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« Localization of potassium channels in spinal cord microglial cells in peripheral nerve injury model »

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Abstract

Introduction

Neuropathic pain is often chronic and hard to treat: its prevalence is 7 to 8% in the general population and higher in patients with chronic post-surgical pain (CPSP). There's a growing interest toward glial cells, which may also contribute to chronic pain. Understanding their role and physiology could lead to new drugs development and better outcome for the patients.

Microglia is the central nervous system (CNS) macrophage: it modulates the neural environment and is involved in neuro-inflammation. In case of peripheral neural lesion, microglial cells at the corresponding spinal cord levels are activated. This phenomenon is related to membrane current changes, potassium currents among others. Thus, we chose to study the microglial potassium channels in order to better understand this process. We focused on $K_v1.3$, $K_v1.5$ and $K_{IR}2.1$.

Material and method

We worked with spared nerve injury (SNI) model in CX3CR1 – Green fluorescent protein (GFP) mutated mice. The animals were sacrificed at different timings after surgery. We used spinal cord complete tissues from L3-L4-L5 levels and studied them with 2 different technics. First we used immunofluorescence to locate the channels at the microglial membrane. Secondly, we used Western-Blot to quantify the potassium channel expression prior to and after microglial activation.

Results

SNI induces a strong microglial proliferation in the spinal cord. The immunofluorescence shows that $K_v1.3$ is localized on microglial cells. The same occurs for $K_{IR}2.1$ in a lesser way, but not for $K_v1.5$. A strong unspecific background signal prevented us from quantifying the channel expression. The Western Blot couldn't be interpreted due to unspecific signal. Further experiments need to be done to find the right conditions to analyze the channels expression in a quantitative way.

Keywords

Microglia, potassium channel, spinal cord

Introduction

Pain

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (1).

We classify pain in several categories according to its mechanism and its duration. The first category is nociceptive pain: it occurs when a traumatism, a thermal or chemical agent injures the body for example. Local receptors on C and Aδ peripheral nerve fibers are triggered and send a pain signal to the brain through the axon of the primary neuron to the spinal cord and then via the spino-thalamic tract to the brain. The second category is inflammatory pain, at the site of an infection for example. Inflammatory mediators can trigger pain receptor as well, sending a signal to the brain through the same route (2). This negative experiences allow us to avoid injuries in similar conditions and help for protecting the body integrity (3) and contributes to wounds healing. The last category is pathological pain, in which we distinguish two main causes. The first is neuropathic pain: it is the result of a lesion of the somatosensory pathway. The second is dysfunctional pain: the nervous system remains physically intact but the physiological information is misprocessed and results in pain sensation.

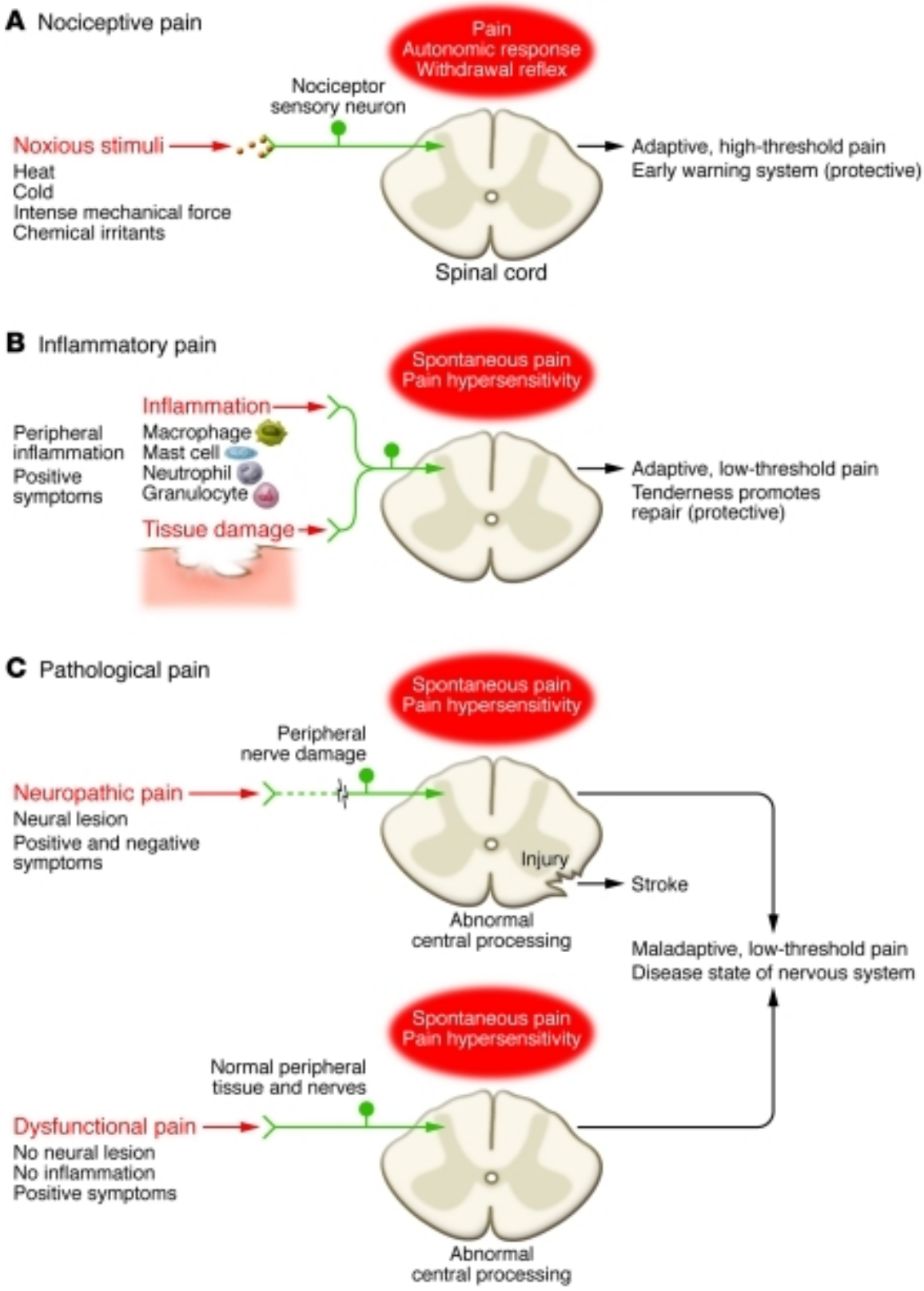


Fig. 1: pain classification (4)

We also distinguish acute pain from chronic pain. In the second situation, pain becomes a disease per se: its duration exceeds what can be normally expected according to the patient's

condition (most authors agree on a 3 month cut-off) and sometimes the original substrate to the pain is not found any longer (1).

Neuropathic pain is caused by a lesion or disease of the somatosensory system. Patients describe it like burning, painful cold, and electric shocks, which are associated with abnormal sensation phenomenon. Two types of abnormal sensations caused by pathological neuron activity may arise: gain of function (paresthesia, hyperalgesia, allodynia) and loss of function ones (hypoesthesia, anesthesia). The DN4 score use theses peculiar characteristics and helps the clinician for neuropathic pain assessment. Neuropathic pain is often chronic and disabling (1). It can occur in various conditions like diabetes, stroke, cancer or postsurgical nerve injury. When axons are damaged and surrounded by inflammation, the sensory neurons become spontaneously active and the pain threshold becomes much lower, resulting in peripheral sensitization. Through retrograde signaling via the wounded axon, environmental and synaptic changes occur in the dorsal horn of the spinal cord at the corresponding levels, which participates in neuropathic pain (5).

