 0.01; ***p < 0.001*). To identify the mechanisms underlying this effect, we i.t. administered DIOA (40 µg), a highly specific KCC2 blocker [31] 50 minutes after PCPZ injection. KCC2 blockade reversed PCPZ effect (**Fig. 3b**, n=6 in each group), which is consistent with the hypothesis of KCC2 mediating the E_{IPSP} shift induced by PCPZ. Taken together these results indicate that PCPZ reduces spasticity essentially through a modulation of KCC2.

PCPZ increases the expression of KCC2 in motoneurons below the SCI.

We used immunohistochemistry to quantify the effect of PCPZ on the expression of KCC2 on lumbar motoneurons after SCI. Peaks of KCC2 labeling were detected in the periphery of cell bodies of sham, SCI and PCPZ treated rats showing a membrane labeling (**Fig. 3c**). Levels of KCC2 labeling surrounding motoneurons were higher in PCPZ-treated compared to vehicle-treated animals (10 µg/kg; i.v. 80 min), but did not return to the level found in sham-operated rats (**Fig. 3c**, Sham vs SCI + prochlorperazine * p < 0,05; SCI vs SCI + prochlorperazine *** p < 0.001; Kruskal–Wallis test, Dunn's post-tests; n = 231 motoneurons in each group).

PCPZ is as effective in reducing spasticity as baclofen.

From a translational perspective, the therapeutic profile of a new antispastic drug cannot be defined solely on the basis a comparison to vehicle-treated rats. We thereby assessed PCPZ potential advantages with respect to existing antispastic drug. The $GABA_B$ receptor agonist, baclofen, is the most effective and widely used drug for the treatment of spasticity after SCI [32-37]. We compared the relative efficacy of PCPZ to that of the "gold standard" baclofen (2mg/kg, i.v) in restoring the RDD. The reduction of the RDD was more pronounced in PCPZ-treated animals at 1 Hz and remained comparable to that of baclofen treated rats at higher frequencies of stimulation (**Fig. 3d**, 2-way ANOVA test with bonferroni post test; ns p > 0,5; *p < 0.5, n = 6 in each group). These results suggest that antispastic effects of PCPZ is at least similar to that of baclofen.

DISCUSSION

We identified PCPZ as a positive modulator of KCC2 capable to restore endogenous inhibition of motoneurons after SCI. PCPZ leads to a hyperpolarizing shift of E_{IPSP} and reduces SCI-induced

spasticity. The observed effect of PCPZ on spasticity likely results from an enhancement of KCC2 function. In line with this assumption, PCPZ increases cell surface expression of KCC2 in motoneurons after SCI and blocking KCC2 prevents the effect of PCPZ to alleviate spasticity. Likewise, the reciprocal inhibition, downregulated after SCI and determined by the level of KCC2 function, [25] is restored by PCPZ.

Deficits in KCC2 function have been documented in brain and spinal cord injuries, [15,38] in temporal lobe epilepsy [39,40] and in neuropathic pain. [41-44] Accordingly, KCC2 appears to be an attractive target to restore endogenous inhibition in pathological conditions. Previous investigations showed KCC2 as a druggable target in modulating $[Cl^-]_i$ for the development of therapeutics. The 5-HT_{2A} high-affinity agonist, TCB-2, reduces spasticity after SCI by boosting the KCC2 function [21] while CLP257, a new KCC2 enhancer, alleviates hypersensitivity in a rat model of neuropathic pain. [20] Unfortunately, these two compounds are not approved human drugs. Conversely, PCPZ is currently licensed for its neuroleptic actions with an adult daily dosage ranging from 15 to 150 mg.[45] Typical antipsychotic drugs such as PCPZ may produce extrapyramidal side-effects at clinically effective doses against schizophrenia. [45] However, the antispastic effect of PCPZ, equivalent to that of baclofen, was achievable with a low human equivalent dose estimated at 1.6µg/kg for a 70 kg human [HED = Animal Dose (mg/kg) x (animal wt/human wt in kg) [0.33]; see Nair and Jacob 2016)]. The phenothiazine ring possesses a high degree of lipophilicity and these drugs easily cross the blood-brain barrier [46-48]. Chlorine substitution of C-2 atom of prochlorperazine confers less anti-psychotic activity than other substituents (X = -SO2NR2 > -CF3 > -CO-CH3 > -Cl). [48] Collectively, the use of PCPZ at lower doses represents an effective and tolerable therapeutic strategy for reducing spasticity, likely better than the common *anti-spastic agent* baclofen which often causes sedation and dizziness. [35,36]

