Basic Science

Acute Baclofen administration promotes functional recovery after spinal cord injury

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Abstract

BACKGROUND CONTEXT: Traumatic Spinal Cord Injury (SCI) leads to severe motor and sensorimotor functional impairments that affect personal and social behaviors. Medical advancements have improved supportive therapeutic measures for SCI patients, but no effective neuroregenerative therapeutic options exist to date. Deficits in motor function are the most visible consequence of SCI. However, other complications, as spasticity, produce a significant impact on SCI patient’s welfare. Baclofen, a GABA agonist, is the most effective drug for spasticity treatment. Interestingly, emerging data reveals that Baclofen can also play a role on neuroprotection and regeneration after SCI.

PURPOSE: The goal of this study was to understand the potential of Baclofen as a treatment to promote recovery after SCI.

STUDY DESIGN: We used a pre-clinical SCI mouse model with the administration of Baclofen 1 mg/Kg at different time-points after injury.

METHODS: Behavior analysis (locomotor and bladder function) were performed during nine weeks of the in vivo experiment. Afterwards, spinal cords were collected and processed for histological and molecular analysis.

RESULTS: Our data showed that Baclofen leads to locomotor improvements in mice when its administered acutely after SCI. Moreover, Baclofen administration also led to improved bladder function control in all experimental groups. Interestingly, acute Baclofen administration modulates microglia activation state and levels of circulating chemokines and cytokines, suggesting a putative role of Baclofen in the modulation of the immune response.

CONCLUSIONS: Although further studies must be performed to understand the mechanisms that underlie the functional improvements produced by Baclofen, our data shed light into the pharmacological potential of Baclofen to promote recovery after SCI.

CLINICAL RELEVANCE: Our outcomes revealed that Baclofen, a well-known drug used for spasticity management, improves the motor performance after SCI in a pre-clinical animal model. Our data opens new avenues for pharmacological strategies design to promote SCI recovery.

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Introduction

Traumatic Spinal Cord Injury (SCI) results in loss of nervous tissue and consequently severe motor and sensory permanent functional impairments that negatively impact the patient’s quality of life. Spontaneous recovery is limited after SCI [1]. Current therapy is based on surgery to decompress and stabilize the spinal cord, spasticity management and rehabilitative care. However, these medical approaches focus on stabilizing the spine and managing SCI consequences with little, if any, improvements in the SCI trajectory. Therefore, the search for new therapeutic strategies to improve SCI patient’s recovery must be a priority.

Spasticity is a neurological impairment that affects the control of muscle tone after an insult, trauma, or injury to the central nervous system (CNS). The management of spasticity is mainly achieved pharmacologically, and Baclofen is the most effective drug for spasticity treatment in SCI patients [2]. Baclofen is a GABA agonist, the major inhibitory neurotransmitter in the CNS, where it plays a key role in neuronal modulation [3]. Presynaptically, Baclofen activates GABA$_B$ receptors leading to a reduction of Ca$^{2+}$ channels conductance. This hyperpolarization inhibits the release of excitatory transmitters as glutamate. As a consequence of the decrease in signal propagation, there is a relief of spasticity [4,5].

Beyond Baclofen action in spasticity, recent studies in lampreys revealed that Baclofen might also promote neuroprotection and neuroregeneration in an SCI context, which can be translated to improved motor outcomes in humans [6–9]. In addition, a study using rats as a model, showed that repeated Baclofen administration starting in the sub-acute period after SCI improves motor dysfunction [10]. Nevertheless, additional in-depth studies must be performed to clarify the potential of Baclofen to promote recovery after SCI, especially in the acute phase. Understanding the mechanisms behind the neurological outcomes promoted by Baclofen using preclinical models is essential to introduce this approach as a therapeutic option for SCI patients.

Here, we investigated the role of Baclofen in the promotion of neurological recovery after a SCI. Using a SCI mouse model, we administrated Baclofen at different timepoints after the injury. Our data shows that the acute administration of Baclofen after SCI leads to locomotor recovery and an improved bladder function control in mice. Interestingly, Baclofen did not prevent demyelination nor increase the number of a-motor neurons in the spinal cord but rather modulated the immune response in the SCI lesion site.