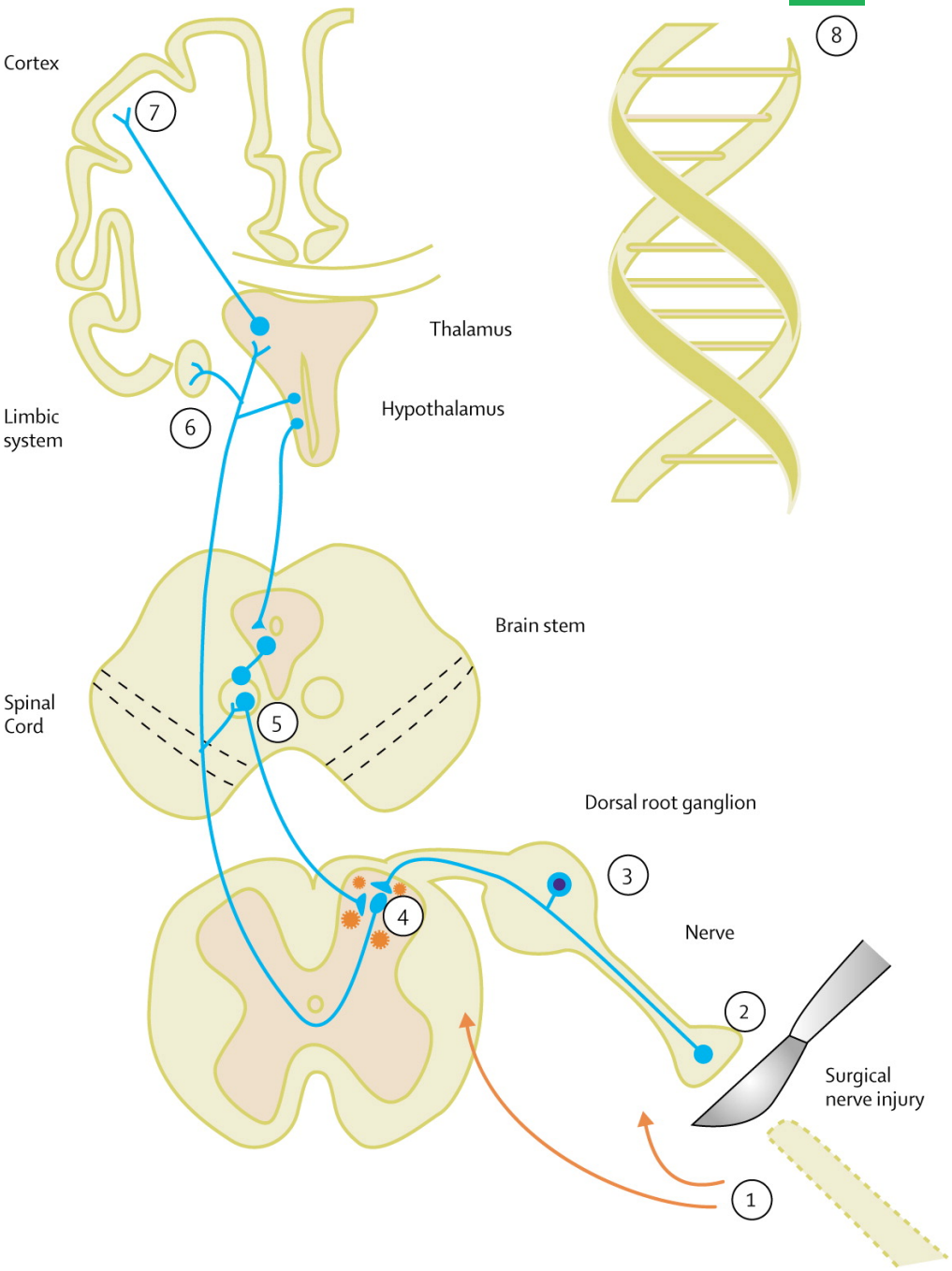


Fig. 2: sensitization mechanisms after surgical nerve injury (6)

Sites and mechanisms responsible for chronic postsurgical neuropathic pain

(1) Denervated Schwann cells and infiltrating macrophages distal to nerve injury produce local and systemic chemicals that drive pain signalling. (2) Neuroma at site of injury is source of ectopic spontaneous excitability in sensory fibres. (3) Changes in gene expression in dorsal root ganglion alter excitability, responsiveness, transmission, and survival of sensory neurons. (4) Dorsal horn is site of altered activity and gene expression, producing central sensitisation, loss of inhibitory interneurons, and microglial activation, which together amplify sensory flow. (5) Brainstem descending controls modulate transmission in spinal cord. (6) Limbic system and hypothalamus contribute to altered mood, behaviour, and autonomic reflexes. (7) Sensation of pain generated in cortex (past experiences, cultural inputs, and expectations converge to determine what patient feels). (8) Genomic DNA predispose (or not) patient to chronic pain and affect their reaction to treatment.

QUESTIONNAIRE DN4 : un outil simple pour rechercher les douleurs neuropathiques

Pour estimer la probabilité d'une douleur neuropathique, le patient doit répondre à chaque item des 4 questions ci dessous par « oui » ou « non ».

QUESTION 1 : la douleur présente-t-elle une ou plusieurs des caractéristiques suivantes ?

	Oui	Non
1. Brûlure	☐	☐
2. Sensation de froid douloureux	☐	☐
3. Décharges électriques	☐	☐

QUESTION 2 : la douleur est-elle associée dans la même région à un ou plusieurs des symptômes suivants ?

	Oui	Non
4. Fourmillements	☐	☐
5. Picotements	☐	☐
6. Engourdissements	☐	☐
7. Démangeaisons	☐	☐

QUESTION 3 : la douleur est-elle localisée dans un territoire où l'examen met en évidence :

	Oui	Non
8. Hypoesthésie au tact	☐	☐
9. Hypoesthésie à la piqûre	☐	☐

Fig. 3: DN4 score used for neuropathic pain assessment in clinical practice (7)

The prevalence of chronic pain with neuropathic characteristics is 7 to 8% of the general population (1). Chronic postsurgical pain (CPSP) occurs in 10-50% of the patients after a common operation (6). According to the type of intervention, up to 70% of the CPSP cases present a neuropathic component (8).

Nowadays, the pharmacological treatments, which mainly target neurons (antidepressants, amine regulators, local analgesics, membrane stabilizer) (9), allows to reach a significant reduction in pain intensity (average 30%) in 50% of the patients (10). This result is not satisfying yet; therefore identifying other targets in pain mechanisms would be of a great interest.

Microglia is generally viewed as the macrophage of the central nervous system (CNS). Both share many properties: microglia has the capacity to phagocyte (11) and to release inflammation mediators such as TNF-α (12) and IL-1β (13). Latest findings suggest that almost the entire population of microglia originate from embryonic macrophages derived from the yolk sac (14).

In physiological conditions microglial cells are quiescent and adopt a ramified morphology. They occupy a given territory that they scan with their processes (15). Under pathological conditions, some molecular pathways are activated, mitogen-activated protein kinases (MAPKs) among others (16)(17). Microglial cells undergo a morphological and functional transformation and reach new states of activation (18)(19). They revert to an amoeboid appearance, become more mobile, release peptides with inflammatory and immuno-regulatory effects and up regulate their expression of cell surface receptors (11). This also occurs in case of peripheral neural lesion: microglial cells in the sensitive area (dorsal horn) of the spinal cord at the corresponding levels are activated, proliferate and modify their gene expression profile (20). This process starts within hours to days (21).

