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Title: A FUNCTIONAL PROGESTERONE RECEPTOR IS REQUIRED FOR IMMUNOMODULATION, REDUCTION OF REACTIVE GLIOSIS AND SURVIVAL OF OLIGODENDROCYTE PRECURSORS IN THE INJURED SPINAL CORD

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Abstract: The anti-inflammatory effects of progesterone have been increasingly recognized in several neuropathological models, including spinal cord inflammation. In the present investigation, we explored the regulation of proinflammatory factors and enzymes by progesterone at several time points after spinal cord injury (SCI) in male rats. We also demonstrated the role of the progesterone receptor (PR) in inhibiting inflammation and reactive gliosis, and in enhancing the survival of oligodendrocyte progenitors cells (OPC) in injured PR knockout (PRKO) mice receiving progesterone. First, after SCI in rats, progesterone greatly attenuated the injury-induced hyperexpression of the mRNAs of interleukin 1beta (IL1beta), IL6, tumor necrosis factor alpha (TNFalpha), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), all involved in oligodendrocyte damage. Second, the role of the PR was investigated in PRKO mice after SCI, in which progesterone failed to reduce the high expression of IL1beta, IL6, TNFalpha and IkappaB-alpha mRNAs, the latter being considered an index of reduced NF-kappaB transactivation. These effects occurred in a time framework coincident with a reduction in the astrocyte and microglial responses. In contrast to wild-type mice, progesterone did not increase the density of OPC and did not prevent apoptotic death of these cells in PRKO mice. Our results support a role of PR in: (a) the anti-inflammatory effects of progesterone; (b) the modulation of astrocyte and microglial responses and (c) the prevention of OPC apoptosis, a mechanism that would enhance the commitment of progenitors to the remyelination pathway in the injured spinal cord.

Suggested Reviewers: Cordian Beyer PhD
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Cover Letter

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August 25 2015
RE: SBMB-D-15-00250

Dear Prof. Adamski:

Thank you for your letter and the comments of Reviewer I and the Editor regarding our manuscript entitled “A FUNCTIONAL PROGESTERONE RECEPTOR IS REQUIRED FOR IMMUNMODULATION, REDUCTION OF REACTIVE GLIOSIS AND SURVIVAL OF OLIGODENDROCYTE PRECURSORS IN THE INJURED SPINAL CORD”.

Following the instructions in your letter, we are pleased to submit a revised version in which we hope comments and criticisms have been adequately answered. We also include a point to point answer to the queries, as requested.

Hoping our manuscript is suitable for publication in your journal ,I remain

Yours truly,

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Reviewer #1:

We thank this Reviewer for important comments and the helpful consideration given to our manuscript. Following his/her comments and queries, we have made several changes to the original version. These are highlighted in yellow throughout the text of the revised version and written in italics as shown below.

Specific comments:

Reviewer: However it is not clear whether the observed changes in progesterone treated animals in fact facilitate remyelination, as authors propose, and improve functional outcome: The measurements of myelination as well functional, behavioral, tests were not applied.

Response: The effects of progesterone on remyelination after SCI have been demonstrated by our group in previous publications (please see ref. [24] Labombarda et al Glia 57(2009) 884-897 and ref. [28] Labombarda et al. J. Neurotrauma 23(2006) 181-192. Progesterone effects on functional outcome after SCI have been demonstrated in our previous publication ref. [22] Garcia-Ovejero et al. J.Neurotrauma 31(2014) 857-871. In the present paper, we have mentioned that the anti-inflammatory effects and effects on OPC survival via PR may explain the cellular basis of remyelination and functional recovery already shown in the mentioned publications. In order to make this point more clearly, the following change was introduced in the revised version (please see page 19 , last line and page 20, lines 1-7):

"In this regard, we have previously shown that progesterone significantly improved motor outcomes from rats with SCI measured by the Basso-Bresnahan-Beattie scale for locomotion and CatWalk gait analysis [22]. We suggest that these behavioral effects of progesterone can be partially explained by the increased density of OPC, their differentiation into oligodendrocytes and the increased expression of central myelin proteins, i.e. myelin basic protein and proteolipid protein [24, 29]."

Reviewer: One intriguing result needs more detailed explanation: How progesterone can down-regulate astrocytes and microglial cells and upregulate oligodendrocyte progenitor cells at the same time? What would be the mechanism for the first and latter? If in case of OPC it is anti-apoptotic influence of progesterone, what is the mechanism of progesterone-induced decrease of astrocytes and microglia? How progesterone is able to exert anti-apoptotic and apoptotic effects simultaneously if it is the case?