Materials and methods

Animals

The Ethical Subcommittee in Life and Health Sciences (SECVS; ID: 018/2019, University of Minho) and the Portuguese Authority (DGAV; ID: 022405) previously approved all experiments. Local regulations on animal care and experimentation (European Union Directive 2010/63/EU) were respected. Female C57BL/6J mice (Charles River, USA), with 10 weeks old, were maintained at the animal facilities of the Institute of Life and Health Sciences (ICVS, Braga, Portugal) under sterile conditions and in light, humidity, and temperature-controlled rooms. Animals had food and water provided ad libitum. The number of animals on this study was determined by G-power software using a medium effect size of 0.5, a confidence coefficient of 0.05 and a statistical power of 0.9 (G$^*$power) [11] with results obtained from a pilot study. In this study, 48 animals were initially used with a distribution of 12 animals per experimental group. Animals were randomly treated with Baclofen or saline and all data collection (behavior and histology) was obtained blindly to the treatment group.

Spinal cord injury surgery and post-operative care

C57BL/6J mouse (Charles River, USA) was maintained under standard laboratory conditions (12 h light: 12 h dark cycles, 22 °C, relative humidity of 55%, ad libitum access to standard food and water). C57BL/6J adult females (10 weeks age) were used for the in vivo study. Animals were handled every other day for 1 week prior to the surgeries, for stress reduction and habituation to the experimenter presence. Animals were anesthetized with an intraperitoneal injection of a mixture containing ketamine (Imalgene, 75 mg/kg, Merial, France) and medetomidine (Dormitor, 1 mg/kg, Pfizer, USA). Once anesthetized, buprenorphine (Bupaq, 0.05 mg/kg) was also administrated subcutaneously for analgesia. To confirm the surgical plane of anesthesia, mice were pinched on the lower limb toes. Mice were placed under a warm lamp before and after surgery and placed on a warm surgical blanket to avoid dropping the body temperature. Vaseline was applied onto the eyes to prevent drying and corneal damage. Surgeries were performed following aseptic procedures. A dorsal midline incision was made at the level of the thoracic spine (T5-T12). The paravertebral muscles were retracted and the spinous and laminar arc of T8-T9 vertebral body was removed,
exposing the spinal cord. Using forceps (Dumont #5/45° angled, FST, CA, USA), the spinal cord was completely compressed for 10 s to produce a severe lesion at T10-T11 spinal cord segment level. Lesions were performed consistently with the same forceps and always by the same experimenter to reduce variability in the applied force. After SCI, the muscle and skin were closed with 4.0 polyglycolic absorbable sutures (Safil, G1048213). Atipamezole (Antisedan/Pfizer) was administered subcutaneously to revert the anesthesia, and animals were allowed to completely recover from the anesthesia under a warm lamp. Animals were then placed on individual cages with hydrogel and moisturized food pellets on the floor to allow easy access to water and food. Post-operative care included buprenorphine (Bupaq, 0.05 mg/kg), vitamins (Duphalyte, Pfizer, New York, NY, USA), saline, and enrofloxacin (Bayer, Leverkusen, Germany) administration, twice a day. Manual voiding of bladders was performed twice a day. During post-operative care, animals were also monitored for body temperature and recovery of general activities. Basso Mouse Scale (BMS) was used 48 hours post-injury (hpi) to confirm the lesion extent reflected by no motor movement in both hind paws (BMS score=0). BMS scoring was performed as a quality control procedure for the surgery to exclude eventual partial lesions.

After 7 days post-injury (dpi) mice were transferred to mice cages to avoid behavior changes due to isolation. The maximum number of animals per cage was determined following the rules of the Ethical Subcommittee in Life and Health Sciences.

