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Progesterone modulates pro-inflammatory cytokine expression profile after spinal cord injury: Implications for neuropathic pain

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ABSTRACT

Neuropathic pain is a frequent complication of spinal cord injury (SCI), still refractory to conventional treatment. Glial cell activation and cytokine production contribute to the pathology of central neuropathic syndromes. In this study we evaluated the effects of progesterone, a neuroactive steroid, on pain development and the spinal expression of IL-1β, its receptors (IL-1RI and IL-1RII) and antagonist (IL-1ra), IL-6 and TNFα, and NR1 subunit of NMDAR. Our results show that progesterone, by modulating the expression of pro-inflammatory cytokines and neuronal IL-1RI/NR1 colocalization, emerges as a promising agent to prevent chronic pain after SCI.

1. Introduction

Neuropathic pain, a frequent complication of spinal cord injury (SCI), is an important contributor to decreased quality of life (Finnerup, 2013). This chronic pain is characterized by the presence of both spontaneous and induced pain. Unfortunately, the incomplete understanding of the mechanisms involved in pain arising after SCI threatens the search of effective medical treatment.

While multiple mechanisms may contribute to neuropathic pain after central nervous system (CNS) lesions (Hulsebosch et al., 2009; Yezierski, 2009), central neuroinflammation is a critical driving force for the development and maintenance of chronic pain (Ji et al., 2013; Walters, 2014).

Spinal glial cells play a central role in the onset of the neuroinflammation. Among the glial mediators released within the CNS, special emphasis has been placed on pro-inflammatory cytokines like interleukin 1β (IL-1β) and tumor necrosis factor α (TNFα), possibly for their involvement in a plethora of CNS diseases and neurotoxic conditions (Burda and Sofroniew, 2014; Vezzani and Viviani, 2015). It has been demonstrated that these cytokines facilitate pain via neural–glial interactions (Kawasaki et al., 2008; Ji et al., 2013). In particular, IL-1β plays a pivotal role in pain mechanisms (Taves et al., 2013; Ren and Dubner, 2015). The actions of IL-1β are regulated by different mechanisms involving a functional receptor (IL-1RI), a decoy receptor (IL-1RII), and a specific endogenous antagonist (IL-1ra) (Dinarello, 1998).

Since IL-1RI is not only found in glial cells but also allocated in neurons (Ravizza and Vezzani, 2006; Gardoni et al., 2011), IL-1β may act directly on neurons to modulate their activity. In fact, IL-1β has been shown to enhance synaptic transmission and neuronal activity in the superficial dorsal horn (Kawasaki et al., 2008; Pedersen et al., 2010). Furthermore, IL-1RI acts as a coordinating factor for the functional interaction between IL-1β and N-methyl-D-aspartate receptor (NMDAR) (Fogal and Hewett, 2008; Zhang et al., 2008), a key player in pain transmission. Therefore, targeting these processes could reduce the excitability of dorsal horn neurons and prevent the development of chronic pain.

Progesterone, a neuroactive steroid, has multiple non-reproductive functions in the CNS, exerting neuroprotective and remyelinating

Abbreviations: (SCI), spinal cord injury; (CNS), central nervous system; (IL-1β), interleukin 1β; (TNFα), tumor necrosis factor α; (NMDAR), N-methyl-D-aspartate receptor; (IL-1RI), IL-1β functional receptor; (IL-1RII), IL-1β decoy receptor; (IL-1ra), IL-1β receptor antagonist; (GFAP), glial fibrillary acidic protein; (iNOS), inducible isoform of the nitric oxide synthase; (COX-2), cyclooxigenase 2; (PG), progesterone; (CTL), control animals; (PCR), polymerase chain reaction; (PBS), phosphate-buffered saline; (PGRMC1), progesterone receptor membrane component 1.

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actions after experimental traumatic brain and spinal cord injuries (De Nicola et al., 2013; Garcia-Ovejero et al., 2014; Geddes et al., 2014; Schumacher et al., 2014). Furthermore, progesterone is emerging as an attractive potential drug for preventing persistent pain conditions (Milani et al., 2010; Coronel et al., 2011a, b, 2014; Dableh and Henry, 2011; Melcangi et al., 2014). In particular, we have recently shown that progesterone reduces the number of GFAP and OX-42 positive glial cells, regulates iNOS and COX-2 expression and modulates the expression and phosphorylation of NMDAR subunits at the dorsal horn level after SCI (Coronel et al., 2011b, 2014).

In the present study, we used molecular, immunohistochemical and behavioral studies to evaluate the impact of progesterone administration on the temporal expression of IL-1β, its receptors IL-1RI and IL-1RII and antagonist IL-1ra, IL-6, and TNFα in the injured spinal cord. These parameters were assessed in injured male rats treated with daily injections of progesterone or vehicle, as well as control animals. Since neuronal activity and pain sensitivity are controlled by these pro-inflammatory mediators, their modulation by progesterone could represent a crucial factor to regulate neuroinflammatory dynamics and prevent pain after SCI.

2. Methods

2.1. Spinal cord injury

All experimental procedures were reviewed and approved by the local Animal Care and Use Committee (Assurance Certificate No. A5072-01) and the Ethical Committee from Instituto de Biología y Medicina Experimental (Buenos Aires, Argentina), and followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). Care was taken to minimize animal discomfort and to limit the number of animals used. Male Sprague–Dawley rats (200–220 g), bred at the colony of the Instituto de Biología y Medicina Experimental, were deeply anesthetized with ketamine (50 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.). In a group of rats, the spinal cord was exposed and unilaterally hemisected at the thoracic T13 level (Labombarda et al., 2008; Coronel et al., 2011b, 2014), as originally described by Christensen et al. (Christensen et al., 1996). In sham-operated animals the spinal cord was exposed but not lesioned. Post-operative care included control of body temperature using an electric heating pad, and antibiotic administration (cephalexin 20 mg/kg/day) for 5 days, starting immediately after surgery. Animals were monitored for eventual infections until the end of the experiment.