Response:

The Reviewer has brought up a very important question. We don´t know if progesterone exerts apoptotic effects on astrocytes and microglia in our model. However, Arnold et al (J.Mol.Endocrinol. 41(2008) 289-300) has shown that progesterone can induce apoptosis in male astrocytes, which could be one explanation for our results. An additional possibility would be the differential effects of spinal cord injury and progesterone on TNFα. On page 18, we have already discussed that " in another neuroinflammation model, TNFα colocalizes

with OX-42+ and GFAP+ cells, implying that microglia, macrophages and astrocytes become sources of this factor [30]. Interestingly, in our transection model, highest levels of TNFα coincided with the peak reaction of astrocytes and microglia. Increases in TNFα have detrimental effects, damaging endothelial cells and promoting the release of inflammatory mediators by reactive macrophages/microglia [8, 53, 54]. Furthermore, it has been demonstrated that TNFα binding to the TNFR1 receptor is responsible for the apoptosis and lack of differentiation of OPC following SCI [8]." Therefore, down-regulation of this factor may account for the oligodendrocyte precursor survival and decrease of astrocytes and microglia.
To shed further light on this point, the following sentences were added to the revised version (page 18, lines 11-18). We also added the Arnold et al. paper to the Reference list.

"*Thus, negative modulation of TNFα expression may be one mechanism to explain why progesterone can down-regulate astrocyte and microglial cells, and up-regulate OPC viability at the same time. Another mechanism,at least for astrocytes, derives from the work of Arnold et al [55]. These authors have shown that progesterone directly induces apoptosis of rodent male astrocytes by decreasing the transcription of mitochondrial fusion and fission genes, a process that influences the outcome of CNS pathology".*

Minor points:
Abstract, page 2, line 16: please first time use full definition for PRKO and acronyms then: "…in injured PRKO mice…" -> "…in injured PR knockout (PRKO) mice…"
Response: We now define PRKO mice as " *progesterone-receptor knock-out mice"* the first time used.

Methods, page 7, line 56: it seems the sentence needs dot or comma "…homogenizer. RNA…"
Response: dot follows homogenizer.

Methods, page 10, line 23: "After immmunostaining, …" -> please remove third "m" and comma is odd here.
Response: the third "*m*" was removed from immmonostaining and also the comma.

Results, page 15, line 1: the sentence is incomplete "In contrast to wild-type mice, progesterone treatment failed…" (in PRKO group?)
Response: We completed the sentence by adding " *in the PRKO group*"(page 15, lines 2-3).

Results, page 15, line 46: the sentence is not clear, word "took" is odd here.
Response: the word "*took*" was deleted:

Discussion, page 17, line 54: SCI., -> remove dot
Response: dot was removed.

Discussion, page 21, line 47: give definition for GR.

Response: GR is now defined as " *glucocorticoid receptor* " on page 22, lines 1-2..

Figure legend 4, page 26, line 41: add "48 hrs" for C-H.
Response: 48 h was added for C-H in the legend to Figure 4.

Please be consistent throughout the text with: either "wild-type" or "wild type"; either "NG2"(in context of positive cells) or "NG2+" or "NG2 +"

Response: we now write "*wild-type*" and "*NG2+ cells*" throughout the text.

Editorial
1. Shorten and revise all figure legends. Figure legends should contain a title and a description to figure.

Response :Legends to all Figures (1-5) have been shortened and represent now a message on outcome. Details of the methodology or experimental approach have been deleted. No abbreviations are employed in the figure titles. All titles have been reduced to about 20 words. We do not repeat information regarding the statistical methods used. Please see legends to Figures 1 – 5 for changes made in response to Editorial queries (highlighted in yellow).

Highligths

- Spinal cord injury generates inflammation and glial cell reaction.
- Progesterone decreases proinflammatory mediators in the injured spinal cord.
- Progesterone down regulates astrocyte and microglia activation
- Progesterone prevents the apoptosis of oligodendrocyte progenitors
- Progesterone effects are absent in progesterone receptor knock out mice.