Drug treatment

After SCI, mice were randomly assigned to each experimental group. We administrated Saline (Saline Solution 0.9%, B. Braun, SKU 06063042) or Baclofen ((±)-β-Aminomethyl-4-chlorobenzepropionic acid, Loresal, (±)-Baclofen, Sigma-Aldrich, CAS number 1134470) at the concentration of 1mg/kg (Fig. S1A), in following experimental groups: Vehicle, SCI mice control group treated with saline within 10 minutes after the lesion (n=12); Acute, SCI mice received a single treatment administration within 10 minutes after the lesion (n=12); Acute + Chronic, SCI mice treated within 10 minutes after the lesion and thereafter once a day for two weeks (n=12); 24 h Delay + Chronic, SCI mice received the first treatment 24h after the lesion and thereafter once a day for two weeks (n=12) (Fig. S1B). The two weeks treatment refers to treatment on the day of surgery and then for the next 13 days (14 days in total). Treatments were administered intraperitoneally (IP). Baclofen concentration of 1mg/kg was chosen because is within the therapeutic range used in humans [12]. Animals’ survival rate was analyzed throughout the experiment (Fig. S1C) and at the end of week nine we had the following animal n numbers: Vehicle, n=11; Acute, n=11; Acute + Chronic, n=9; 24 h Delay + Chronic, n=6. Animals were restrained to facilitate the IP. To exclude the cage input environment, animals from the same cage received different treatments.

BMS

Animals were placed in an open arena, and two independent observers scored their locomotor function according to the BMS scale [13], blinded to the experimental groups. Locomotor behavior (BMS score) was assessed at 48 hpi (hours post-injury) and 7, 14, 21, 28, 35, 42, 49, 56 and 63 dpi (days post-injury). Body weight support was obtained based on animal’s BMS score >4.

Bladder function analysis

The experimental design was performed based on previous studies [14,15]. Once a week, two animals from the same experimental group were placed in the same cage overnight. Before that, bladders were completely void. Water volumes in the bottles were measured before and after the experiment to assess the water intake. To avoid variability from bottles water leaks, a thinner teat was used during this experimental assessment. Additionally, the same volume of water was introduced in all the bottles (100 ml). The mean of water intake per animal was calculated. Bladders were voided to a beaker and the urine was weighted. A ratio between water intake and urine was performed to assess the bladder control of the different experimental groups. If the amount of collected urine was <0.2 g we considered that the animal regains bladder control [16,17].

von Frey test

To evaluate allodynia in mice we used the “up-down method” of von Frey test for approximation of the 50% withdrawal threshold to determine the weight of stimulus that leads to a response 50% of the times [18]. A nylon filament is applied under the paw of the mice and paw withdrawal threshold was defined as the mean of three readings. This protocol was performed at the end of the experiment.

Tissue processing

Mice were anesthetized and transcardially perfused with 20 ml of cold phosphate-buffered saline (PBS) and then with 20 ml 4% paraformaldehyde/PBS (PFA). Spinal cords were dissected and post-fixed for 24 h in 4% PFA. The spinal cords were then cryoprotected in a 30% sucrose solution for 24 h and involved in optimal cutting temperature compound (OCT, ThermoFisher Scientific, Massachusetts, USA), snap-frozen in liquid nitrogen, and stored at −20°C. Regarding tissue sectioning, transversal-section of 20 μm was performed using a cryostat (Leica CM1900, Leica-Biosystems, Nussloch, Germany) and thaw-mounted onto charged microscope slides (Superfrost Plus, Thermo Fisher
Scientific, MA, USA). All histological procedures and evaluations were performed blindly to the treatment groups.

**Immunohistochemistry**

Spinal cord tissue was analyzed by collecting photomicrographs every 200 μm both rostrally and caudally from the lesion epicenter, at T8 level. The lesion epicenter was considered the area ranging from -600 to 600 μm surrounding the epicenter. The most rostral area analyzed extended from -2000 to -600 μm from the epicenter and the most caudal area analyzed extended from 600 to 2000 μm.

For immunofluorescence staining, sections of the spinal cords were washed in PBS, permeabilized with 0.2% Triton X-100 for 10 min and blocked with 2% fetal calf serum (FCS) in 0.2% Triton X-100 for 30 min. Afterwards, the following primary antibodies were incubated overnight at room temperature (RT): mouse anti-NeuN (1:200; Millipore, Darmstadt, German) and rabbit anti-Iba-1 (1:1000; Wako Osaka, Japan). On the following day, sections were washed in PBS 3 times 10 min. Afterwards, the following secondary antibodies were incubated for two hours at room temperature (RT): Alexa FluorTM 594 goat anti-mouse IgG (1:1000; Invitrogen, Paisley, UK) and the secondary antibody Alexa FluorTM 488 goat anti-rabbit IgG (1:1000; Invitrogen, PA). Sections were counterstained with 4',6-diamidino-2-phenylindol (DAPI) for 30 min (1:1000; Sigma-Aldrich, Missouri, USA), washed with PBS 3 times 10 min and mounted with Immu-Mount® (Thermo Fisher Scientific, Massachusetts, USA).