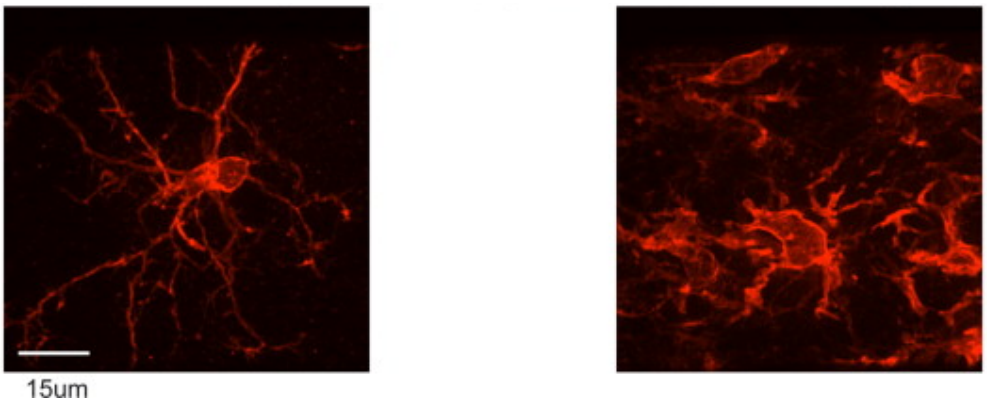
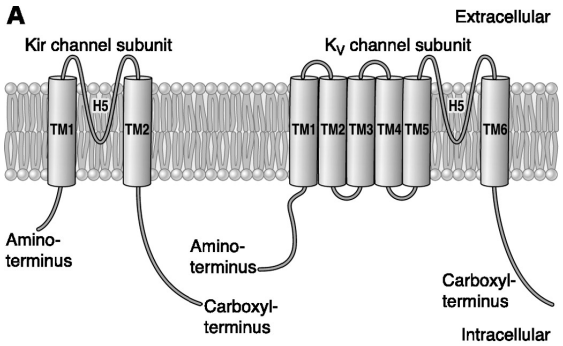


Fig. 4: fluorescent microscopy photography of spinal cord microglial cells prior to (L) and after (R) peripheral nerve injury (20)

It is now generally accepted that microglia is implicated in neuropathic pain. At first, a correlation between nerve injury and glial activation has been observed (22). A causal

relation has been then demonstrated: microglial activation participates in neuropathic pain mechanisms in several ways and can alone trigger allodynia (23). The activated microglial cells can release inflammatory mediators, growth factors, influence the neurons environment, play a role in neural plasticity or modulate the second sensory neuron excitability in the spinal cord (20)(21)(24). Several authors have also demonstrated that glial inhibitors can prevent or attenuate abnormal pain symptoms following nerve injury in animal models (23)(25). Thus, better understanding the activation process of spinal cord microglia could help provide some leads to new neuropathic pain treatment development.

« Potassium channels play a crucial role in determining the resting membrane potential, time course, amplitude and polarity of electrical changes in most types of cells » (26). It was demonstrated that potassium membrane currents vary with brain microglial activation. They have a causal effect on this process, and are also implicated in the functions of these cells (27)(28)(29)(30). At quiescent state, in vivo microglia has a high membrane resistance and little voltage-gated membrane currents (11). In the brain, 12 hours after activation appears an inwardly rectifier potassium current (K_{IR}) at first. In a second time after 24 hours, the microglial membrane potassium current is predominantly outward, which lasts up to seven days. The first current (K_{IR}) could be seen as an early marker of activation, and the appearance of the outward potassium current as a peak of activity marker (31).



4 subunits assembly to form a 1-pore channel

Fig. 5: Schematic view of potassium channel subunits (32)

The K_{IR} current is mediated by the $K_{IR}2.1$ family (11). This current becomes more important during microglial activation and is implicated in cell morphological changes, migration ability (33) and calcium metabolism (11)(34). The K_{DR} current is mediated mainly by $K_v1.3$

and $K_v1.5$ (27)(35). The expression of these channels increases during the microglial activation process (36). They are mandatory for microglia to become fully activated and functional (29). They play a role in neurotoxicity (37), respiratory burst and secretion of reactive oxygen species (ROS), and proliferation (38). Blocking these channels could attenuate or abolish the contribution of microglia in neuroinflammation or pain (39).

Spared nerve injury model

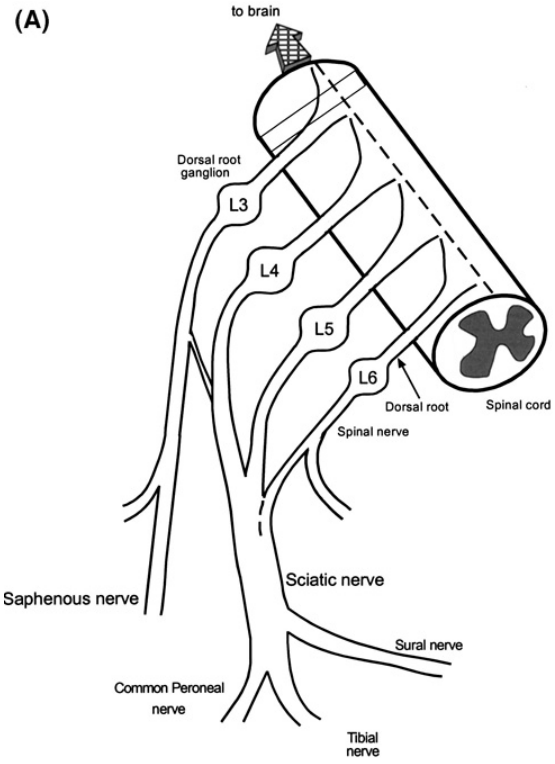


Fig. 6: schematic view of the sciatic nerve and his branches (40)

Exploring neuropathic pain mechanisms requires animal models. The spared nerve injury (SNI) model is one among several others, which result in partial denervation. 2 (Common peroneal and tibial) from the 3 branches of the sciatic nerve are cut during a surgical procedure while the last one (sural) remains intact. It produces quick and long lasting behavioral modifications: the process starts within hours and lasts over months. This model allows us to study the spinal cord and its microglial population at the levels corresponding with the peripheral nerve injury (L3-L5). This part of the spinal cord is of great interest

because physiological changes occur on the side corresponding to the lesion (ipsilateral) while the other (contralateral) one may be used as a control.

Objectives

In this thesis, our aim was to study the potassium channels in spinal cord microglia. We worked with SNI model in mice, at different timings from the neural lesion. We did it in 3 steps. First a literature search was made to define which potassium channels are of interest. Second we used immunofluorescence to locate the channels, focusing on the ipsilateral dorsal horn of the spinal cord at L3-L4-L5 levels. And last we performed Western-Blots on spinal cord tissues to observe the potassium channel expression level at different time points from the neural lesion.

Material and method

Mice and tissues

For our experience, we used CX3CR1 – Green fluorescent protein (GFP) mutated C57BL/6J mice. The mutated gene sequence was implanted via viral vector at early embryonic stage. CX3CR1 is a gene, which codes for a chemokine (fractalkin) receptor found in lymphocytes and monocytes. It plays a role in survival or migration. In the CNS it is also found exclusively in microglia and is involved in brain colonization mechanisms during embryologic development (41). One of the alleles remained intact while the other was mutated: the GFP gene was inserted in front of the promoting sequence of CX3CR1, thus ensuring microglia to express GFP and emits green light on a 475 nm wavelength when exposed to exciting light. The mice underwent surgery according to the SNI model and where then sacrificed at different timings: after 2 days (D2) and 7 days (D7). We also used samples from naive animals or animals that underwent sham surgery as controls. The spinal cord was dissected, fixated in Tissutec® and kept at – 70 °C for storage. Slices were then obtained using the Cryostat® at L3- L4 - L5 spinal cord levels and fixated on slides.

