

# Total rupture of achilles tendon induces inflammatory response and glial activation on the mice spinal cord

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## Research Article

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# Abstract

Rupture of Achilles tendon is a common accident affecting professional and recreational athletes. Acute and chronic pains are symptoms commonly observed in ruptured patients. Despite that, no studies have described whether Achilles tendon rupture is able to promote disorders in CNS. Based in these finds, the current study aimed to evaluate nociceptive alterations and inflammatory response in L5 lumbar segment of Balb/c mice spinal cord after Achilles tendon rupture. We demonstrated increased algesic response in the paw of ruptured group on the 7th and 14th days post tenotomy when compared with control group. This phenomenon was accompanied by over expression of COX-2 and NOS-2 as well as hyperactivation of astrocytes and microglia in nociceptive areas of L5 spinal cord as evidenced by intense GFAP and IBA-1 immunostaining, respectively. Biochemical studies also demonstrated increased levels of nitrite in the L5 spinal cord of tenotomized animals when compared with control group. Thus, we have demonstrate for the first time that total rupture of the Achilles tendon induces inflammatory response, nitregeric and glial activation in the CNS at L5 spinal cord region.

## Introduction

Rupture of Achilles tendon is a common injury observed in recreational and professional athletes<sup>1-3</sup>. Partial or total rupture of the Achilles tendon demands special attention since prolonged periods of tissue impairment can induce permanent alterations in the normal gait pattern of patients<sup>4</sup>. The treatment of ruptured Achilles tendon is generally made by surgical intervention being time of recovery variable among individuals<sup>5,6</sup>. As widely described in the literature, the time elapsed from tendon rupture to surgical repair is predictive of Achilles tendon recovery<sup>7</sup>. Different studies show that localized pain and changes in gait pattern are symptoms commonly described in injured subjects<sup>8,9</sup>. In fact, the biochemical and histological alterations in the local of ruptured Achilles tendon are well documented, however, little is known about the impact of this injury on the central nervous system<sup>10-12</sup>.

The Achilles tendon is connected to gastrocnemius and soleus muscles<sup>13</sup>. Under normal conditions the tendon has few nerves, located in an adjacent structure (paratenon) forming nerve plexuses, where small branches follow which penetrate into the sheath that covers the tendon (epitenon)<sup>12,17</sup>. Notably, in pathological conditions, an intense nerve fiber growth occurs (sensory and Autonomic) between the collagen fibers that suggest an important role in the regulation of pain, inflammation and tissue repair<sup>14-16</sup>.

Neuronal cells which process information from the Achilles tendon are localized in the lumbar intumescence at the L5 segment<sup>18,19</sup>. Few studies have evaluated how the spinal cord responds to injury in the Achilles tendon<sup>20,21</sup>. Furthermore, it remains unclear if Achilles tendon injuries are able to trigger inflammatory response on the spinal cord of injured patients. As previously demonstrated inflammatory activation in injured spinal cord can be monitored by glial or neuronal activation<sup>22,23</sup> as well as by measurement *in loco* of inflammatory mediators such cyclooxygenase-2 (COX-2) and inducible nitric

oxide synthase-2 (NOS-2) enzymes. Astrocytes and activated microglia produce inflammatory mediators such as prostaglandins and nitric oxide (NO) which are important indicators of CNS injury<sup>24,25</sup>. The production of these inflammatory agents is frequently evaluated by local expression of NOS-2 and COX-2 which are enzymes responsible for their synthesis<sup>24, 26</sup>. The current study aimed to describe, by biochemical and histological evaluation, the effect of Achilles tendon rupture on the inflammatory response and pain response in spinal cord of tenotomized mice.

## Results

### *Rupture of Achilles tendon potentize algescic response in mice paw.*

We used the paw withdrawal threshold (PWT) in order to evaluate the effect of Achilles tendon rupture on the algescic response of ipsilateral paw of ruptured mice. As demonstrated in Fig. 1, animals submitted to total tenotomy of Achilles tendon showed significant decrease in PWT values for ipsilateral paw ( $0.05 \pm 0.18$  g) when compared with control group ( $3.5 \pm 1.0$  g) at 7th day post lesion. Similar results were observed at 14th day post lesion, the rupture group showed low values of PWT ( $0.1 \pm 0.07$  g) in relation with control group ( $3.3 \pm 1.1$  g). The baseline test has demonstrated no significant difference among the animals before experimental procedures

### *Effect of Achilles tendon rupture on the microglial activation in the L5 spinal cord segment.*

Immunolabeling analysis in L5 spinal cord shows remarkable staining for GFAP protein in control group (Fig. 2). This phenomenon can be evidenced in Figs. 2A and 2B which also demonstrate that ruptured animals show an increased number of GFAP positive cells on the 7th ( $n = 910 \pm 29$  cells/ $\mu\text{m}^2$ ) and 14th ( $n = 860 \pm 20$  cells/ $\mu\text{m}^2$ ) days post-surgery when compared with control group ( $n = 412 \pm 54$  cells/ $\mu\text{m}^2$ ). In addition, our data has shown that Achilles tendon rupture induces microglial activation in the L5 spinal cord of the experimental groups. As described in Fig. 3, the control group presents fewer IBA-1 positive cells ( $n = 60 \pm 7$  cells/ $\mu\text{m}^2$ ) while tenotomized animals present an increased number of IBA-1 cells stained in the L5 spinal cord on the 7th day post-surgery ( $n = 100 \pm 2$  cells/ $\mu\text{m}^2$ ) and 14th days post-surgery ( $n = 500 \pm 70$  cells/ $\mu\text{m}^2$ ). Spinal cord glial reactivity was predominant on the ipsilateral side to tendon rupture and focused in the dorsal horns.

We have counted of GFAP and IBA-1 positive cells in the dorsal horn of control and tenotomized animals. As described in Fig. 4A, tendon rupture induced an increase in the number of GFAP positive cells at 7th ( $267 \pm 73$  cells/ $\mu\text{m}^2$ ) and 14th ( $211 \pm 18$  cells/ $\mu\text{m}^2$ ) day post-rupture when compared with non-ruptured animals ( $155 \pm 16$  cells/ $\mu\text{m}^2$ ). On the other way, as evidenced in Fig. 4B increased number of IBA-1 positive cells was observed only at 14th days post rupture (control =  $20 \pm 2.7$  cells/ $\mu\text{m}^2$  vs rupture group =  $158.75 \pm 35.5$  cell/ $\mu\text{m}^2$ ). It also was performed determination of GFAP and IBA-1 positive cells present in dorsal horn ipsilateral and contralateral side of spinal cord as demonstrated in Fig. 5. Our results show that ipsilateral side of rupture already presented increased number of GFAP positive cells at 7th days post-surgery when compared with contralateral side of lesion (ipsilateral =  $305 \pm 49.4$  vs contralateral =

183 ± 14.1 cell/μm<sup>2</sup>) and no significant difference was observed at 14th days post surgery (Fig. 5A). On the other way, rupture of Achilles tendon did not have altered the number of IBA-1 positive cells at 7th days post-surgery, but at 14th days post-surgery, these values were significantly increased when compared with contralateral side (ipsilateral = 178.5 ± 17.6 vs contralateral = 104 ± 5.6) (Fig. 5B).