**White matter sparing**

Myelin quantification was performed as previously described [15], every 200 μm both rostrally and caudally from the lesion epicenter, -2000 μm rostrally to the lesion site and 2000 μm caudally to the lesion site. Spinal cord transversal cross-sections were stained with FluoroMyelinTM Green (F34651, Thermo Fisher Scientific, Massachusetts, USA) for 1 hour at room temperature. After obtaining micrographs with an Olympus Widefield Upright Microscope BX61, Tokyo, Japan, photos were analyzed using the Image J software. The percentage of spared myelin was measured on the entire spinal cord slice using a lower magnification. Freehand selection tool from Image J software was used to measure the stained myelin area. Percentage of spared myelin was normalized for the total cross-sectional area of the spinal cord slide.

**Quantification of motor neuron**

Motor neuron quantification was performed as previously described [19], every 200 μm both rostrally and caudally from the lesion epicenter, -2000 μm rostrally to the lesion site and 2000 μm caudally to the lesion site. Spinal cord transversal cross-sections were stained with the primary antibody anti-NeuN (1:200; Millipore, Darmstadt, German) and the secondary anti-body Alexa FluorTM 594 goat anti-mouse IgG (1:1000; Invitrogen Paisley, UK). After obtaining micrographs through Olympus Widefield Upright Microscope BX61, Tokyo, Japan, photos were opened with the Image J software. The multipoint tool was used for NeuN+ cells counting. Data plotted in the graphs represents NeuN+ cells located in the spinal cord the ventral horn.

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**Quantification of resting microglia**

Resting microglia quantification was performed as previously described [20] every 200 μm both rostrally and caudally from the lesion epicenter, -2000 μm rostrally to the lesion site and 2000 μm caudally to the lesion site. Spinal cord transversal cross-sections were stained with the primary antibody rabbit anti-Iba-1 (1:1000; Wako Osaka, Japan) and the secondary antibody Alexa FluorTM 488 goat anti-rabbit IgG (1:1000; Invitrogen Paisley, UK). Resting and activated microglia were classified based on the following criteria: Resting glia displayed small compact somata bearing long, thin, ramified processes. Activated microglia exhibited marked cellular hypertrophy. These phenotypic differences allow to identify the two microglia subpopulations with the same labelling. Freehand selection tool from Image J software was used to assess the resting microglia area. Percentage of resting microglia was normalized for the total cross-sectional area of the spinal cord slide.

**Imaging**

Spinal cord sections were scanned for positive signal, and non-overlapping images were acquired using the Olympus Widefield Upright Microscope BX61, Tokyo, Japan. Photomicrographs were obtained every 200 μm for -2000 μm rostrally and 2000 μm caudally to the lesion site. Images were processed using Image J software. Brightness/contrast and color balance adjustments were always applied to the entire image. All histological procedures and evaluation were performed blindly to the treatment groups. A total of six spinal cords per experimental group were analyzed.

**Chemokine and cytokine array**

Two and six days after the injury blood was collected using the tail vein sampling method from mice of the Vehicle and Acute experimental groups. Samples were collected to an Eppendorf without any anticoagulant and lefted for 30 min in standing position. Samples were centrifuged for 15 min at 13,000g at room temperature. Serum was collected to an Eppendorf and stored at -80°C. To assess circulating chemokines and cytokines, the serum from five animals from each experimental group was pooled. Two technical replicates were performed. The RayBio C-Series
Mouse Inflammation Antibody Array 1 Kit (AAM-INF-1-2, RayBiotech, GA, USA) was used in accordance with the manufacturer’s recommendations.