Immunofluorescence

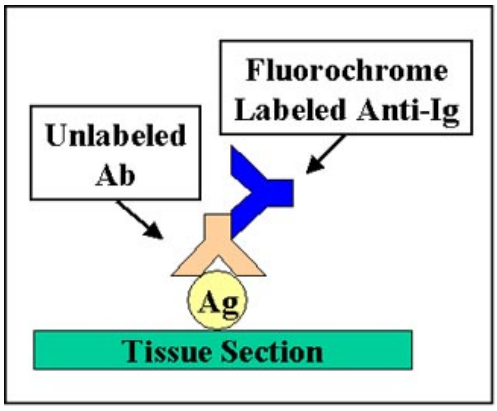


Fig. 7: immunofluorescence scheme (42)

Immunofluorescence is a molecular biology technique using the specific antigen-antibody interaction to study some previously chosen antigens at cell membranes. In secondary or

indirect immunofluorescence, 2 antibody types are needed. The primary antibody has a variable region specific to the chosen antigen and is used to target it. The secondary antibody is used to label the primary antibody (see fig. 5): it targets a whole subtype of antibodies and is coupled to a fluorochrome. Once the interactions occurred, we can use a fluorescence microscope to observe the slides. The microscope emits fluorescent light, which excites the fluorochrome allowing it to emit light at a given wavelength. This signal is detected by the microscope and allows us to observe the location of the labeled antigen, or the co-localization of several antigens. We used two sets of primary antibodies against $K_{IR}2.1$, $K_v1.3$ and $K_v1.5$. The first one contains immunoglobulin (Ig) G1 isotype provided by Neuromab, California (www.neuromab.org) and was produced in mice. The second set was provided by Alomone labs (www.alomone.org), contains polyclonal Ig and was produced in rabbits.

We separated the spinal slices on the slides in different areas with a hydrophobic pen to obtain separated pits to vary the antibody concentration. The slides incubated then in a 1X phosphated buffer saline solution (PBS 1X). To diminish the unspecific protein-antigen interaction, the slides were incubated in a blocking buffer solution with normal goat serum (NGS 10%) or bovine serum albumin (BSA 1%). They were then incubated with solutions containing the primary antibodies at various concentrations, washed with PBS 1X and incubated with the secondary antibodies (goat against mouse for the first set; donkey against rabbit for the second one) conjugated to a fluorochrome. As controls, we also labeled other antigens such as NEUN and α-CGRP, which are found on neurons, and GFAP, which is found on astrocytes. The slides were then mounted and ready for fluorescent microscopy.

Western blot is a technique used in molecular biology to study specific proteins. The tissue sample is grinded and the cells are lysed using a lytic buffer. The resulting mixture is then dropped in wells carved in a migrating gel. Electric current is used to drive protein migration across the gel, according to the protein size. This way, size sorts the proteins: the smaller they are, the further they go. Once this process is completed, the proteins are transferred to a solid membrane using electric current. The primary antibody specific to the proteins of interest is then incubated on the membrane. We label it with a secondary antibody, which is coupled to an enzyme that will allow detection when exposed to the substrate by emitting photons (See fig. 7).

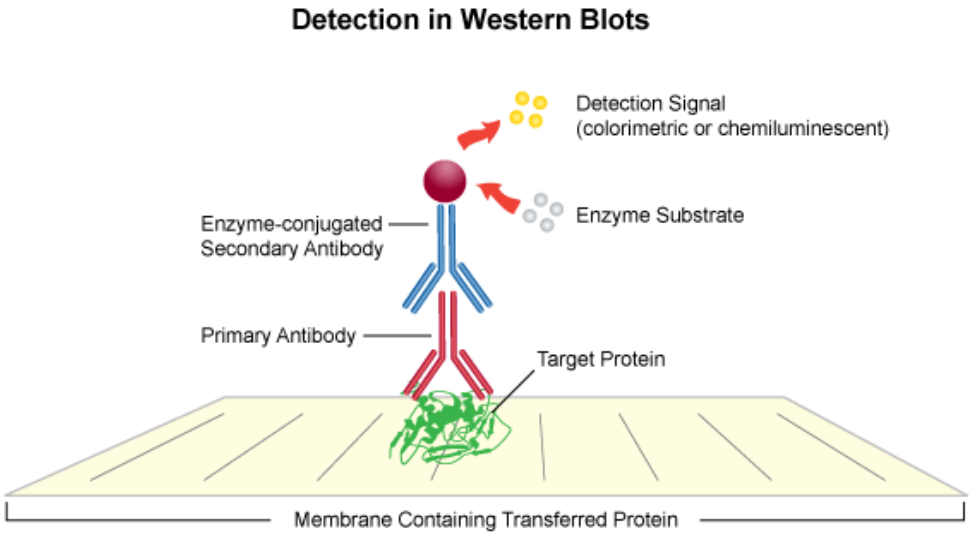
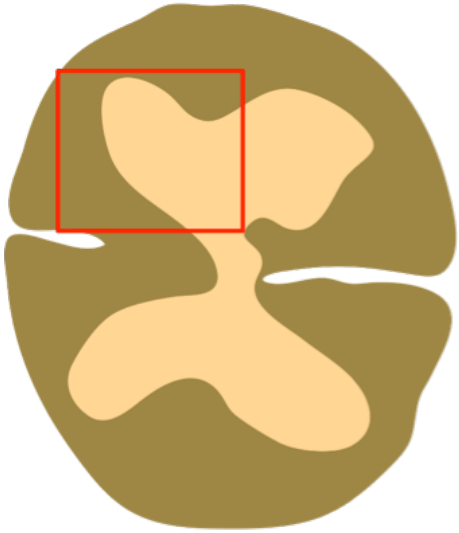


Fig. 8: detection in Western Blots scheme (43)

We performed Western blots on grinded spinal cord tissue at L3 – L4 – L5 levels. We used the Bradford protein assay method to determine the concentration of protein in the mixture, and ensure that 40 µg were dropped in each well. Our SNI samples were taken at D2. We chose this time point because it was relatively close to the injury and we wanted to see the effects before the healing process would start. As control we used heart muscle tissue, which express $K_v1.5$ and $K_{ir}2.1$ (44).

Immunofluorescence

Fig. 9: scheme of a spinal cord transverse section. The sections were always positioned with the ipsilateral side top and the contralateral bottom. The red rectangle shows the area of interest (dorsal horn ipsilateral to the neural lesion) where the pictures were taken.



Ipsilateral side

Contralateral side

Fig. 10: epifluorescent microscopy, zoom 20x, green filter showing GFP on CXCR1 expressing cells. A: Naive. B: SNI D2. C: SNI D7.

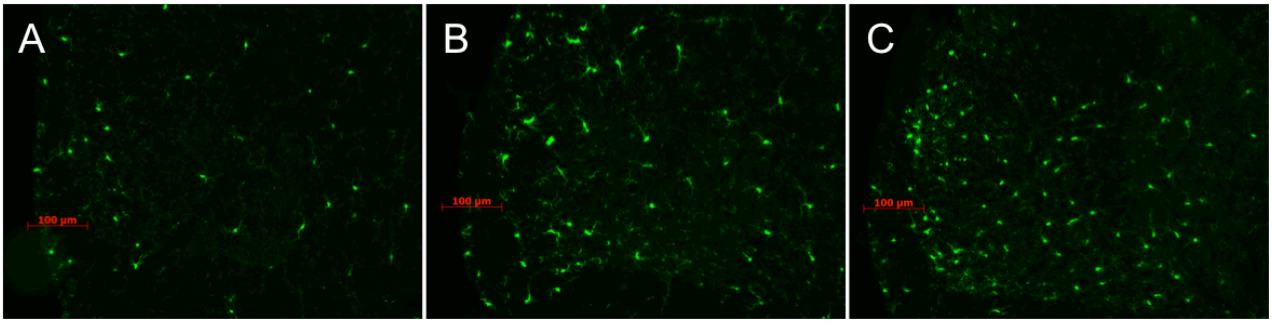


Fig. 11: epifluorescent microscopy, 3-color merge. Green: GFP, Blue: GFAP, Red: NEUN. L: zoom 10x; R: zoom 20x. It is noticeable that the microglial cell population on the ipsilateral side outnumbers the one on the contralateral side. The rectangle shows the area of interest on which we focused to take the following pictures.