2.2. Progesterone administration

Injured animals received daily subcutaneous injections of natural progesterone (Sigma, Saint Louis, MO, USA; P8783, 16 mg/kg/day; HX+PG) or vehicle (Ricine oil, Ewe, Sanitas Argentina, Buenos Aires, Argentina; HX) (Coronel et al., 2011b, 2014). Progesterone was administered immediately after performing the lesion and once a day thereafter until the animals were euthanized (either 1, 14 or 28 days after injury). We have previously tested this dose of progesterone in several animal models of nervous system injury (Labombarda et al., 2009; Coronel et al., 2011a, b, 2014; Garcia-Ovejero et al., 2014). Sham-operated animals receiving oil were used as control animals (CTL). Therefore, the study included three experimental groups: animals subjected to the spinal cord injury receiving oil (HX group), injured animals treated with progesterone (HX+PG group) and sham-operated animals receiving oil (CTL group).

2.3. Assessment of pain behaviors

Behavioral testing was performed by a blinded observer. The animals were tested 1 day before surgery, in order to obtain normal baseline values, and at different time points (days 1, 7, 14, 21 and 28) after SCI or sham-operation, as previously described (Coronel et al., 2011b, 2014). Only rats showing normal responses to mechanical and thermal stimulation before surgery were included in the experiments. At least 13 animals were included in each experimental group. They were placed in transparent testing chambers and allowed to acclimate for 15 min before testing.

2.3.1. Mechanical allodynia

Paw mechanical sensitivity was assessed by evaluating the response to normally innocuous mechanical stimuli using a series of 8 calibrated von Frey filaments (1, 2, 4, 6, 8, 10, 15, 26 g, Stoelting, Wood Dale, IL, USA). Each hair was delivered three times with 5 s intervals. The lowest force at which application elicited a brisk paw withdrawal was taken as the mechanical response threshold. A paw withdrawal reflex obtained with 6 g or less was considered as an allodynic response. Values shown in Fig. 1a correspond to the mean ± SEM. As previously reported, results were analyzed using the Friedman repeated measures of analysis of variance followed by multiple comparison test (Coronel et al., 2011b, 2014).

2.3.2. Cold allodynia

Cold sensitivity of the hind paw to acetone (Choi test) was quantified by paw withdrawal frequency. Thus, 100 μl of acetone was applied to the plantar surface of the paw using a plastic tubule connected to a 1 ml syringe. Acetone was applied five times to each paw at an interval of at least 5 min. The number of brisk foot withdrawals was recorded. Values shown in Fig. 1b correspond to the mean ± SEM. As previously reported, results were analyzed using the Friedman repeated measures of analysis of variance followed by multiple comparison test (Coronel et al., 2011b, 2014).

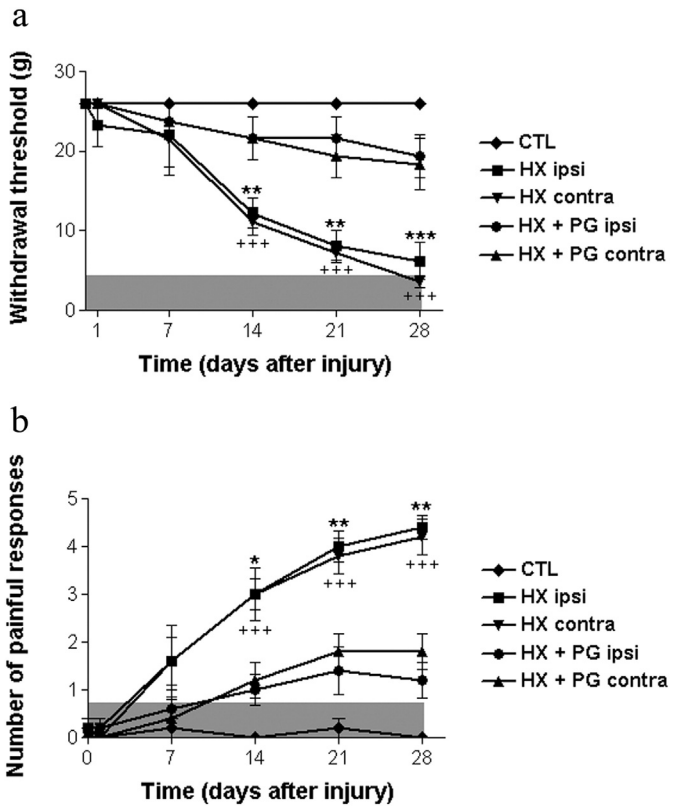

Fig. 1. Spinal cord injury induced the development of mechanical (a) and thermal (b) allodynia in both the ipsilateral and contralateral hind paws. Progesterone administration was able to prevent these pain-related behaviors (a, b). The following symbols were used to represent p values: $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ when comparing HX vs HX+PG, and $^{+}p < 0.05$, $^{++}p < 0.01$ and $^{+++}p < 0.001$ when comparing HX vs CTL. Shaded areas in both graphs represent the period of progesterone administration.