## Inflammatory activation in L5 spinal cord induced by tendon rupture

In order to evaluate if glial activation in the L5 spinal cord of the tenotomized group is associated with expression of proteins mediating inflammatory response, immunofluorescence for COX-2 and NOS-2 proteins were analyzed as described in the methods. As observed in Fig. 6, no staining for COX-2 were found in L5 spinal nerve of tenotomized animals on the 7th day post injury. However, Achilles tendon rupture has induced intense expression of COX-2 in the spinal cord on the 14th day post rupture (Fig. 6). Similarly, the expression of NOS-2 in the L5 spinal nerve was only detected on the 14th day post rupture (Fig. 7). Immunoreactivity of these inflammatory mediators (NOS-2 and COX-2) are focused on dorsal horns of the spinal cord and ipsilateral side to tendon rupture. Nitrite production in the L5 spinal cord was also evaluated in control and rupture groups. Our data revealed that ruptured animals presented increased levels of nitrite in the L5 spinal cord when compared with control. Our results showed elevated levels of nitrite in the L5 spinal nerve on the 7th day post Achilles tendon rupture (Fig. 8).

## Discussion

The current study had demonstrated for the first time that total rupture of Achilles tendon evokes microglial activation and inflammatory response in the L5 spinal cord. Although it is widely described that total rupture of Achilles tendon promotes a painful recovery in injured patients, there is little data in the literature describing the effect of tendon injury on the central nervous system<sup>21,30,31</sup>. As previously described, rupture of the Achilles tendon triggers an *in loco* activation of inflammatory response which is characterized by hypercellularity, intense enervation and local angiogenesis<sup>[31, 32, 33]</sup>.

The L5 spinal cord segment is a region of the CNS responsible for receiving input from the Achilles tendon-muscle complex<sup>34-36</sup>. The remarkable time-dependent astrogliosis and microglial activation observed in this region suggest that Achilles tendon rupture elicits activation of a neuroinflammatory response in the spinal cord at L5 segment. In addition, it was demonstrated in the behavioral test significant decrease in PWT values for ipsilateral paw which was sustained until o 14th dpt. The hallmarks of neuroinflammation are the activation and infiltration of leukocytes, activation of glial cells, and increased production of inflammatory mediators<sup>37,38</sup>. The interactions between inflammation and pain are bidirectional, nociceptive sensory neurons not only respond to immune signals, but also directly modulate inflammation<sup>38</sup>.

Numerous non-neuronal cell types influence pain sensation, including immune, epithelial, mesenchymal and glial cells<sup>37</sup>. It is well documented that microglial activation represents an important response to neuronal injuries<sup>39,40</sup>. In accordance with our findings, previous studies already have described activation of microglial cells in damaged spinal cord of humans and animal models of spinal nerve injury<sup>25,41</sup>.

Several studies about peripheral nerve injury and spinal cord injury hypothesized that astrocytic reactivity occurs secondary to the microglial reactivity. Our results show evidence that astrocytes are involved in the maintenance, but not in the development of pain, being this function attributed to microglia. In a comparative study between two injury models, Romero-Sandoval, et al (paw incision and L5 nerve injury), showed that glial expression pattern is differentiated in each condition and divergent in the temporal course, suggesting that the onset and intensity of IBA-1 and GFAP expression are related to the cause of the primary lesion. Our data along with these findings show the dynamic and plastic nature of glial cells under pathological conditions, and that glial reactivity may demonstrate distinct temporal patterns of expression, depending on the lesion<sup>42</sup>.

GFAP overexpression and inflammatory response are events correlated to the impairment in spinal cord normal physiology as well as to the onset of hyperalgesia<sup>22,43,44</sup>. The immunostaining sites on the spinal cord are relevant since the posterior region (dorsal horns) of the spinal cord are accountable for conduction of sensory stimuli. Beyond that, increased glial activation on the ipsilateral side of the dorsal horn, as well as decreased mechanical sensitivity in the post-rupture time course supports that longer hyperalgesic events associated with tendon rupture could be related with inflammatory activation in the spinal cord. The response of astrocytes may be triggered by progressive nerve degeneration and can be responsible for initiation of acute pain while microglial activation could mediate inflammatory response. However, posterior experiments need to be performed to ratify this hypothesis.

Inflammatory response in the L5 spinal cord induced by Achilles tendon rupture was confirmed by *in loco* expression of COX-2 and NOS-2 on the 7th and 14th day post tendon injury. Immunostaining results were also supported by biochemical findings that have demonstrated significant elevation of nitrite levels in the lumbar spinal cord of animals submitted to Achilles tendon rupture. It was widely described in the literature that COX-2 and NOS-2 expressions are intimately related with overproduction of prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) and nitric oxide (NO) which are important inflammatory mediators<sup>45-48</sup>. PGE<sub>2</sub> and NO production are also evidenced in different kinds of spinal cord and nerve injuries and previous studies point to a close correlation between these mediators and injury in motor performance<sup>49-51</sup>. As presented in Fig. 9, our hypothesis is that Achilles tendon triggers glial activation and production of inflammatory mediators in spinothalamic tract of L5 spinal cord which favors the generation of acute and neuropathic pain. However, it is important to highlight that we do not discard that additional studies on Neuro-glial interactions are essentials to understand the exact mechanism involved in acute and chronic pain evoked by Achilles tendon rupture since neurons are also able to express NOS-2 and COX-2.

Taken together our findings demonstrate that total rupture of Achilles tendon directly affects the CNS on the L5 spinal cord level.

## Methods

### Animals and Experimental design

60 Balb/c mice (Males, at 6–8 weeks old, weighing 25–30 g) were provided by animal facilities from the Federal University of Pará (UFPA) and kept in polypropylene cages at 25°C in controlled dark/light cycle (12:12) with food and water *ad libitum*. Animals were anesthetized by intraperitoneal injection of ketamine/xylazine solution (90/5 mg/kg). Tibia region of the right paw of the animals was manually tricotomized and a longitudinal skin incision (about 0.5 cm) to access the Achilles tendon. The animals (n = 6 per group) were separate in control group (CG) which was not submitted to surgical procedure and ruptured group (RG) which Achilles tendon was transected at 0.5 cm from it calcaneal insertion. These steps were followed by local asepsis and skin suture using a 4.0 nylon monofilament. The experimental animals were returned to home cages without movement restriction. The spinal cord of control and ruptured animals on the 7 and 14 days post surgery were collected after tissue fixation by transcardiac perfusion with saline solution (0,9%) and 4% paraformaldehyde (PFA). The lumbar region of the spinal cord was removed by laminectomy and was then post-fixed with 4% PFA for 24 hours. Afterwards L5 level was identified and sectioned at 10 µM utilizing a Leica cryostat (model CM3050).