A FUNCTIONAL PROGESTERONE RECEPTOR IS REQUIRED FOR IMMUNOMODULATION, REDUCTION OF REACTIVE GLIOSIS AND SURVIVAL OF OLIGODENDROCYTE PRECURSORS IN THE INJURED SPINAL CORD

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ABSTRACT

The anti-inflammatory effects of progesterone have been increasingly recognized in several neuropathological models, including spinal cord inflammation. In the present investigation, we explored the regulation of proinflammatory factors and enzymes by progesterone at several time points after spinal cord injury (SCI) in male rats. We also demonstrated the role of the progesterone receptor (PR) in inhibiting inflammation and reactive gliosis, and in enhancing the survival of oligodendrocyte progenitors cells (OPC) in injured PR knockout (PRKO) mice receiving progesterone. First, after SCI in rats, progesterone greatly attenuated the injury-induced hyperexpression of the mRNAs of interleukin 1β (IL1β), IL6, tumor necrosis factor alpha (TNFα), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), all involved in oligodendrocyte damage. Second, the role of the PR was investigated in PRKO mice after SCI, in which progesterone failed to reduce the high expression of IL1β, IL6, TNFα and IκB-α mRNAs, the latter being considered an index of reduced NF-κB transactivation. These effects occurred in a time framework coincident with a reduction in the astrocyte and microglial responses. In contrast to wild-type mice, progesterone did not increase the density of OPC and did not prevent apoptotic death of these cells in PRKO mice. Our results support a role of PR in: (a) the anti-inflammatory effects of progesterone; (b) the modulation of astrocyte and microglial responses and (c) the prevention of OPC apoptosis, a mechanism that would enhance the commitment of progenitors to the remyelination pathway in the injured spinal cord.

Key words: astrocytes; knockout mice.; microglia ; neuroinflammation; progesterone receptor; spinal cord injury;

1. INTRODUCTION

Cells in the spinal cord respond differently to spinal cord injury (SCI), with some becoming unfortunate targets while others playing an effector role. The first category includes neurons and oligodendrocytes. Neurons suffer necrosis, apoptosis, oxidative damage, and chromatolysis [1, 2] concomitant with the activation of several molecules associated with neuropathology [3, 4] SCI also causes oligodendrocyte death by apoptosis and the release of proinflammatory mediators, mechanisms leading to axonal demyelination and functional impairment [1, 5]. Oligodendrocyte loss is followed by a wave of oligodendrocyte precursor cell (OPC) proliferation, although in the absence of adequate support their survival and differentiation is compromised [6, 7]. In contrast to neurons and oligodendrocytes, astrocytes and microglia become activated after SCI and produce proinflammatory mediators, oxygen free radicals and neurotoxic levels of nitric oxide as part of a process known as reactive gliosis [8, 9]. Astrocytes, change their phenotype with strong expression of glial fibrillary acidic protein (GFAP), vimentin and S100β, and they show early hypertrophy and late proliferation depending on the severity of the lesion [9]. Marked changes also occur in microglia, which present a more reactive, proinflammatory phenotype [10]. Reactive astrocytes and microglia release proinflammatory mediators which reciprocally regulate each other, producing a feed-forward mechanism that propagates secondary injury and inflammation after spinal cord trauma [8, 11].

In this context, therapies aimed at blocking the innate immune response and holding back the glial reaction may be relevant for preserving functions of the damaged spinal cord. For example, the glucocorticoid methylprednisolone

(MP) has been used as a standard therapy for humans with SCI. Clinical trials (NASCIS II and III) have demonstrated therapeutic benefits for these patients [12]. However, the true efficacy of MP in addition to adverse effects has raised questions regarding the value of this steroid for SCI [13, 14]. Progesterone emerges as much more promising candidate for SCI considering its potent neuroprotective, promyelinating, anti-inflammatory and anti-nociceptive effects [15-21].

Improved functional recovery resulting from progesterone treatment of rats with SCI has been demonstrated by the improved motor outcome in the Basso-Bresnahan-Beattie scale for locomotion and CatWalk gait analysis [22]. At the neurochemical level, a strong myelinating drive follows progesterone treatment of injured rats. As in models of experimental demyelination, the steroid enhances the density of OPC, promotes their differentiation into myelinating oligodendrocytes, and increases the expression of central myelin proteins after SCI [23-25]. In this regard, restraining oligodendrocyte death improves recovery after SCI [26]. Besides exerting direct effects on neurons and oligodendrocyte lineage cells [27, 28], progesterone´s neuroprotective and remyelinating actions are associated with inhibition of the activation and proliferation of astrocytes and microglia [29, 30].

The effects of progesterone and its metabolites in the nervous system are pleiotropic and mediated by the classical progesterone receptor (PR), membrane progesterone receptor component 1 (PGRMC1), membrane progesterone receptors (mPR) and neurotransmitter receptors [15, 31-34]. Recently, the roles of progesterone binding to the classical PR or after conversion to allopregnanolone have been reconsidered for neurological

disorders [35, 36]. Therefore, it was important to determine whether PR is required for the effects of progesterone on inflammatory and glial responses after SCI.