2.4. Tissue preparation for real time-polymerase chain reaction (PCR) or ELISA test

Either 1, 14 or 28 days after SCI animals receiving progesterone or vehicle, as well as CTL animals, were killed by decapitation after being deeply anesthetized with chloral hydrate (800 mg/kg, i.p.). Spinal lumbar segments caudal to the injury site (L4–5) and equivalent regions from CTL animals were immediately removed and the dorsal spinal halves were dissected (Coronel et al., 2011b, 2014). Tissues were frozen and stored at −70 °C until further studies were performed. Samples from the different experimental groups were run at the same time.

2.5. Real time-PCR

Spinal dorsal halves were collected as described above (n = 7 in each group). RNA was extracted using TRIzol (Invitrogen, USA), as previously described (Coronel et al., 2011b, 2014). Nucleotide sequences of forward and reverse primers used for amplification are listed in Table 1. The change in the target mRNA was calculated using the $2^{-(\Delta\Delta Ct)}$ method (Livak and Schmittgen, 2001) and expressed as fold-increase relative to control values. Seven animals were included in each experimental group and samples were run in triplicate. Data shown in Figs. 2 and 3 correspond to the mean ± SEM of mRNA levels relative to control values (CTL: sham-operated animals receiving oil). Statistical analysis was performed by applying two-way analysis of variance (ANOVA) and Bonferroni multiple comparison post-test.

2.6. ELISA

Spinal dorsal halves were collected as described above (n = 6 in each group). Protein concentration was measured using Bradford Reagent (Sigma) and IL-1β levels were determined using an ELISA Kit (Invitrogen), following the instructions provided by the manufacturer. Six animals were included in each experimental group and all measurements were performed in duplicate. IL-1β levels were calculated and expressed as pg/μl/μg protein. Data shown correspond to the mean ± SEM of each experimental group. Statistical analysis was carried out by applying one-way ANOVA, followed by Newman–Keuls post-hoc test.

2.7. Tissue preparation for immunofluorescence

Twenty eight days after spinal hemisection, animals receiving progesterone or vehicle, as well as CTL animals (n = 5 in each group), were deeply anesthetized with an overdose of chloral hydrate (800 mg/kg i.p.) and perfused through the heart with 60 ml of 0.9% NaCl, followed by 60 ml of fixative (4% paraformaldehyde in 0.16 M phosphate buffer, pH 7) at 4 °C (Coronel et al., 2011b, 2014). Spinal lumbar segments caudal to the injury site (L4–L5) and equivalent regions from CTL animals were removed and post-fixed in the same fixative for 90 min at 4 °C. Tissues were then rinsed in 20% sucrose in phosphate buffer (pH 7.2) and stored in the same solution at 4 °C. Thereafter, tissues were embedded in OCT compound (Tissue Tek, Miles Laboratories, USA) and cut transversally at 14 μm thickness in a cryostat (HM505N, Microm, Germany).

Table 1
Foward and reverse primers sequences.

Gene	Primer sequence	Reference
IL -1β	F: 5′ CACCTCTCAAGCAGAGCACAG 3′ R: 5′ GGGTTCCATGGTGAAGTCAAC 3′	Peinnequin et al., 2004
IL-1RI	F: 5′ GTTTTTGGAACACCCTTCAGCC 3′ R: 5′ ACGAAGCAGATGAACGGATAGC 3′	Peinnequin et al., 2004
IL -1RII	F: 5′ CATTCAGACACCTCCAGCAGTTC 3′ R: 5′ ACCCAGAGCGTATCATCCTTCAC 3′	Peinnequin et al., 2004
IL-1ra	F: 5′ AAGACCTTCTACCTGAGGAACAACC 3′ R: 5′ GCCCAAGAACACATTCCGAAAGTC 3′	Peinnequin et al., 2004
TNFα	F: 5′ TCGTAGCAAACCACCAAGCA 3′ R: 5′ CCCTTGAAGAGAACCTGGGAGTA 3′	Jin et al., 2008
IL-6	F: 5′ AAGTCGGAGGCTTAATTACATATGTTC 3′ R: 5′ TGCCATTGCACAACTCTTTTCT 3′	Rothman et al., 2009
CyCB	F: 5′ GTGGCAAGATCGAAGTGGAGAAAC 3′ R: 5′ TAAAAATCAGGCCTGTGGAATGTG 3′	Gen Bank Accession Number NM_022536