**RT-PCR**

After animal’s euthanasia, one centimeter of spinal cord around the lesion was collected and stored at - 80°C. To extract the total ribonucleic acid (RNA), samples were homogenized in TripleXtractor (GB23.0050, Grisp, Porto, Portugal), and processed following the manufacturer’s instructions. Synthesis of complementary deoxyribonucleic acid (cDNA) was performed using Xpert cDNA Synthesis instructions. Synthesis of complementary deoxyribonucleic acid 3-phosphate dehydrogenase (Gadph), hypoxanthine-guanine phosphoribosyl-transferase (Hprt), and 18S expression levels were analyzed by real-time qPCR using the Xpert Fast SYBR Mastermix (GE22.0100, Grisp, Porto, Portugal) from 1.5 μg of total RNA template. mRNA levels were analyzed by real-time qPCR using the Xpert Fast SYBR Mastermix (GE22.0100, Grisp, Porto, Portugal) according to the manufacturer’s instructions and using the oligonucleotides primers indicated in Table. qPCR reactions were performed in a 7500 Fast Real-Time PCR system (Thermo Fisher Scientific, MA, USA). Melting curve analysis was used to assess the specificity of the gene amplification. Gene expression was normalized to the mean expression level of the three housekeeping genes: glyceraldehyde 3-phosphate dehydrogenase (Gadph), hypoxanthine-guanine phosphoribosyl-transferase (Hprt), and 18S gene expression.

**Oligonucleotide primers**

**Quantification and statistical analysis**

Statistical analysis was performed using GraphPad Prism ver. 8.0. Data from BMS and Bladder control was assessed by a repeated measure Two-way ANOVA test. Differences between groups were compared with the post hoc Bonferroni test. Unpaired Student’s t-test was used when comparing two experimental groups. Statistical difference was defined at p<.05 (95% confidence level). Data is presented on text as group mean ± standard error of the mean (SEM). Details on statistical parameters, including sample numbers and precision measures (eg, mean and p-values) are described in the figure legends or in the main text.

**Results**

Baclofen administration improves locomotor function and bladder function control in a SCI mouse compression model

To understand the effects of Baclofen after a SCI we set up an experimental protocol with nine weeks long and different time-points of Baclofen administration after SCI (Fig. S1A; Fig. S1B and Fig. 1A). The experimental procedure started with twelve animals per experimental group. During the experiment, animals from all the experimental groups died, with a higher number in the 24 h Delay + Chronic experimental group (Fig. S1C).

Before the injury all mice presented a normal locomotor behavior. After the injury, all mice presented a complete paralysis of the hindlimbs (Fig. 1B). After three weeks post-injury, the mice from the Acute experimental group presented a higher BMS in comparison with the Vehicle group, which is maintained during the nine weeks of the experiment. This difference is significant relevance between week four and six of the experiment (Fig. 1B). The mice from the Acute + Chronic experimental group also presented a significantly higher BMS score than the Vehicle group, namely in the weeks six and seven after injury (Fig. 1B). The mice from the 24h Delay + Chronic group showed a locomotor behavior similar to the Vehicle group (Fig. 1B).

The analysis of the mice with weight support over the nine weeks showed that the experimental groups Acute and Acute + Chronic had a significantly higher percentage of animals reaching weight support when compared with the Vehicle treated group. At the end of the experiment, over 60% of the animals from these experimental groups showed weight support. Mice from 24h Delay + Chronic experimental group presented the same results as mice from the Vehicle treated group (Fig. 1C).

Based on previous experiments [14,15], bladder function analysis was performed by the calculation of the ratio between the mice water intake overnight and the amount of urine collected the day after. From 4 weeks after the injury, we observed that the mice from all the experimental groups treated with Baclofen presented a higher ratio when compared with the Vehicle treated group (Fig. 1D). From week four of experiment until week eight the Acute and 24 h Delay + Chronic experimental groups presented a significant increase in the bladder control. The Acute + Chronic experimental group showed better performance from week seven until the end of the experiment (Fig. 1D). In a parallel analysis, we considered that mice with an amount of collected urine under 0.2g regained the total bladder function control. This analysis showed that Acute and 24h Delay + Chronic experimental groups treated with Baclofen have a higher percentage of mice that fully regain bladder control when compared with the Vehicle treated group (Fig. 1E).