### Von Frey behavioral test (mechanical sensitivity)

The hind paw withdrawal threshold was determined using von Frey hairs ranging from 0,02 to 10 g. All experimental procedure was performed as a blind test such that the experimenter was not aware of the group (control or ruptured mice) that were being tested. The protocol used in current study was made in accordance with Chaplan et al., with few variations<sup>27</sup>. The tests for control and ruptured groups began after 5 minutes of habituation. The series of von Frey hairs was applied from below customized platform as following. Ipsilateral hind paw of control or ruptured mice were pressed with one of a series of filaments gradually increasing stiffness (0.02-10 g) applied to the plantar surface for 5–6 s for each filament. Ipsilateral hind paw of control or ruptured mice were pressed with one of a series of filaments gradually increasing stiffness (0.02-10 g) applied to the plantar surface for 5–6 s for each filament. Each filament was applied 10 times and the minimum value that caused at least 3 responses which were recorded as paw withdrawal thresholds (PWT). Acute withdrawal, bite, licking or shaking the ipsilateral posterior limb and vocalization were considered positive signs of withdrawal. The average of these values was used for data analysis. The withdrawal threshold was determined to each animal before surgery, 7 and 14 days after surgery in independent groups.

### Immunostaining assays

Slices of L5 spinal cord were washed twice with pH 7.2 phosphate buffer (PBS) for 30 min and incubated in ammonia chloride solution (pH 8.0) for 40 min. The tissues were permeabilized with Triton X-100 and 3% BSA solution at room temperature for 1 hour. This step was followed by overnight incubation with primary anti-GFAP (H-50 Santa Cruz Biotechnology, 1:200); anti-Iba-1(ab5076, Abcam, 1:200), anti-NOS-2 (Santa Cruz Biotechnology, 1:200) and anti-COX-2 (SAB42,Sigma-Aldrich 1;200) at 4°C and posterior

washes with pH 7.2 PBS. The slices used to evaluate GFAP and IBA-1 expression were incubated for 2 hours with secondary antibody conjugated with peroxidase and revealed using DAB peroxidase substrate. NOS-2 and COX-2 expression were evaluated by immunofluorescence after incubation for 2 hours with secondary anti-body Alexa Fluor 488 or Alexa Fluor 594 (Santa Cruz Biotechnology, 1:1000) at room temperature. Cells nuclei were stained with DAPI probe (1:1,000). Finally, tissue sections were washed and mounted on lamina containing N-propyl-gallate and then visualized by fluorescence microscopy (Zeiss Mod). Glial cell reactivity quantification (GFAP and IBA-1) was performed by manual counting, using 4 images of each field (1.530 mm<sup>2</sup>) of the L5 spinal cord segment, on ImageJ® software. The spinal cord was demarked and the cell number stained to GFAP and IBA-1 present in dorsal and ventral horns as well as in the ipsilateral and contralateral side of lesion was determinate<sup>28</sup>. Data were expressed in mean and standard deviation.

## **Biochemical analysis**

Nitric oxide production in the L5 spinal cord was measured by determination of nitrite levels as previously described by Darmani et al<sup>29</sup>. Control (n = 6) and rupture mice (n = 6) had their lumbar spinal cord dissected at L5 level as described above. The tissues were homogenized in saline solution and then 700 µl were centrifuged and the supernatant was collected. After that, 500 µl of sample were mixed with the same volume of Griess reagent (1% sulfanilamide, 0.1% naphthalene and 5% phosphoric acid). Absorbance values were read at 540 nm and referred to the nitrite standard curve. Nitrite levels in the samples were normalized by the concentration of protein found and protein content determined by Bradford method (Bradford 1976).

## **Statistical analysis**

The appropriated statistical tests were selected after data analysis of hyperactivity cell count and behavioral tests using Kolmogorov-Smirnov normality test. Data were expressed in mean and standard deviation and the difference between control and ruptured groups was evaluated using one-way ANOVA test followed by Tukey post-hoc tests. For behavioral data, two-way ANOVA test followed by Tukey post-hoc was performed. Statistical analyses were made with BioStat 5.0 and  $p \leq 0.05$  was considered as significant.

## **Ethics statement**

The animal experiments were handled in strict compliance with the guidelines of Brazilian law Number 11.794/2008 for the care and use of animals for scientific purposes. The protocol was previously approved by Ethical Committee for Care and use of laboratory Animals (CEUA) from UFPA (Protocol number 8179020318). The study was carried out in compliance with the ARRIVE guidelines.

## **Declarations**

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## Competing interests

The authors declare no competing interests.

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## Author Contributions:

AH designed the experiments. DP, MS, AM, TM and MK collected and analyzed the data. AH and DP wrote the manuscript. SM, LL, CB, RB, EB, AP, KO and BV provided laboratory facilities, materials, reagents, and mice. All authors have approved the manuscript for publication in Scientific Reports journal.