2.8. Immunofluorescence procedure

As previously reported (Coronel et al., 2011b), sections were mounted onto positively charged microscope slides, allowed to dry for at least 1 h and rinsed twice in phosphate-buffered saline (PBS). After preincubation in 10% goat serum for 10 min at 37 °C, sections were incubated overnight at 4 °C with antibodies raised against NR1 (mouse, Upstate-Millipore, USA) and IL-1RI (rabbit, Santa Cruz Biotechnology, USA), both diluted in PBS containing 2% goat serum and 0.2% Triton X-100. The sections were then rinsed twice in PBS containing 0.1% Triton X-100 and incubated for 1 h at room temperature with goat anti-rabbit secondary antibody conjugated with Alexa 488 (Molecular Probes, Invitrogen, USA) and goat anti-mouse secondary antibody conjugated with Alexa 555 (Molecular Probes, Invitrogen, USA). The sections were given three rinses in PBS and coverslipped using Fluoromount G (Southern Biotech, USA) as mounting media. In order to confirm the neuronal localization of IL-1RI, cells were double labeled with an antibody directed against the specific neuronal marker NeuN (mouse, Upstate-Millipore, USA). Negative controls were prepared omitting the primary or secondary antibodies. Sections were examined under a Zeiss Axioplan fluorescence microscope (Zeiss, Germany) and a Nikon Eclipse E-800 confocal scanning laser microscope (Nikon, Japan), with which images were taken. The microscope illumination and data acquisition parameters were fixed throughout the entire analysis. As previously reported, digital images were visualized and further analyzed using a computer assisted image analysis system (Bioscan Optimas II software) (Coronel et al., 2011b, 2014). Counting and data processing were performed by a blind observer. The number of neuronal profiles exhibiting IL-1RI and/or NR1 immunoreactive (IR) signal was determined in the superficial laminae (lamina I and II) of the dorsal horn, identified by cytoarchitectonic criteria (Molander and Grant, 1995). Immunostained profiles were counted in randomly, systematically sampled sections throughout the lumbar L4–L5 segments of the spinal cord. In order to avoid double-counting immunopositive cells, the quantification was performed in sections at least 160 μm apart (every 10th section, 10 sections per spinal cord) (Coronel et al., 2011b, 2014). The mean number of IL-1RI immunopositive (+) neuronal profiles per section was determined for each animal. These values were averaged within each experimental group, presented as group data and expressed as the mean number of IL-1RI-IR neuronal profiles per unit area (10^5 μm^2). For this purpose, and as previously described (Coronel et al., 2011b, 2014), the mean area of the superficial laminae was determined (0.098 ± 0.008 mm^2) using a computer assisted image analysis system (Bioscan Optimas II software). As previously reported, the mean area of each of the dorsal horn regions evaluated did not change across the different experimental groups (Coronel et al., 2011b). After counting double-labeled cells, the number of IL1-RI+/NR1+ cells was determined and expressed as percentage of the total number of NR1+ neurons in the area under study. These values were averaged within each experimental group and presented as group data. Five animals were included in each experimental group. In Fig. 4 data show mean ± SEM. Statistical analysis was carried out by applying one-way analysis of variance and Newman–Keuls multiple comparison post-test.

3. Results

3.1. Behavioral evaluation of neuropathic pain: mechanical and cold allodynia after spinal cord injury and progesterone administration

In line with previous observations (Christensen et al., 1996; Labombarda et al., 2008; Coronel et al., 2011b, 2014), animals subjected

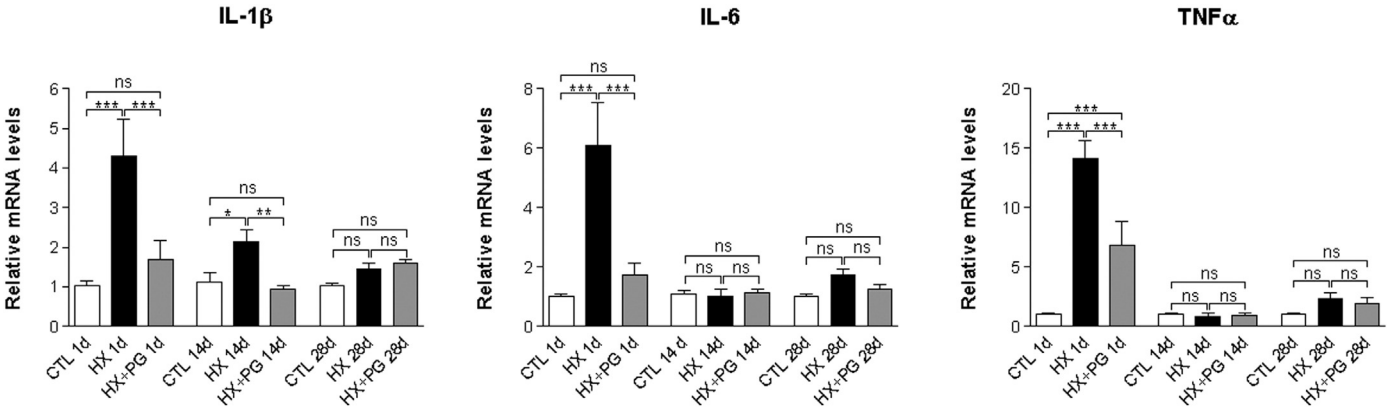

Fig. 2. IL-1β, IL-6 and TNFα relative mRNA levels detected in the lumbar dorsal spinal cord 1, 14 and 28 days after spinal cord injury. Note the significant increase in the mRNA levels corresponding to the three cytokines observed in the acute phase after injury. Interestingly, during progesterone administration this injury-induced early increase in cytokine expression was not observed. In the chronic phase, IL-1β, IL-6 and TNFα mRNA levels were low in both treated and non treated hemisected rats, and similar to those detected in control animals. Symbols that represent p values: ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

to a spinal cord hemisection showed guarding behaviors and changes in the posture such as plantar flexion and toe-clenching. A progressive decrease in mechanical withdrawal threshold was observed in both the ipsilateral and contralateral hind paws (Fig. 1a, $p < 0.01$ vs CTL at day 14) and allodynic values were detected 21 and 28 days after injury (Fig. 1a, $p < 0.001$ vs CTL at both 21 and 28 days). When cold sensitivity was assessed, a similar behavioral pattern was obtained: there was a gradual and clear increase in the number of positive nociceptive responses in both hind paws starting 14 days after injury (Fig. 1b, $p < 0.001$ vs CTL), with the highest number of allodynic responses detected at days 21 and 28 (Fig. 1b, $p < 0.001$ vs CTL in both cases). It should be noted that in both tests paw withdrawals were accompanied by active attention to the stimulus, abrupt head turning and attack, vocalization, and/or body reposturing. These aversive behaviors indicate that noxious stimuli were detected supraspinally.

Injured animals receiving progesterone did not develop mechanical allodynia (Fig. 1a, ($p < 0.01$ vs HX at day 21, $p < 0.001$ vs HX at day 28) and showed reduced sensitivity to cold stimulation (Fig. 1b, $p < 0.05$ vs HX at day 14, $p < 0.01$ vs HX at days 21 and 28), which was consistent with our previous results (Coronel et al., 2011b, 2014).