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<th>Table</th>
<th>Oligonucleotide primers</th>
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<tr>
<td>Gene</td>
<td>Forward</td>
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<tr>
<td>Ihh</td>
<td>5’-GTAAACCCTTTGAAACCACCATT-3’</td>
</tr>
<tr>
<td>Gaddb</td>
<td>5’-GGCCCATGCAAGGCTGAA-3’</td>
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<tr>
<td>Hprt</td>
<td>5’-AGTCTGGTAAAGACGCTCTC-3’</td>
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<td>Glip5</td>
<td>5’-CGAGAGAAAACCACATCATCCTT-3’</td>
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<tr>
<td>Myelin</td>
<td>5’-AGCAACACACTCCTCCCTCCA-3’</td>
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<td>TGF-β</td>
<td>5’-CACCGAAGGAGCCTGATGA-3’</td>
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<td>IFNα</td>
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<tr>
<td>GABAb2</td>
<td>5’-CTTCTCGGAGCTCAGCGGTCA-3’</td>
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Mice sensory behavior was assessed through von Frey Test, only at nine weeks post-injury to avoid animals’ habituation to the test. Our results did not show differences between mice treated with Baclofen and Vehicle group (Fig. 1F). Interestingly, the analysis of gene expression of the two subunits of the GABAB Receptor in the spinal cord showed that the acute administration of Baclofen tendentially increases the expression of both GABAB1 and GABAB2 subunits in the spinal cord (Fig. S1D).

Summing up, our results show that the acute administration of Baclofen after a SCI improves locomotor behavior, increasing the percentage of mice that acquire body weight support. Our data also reveals that Baclofen administration increases bladder function control in all treated groups.

**Baclofen does not prevent demyelination after SCI**

To assess if the administration of Baclofen could prevent demyelination after a SCI, we perform a staining using...
FluoroMyelin green fluorescence to assess the white matter sparing. White matter area was quantified in the total cross-sectional area of the spinal cord (Fig. 2A).

The white matter sparing quantification was performed 2 mm rostrally and 2 mm caudally to the lesion site, nine weeks after the injury. Our data showed no differences in the white matter sparing levels between the different experimental groups (Fig. 2B). A general quantification was performed by the calculation of the area under the curve from graph with total white matter sparing levels. This result also did not reveal differences between the treated mice and the Vehicle treated group (Fig. 2C). A deepen analysis of the rostral region (-1.2 mm to -0.6 mm), epicenter (-0.6 mm to 0.6 mm) and caudal region (0.6 mm to 1.2 mm) also revealed no statistical differences between the different experimental groups (Fig. 2D).

Overall, our results show that the administration of Baclofen does not prevent the de-myelination in the surrounding area of the lesion after a SCI.

Baclofen administration does not increase the number of α-motor neurons after SCI

The role of Baclofen in preventing neuronal death was previously shown in lampreys after SCI [6]. To assess if Baclofen administration could lead to α-motor neurons preservation after SCI, we performed an immunostaining with anti-NeuN antibody for the four experimental groups (Fig. 3A). The α-motor neurons were quantified in the ventral horn of the spinal cord, 2 mm rostrally and 2 mm caudally to the lesion site, nine weeks after the injury, as previously described [19] (Fig. 3B). The quantification of α-motor neurons revealed that there were no differences between the experimental groups (Fig. 3B). The analysis of the area under the curve from the graph with the total α-motor neurons quantification is similar between the experimental groups with a slight increase in the mice from the 24h Delay + Chronic group, when compared with the Vehicle treated mice (Fig. 3C). A detailed analysis of the rostral region the lesion (-1.2 mm to -0.6 mm), epicenter
Fig. 3. Baclofen does not increase the number of α-motor neurons. (A) Spinal cord transversal cross-sections at -1.2, 0.6, epicenter, 0.6 and 1.2 mm from the lesion site, nine weeks after injury for Vehicle, Acute, Acute + Chronic and 24h Delay + Chronic experimental groups, stained with the anti-body anti-NeuN. Magnification of representative regions for NeuN+ cells. (B) Quantification of the motor neurons in the spinal cord ventral horn every 200 μm, 2 mm rostrally and 2 mm caudally to the lesion epicenter. (C) Graph showing the area under the curve of results showed on graph from b. Area was calculated using GraphPadPrism ver.8.0. (D) Quantification of NeuN+ cells in the Rostral region (-1.2 to -0.6 mm), Epicenter (-0.6 to 0.6 mm) and Caudal region (0.6–1.2). Vehicle treated group n=6; Acute group n=6; Acute+Chronic group n=6; 24h Delay+Chronic group n=6 Values shown as mean±SEM. Scale bars=100 μm.
Acute administration of Baclofen modulates the immune response after SCI

To understand the role of Baclofen in the inflammatory response after a SCI we performed immunostaining using the anti-Iba-1 antibody. Using this marker, we quantified the area covered by resting microglia and normalized it per the total cross-sectional area of the spinal cord (Fig. 4A).