The mechanism by which PCPZ modulates KCC2 function in motoneurons has not been investigated in the present study. The therapeutic potential of PCPZ in reducing psychosis has been mainly attributed to its anti-dopaminergic action. [49,50] However, the main source of dopamine in the mammalian spinal cord arises from the diencephalon and disappears after SCI. [51,52] Furthermore, dopamine appears to be anti-spastic by restoring the RDD in chronic SCI rats, [53] thereby suggesting that inhibition of dopaminergic receptors unlikely accounts for the upregulation of KCC2. Antipsychotic drugs have been also found to display anti-serotoninergic actions most notably through an inverse agonist activity of 5-HT_{2C} receptors. [54]. Interestingly, 5-HT_{2C} receptors in motoneurons become constitutively active after SCI and contribute to muscle spasms. [55] We recently showed that blocking 5-HT_{2B}Rs and 5-HT_{2C}Rs hyperpolarized E_{IPSP} in rats with SCI. [21] However, PCPZ is one of the typical antipsychotic devoided of inverse agonist activity [54] and thus unlikely modify KCC2 function via 5-HT_{2C} receptor-mediated mechanisms.

PCPZ may also interact indirectly via some intermediate signaling molecules, as suggested by the potent inhibitory action of phenothiazines on Ca^{2+}-dependent PKC [56]. The phosphorylation of KCC2 by PKC constitutes a powerful and dynamic mechanism to influence KCC2 function. [57] Following SCI, a tonic activation of a Ca^{2+}-dependent PKC signaling pathway alters motoneuronal Cl^- homeostasis by depolarizing shift E_{IPSP}.[21] A similar relationship between Ca^{2+}-dependent PKC isozymes and the positive shift of E_{Cl} has been pointed out in hippocampal neurons. [58] Therefore, the potency of PCPZ in restoring KCC2 function may be connected with its inhibitory action on Ca^{2+}-dependent PKC. [56]

In sum, we provide strong preclinical evidence for translation to chronic SCI subjects, a process that will be facilitated as PCPZ is already an approved compound and has been recently patented for treating spasticity (WO 2015135947 A1). [59]

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Author Disclosure Statement

No competing financial interests exist.

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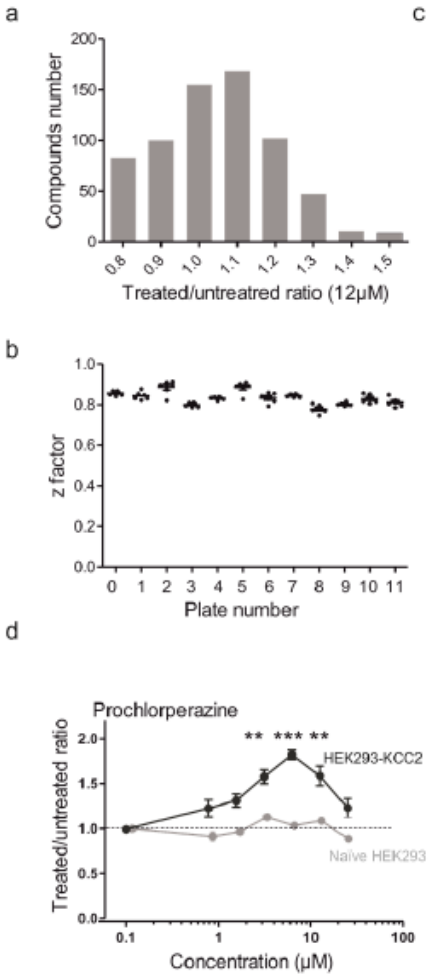
TABLE 1
Maximal amplitudes of M and H waves (mV)