3.2. Effect of progesterone administration on IL-1β, IL-6 and TNFα mRNA levels after spinal cord injury

One day after spinal injury, IL-1β, IL-6 and TNFα mRNA levels were significantly raised over CTL values (Fig. 2, $p < 0.001$ vs CTL in all cases). At this time point, injured animals receiving progesterone showed significantly lower levels of the three cytokines mRNAs, as compared to vehicle-treated injured animals (Fig. 2, $p < 0.001$ vs HX in all cases).

Fig. 2 also depicts that IL-1β expression was significantly elevated even 14 days after injury, as compared to CTL animals (Fig. 2, $p < 0.05$), but dropped to basal levels at the chronic time point (28 days). Both IL-6 and TNFα mRNAs returned to baseline levels at and beyond 14 days post-injury (Fig. 2, $p > 0.05$ vs CTL in both cases). Thus, 28 days after injury, the spinal dorsal cord from HX and HX + PG animals presented a cytokine expression profile similar to that observed in CTL animals (Fig. 2, $p > 0.05$ in all cases).

It should be mentioned that IL-1β mRNA levels correlated with cytokine protein levels, determined using a commercial ELISA kit. For instance, an increase in spinal IL-1β protein levels was detected in lesioned animals, 1 day after injury, which returned to basal levels in progesterone-treated rats (CTL: 0.088 ± 0.013, HX: 0.158 ± 0.017, HX+PG: 0.092 ± 0.009 pg/μl/μg protein; $p < 0.01$ for both HX vs CTL and HX vs HX+PG).

3.3. Effect of progesterone administration on IL-1RI, IL-1RII, IL-1ra mRNA levels after spinal cord injury

The expression of specific receptors is critically involved in the functional properties of IL-1β. Thus, we studied the mRNA levels of the functional receptor (IL-1RI), the decoy receptor (IL-1RII) and the specific receptor antagonist (IL-1ra), all of which regulate the actions of IL-1β. One day after injury, no differences were detected in IL-1RI expression

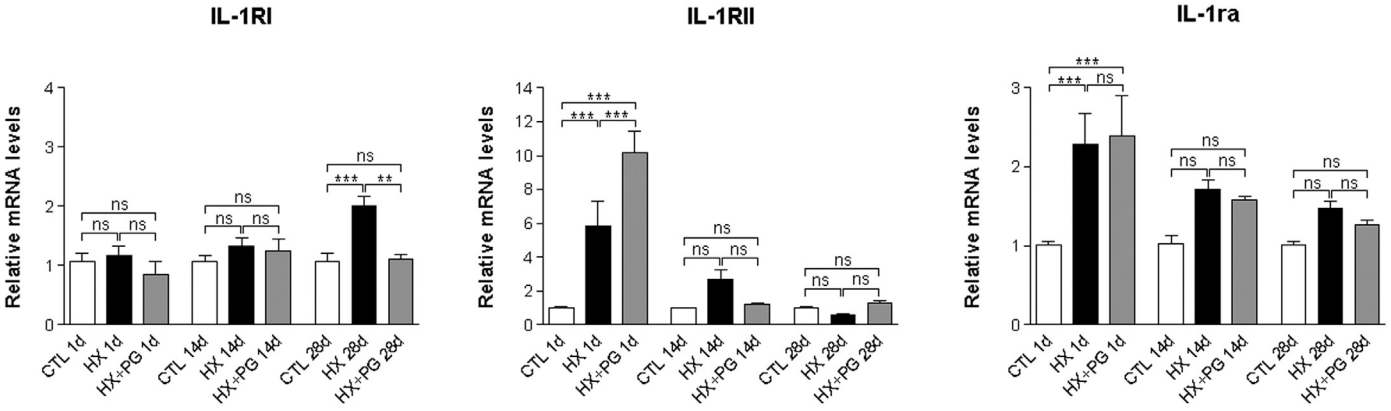

Fig. 3. IL-1RI, IL-1RII and IL-1ra relative mRNA levels detected in the lumbar dorsal spinal cord 1, 14 and 28 days after spinal cord injury. Note the increase in IL-1RII and IL-1ra expression observed in both treated and non-treated injured animals in the acute phase after spinal cord injury. In the chronic phase, the expression of IL-1RI was significantly up-regulated in lesioned animals. Interestingly, animals receiving progesterone showed baseline IL-1RI mRNA levels. Symbols used to represent p values: ns $p > 0.05$, **$p < 0.01$ and ***$p < 0.001$.