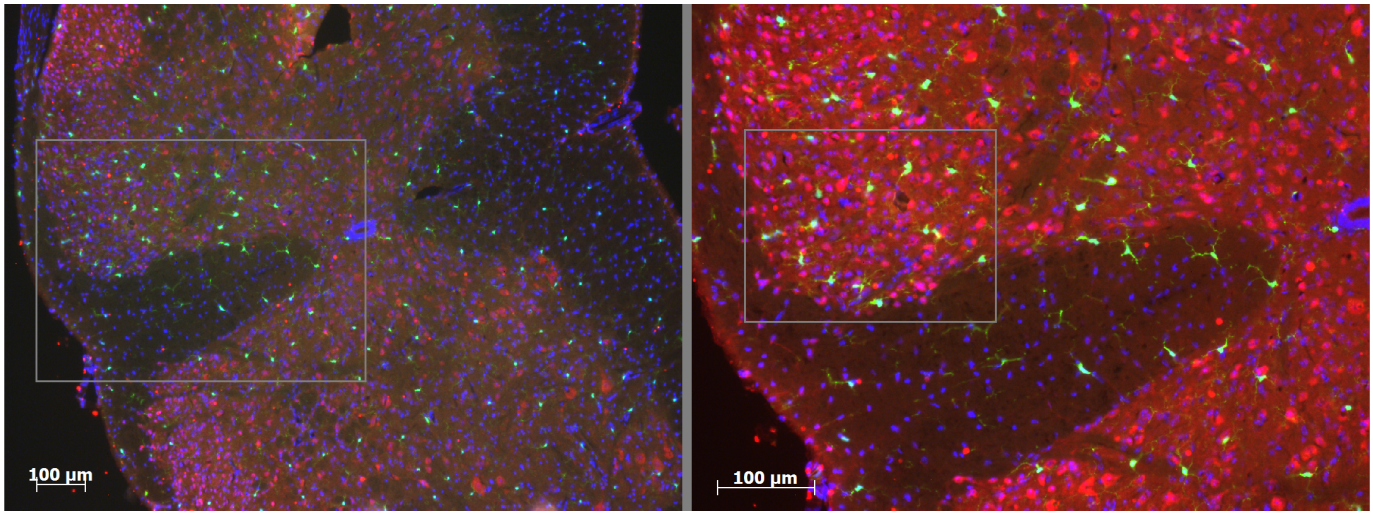


Fig. 12: K_v1.3, epifluorescent microscopy, SNI D2, zoom 40x. A: green filter showing GFP in microglial cells. B: red filter showing K_v1.3. C: merge green + red, it appears in yellow when both colors are co-localized.

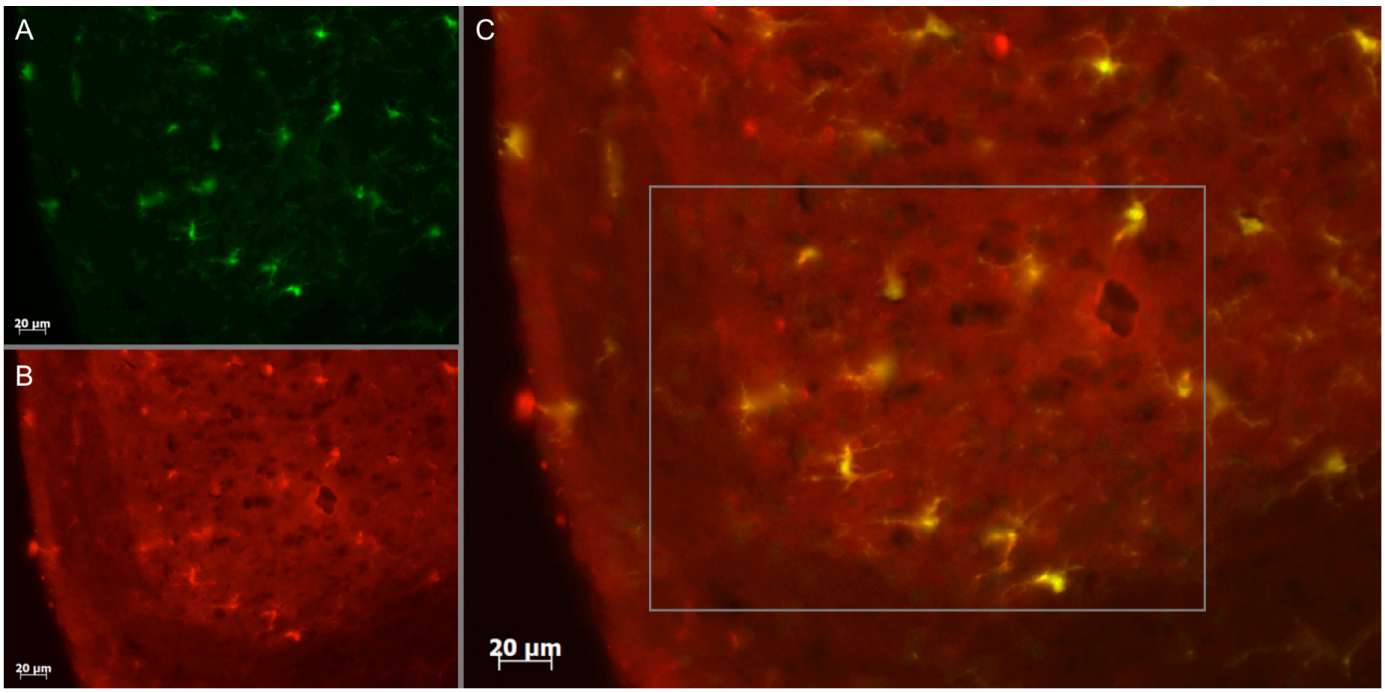


Fig. 13: K_v 1.3, confocal microscopy. Top: SNI D2, zoom 40x. A: in green GFP. B: in red K_v1.3. C: merge green + red. Bottom: SNI D7, zoom 40x. A: in green GFP. B: in red K_v1.3. C: merge green + red.