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## Figures

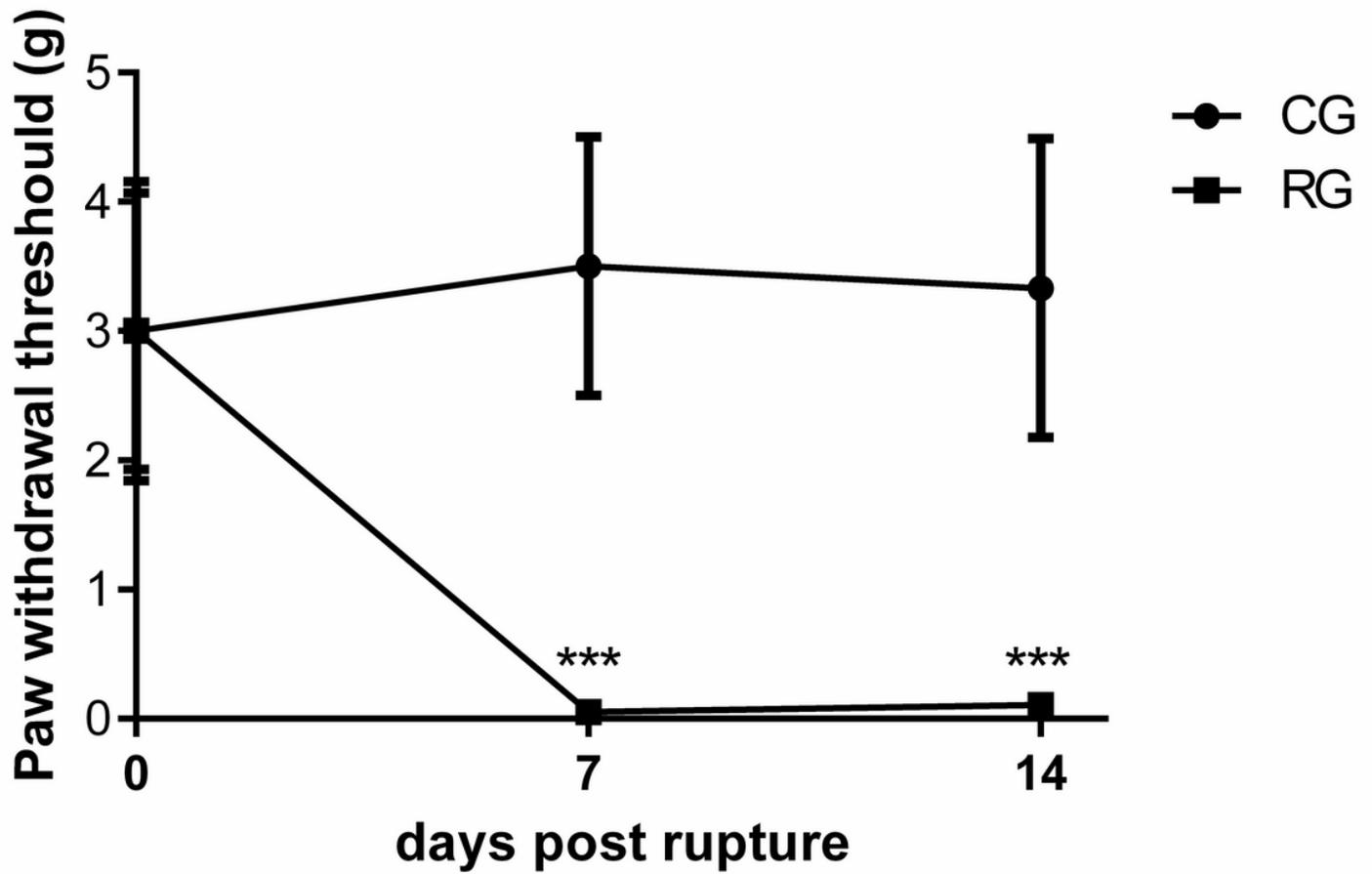
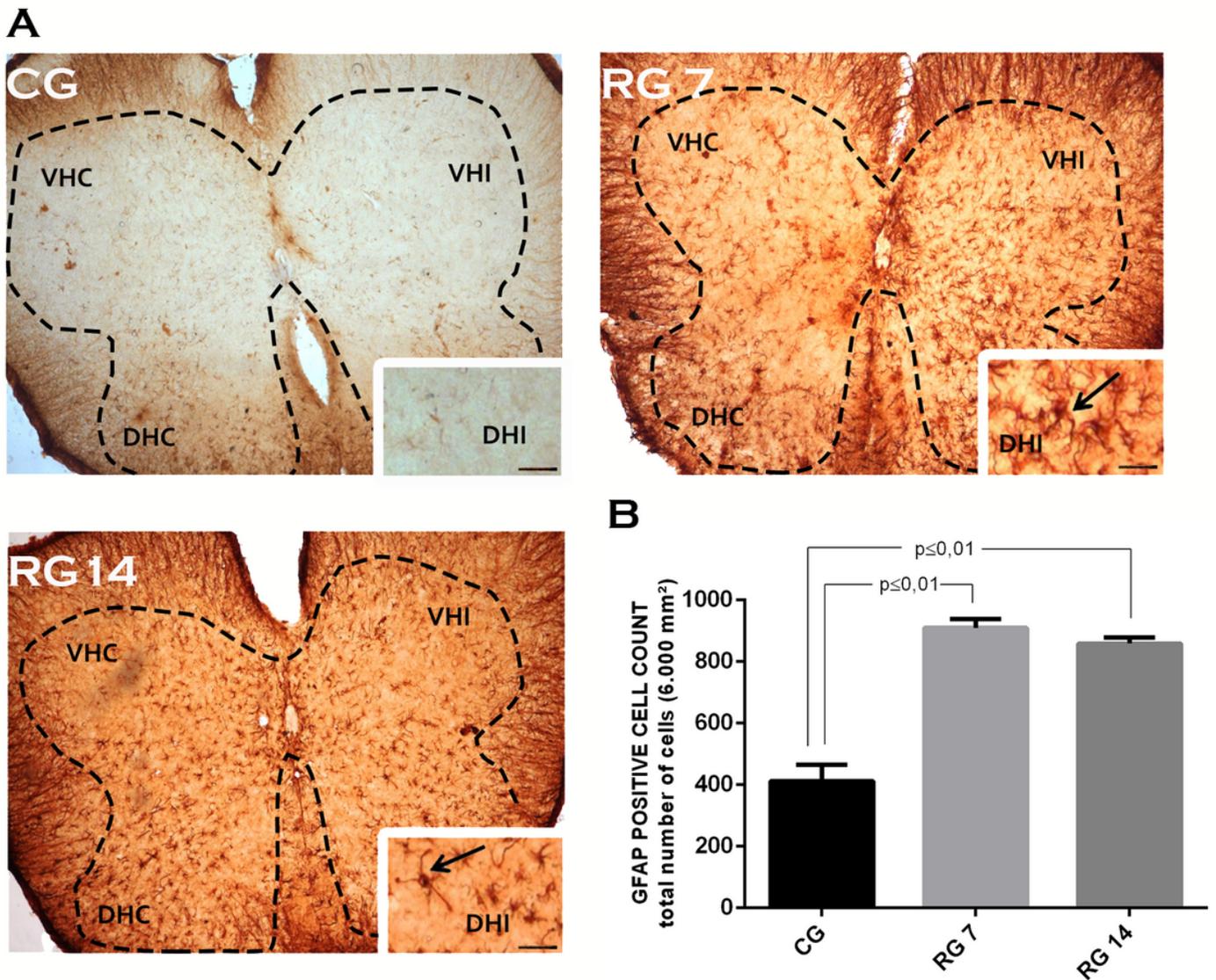