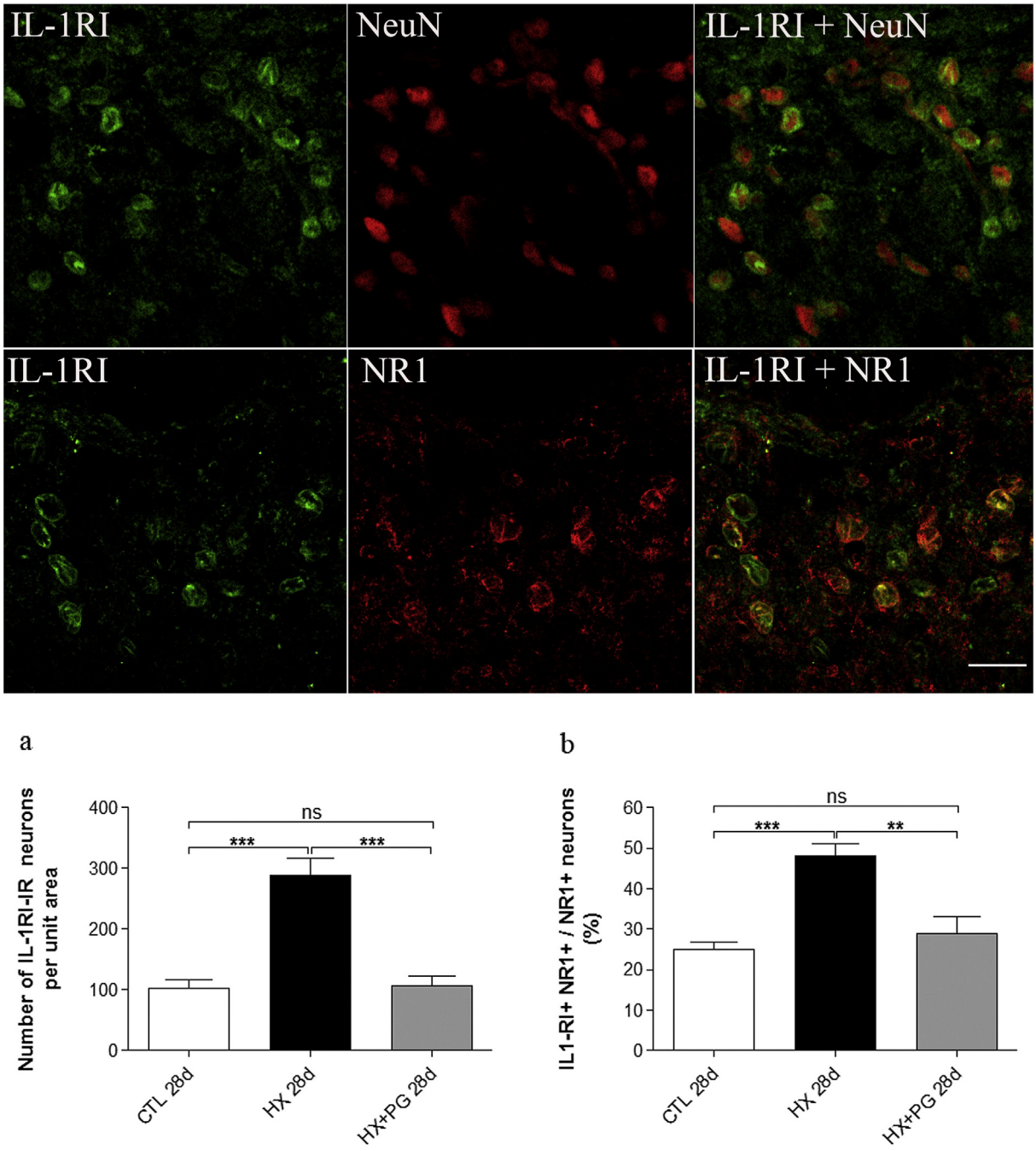

Fig. 4. IL-1RI co-localization with NR1, the functional subunit of NMDAR, in dorsal horn neurons. Representative photomicrographs showing IL-1RI/NeuN (upper panel) and IL-1RI/NR1 (lower panel) immunoreactive (IR) cells within the superficial dorsal horn (laminae I–II), 28 days after spinal cord injury. Scale bar: 40 μm. Number of IL-1RI positive neurons per unit area (a) and percentage of IL-1RI + /NR1 + neurons (b) within the superficial dorsal horn of animals corresponding to the different experimental groups, 28 days after injury. Quantitative analysis showed increased numbers of IL-1RI positive neurons and an increased number of cells coexpressing IL-1RI and NR1 in lesioned animals. In both cases, significantly lower counts were obtained in injured animals receiving progesterone. The following symbols were used to represent p values: ns $p > 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Color figure is available in the online version of this article.

(Fig. 3, $p > 0.05$ between any of the three experimental groups). However, injured animals showed a significant increase in the mRNA levels of the decoy IL-1RII receptor, and a concomitant increased IL-1ra expression (Fig. 3, $p < 0.001$ vs CTL in both cases). At this time point, HX + PG animals presented a further increase in IL-1RII expression (Fig. 3, $p < 0.001$ vs HX), while maintaining high IL-1ra mRNA levels (Fig. 3, $p > 0.05$ vs HX).

CTL, HX and HX + PG groups displayed a similar pattern of receptor expression 14 days after injury (Fig. 3, $p > 0.05$ in all cases). Interestingly, only HX animals displayed a significant 2-fold increase in IL-1RI mRNA levels 28 days post-injury (Fig. 3, $p < 0.001$ vs CTL). HX + PG animals did not exhibit IL-1RI mRNA up-regulation, maintaining the expression levels observed in CTL animals at this chronic time-point (Fig. 3, $p < 0.01$ vs HX, $p > 0.05$ vs CTL).

3.4. Co-localization of IL-1RI and NMDAR NR1 subunit in dorsal horn neurons

As stated in the previous section, IL-1RI mRNA was up-regulated in the chronic phase after SCI. Relevant to the role of IL-1RI in neuronal

signaling, we then evaluated whether IL-1RI was expressed in laminae I–II dorsal horn neurons. Neuronal expression of IL-1RI was confirmed by double-immunostaining, which showed receptor co-localization with the neuronal marker NeuN (Fig. 4, upper panel photomicrographs).

In addition, double immunofluorescence labeling also demonstrated that in spinal dorsal horn neurons IL-1RI co-localized with NR1, the functional subunit of the NMDAR (Fig. 4, lower panel photomicrographs).