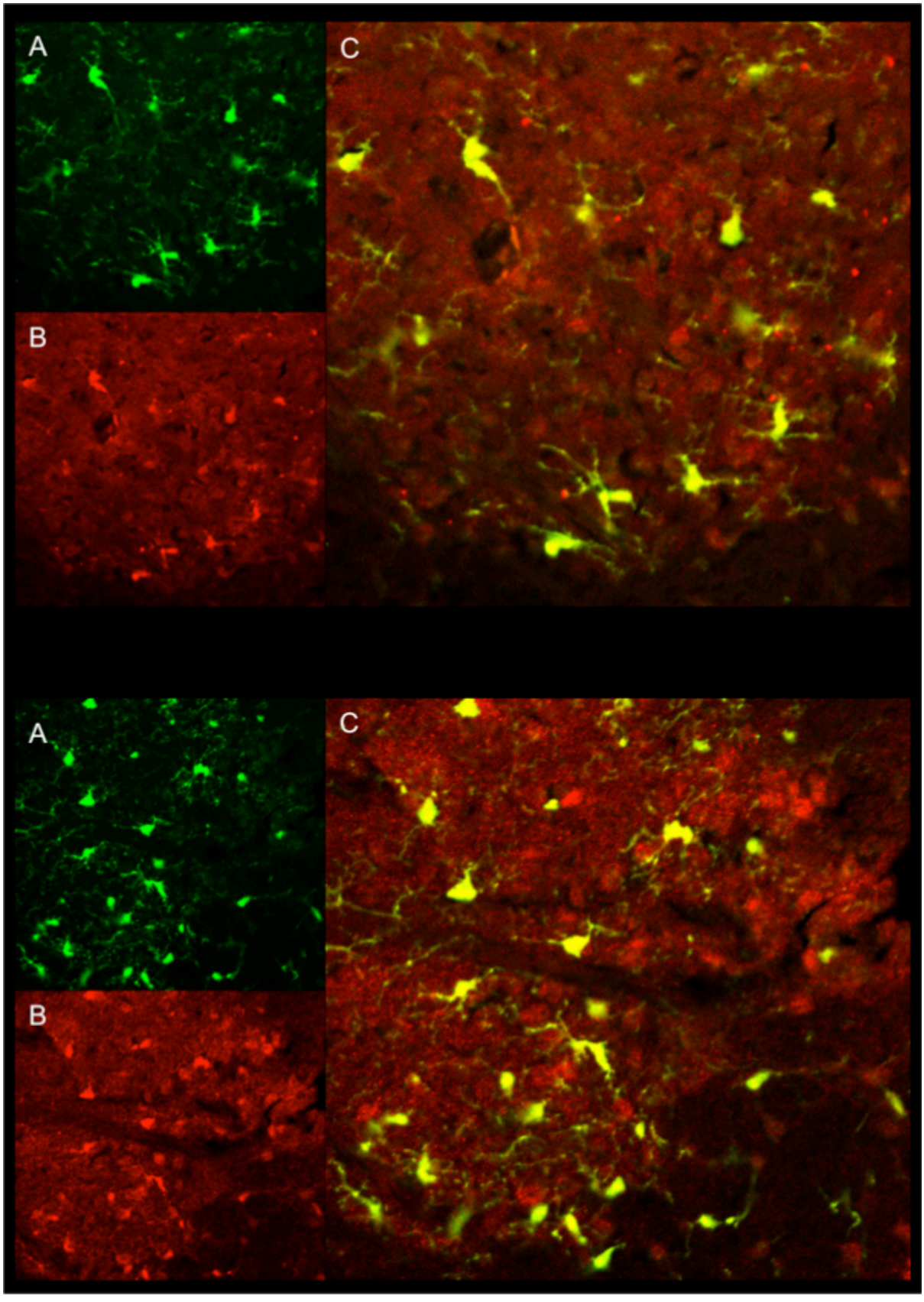


Fig. 14:K_v 1.5, confocal microscopy. Top: SNI D2, zoom 40x. A: in green GFP. B: in red K_v1.5. C: merge green + red. Bottom: SNI D7, zoom 40x. A: in green GFP. B: in red K_v1.5. C: merge green + red.

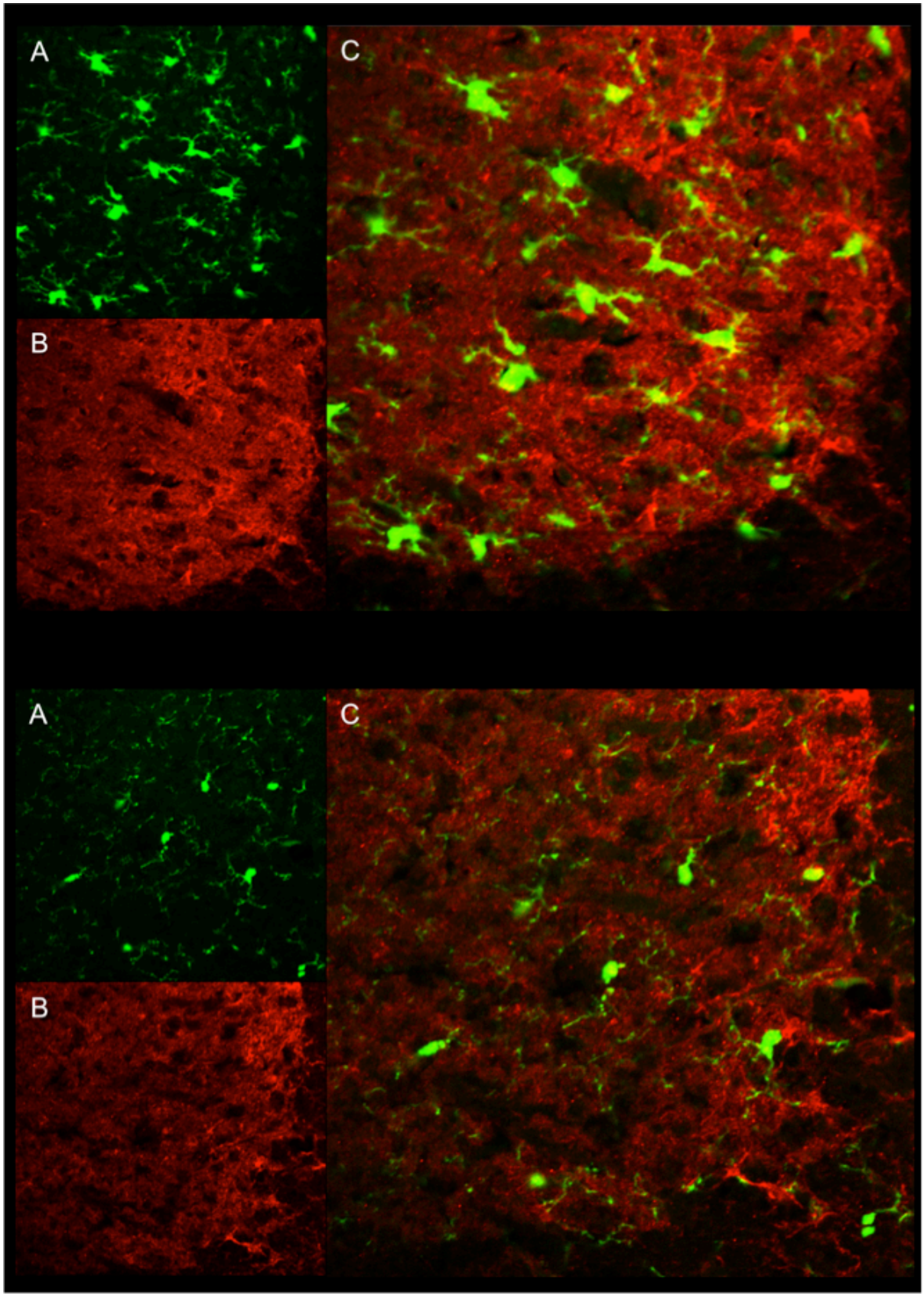


Fig. 15: K_{ir}2.1, confocal microscopy. Top: SNI D2, zoom 40x. A: in green GFP. B: in red K_{IR}2.1. C: merge green + red. Bottom: SNI D7, zoom 40x. A: in green GFP. B: in red K_{IR}2.1. C: merge green + red.