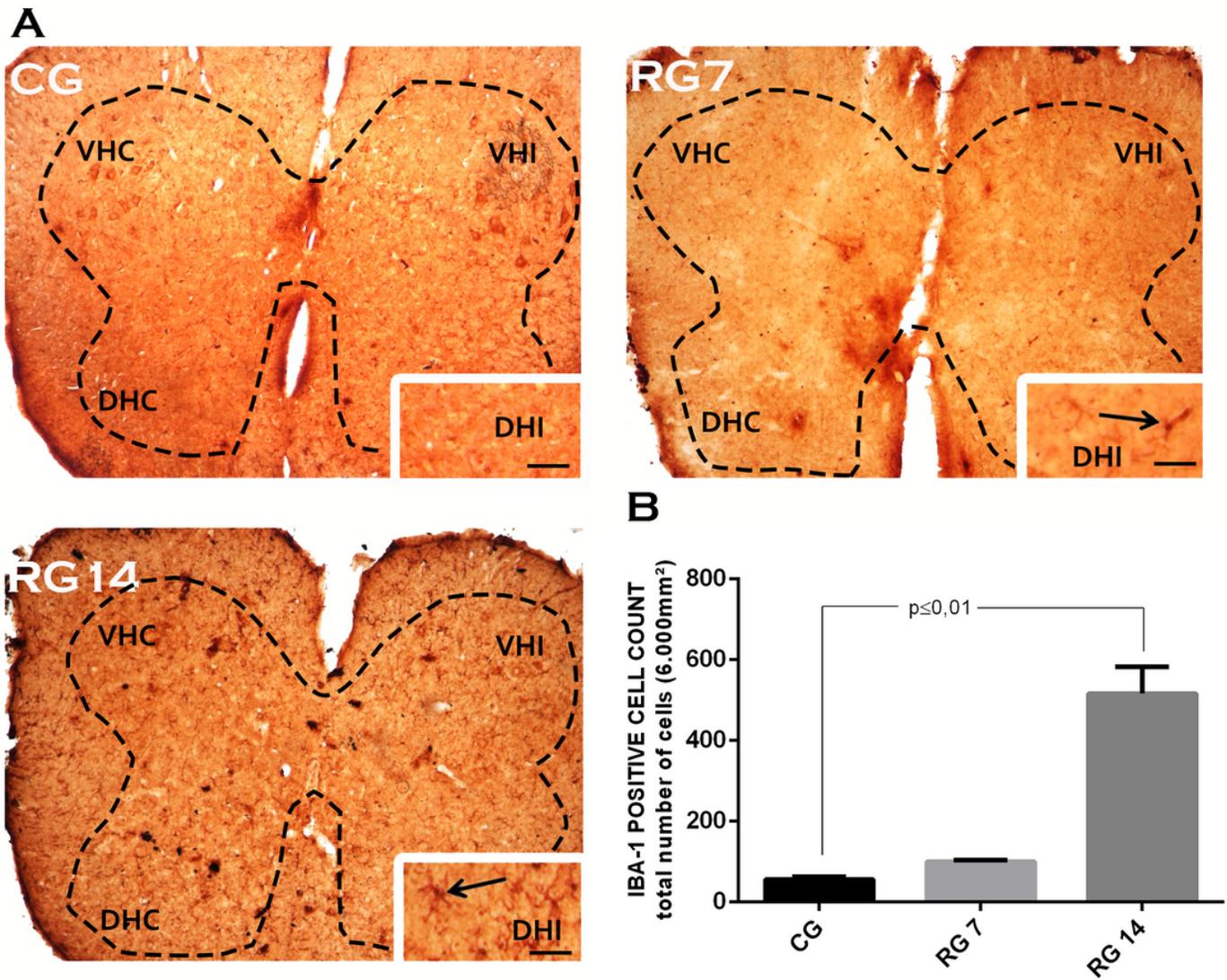
Figure 1

Effects of Achilles tendon rupture in mechanical nociceptive threshold. CG control group and RG rupture group analyzed on 0 before surgery, 7th and 14th days post Achilles tendon tenotomy. Data are presented as mean  $\pm$  SD. \*\*\* Significant difference ( $P < 0.01$ ) between the CG versus RG,  $n = 6/\text{group}$ , assessed by test two-way ANOVA followed by Tukey's post hoc comparisons



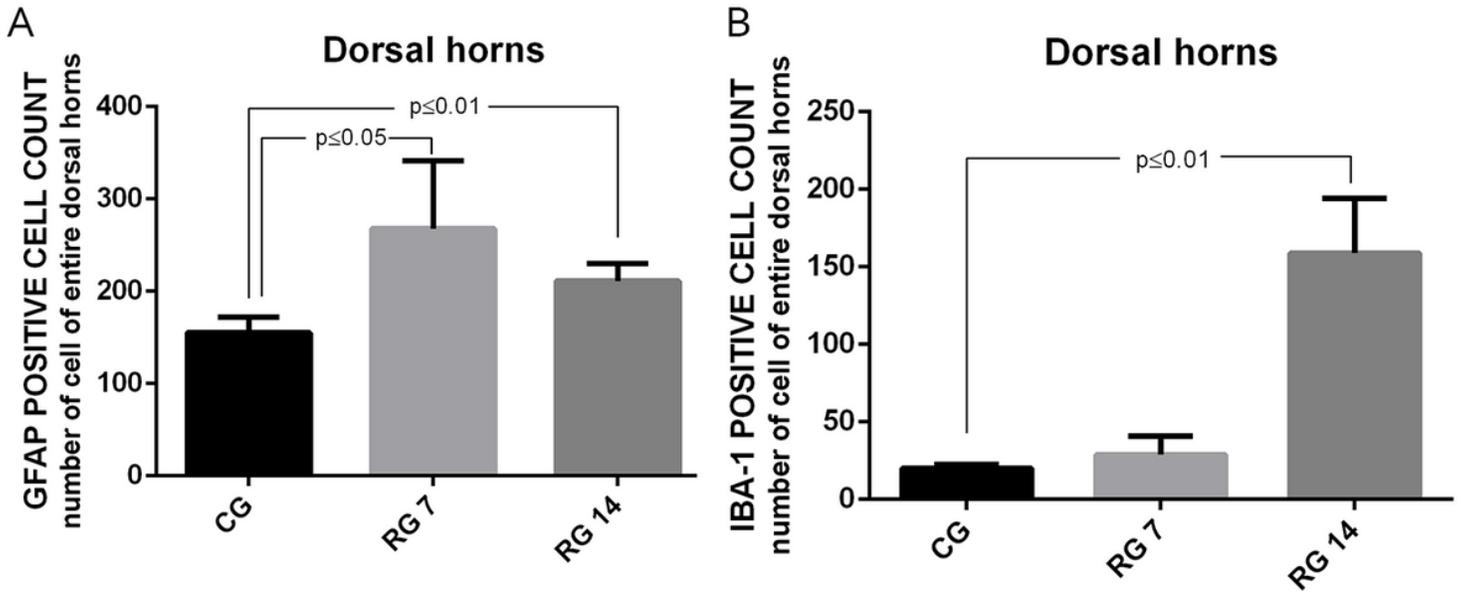
**Figure 2**

Effects of Achilles tendon rupture in Astrocytes reactivity of spinal cord. A- GFAP immunostaining of transversal sections of mice spinal cord (segment L5) on the 7th and 14th days post Achilles tendon tenotomy (dpt). CG control group, RG 7 rupture group analyzed on the 7th dpt. RG 14 analyzed 14th dpt. B- GFAP positive cell count in the L5 spinal cord. Data are presented as mean  $\pm$  SD,  $p < 0.01$ ,  $n=6$ /group, assessed by one-way ANOVA followed by Tukey's post hoc comparisons. VHC- ventral horn contralateral, VHI- ventral horn ipsilateral, DHC- dorsal horn contralateral and DHI- dorsal horn ipsilateral. Black arrows in the enlarged images show strong astrogliosis in the dorsal horn of the ipsilateral side to injury. Scale bar = 100 $\mu$ m