Quantitative analysis demonstrated that, 28 days after SCI, there was not only a significant increase in the number of IL-1RI positive neurons (Fig. 4a, $p < 0.001$ vs CTL) but also a higher percentage of NR1 positive cells co-labeled with IL-1RI (Fig. 4b, $p < 0.001$ vs CTL). Interestingly, animals receiving progesterone administration showed a lower number of IL-1RI positive neurons in the superficial laminae of the spinal cord (Fig. 4a, $p < 0.001$ vs HX, $p > 0.05$ vs CTL), as well as a reduced percentage of IL-1RI +/NR1 + dorsal horn cells (Fig. 4b, $p < 0.01$ vs HX, $p > 0.05$ vs CTL).

4. Discussion

Reactive gliosis along with the release of pro-inflammatory cytokines is a hallmark of human and experimental CNS injury (Yang et al., 2004, 2005; Fleming et al., 2006; Burda and Sofroniew, 2014) and represents one of the main mechanisms contributing to the genesis and maintenance of chronic pain after SCI (Hulsebosch et al., 2009; Gwak et al., 2012). In fact, it is now becoming clear that cytokines are key signaling molecules between glia and neurons, capable of modulating neuronal function (Viviani et al., 2014; Vezzani and Viviani, 2015) and enhancing pain transmission (Guo et al., 2007; Gwak et al., 2012; Ji et al., 2014; Tiwari et al., 2014).

In this regard, we have recently reported an increased number of GFAP immunoreactive astrocytes and OX-42 immunoreactive microglial cells detected in the dorsal horn of animals subjected to a spinal cord lesion, both in the acute and the chronic phase after injury (Coronel et al., 2014). Lesioned animals also showed an increase in the expression and activity of the pro-inflammatory enzymes COX-2 and iNOS, and developed both mechanical and thermal allodynia (Coronel et al., 2014).

As predicted, we found here that SCI also triggers a robust and transient induction of IL-1β, IL-6 and TNFα in the dorsal spinal cord (Yang et al., 2004; Fleming et al., 2006; Hulsebosch et al., 2009). In particular, one day after injury, a significant and persistent rise in IL-1β mRNA levels was observed, concomitant with a significant increase in the expression of both IL-1ra and IL-1RII. In good agreement with other experimental (Liu et al., 2008) and clinical (Bellehumeur et al., 2009) conditions, expression of IL-1β over basal levels may trigger the up-regulation of counteracting regulatory molecules such as the IL-1RII decoy receptor, and/or IL-1ra, as part of an endogenous protective mechanism intended to minimize IL-1β inflammatory actions following SCI (Liu et al., 2008) and avoid chronic pain development (Tan et al., 2009). However, this early protective response may not be sufficient, since the injured animals developed neuropathic pain behaviors in the chronic phase after injury. In this regard, the local release of IL-1β has been associated with spinal long-term potentiation (Kawasaki et al., 2008; Pedersen et al., 2010) and synaptic strength (Chirila et al., 2014) implicated in persistent pain development (Kawasaki et al., 2008).

Interestingly, our results also show that progesterone administration after SCI resulted in reduced mRNA levels of the 3 pro-inflammatory cytokines in the acute phase after injury, and caused a further increase in IL-1RII expression, sustaining the injury-induced high levels of IL-1ra expression, as compared to vehicle treated animals. These actions exerted by progesterone may be crucial for avoiding the long-term consequences of the neuromodulatory cascade triggered by cytokines.

Progesterone, a well-known suppressor of the inflammatory response, exerts a strong impact on reactive gliosis following CNS injury (Garay et al., 2011; Labombarda et al., 2011; De Nicola et al., 2013; Coronel et al., 2014). In fact, our previous reports show that progesterone treatment resulted in reduced expression and activity of the pro-inflammatory enzymes COX-2 and iNOS in the dorsal horn after SCI, by decreasing the transactivation of NF-κB, a key factor for cytokine and pro-inflammatory enzyme expression, and by lowering the number of reactive glial cells (Coronel et al., 2014), a crucial source of inflammatory mediators. Moreover, and in line with the proposed role for these molecules, injured animals receiving progesterone did not develop allodynic behaviors after injury. Thus, progesterone, by modulating these multiple and acute neuroimmune events may have a powerful impact on the prevention of neuropathic pain after SCI.

However, progesterone has been shown to prevent the development of neuropathic pain after nervous system injury only if the treatment is started early and is maintained for a critical period after peripheral nerve (Dableh and Henry, 2011) or spinal cord (Coronel et al., 2014) injuries. These findings suggest that several other maladaptive mechanisms arising through the chronic phase after SCI (Tenorio et al., 2013) may require longer administration of the steroid.

Here we have also observed that in the chronic phase after injury, and coincident with the presence of allodynic behaviors, the SCI group presented basal cytokine expression levels, which appear to be in divergence with the still increased number of spinal glial cells we have previously observed at this time point (Coronel et al., 2014). However, a glial activation profile with low pro-inflammatory cytokine production has already been described (Lee et al., 2010). Furthermore, since these cytokines act in an autocrine and paracrine manner, they might not need to be produced in large amounts to be functional, and as previously proposed (Viviani et al., 2014), the final consequences of the neuroinflammatory process in the CNS may rely on a delicate balance between the production of cytokines and the ability of neurons to sense them through the expression of specific receptors.

In this regard, a key issue concerning glial control of pain is how glial mediators regulate synaptic transmission (Viviani et al., 2014; Vezzani and Viviani, 2015). Currently available evidence shows that several mechanisms may explain the convergence between cytokines and glutamatergic systems, including the hyperactivation of NMDAR (Fogal and Hewett, 2008; Zhang et al., 2008; Vezzani and Viviani, 2015), a pivotal player in pain transmission.