		M-Max						H-max					
		Before inject.	T0	T20	T40	T60	T80	Before inject.	T0	T20	T40	T60	T80
PCPZ	Mean	5.5	5.93	5.50	5.44	5.38	5.33	1.28	1.49	1.38	1.36	1.31	1.54
	SEM	0.37	0.44	0.43	0.43	0.39	0.41	0.13	0.11	0.11	0.12	0.08	0.14
DMSO	Mean	6.48	6.60	6.06	5.89	6.30	6.22	1.81	1.74	1.49	1.61	1.74	1.55
	SEM	0.73	0.55	0.63	0.58	0.49	0.50	0,31	0.20	0.22	0.29	0.32	0.21
PCPZ + DIOA	Mean	8.79	8.20	8.62	8.46	8.22	7.98	2.70	2.61	2.51	2.40	2.39	2.43
	SEM	1.63	1.43	1.21	1.26	1.47	1.48	0.33	0.26	0.23	0.25	0.33	0.30
PCPZ	Mean	5,76	5.46	4.91	5.03	4.95	4.63	1.52	1.50	1.58	1.40	1.38	1.35
	SEM	0.68	0.58	0.47	0.60	0.61	0.81	0.16	0.17	0.27	0.17	0.16	0.17
Baclofen	Mean	6,10	5.84	5.46	5.15	5.28	5.20	1,80	1.67	1.37	1.26	1.213	1.29
	SEM	0,73	0.55	0.80	0.53	0.64	0.60	0.30	0.21	0.26	0.13	0.11	0.11

FIGURES



c

Phenothiazine piperazine	µM	Ratio	TC 50
Prochlorperazine	3	1,57	>200µM
Thiethylperazine	6	1,38	30µM
Perphenazine	6	1,15	50µM
Trifluoperazine	6	1,10	>200µM
Fluphenazine	12	1,4	50µM
Perazine	12	1,35	>200µM
Acetophenazine	12	1,24	80µM
Thioproperazine	12	1,21	80µM

Figure 1: Prestwick library screening on KCC2 using the thallium flux assay in HEK-293 cells. (a): Distribution of identified hits in a total of 880 compounds. The x-axis represents the potentiation activity of the hits on the KCC2-mediated fluorescence signal. Molecules with a KCC2 activity below 80% of the control level are not shown. (b): Z factors of the thallium flux assay. (c): Dose-response curve of prochlorperazine on KCC2 function, in KCC2 expressing (black) and in naïve (grey) HEK293 cells. K+ uptakes were measured in triplicate at different concentrations of drug ranging from 0.1 to 25 µM. Flux was expressed as a percentage of control (flux without drug). (d): Chemical structures of selected compounds with their minimal effective concentration (µM) on the activity of KCC2 (expressed in % of control) and their toxic concentration for half of the sample population (TC50).