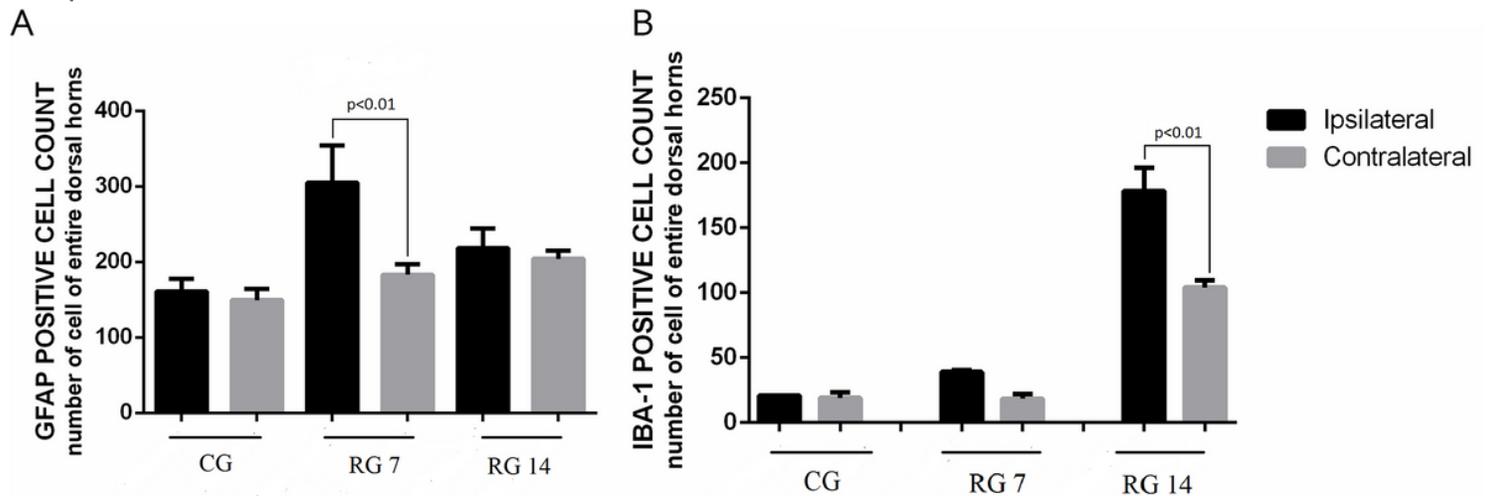
**Figure 3**

Effects of Achilles tendon rupture in microglial reactivity of spinal cord. A- IBA-1 immunostaining of transversal sections of mice spinal cord (segment L5) on the 7th and 14th days post Achilles tendon tenotomy (dpt). CG control group, RG 7 rupture group analyzed on the 7th dpt. RG 14 analyzed on the 14th dpt. B- IBA-1 positive cell count in L5 spinal cord. Data are presented as mean  $\pm$  SD,  $p < 0.01$ ,  $n=6/\text{group}$ , assessed by one-way ANOVA followed by Tukey's post hoc comparisons. VHC- ventral horn contralateral, VHI- ventral horn ipsilateral, DHC- dorsal horn contralateral and DHI- dorsal horn ipsilateral. Black arrows in the enlarged images show strong microgliosis in the dorsal horn of ipsilateral side to injury, mainly on RG 14. Scale bar = 100 $\mu\text{m}$



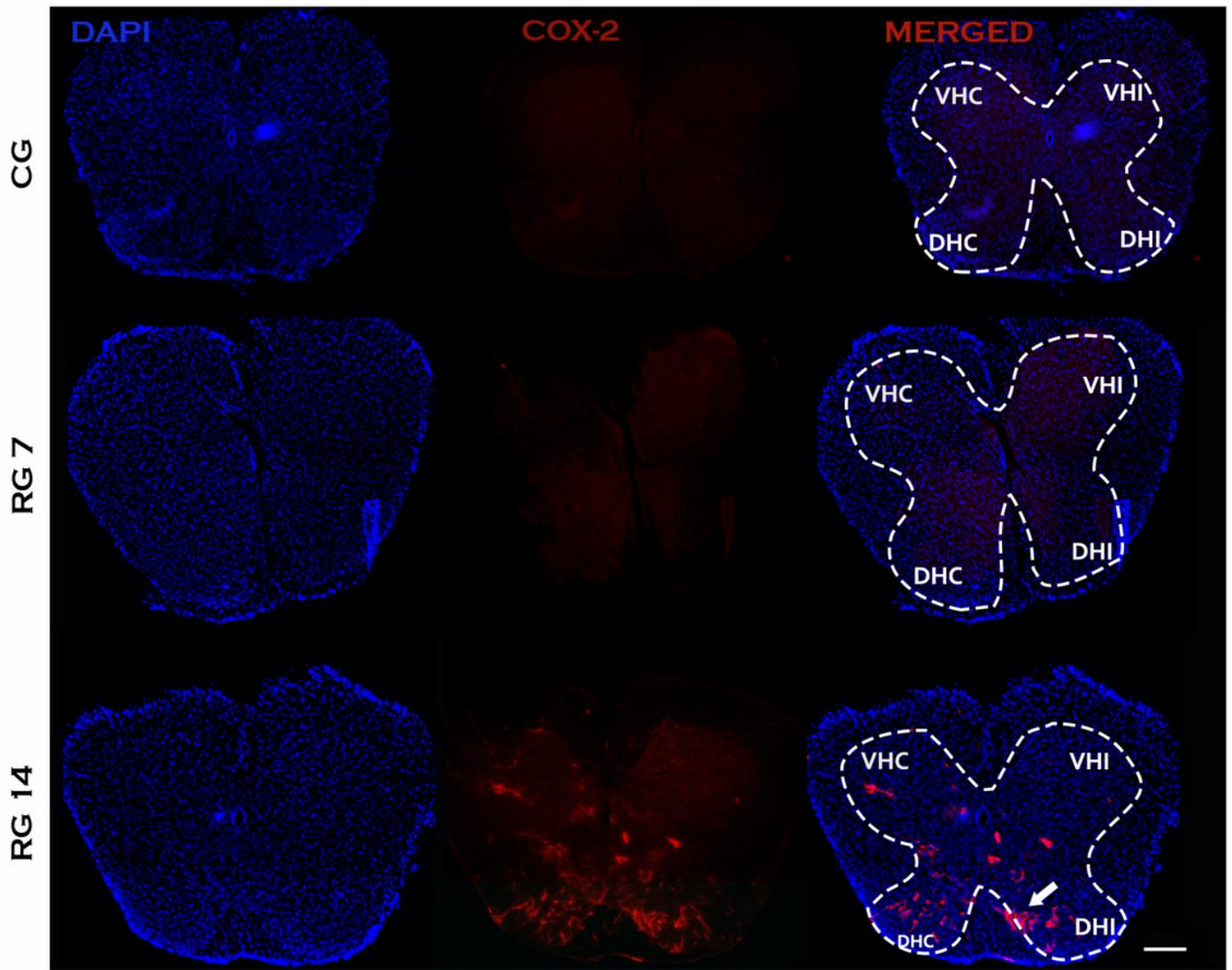
**Figure 4**

Effects of Achilles tendon rupture in glial reactivity of dorsal horns. A- GFAP positive cell count only dorsal horns of L5 spinal cord. B- IBA-1 positive cell count only dorsal horns of L5 spinal cord. Data are presented as mean  $\pm$  SD,  $p < 0.01$ ,  $n=6$ /group, assessed by one-way ANOVA followed by Tukey's post hoc comparisons