Several associated pain conditions show alterations in the expression and/or phosphorylation of NMDAR subunits in the dorsal spinal cord (Grossman et al., 2000; Caudle et al., 2003; Tomiyama et al., 2005), facilitating pain transmission (Guo et al., 2002; Caudle et al., 2005). Moreover, we and others have shown that increased phosphorylation of the NR1 subunit, a critical step to modulate this receptor activity, correlates with the presence of neuropathic behaviors after both spinal cord (Caudle et al., 2003; Coronel et al., 2011b) and peripheral nerve lesions (Ultenius et al., 2006; Coronel et al., 2011a).

In particular, IL-1β has been involved as a central modulator of glutamatergic response (Fogal and Hewett, 2008; Kawasaki et al., 2008; Zhang et al., 2008; Gardoni et al., 2011). IL-1β, via IL-1RI signaling, facilitates NMDAR activation in neurons by increasing NMDAR subunit phosphorylation (Guo et al., 2007), enhancing the NMDAR-induced Ca^{2+} influx and NR2B phosphorylation (Viviani et al., 2003) and modulating NMDAR expression and membrane distribution (Viviani et al., 2003, 2006; Gardoni et al., 2011).

Relevant to our work, spinal IL-1RI up-regulation has been previously observed after peripheral inflammation (Samad et al., 2001), in spinal motoneurons and glial cells of the contused spinal cord (Wang et al., 2006), in the dorsal horn in a model of bone cancer pain (Zhang et al., 2008) and in hippocampal neurons after status epilepticus (Ravizza and Vezzani, 2006). However, neuronal IL-1RI expression during experimental pain development after SCI has not been previously explored.

In this study, we have found an increased number of IL-1RI/NR1 positive neurons in the dorsal horn after SCI which resulted coincident with the establishment of allodynic behaviors. These observations are in good agreement with our previous results showing increased NR1 mRNA levels and higher number of pNR1 immunoreactive neurons in

the dorsal horn of allodynic animals (Coronel et al., 2011b), supporting a potential interaction between IL-1β signaling and NMDAR activation. Thus, it is possible that, in these conditions, even basal IL-1β levels found in the chronic phase after SCI may be enough to maintain the hyperexcitability of local neurons and contribute to neuropathic pain.

Remarkably, our results also show that at the chronic time-point after SCI, animals receiving progesterone exhibited a significantly lower number of IL-1RI/NR1 positive neurons, and did not display aversive responses to mechanical and cold stimuli. Thus, sustained progesterone administration might maintain neuronal IL-1RI expression and/or its membrane localization at control basal levels, diminishing the responsiveness of dorsal neurons to IL-1β and contributing to attenuate pain behaviors.

Active research over the past decades has established that progesterone has multiple functions beyond reproduction (Schumacher et al., 2008; Stein et al., 2008; De Nicola et al., 2013). Compelling evidence supports the concept that progesterone actions in the CNS include neuroprotection, myelin formation, control of inflammation, regulation of glial cell function, neurotransmission and pain, among others (De Nicola et al., 2009, 2013).

It is well-known that many of these actions are mediated via classical progesterone receptors (PR) acting like ligand-dependent transcription factors, both in neurons and glial cells (De Nicola et al., 2013; Schumacher et al., 2014). Although this study did not unravel the mechanisms underlying progesterone actions during the neuropathic condition after SCI, PR has been shown to play a decisive role in the modulation of cytokine production in endothelial cells (Goddard et al., 2013) and, as we have previously shown, may be involved in decreasing spinal NF-κB transactivation (Coronel et al., 2014), critical for the modulation of cytokine expression and their signaling cascade. Notably, recent work from our laboratories has shown that a functional PR is required for progesterone modulation of cytokine expression after SCI (Labombarda et al., 2015), but deserves further investigation in the context of pain conditions.

Moreover, IL-1RI expression has been shown to be affected by hormonal environment both in experimental (Seo et al., 2012) and clinical (Lawson et al., 2008) conditions. IL-1RI transcriptional regulation is quite complex and may involve the participation of GATA proteins and other transcription factors, including Sp-1, AP-1 and CREB (Gächter et al., 1998; Iwahana et al., 1999). Thus, several transactivating mechanisms likely involving PR and its cross talk with these transcription factors may modulate the expression of IL-1RI and related cytokines during progesterone administration.

In addition, new spinal membrane progesterone receptors (mPRs) and a membrane progesterone binding protein (PGRMC1) have been described in spinal cord neurons and glial cells (Labombarda et al., 2010), opening exciting perspectives for pain modulation. Progesterone can also modulate neurotransmitter receptors, antagonizing neuronal sigma 1 receptors and modulating glutamate signaling (Schumacher et al., 2008). Besides, progesterone can be converted to its reduced metabolite allopregnanolone, a potent modulator of the GABA type A receptor (Schumacher et al., 2007; Faroni and Magnaghi, 2011) that is emerging as a safe therapy for neuropathic pain (Patte-Mensah et al., 2013). Moreover, progesterone has been shown to modulate toll-like receptor/NFkB signaling pathway (Wang et al., 2011) strongly involved in inflammatory events and gliosis after SCI (Kigerl et al., 2007; Coronel et al., 2011b; Labombarda et al., 2011), demyelinating diseases (Garay et al., 2011) and pain conditions (Sorge et al., 2011). Such multiple interactions and cross-road signaling pathways may be relevant in the control of pain processing and deserve further investigation.

In conclusion, the present results may contribute to a better understanding of the modulation of neuroinflammatory dynamics during progesterone administration and may help to improve the design of steroid-based therapies to prevent pain after central injury.

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