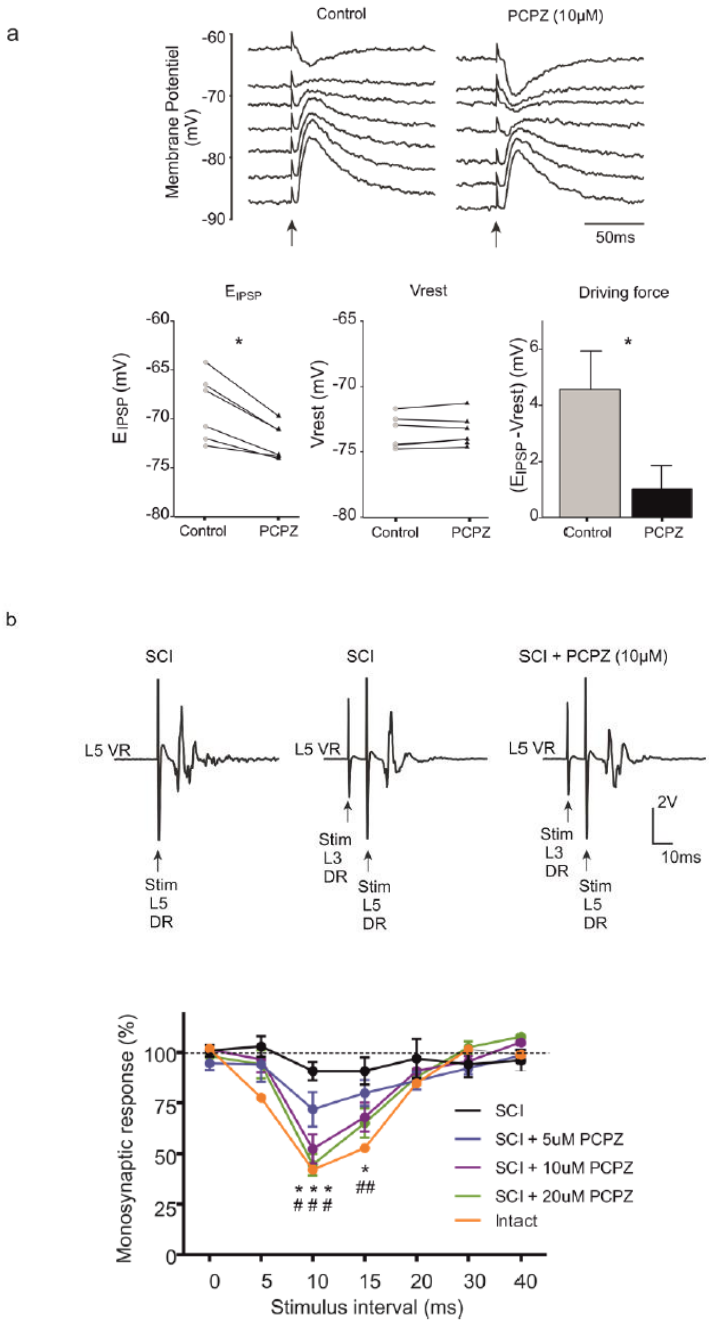


Figure 2: Prochlorperazine hyperpolarizes EIPSP and increases the strength reciprocal inhibition. (a) Top: Inhibitory post-synaptic potentials (IPSPs) evoked by stimulation of the ventral funiculus of the spinal cord (arrow) at different holding potentials in a motoneuron from a P6 intact rat before and after adding PCPZ (10 µM). Bottom: EIPSP, Vrest and driving force (EIPSP-Vrest) measured in six motoneurons before and after PCPZ. Measurements were done 20-25 minutes after adding PCPZ. *P < 0.05 (Wilcoxon paired test). (b) Top: Representative traces of extracellular recordings from a L5 ventral root (VR) of a P6 rat with SCI. Traces show the responses to the L5 dorsal root (DR) stimulation without (left) or with a conditioning stimulation of the L3 DR before (middle) or after (right) adding PCPZ (10 µM). Bottom: Amplitude of the response plotted against the interstimulus interval. Statistical differences between SCI and PCPZ conditions are noted above delays (Wilcoxon test; * p<0.05, 10uM vs. SCI; *** p<0.001, 10uM vs. SCI; ## p<0.05, 20uM vs. SCI; # # # p<0.001, 20uM vs. SCI).