In order to elucidate the immunomodulatory role of progesterone after SCI and the involvement of PR in the modulation of inflammatory responses and reactive gliosis and in promoting the survival of OPC, we performed several experiments. We first studied the time-course of the effects of progesterone on the expression of inflammatory enzymes, proinflammatory mediators and their regulatory molecules in rats with SCI. We also used wild-type and PRKO mice with SCI to elucidate: (a) the role of PR for the regulation of proinflammatory mediators and for astrocyte and microglial responses, and (b) the role of PR for OPC viability.

2. MATERIALS AND METHODS

2.1 Progesterone treatment and spinal cord transection

Male Sprague-Dawley rats (250-300 g) were anesthetized with a mixture of ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and their spinal cords transected at the thoracic level (T10) as described by Labombarda et al. [29] [24]. PRKO mice (PR^{lacz} mice on a C57BL6/129SvEv background) [37] and wild- type mice originally obtained from Baylor College of Medicine (Houston, TX, USA) were bred and maintained at the animal facility of the Instituto de Biologia y Medicina Experimental. In these mice, both PRA and PRB isoforms, transcribed from a single gene, are inactivated. PRKO mice were identified following an established genotyping procedure [37]. Three-month-old male mice were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and xylazine (9 mg/kg, i.p.), and their spinal cords were transected as described above for the rat experiments. After SCI, animals were housed singly. Urinary bladders were manually expressed twice a day, and infections were prevented by administration of cefalexine (20mg/kg daily) starting immediately before surgery. In sham-operated animals (CTL), skin and muscle surgery was performed but the spinal cord was not cut. Animals with SCI received daily sc injections of vegetable oil (vehicle) or 16 mg/kg progesterone (Proluton, Schering, Argentina) and were killed 6 h, 24 h, 48 h, 3 or 21 days following surgery. The first progesterone injection was given immediately after injury. Thus, 3 groups of animals were prepared per time period: CTL, SCI and SCI + progesterone treatment. A CTL + progesterone group was not included because

morphological, neurochemical and molecular evidence have confirmed the absence of progesterone effects in the intact spinal cord [38] .

The dose of progesterone chosen prevents oedema, neuronal loss, and improves cognitive responses following brain contusion [21]. In the damaged spinal cord, this progesterone dose reduces secondary damage, preserves white matter, improves locomotor outcome, promotes remyelination, and modulates glial cells involved in the inflammatory response [22, 29]. The periods of time (6 h, 24 h, 3 and 21 days) were chosen to compare rapid or long-term effects of SCI and steroid treatment on proinflammatory mediators. The Animal procedures described for rats and mice followed the NIH Guide for the Care and Use of Laboratory Animals (Assurance Certificate N A5072-01 to Instituto de Biología y Medicina Experimental) and received approval of the Institute's Animal Care and Use Committee and the CICUAL of the Faculty of Medicine, University of Buenos Aires. Efforts were made to keep the number of lesioned animals to a minimum.

2.2 Real time PCR for semi-quantitative determination of mRNA expression of proinflammatory mediators after spinal cord injury

Six and 24 h, 3 and 21 days after SCI, animals receiving progesterone or vehicle as well as CTL animals (n=8 animal per group), were deeply anesthetized with chloral hydrate (800mg/kg ip) and killed by decapitation. Spinal cord tissue localized immediately rostral to the lesion site, and equivalent regions form CTL animals were removed and homogenized with a Polytron homogenizer. RNA was extracted and subjected to reverse transcription as previously described [24]. Relative gene expression was determined using the

ABI PRISM 7500 sequence Detection System (Applied Biosystems, Foster City, CA). Sequence of primers for mice was designed using the web site http://www.ncbi.nlm.nih.gov/tools/primer-blast. Primer sequences for rat and mice are listed in Tables 1 and 2, respectively. Cyclophilin B (Cyc B) was chosen as the housekeeping gene based on the similarity of mRNA expression across all samples templates. Linearity and efficiency of PCR amplification were validated before quantification. Relative gene expression was calculated using the method described by Pfaffl et al. [39] and it was determined for each target gene as fold induction with respect to its respective control. For amplification 2ng cDNA /μl of reaction was used and PCR was performed in triplicate under optimised conditions: 95°C at 10 min followed by 40 cycles at 95°C for 0.15 sec and 60°C for 1 min.