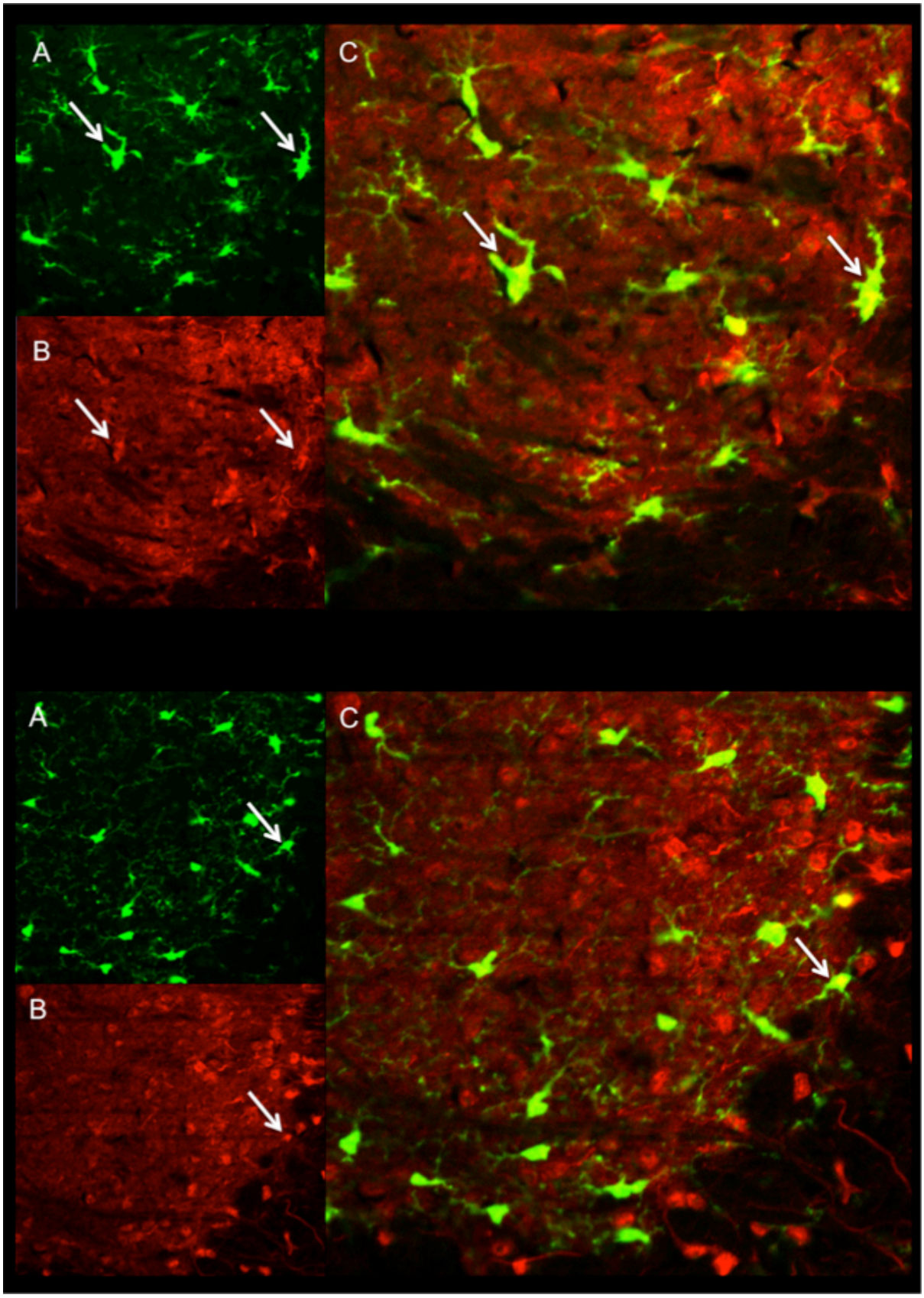


Fig. 16: epifluorescent microscopy, SNI D2, zoom 40x. A: red filter showing K_v 1.5. B: blue filter showing GFAP (astrocytes). C: merge red + blue, it appears magenta when both colors are co-localized.

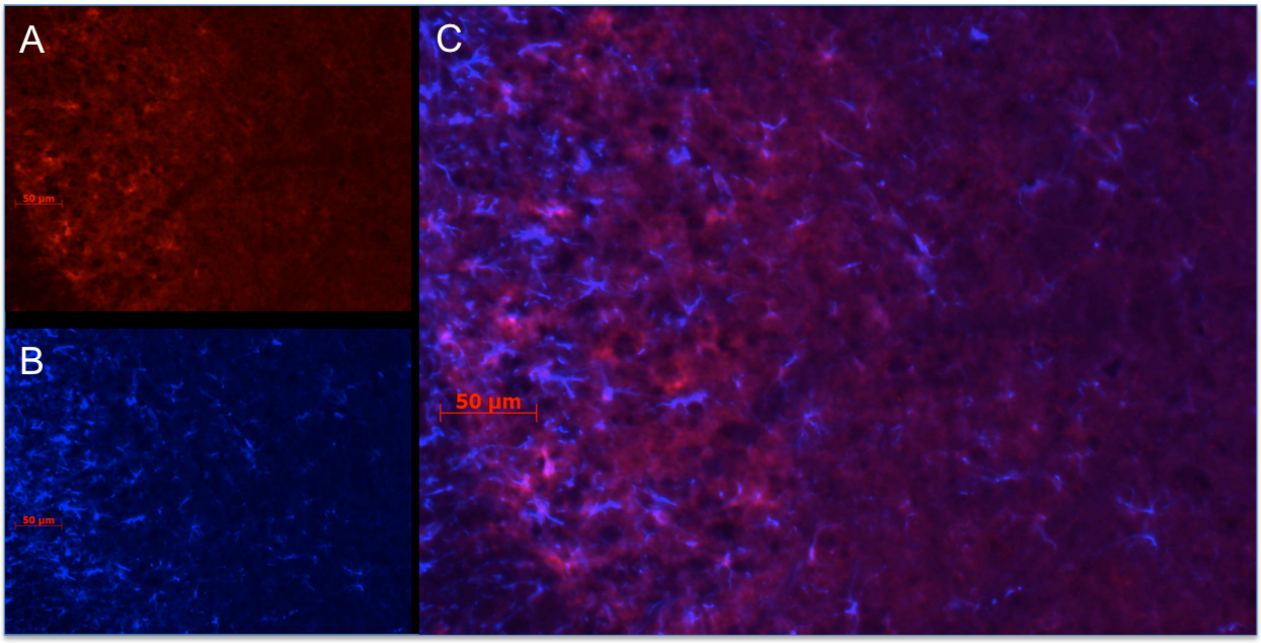


Fig. 17: western blot series 1 with the first set of primary antibodies. L: ladder; SNI: spared nerve injury at D2; Sh: sham; Ctrl: control (heart muscle). Expected antibody molecular weights: $K_v1.3$: 70 kDa; $K_v1.5$: 70 kDa; K_{ir} 2.1: 55 kDa. Left: low exposure. Right: high exposure.
Already at low exposure time, a light strip can be seen around 70 kDa where $K_v1.3$ was labeled (blue arrow) and expected according to the ladder. However, the weakness of this signal compared to an unexpected strip around 60 kDa makes us doubt about the reliability of this result. The controls worked, showing 2 distinct strips around 55 and 70 kDa, which were expected according to the manufacturer's data. The tracks where $K_v1.5$ and $K_{IR}2.1$ where labeled don't give any results at low exposure. At high exposure, the results show non-specific strips at unexpected molecular weights and thus cannot be interpreted.