The quantification of resting microglia was performed 2 mm rostrally and 2 mm caudally to the lesion site, nine weeks after the injury as previously described [20]. Our results showed that mice from the Acute experimental group have a higher percentage of resting microglia when compared with the Vehicle treated group (Fig. 4B). The quantification of the area under the curve from the graph with the total quantification of resting microglia also revealed that the Acute experimental group has higher global resting microglia in comparison with the Vehicle treated group (Fig. 4C). The analysis of the rostral region to the lesion (-1.2 mm to -0.6 mm) showed no differences between the different experimental groups. However, in the region surrounding the lesion epicenter (-0.6 mm to 0.6 mm) and caudal to the lesion (0.6 mm to 1.2 mm) the Acute experimental group showed a higher percentage of resting microglia when compared with the Vehicle treated group (Fig. 4D).

To perform a deeper characterization of the immune response after a SCI we collect blood of animals from Acute and Vehicle experimental groups, two and six days after the injury. These time-points allow to understand the role of Baclofen at an earlier period after a SCI. The animal’s serum was analyzed through an Inflammation Array Kit (Fig. S2). Our data revealed that the circulating chemokines and cytokines of the Acute experimental group decreased over time. In contrast, the circulating chemokines and cytokines from the Vehicle group tend to increase from two to six days after the injury (Fig. 5A). For instance, the MIP-1α and CXCL5 pro-inflammatory chemokines greatly increased in the Vehicle experimental group from two to six days after the injury and the opposite was observed in Acute experimental group (Fig. 5A’).

Quantification of gene expression from the spinal cord epicenter collected nine weeks after the SCI, revealed no differences between Acute and Vehicle experimental groups regarding the expression of TNF-α and TGF-β genes (Fig. 5B). Our data revealed that acute administration of Baclofen modulates the activation state of microglia after SCI even nine weeks after the lesion. In addition, at early time-points after the lesion, the acute Baclofen administration leads to a decrease of the circulating chemokines and cytokines over time.

Discussion

In the present study we describe the role of Baclofen in functional recovery after SCI using a rodent model. We administered the same Baclofen concentration at different time-points after the injury to understand its time-window of efficacy. Our data revealed that an acute administration is more effective, producing better functional results than an administration starting 24 hours post-injury. Indeed, the group with delayed administration even had a higher death rate. This could be explained by the effectiveness of Baclofen to be more targeted to early time-points after injury and delay the first drug delivery could be producing negative outcomes. Moreover, a consecutive animal’s manipulation for two weeks to perform the chronic treatment could lead to additional deaths.

Interestingly, the acute administration of Baclofen can also be correlated with neurological improvements in humans with SCI [9], which agrees with our data. This supports that our model is reliable and meaningful to perform deepen studies regarding the effect of acute administration of Baclofen in the SCI context.