2.3 Tissue preparation for immunohistochemistry and TUNEL reaction

CTL, SCI and SCI+ progesterone wild-type and PRKO mice (n=6 mice per group) were used 6h, 24h and 48h following surgery or sham-operation. Mice were deeply anesthetized as specified above and intracardially perfused with 0.9 % NaCl, followed by ice-cold 4 % paraformaldehyde (PFA). Tissue blocks of 3 mm immediately rostral to the lesion site and equivalent regions from CTL mice were embedded in optimum cutting temperature compound (Tissue Tek, Miles Lab, Elkhart, IL, USA), post-fixed in the same fixative, rinsed in 20% sucrose, frozen on dry ice, cut into 20-μm thick serial coronal sections on a cryostat, and sequentially collected on slides.

2.4 Immunohistochemistry of astrocytes, microglia and OPC

Immunostaining of the 3 cell types was performed according to previously published protocols [24] using primary antibodies against GFAP for astrocytes (1:250 rabbit-policlonal antibody, G-9269 Sigma, St Louis, USA), Ox-42 for microglia (1: 100 mouse-monolconal antibody, Chemicon) and NG2 proteoglycan for OPC (1: 500 rabbit-policlonal antibody, Chemicon). After incubation with secondary antibodies (1:200 biotinylated anti-rabbit and anti-mouse immunoglobulin G, Vector Laboratories) coronal serial sections were incubated with avidin-biotin complex (ABC) for 30 min (ABC kit, Vector Laboratories) and finally revealed with diaminobenzidine tetrachloride (0.50 mg/mL, Sigma, St. Louis, MO) in the presence of 0.01% H_2O_2 for 7 min in the dark. The sections were given a final rinse in phosphate buffered saline, dehydrated in graded ethanols and xylene, and mounted with Permount. Control experiments were performed in parallel and involved the incubation of tissue without primary antibodies in order to rule out non-specific staining. Samples from the different experimental groups were run at the same time.

2.5 Detection of OPC apoptosis by TUNEL assay

For detection of apoptotic cells, serial sections were assayed using the terminal deoxynucleotidyl transferase-mediated UTP nick-end labeling, according to the In situ Cell Death Detection kit-POD (cat. 116848117910, Roche, USA). Sections were first incubated for 2 h in 0.1% sodium citrate, 0.1% TritonX100 for permeabilization, followed by the TUNEL reaction mixture for 60 min at 37 ºC and finally washed with PBS. In order to identify apoptotic OPC, a double-labeling procedure with a rabbit anti-NG2 antibody (1:500, Chemicon)

was performed. Briefly, slices were first blocked with 10% goat serum in 0.1% Triton X100 during 30 min at room temperature and then incubated with a 1/500 dilution of the anti-NG2 serum prepared in 1% goat serum, 0.1% Triton X100 overnight at 4ºC. After several washes in PBS, sections were exposed to a 1/1000 dilution of the second antibody anti-rabbit Alexa 555 (Molecular probes, Invitrogen), washed and mounted with Flouoromount.

2.6 Quantitative analysis using computerized stereology

Serial sections taken every 0.3 mm from a 3mm-long block rostral to the injury were subjected to immunostaining with different antibodies or TUNEL assay for detection of apoptotic cells. After immunostaining, cross sections were examined at 60x magnification by light microscopy equipped with a digital camera Panasonic GPKR222 connected to an Olympus BH2 microscope. Image analysis was performed using Image J NIH image analysis software. The number of total immunopositive cells was estimated using the optical dissector method using total section thickness for dissector height and a counting frame of 50 μm x 50 μm [40]. Section thickness was estimated using the fluorescent properties of hematoxylin in counterstained sections examined under with a Nikon Eclipse E 800 confocal laser microscope. Knowing the volume of the dissector in each section (30000 μm^3), the number of immunopositive cells/mm^3 was calculated for each cell type. A total of 240 counting frames (24 per section covering dorsal, lateral and ventral white matter funiculus) were assessed per animal. TUNEL+ apoptotic cells were detected in a Nikon Eclipse E 800 confocal laser microscope, and the number of double-labeled NG2+ cells/ TUNEL+ cells and total NG2+ cells was quantified in a Z-stack, using the optical dissector method described above for immunopositive cells. For these

experiments, we calculated the number of double stained Tunel+ / NG2+ cells/mm^3 and the % Tunel + NG2+ cells over the total number of NG2+ cells, to provide an index of apoptosis. All slides were assessed blindly with respect to treatment. Results were expressed as the number of cells / mm^3 (mean ± S.E.M.).

2.7 Statistical analysis

Two-way ANOVA followed by the Bonferroni post- test was used for almost all statistical analysis. Changes in the number of NG2+ cells in PRKO mice, was analysed by one-Way ANOVA followed by the Newman-Keuls test. Statistical analyses were performed with Prism 5 GraphPad software (San Diego, CA, USA). Significance was set at $p<0.05$. The number of animals served as the n number.