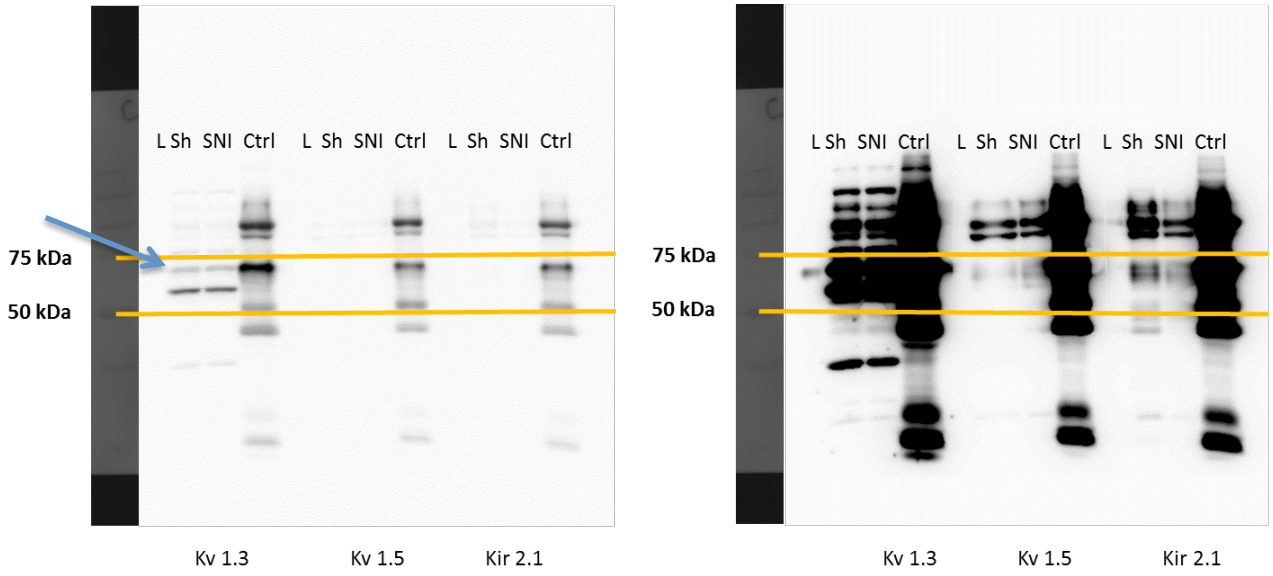
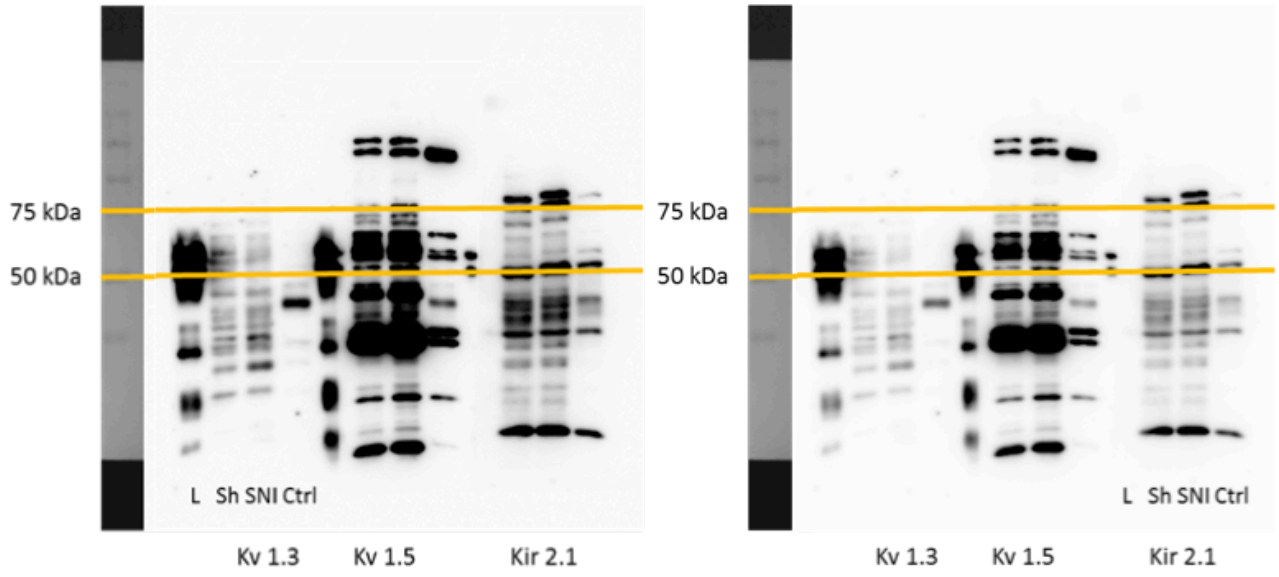


Fig. 18: western blot series 2 with the second set of primary antibodies. L: ladder; SNI: spared nerve injury at D2; Sh: sham; Ctrl: control. Expected antibody molecular weights: $K_v1.3$: 70 kDa; $K_v1.5$: 70 kDa; K_{ir} 2.1: 65 kDa.
The results of this Western blot cannot be interpreted due to lack of specificity.



Discussion

Our results show that SNI induces microglial proliferation (fig. 11). We could observe already at day 2 a clear increase of the microglial cell number in the ipsilateral dorsal horn of the spinal cord between naïve and SNI tissues, which doesn't occur on the contralateral side. This finding is in agreement with the results of precedent experiments reported in the literature (21) (45).

We found in the literature that $K_v1.3$ happens to be co-localized with microglia and up-regulated after activation in the brain (30) (36). Our pictures show that $K_v1.3$ also happens to be co-localized with the microglial cells in the spinal cord. In a lesser way, the same occurs with $K_{IR}2.1$. It is not possible to say if the channel expression increases after neural lesion on the basis of our findings. A way to settle this could be to count the fluorescent signal and compare between naïve and SNI tissue. This couldn't be done in our pictures because of the strong background signal. Antibodies against the potassium channels seem less specific than expected: we get non-specific background labeling on every slice, which gives a strong noise on the pictures. We tried various antibody titers and different sera (goat, bovin) in our blocking buffer and managed to get a good fluorescent signal but couldn't attenuate the noise on the pictures. For all the experiments, we worked with complete tissue slices containing various cell types and extracellular proteins. Cross-reactivity of the antibodies with other proteins, or the presence of the antigens on several cell types may contribute to this issue. The right immunofluorescence conditions could be found by working with transfected cells as positive control, which would surely express the channels, or oppositely channel knockout cells as negative control. Another way to test the specificity would be to use a second antibody, which targets another epitope on the channel to perform a double labeling. And finally, working with free-floating slices could allow better antibody-antigen interactions.

We don't find much co-localization between $K_v1.5$ and the microglial cells on our pictures. This goes against Yamada's findings (36) but his tissue samples comes from the brain. Immunofluorescence could be tested with positive controls and under various conditions to assess weather if Kv1.5 expression is different in spinal cord microglia, or if the problem comes from the antibodies.

As found in the literature, $K_v1.5$ is also present in astrocytes in the brain (46) and the spinal cord (47). We were wondering if this could interfere with our experiments, as in some of our pictures the antibodies seemed to label some cells that weren't microglia. But the pictures where we labeled GFAP and the potassium channels show very few co-localizations between the astrocytes and $K_v1.5$. They could be expressed in astrocytes in fewer quantities than on the microglial cells, or under different conditions. Thus, they could contribute to the background signal that we get on our pictures.
$K_{ir}2.1$ is also found in spinal cord neurons and astrocytes (48). Kv1.3 is found in astrocytes but not expressed at the cell surface (49). Further experiments could be made to establish if this contributes to the background signal we get on our pictures.

The Western blots don't show clear results: the signals obtained lacks specificity. We used heart tissue as control, which also failed to show the results that we expected. An explanation could be that we used whole heart grinded tissue, nevertheless the channels are mainly found in the muscle. This could result in diluting the tissues of interest and scramble the signal, making our control unreliable. Isolating the microglial cells before the Western blot instead of using complete spinal cord tissue samples would allow us to increase specificity and to focus on microglial protein expression.

Conclusion

We find colocalization between microglia and 2 of the 3 chosen channels. As it could be expected with immunohistochemistry, the specificity of the antibodies seems to be our main issue in the experiments. More trouble shooting needs to be done to assess the specificity of the antibodies and to find the right conditions to count the fluorescent signal. This would allow studying the channels in a quantitative way and finally determining the kinetic of their expression.

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