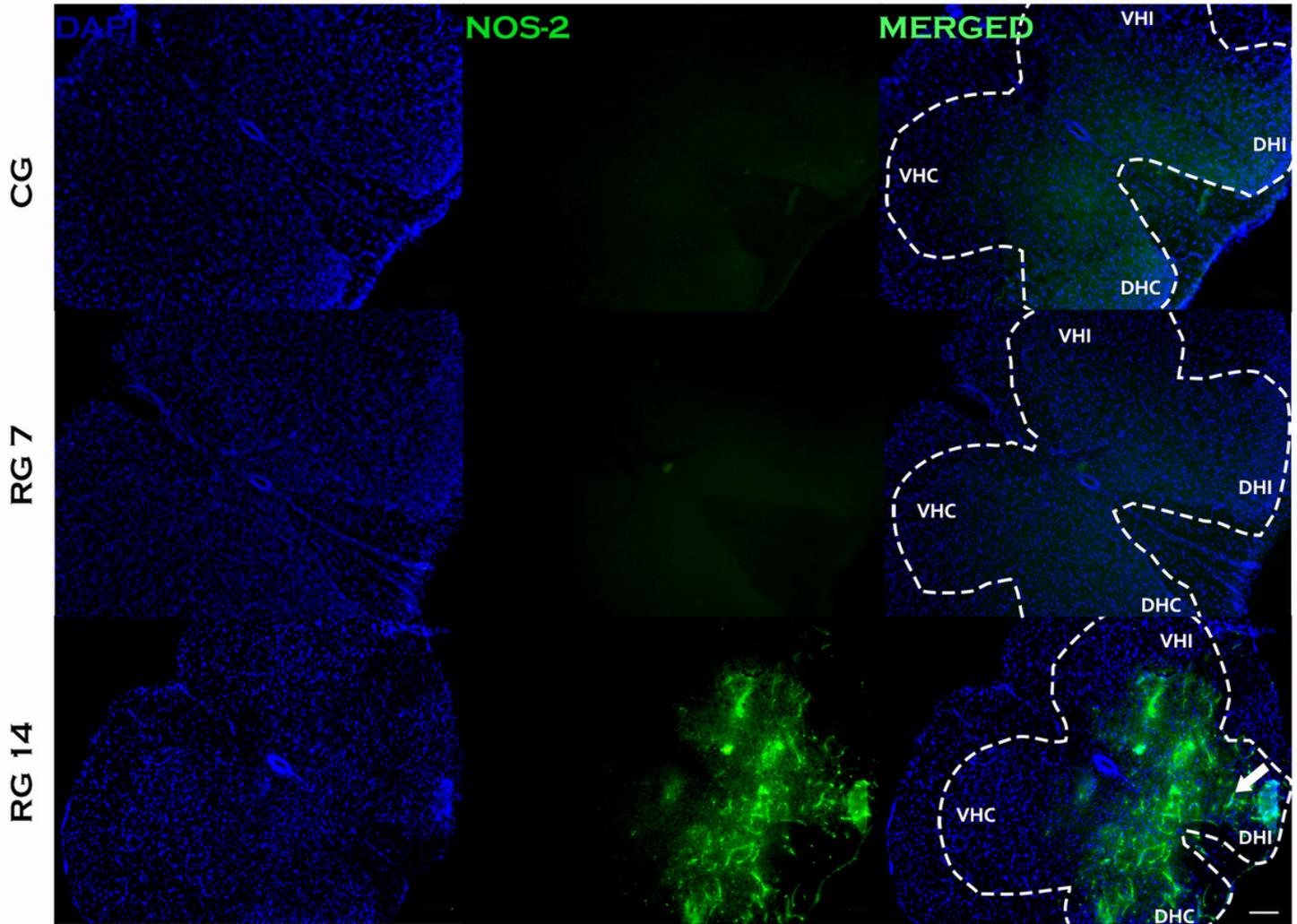
**Figure 5**

Effects of Achilles tendon rupture in glial reactivity between the ipsi and contralateral sides. A- GFAP positive cell count only dorsal horns of L5 spinal cord. B- IBA-1 positive cell count only dorsal horns of L5 spinal cord. Data are presented as mean  $\pm$  SD,  $p < 0.01$ ,  $n=6$ /group, assessed by one-way ANOVA followed by Tukey's post hoc comparisons