3. RESULTS

3.1 Progesterone effects on proinflammatory cytokines and enzymes after SCI

Gene expression of proinflammatory cytokines and enzymes was analyzed in a time-course manner to disclose (a) early and late changes following SCI and (b) their response to daily progesterone treatment in rats killed at several times after surgery (Figure 1, A – H). As shown in figure 1, the highest increase in mRNA of the proinflammatory cytokines and enzymes occurred 6 h after SCI, compared to later time points. The highest induction after SCI was observed for iNOS (G, ~ 350-fold), followed by IL6 (F,~87-fold), IL1β (A, ~60-fold), COX-2 mRNA (H,~ 35-fold) and TNFα mRNA (E, ~27-fold) compared to their non-lesioned controls. Changes of lesser magnitude but still significant from non-injured rats were registered at day 1 post-injury for IL1β, TNFα, IL6, and COX2 mRNA (Figure 1, A, E, F, H). Three and 21 days after SCI, proinflammatory cytokines and enzymes returned to control levels, with the exception of IL1β mRNA, which remained higher than in controls at day 3 (A). Prominent responses to SCI were also obtained for the IL1β receptors. This applied to IL1β R1 (B), a receptor connected to inflammatory and immune gene expression [41], IL1β R2 (C) a decoy and negative regulator of the cytokine, and the antagonist IL1β Ra [42] (D). The mRNAs of these molecules were upregulated at 6 h and 1 day post-injury, while IL1β Ra still remained higher than control at day 3 (D).

Progesterone treatment resulted in a generalized reduction in mRNA of the proinflammatory cytokines and enzymes upregulated by SCI (Figure 1). At the 6 h time point, progesterone-receiving SCI rats showed a ~ 50% reduction

of IL1β and TNFα mRNA (A, E), a less pronounced reduction of IL6 mRNA (F) and a dramatic inhibition of the enzymes iNOS and COX2 mRNA (G and H). Progesterone down-regulation persisted at later time points for IL1β, 1 and 3 days post-injury, (A) and COX2 mRNA, 1 day post-injury (H), but was no longer observed for TNFα, IL6, and iNOS (E, F, G). Regarding the IL1β binding molecules, progesterone greatly inhibited the mRNAs of IL1β R1 receptor at day 1, showed no effect on IL1β R2 but up-regulated the IL1β Ra antagonist at day 1, indicating the steroid regulation of the interleukin autoregulatory pathway.

In conclusion, progesterone opposed gene expression of proinflammatory cytokines and enzymes in the spinal cord of rats with SCI. Data also indicated that the steroid prevented the inflammatory effects of IL1β, by antagonizing its inflammatory receptor IL1β R1 and stimulating the cytokine´s inhibitor IL1β Ra.

3.2 The role of PR in proinflammatory cytokine expression and reactive gliosis after SCI

The role of PR was investigated in PRKO mice lacking both isoforms of the receptor [37]. The mRNA levels of IL1β, TNFα, and IL6 were assessed in wild-type and PRKO mice 6 h after SCI or sham operation with or without progesterone treatment (Figure 2). As shown before in rats, SCI strongly upregulated IL1β, TNFα, and IL6 mRNA in mice. The increase in the cytokines occurred for both wild-type and PRKO mice (Figure 2, A - C).. However, wild-type and PRKO mice strikingly diverge in the response to progesterone. Whereas in wild-type mice progesterone significantly inhibited the mRNAs of IL1β, TNFα and IL6, the steroid was totally ineffective in the PRKO mice (Figure

2 A – C), supporting the prerequisite of the PR for progesterone´ s anti-inflammatory effects.

We also evaluated the role of PR in mRNA expression of the NF-κB inhibitor, IκB-α, which represents an index of NF-κB transactivation [43]. This strategy was previously used to study progesterone effects in the dorsal spinal cord [44]. The results of figure 2 show that SCI significantly induced IκB-α mRNA, indicating that NF-κB transactivation was stimulated in both wild-type and PRKO mice (Figure 2 D, $p<0.05$ or less, CTL vs SCI). Interestingly, progesterone treatment of wild-type mice decreased transactivation of NF-κB, as shown by the inhibition exerted on IκB-α expression (Figure 2 D, $p<0.05$ SCI+progesterone vs SCI). Similar to cytokines, inhibition of IκBα mRNA by progesterone required PR because it was not observed in PRKO mice.