Our results showed that the acute administration of Baclofen leads to improvements in locomotor function with interesting results in the body weight support of treated mice. The Acute experimental group have a higher motor performance in the first four weeks after the lesion, when compared with the other treated experimental groups. This could be due to the Baclofen modulation in the lesion site immediately after the injury, which produces a significant improvement in the locomotor behavior of these animals. Nevertheless, at week nine after the injury, all the experimental groups reach the same motor performance. This observation could be explained by the spontaneous recovery previously observed in the adult rodents after several weeks after a spinal cord injury [21]. In addition, we observed that all the animals treated with Baclofen regained some level of bladder function control. Although Acute + Chronic experimental group presents a similar percentage of animals that reacquire the bladder control to the Vehicle treated group, it is important to note that the threshold to considered as fully regain of bladder function was an amount of urine below 0.2g. A considerable part of the animals from this experimental group presents a high ratio water intake/collection urine, nevertheless the amount of collected urine is not below of 0.2g and were not considered as having a full regain of bladder control. For this reason, Acute + Chronic mice present a low percentage of full bladder function recovery. Interestingly, the experimental group
Fig. 4. Acute administration of Baclofen modulates the activation state of microglia. (A) Spinal cord transversal cross-sections at -1.2, 0.6, epicenter, 0.6 and 1.2 mm from the lesion site, nine weeks after injury for Vehicle, Acute, Acute + Chronic and 24h Delay + Chronic experimental groups, stained with microglia marker Iba-1. Magnification of representative regions for resting and active microglia. (B) Percentage of resting microglia for 2mm rostrally and 2mm caudally to the lesion site, every 200 \( \mu \text{m} \) for the four experimental groups. (C) Graph showing the area under the curve of the results showed on graph from B. Area was calculated using GraphPadPrism ver.8.0. (D) Percentage of resting microglia in the regions Above Lesion (-1.2 to -0.6 mm), Epicenter (-0.6 to 0.6 mm) and Below Lesion (0.6 to 1.2). Vehicle treated group n=6; Acute group n=6; Acute+Chronic group n=6; 24h Delay+Chronic group n=6 Values shown as mean±SEM. \( \uparrow \)p-value <.05 Vehicle versus Acute, according to an unpaired Student t-test (B); *p-value <.05; ****p-value <.0001, Vehicle versus Acute, according to an unpaired Student’s t-test (C, D) Scale bars=100 \( \mu \text{m} \).
24h Delay + Chronic does not present improvements at the locomotor level, but mice from this experimental group showed a high bladder function recovery. These results are encouraging since Baclofen could be playing different roles in an injury time-dependent manner.

Our data showed no differences in white matter sparing levels (demyelination) between the different experimental groups, and the number of α-motor neurons in Baclofen treated animals is similar to the Vehicle treated group. Although our data revealed positive behavioral outputs
that the acute administration of Baclofen increases resting microglia nine weeks post-injury. Interestingly, this result is observed when Baclofen is administered only acutely and not when it is administered acute + chronically after the lesion, suggesting that tight temporal control is needed to regulate these processes. Our data also revealed that Baclofen administration modulates the circulating chemokines and cytokines at early time-points after injury. Although the chemokines and cytokines levels are higher in Acute animals, the levels decrease from two days after injury to six days after injury. In contrast, in Vehicle animals, the chemokines and cytokine levels increase overtime. This data revealed that Baclofen is playing a key, but unknown, role in the modulation of the immune response after SCI, that at later time-points results in more resting microglia.

Since GABA<sub>B</sub> receptors are present in microglia [26], Baclofen could play a direct role in modulating the microglia activity state. Baclofen could be regulating the immune response after the SCI, preventing further damage, and promoting the functional recovery that we observed in our in vivo experiments. Indeed, it was shown that Baclofen administration to microglia cell cultures leads to a decrease of inflammatory mediators such as P38 MAP Kinases [27]. Moreover, the GABAergic system was recently described as a key target to modulate neuroinflammation [28]. The mechanisms behind our observations are not fully understood, and further studies must be performed to understand it. Nevertheless, our observations are aligned with emerging data showing that GABAergic drugs can mitigate the neuroinflammation. The control of the exacerbated inflammation by Baclofen after a SCI could lead to a more permissive environment favoring the functional recovery showed by our data.

The present findings demonstrate that Baclofen, an anti-spasticity drug currently used in the clinical setting, can also lead to functional improvements in a SCI context. We observed that the acute administration of Baclofen increases motor performance and bladder function control with no effect in the prevention of myelin degradation or the survival of α-motor neurons. Surprisingly, the acute administration of Baclofen modulates the immune response after SCI which could be leading to a more permissive environment (Fig. 6).

Our data highlights the pharmacological potential of Baclofen in a SCI context. Further studies to determine if a higher concentration of Baclofen produces better results and to assess the cellular and molecular mechanisms behind the functional improvements must be performed. Nevertheless, our data unveil the potential use of Baclofen, a cheap and well-known drug, for SCI functional recovery.

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Supplementary materials
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References