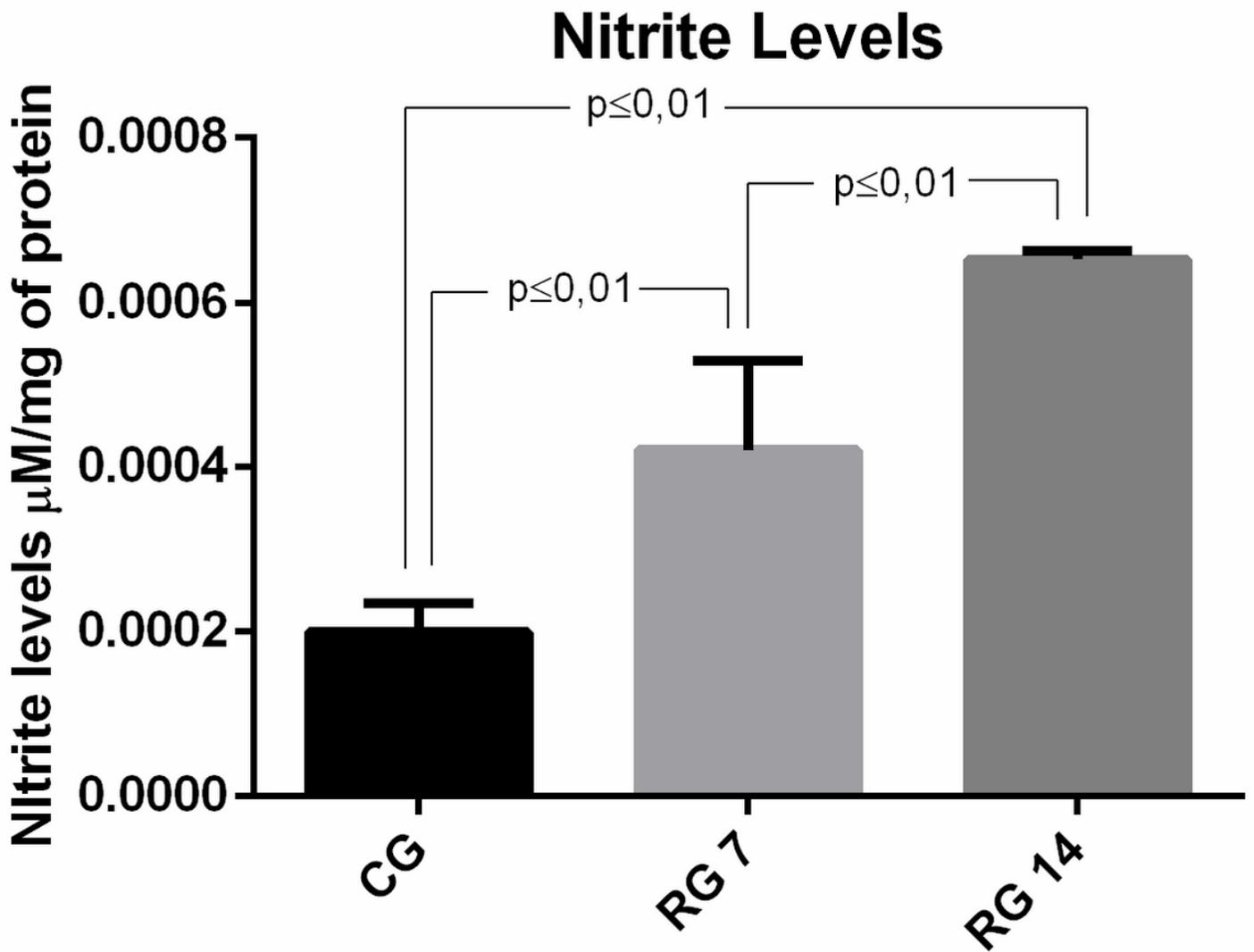
**Figure 6**

Inflammatory response on spinal cord induced by Achilles tendon rupture. Immunofluorescence to COX-2 of transversal sections of mice spinal cord (segment L5) on the 7th and 14th days post Achilles tendon tenotomy (dpt). CG control group, RG 7 rupture group analyzed on the 7th dpt. RG 14 analyzed on the 14th dpt. The nuclei of cells were stained with DAPI. VHC- ventral horn contralateral, VHI- ventral horn ipsilateral, DHC- dorsal horn contralateral and DHI- dorsal horn ipsilateral. White arrow show strong reactivity in the dorsal horn of ipsilateral side to injury. n=6/group. Scale bar = 100 $\mu$ m



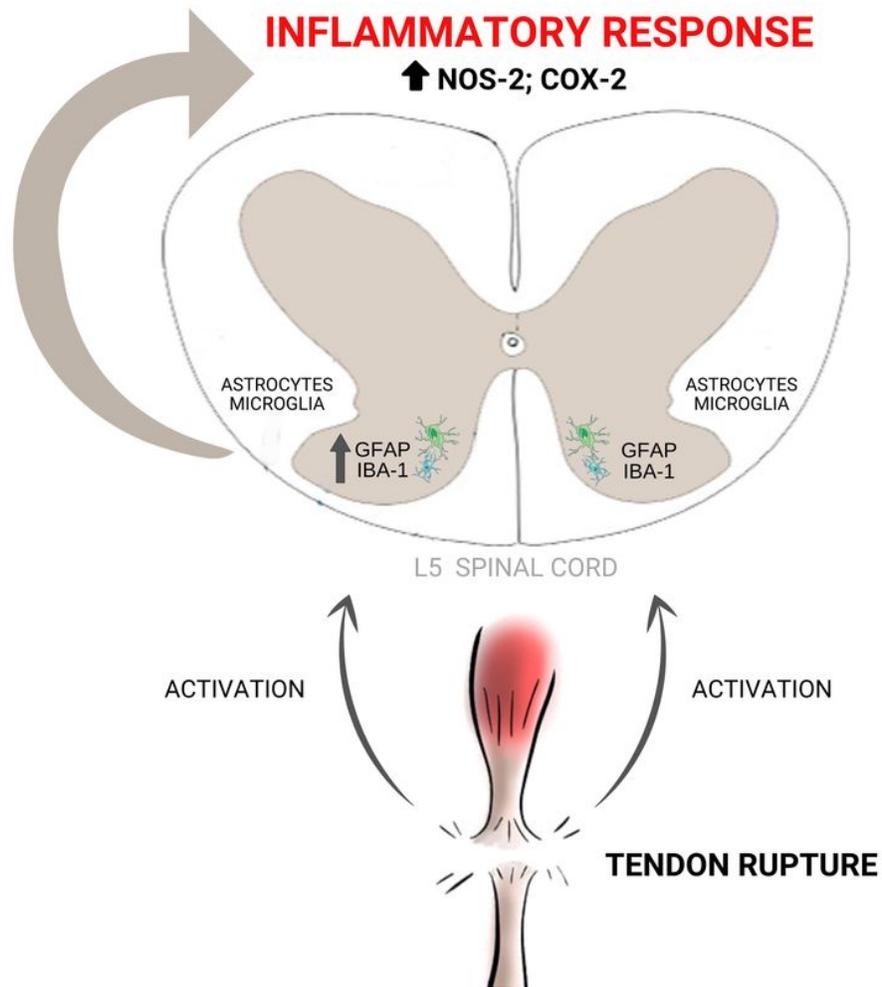
**Figure 7**

Inflammatory response on spinal cord induced by Achilles tendon rupture. Immunofluorescence to NOS-2 of transversal sections of mice spinal cord (segment L5) on the 7th and 14th days post Achilles tendon tenotomy (dpt). CG control group, RG 7 rupture group analyzed on the 7th dpt. RG 14 analyzed on the 14th dpt. The nuclei of cells were stained with DAPI. VHC- ventral horn contralateral, VHI- ventral horn ipsilateral, DHC- dorsal horn contralateral and DHI- dorsal horn ipsilateral. White arrow show strong reactivity in the dorsal horn of ipsilateral side to injury. n=6/group. Scale bar = 100µm



**Figure 8**

Effect of tendon rupture on the nitrite CNS levels of tenotomized mice. Nitrite quantification of spinal cord on the 7th and 14th days post Achilles tendon tenotomy (dpt). CG control group, RG 7 rupture group analyzed on the 7th dpt. RG 14 analyzed on the 14th dpt. The lumbar intumescence was removed to assess the nitrite levels in the tissue by Griess method. Data are presented as mean  $\pm$  SD, \* $p < 0.01$ ,  $n=5-6$ /group, assessed by one-way ANOVA followed by Tukey's post hoc comparisons



**Figure 9**

Representative image of inflammatory response in the spinal cord after total Achilles tendon rupture.