To test whether changes in the expression of cytokines and proinflammatory enzymes in response to SCI and treatments reflected changes in glial cell reactions, astrocytes and microglial cells were counted at 6 h post-injury by stereology. Figure 3 shows the effects of SCI and progesterone treatment on the number of GFAP+ and OX-42+ cells from wild-type and PRKO mice. SCI produced a strong increase in the density of GFAP+ astrocytes and OX-42+ microglial cell in both wild-type and PRKO mice (Figure 3 A-B $p<0.001$ SCI vs. CTL for both cell types) Six hours after progesterone treatment of injured wild-type mice, both astrogliosis (Figure 3A) and microgliosis (Figure 3B) were significantly down-regulated ($p<0.001$, SCI vs. SCI+PROG). Similar responses to SCI and progesterone treatment occurred for rats at 6h (data not shown) and at later time periods following SCI [29].

In contrast to wild-type mice, progesterone treatment failed to decrease the number of GFAP+ (Figure 3A) and OX-42+ cells (Figure 3B) in the PRKO group. The photomicrographs at the bottom of figure 3 represent the profile staining of GFAP+ astrocytes and OX-.42 + microglia in wild-type (Figure 3 C-H) and in PRKO mice (Figure 3F-K). Thus, SCI stimulated astrogliosis and microgliosis in both mice with intact or nonfunctional PR, whereas progesterone treatment reduced the number of reactive astrocytes and microglia in wild-type but not PRKO mice.

3.3 The role of PR on the survival of NG2+ oligodendrocyte precursor cells

In view of the reported susceptibility of OPC to proinflammatory mediators [11, 45, 46] and their responsiveness to SCI and progesterone treatment [24, 28], we decided to test the role of PR in the number and survival of these progenitor cells, evaluated by a stereological method. As shown in figure 4A, the number of NG2+ cells per mm^3 was increased in wild-type mice 24 and 48 h following SCI ($p<0.001$ SCI vs. CTL mice). Twenty four hours after lesion, progesterone treatment showed no additional effect, whereas the abundance of NG2+ cells was higher at 48 h in the group receiving progesterone (Figure 4A, $p<0.001$ SCI vs SCI + progesterone). Taken into consideration that progesterone effects on NG2+ cells were only observed at 48 h, this time point was chosen to study if the PR was required for this effect in PRKO mice. Data presented in figure 4B show that in PRKO mice, progesterone no longer stimulated an increase in NG2+ cell number. The photomicrographs of figure 4 represent the NG2+ cell population in CTL, injured and progesterone-treated wild-type (Figure 4 C-E) and PRKO (Figure 4 F-H)

mice, 48 h post-lesion. The progesterone-enhancing effect on the number of NG2+ cells in wild-type mice with SCI is clearly observed.

Finally, we investigated if the increase in NG2+ cell density induced by progesterone administration could be due to a blockage of apoptotic cell death. Counting of double-labeled apoptotic OPC (TUNEL+ / NG2+ cells) at 48 h following SCI, showed marked differences between the wild-type and PRKO groups (Figure 5A). In wild-type mice, progesterone inhibited apoptosis of NG2+ cells by 20% ($p<0.05$ vs. SCI vs SCI+progesterone). In PRKO mice, instead, progesterone treatment had no effect. A comparable result was obtained when the ratio of apoptotic cells over the total number of NG2+ cells was calculated (Figure 5B). The bottom photomicrographs are laser confocal microscopy-based images of wild-type mice showing NG2+ cells (Figure 5D, red), TUNEL + apoptotic cells (Figure 5E, green) and their merge (Figure 5C). TUNEL + / NG2+ cells were absent in CTL mice (Figure 5F), they were frequent in SCI mice (Figure 5G) but considerably reduced in wild-type mice with SCI receiving progesterone (Figure 5H).

4. DISCUSSION

The present investigation supported the strong inhibition of progesterone on neuroinflammation, glial responses and enhanced OPC survival after SCI. A major finding was the requirement of the PR for the mentioned progesterone actions.

4.1 Progesterone effects on the inflammatory response and reactive gliosis

As already shown in cases of CNS injury, a fast response of the acute-phase proinflamamtory cytokines IL1β and IL6 followed SCI in rats (reviewed in [3]). Both microglia and astrocytes are known sources of IL1β and IL6 after SCI [47]. Once released, IL1β promotes activation, proliferation and production of neurotoxic mediators by astrocytes [48]. The peak production of this cytokine at 6h after SCI coincided with the strong microgliosis and astrogliosis characterizing SCI [29]. Furthermore, up-regulation of the IL1βR1 mRNA at 6 h and 1 day following SCI, supports that signalling pathways for IL1β were boosted [41]. However, the damaging effect of IL1β may be attenuated, because the ligand-sequestering IL1β R2 receptor and the IL1β Ra antagonist also peaked at the same time. In the case of IL6, increased mRNA following SCI may originate from neuronal, astrocyte, microglia or endothelial sources [49]. IL6 plays a pathological role by worsening inflammation, demyelination and axonal damage in the spinal cord and several neuropathological disorders [50]