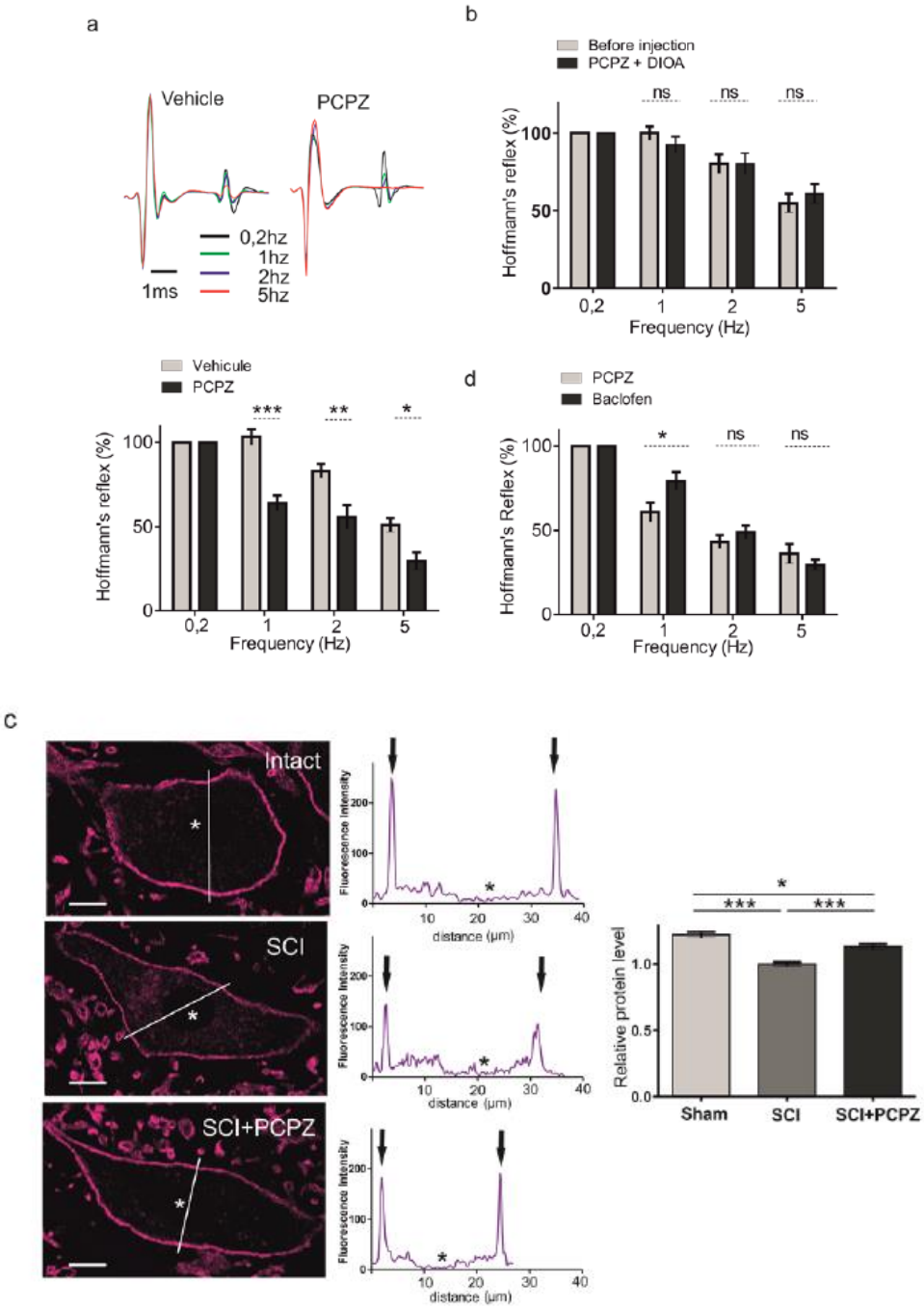


Figure 3: PCPZ decreases spasticity in rats with SCI via KCC2. (a) Prochlorperazine increases the RDD of the H reflex in chronic SCI adult rats. Top: M an H waves evoked in control (vehicle) and 80 min after PCPZ i.v injection (10 µg / kg). Each trace is the mean response to 3 last consecutive stimulations at 0.2, 1, 2, or 5 Hz. Bottom: Mean relative amplitudes of the H reflex after (80 min) the injection of vehicle (grey) or PCPZ (black, n= 9 rats with vehicle; n= 8 rats with PCPZ). (b): Pharmacological blockade of KCC2 by DIOA, (i.t. injection) 50 minutes after i.v. injection of PCPZ, inhibits the PCPZ-induced increase of the RDD (n=6 rats in each group). Mean relative amplitudes of the H reflex before injection (grey) or after (80 min) the injection of PCPZ + DIOA. (c): Left: Example single optical sections showing immunostaining of KCC2 of lumbar motoneurons (L4–L5) in sham-operated (intact), in vehicle-treated SCI rats (SCI) and PCPZ-treated SCI rats (SCI+PCPZ). Scale bars, 10 µm. Line scans illustrate the KCC2 distribution profile. Arrows indicate peaks of KCC2 immunofluorescence, stars indicate nuclei position.

Right: Relative immunostaining intensities of the plasma membrane obtained with KCC2 antibody in sham-operated rats (white) and SCI rats vehicle-treated (grey) or PCPZ-treated (black) and normalized to vehicle-treated SCI rats. (n = 231: 77 cells per rat, 3 rats per group). Data are mean ± s.e.m. *, p < 0,05; ***p < 0.001 (Kruskal–Wallis test, Dunn's post tests). (d): PCPZ is as effective in reducing spasticity as baclofen. Mean relative amplitudes of the H reflex after (80 min) of PCPZ (grey) or baclofen injection (black, n= 6). 2-way ANOVA test with bonferroni post test; ns p > 0,5; *p < 0.5; ** p < 0.01; ***p < 0.001).