Wang et al [51] have previously shown that TNFα is an acute phase proinflammatory molecule following traumatic SCI, in agreement with our results showing increased TNFα mRNA expression at 6 h and 1 day after SCI. The inflammatory phase of SCI partly consists in the accumulation of microglia /

macrophages showing both M1 and M2a phenotypes. In this sense, TNFα better characterizes the M1 phenotype [52]. In another neuroinflammation model, TNFα colocalizes with OX-42+ and GFAP+ cells, implying that microglia, macrophages and astrocytes become sources of this factor [30]. Interestingly, in our transection model, highest levels of TNFα coincided with the peak reaction of astrocytes and microglia. Increases in TNFα have detrimental effects, damaging endothelial cells and promoting the release of inflammatory mediators by reactive macrophages/microglia [8, 53, 54]. Furthermore, it has been demonstrated that TNFα binding to the TNFR1 receptor is responsible for the apoptosis and lack of differentiation of OPC following SCI [8] suggesting that TNFα could be among the players causing the loss of OPC. Thus, negative modulation of TNFα expression may be one mechanism to explain why progesterone can down-regulate astrocyte and microglial cells, and up-regulate OPC viability at the same time. Another mechanism, at least for astrocytes, derives from the work of Arnold et al [55]. These authors have shown that progesterone directly induces apoptosis of rodent male astrocytes by decreasing the transcription of mitochondrial fusion and fission genes, a process that influences the outcome of CNS pathology.

Spinal cord transection also increased the mRNAs of the pro-inflammatory enzymes iNOS and COX2. Induction of NOS in astrocytes and microglia produces neurotoxic levels of NO [4], which can impair mitochondrial respiration, block the electron transport carriers and increase the synthesis of reactive oxygen species [56]. These changes also promote death of OPC and oligodendrocytes with concomitant demyelination [57, 58]. In turn, the SCI-induced increase in COX-2 converts arachidonic acid into prostaglandins, which

may originate from invasive immune cells that penetrate the impaired blood-brain barrier [59]. Moreover, prostaglandins are strong oxidants, increasing peroxidation of membrane lipids and resulting in cell death [60]. Therefore, it is likely that excess production of IL1β, TNFα, IL6, iNOS and COX-2 jointly contribute to white matter demyelination and oligodendrocyte loss reported in SCI [28].

It is worth mentioning that proinflammatory cytokines and enzymes show a strong dependency on NFκB. This transcription factor controls the expression of IL1β, IL-6, TNFα, iNOS and COX-2 after injury [61]. NF-kB induces *de novo* synthesis of IκB-α mRNA, its inhibitory protein, thus representing a negative feedback. Hence qPCR quantification of IkB-α mRNA represents a potent and sensitive method for evaluating NF-kB transactivation [43]. Thus, in this study the coincidence of proinflammatory mediators with the maximal expression of IkB-α mRNA supports an involvement of NFκB in this process after SCI.

From a translational perspective, it was highly rewarding that up-regulation of the interleukins, proinflammatory factors and enzymes, was greatly attenuated by treatment with progesterone. The effects of progesterone on the inflammatory responses were paralleled by a decrease in reactive astro- and microgliosis. It is worth mentioning that the modulation of IL1β-related molecules showed a differential pattern. Whereas IL1βR1 mRNA was suppressed by day 1 post-injury, the IL1β Ra antagonist was increased at this time by progesterone. This dual effect might be biologically relevant to attenuate the deleterious effects of IL1β [42]. The progesterone-mediated modulatory actions of the expression of the interleukins, TNFα and the proinflammatory enzymes might be crucial for the recovery of function following SCI. In this

regard, we have previously shown that progesterone significantly improved motor outcomes from rats with SCI measured by the Basso-Bresnahan-Beattie scale for locomotion and CatWalk gait analysis [22]. We suggest that these behavioral effects of progesterone can be partially explained by the increased density of OPC, their differentiation into oligodendrocytes and the increased expression of central myelin proteins, i.e. myelin basic protein and proteolipid protein [24, 29].

4.2 OPC survival following SCI is a PR-dependent event

Although SCI is followed by a wave of OPC proliferation, many of these cells die from apoptosis [6]. However, progesterone treatment increases OPC cell density over levels in rats with SCI [24], suggesting a pro-survival mechanism. To address this issue, we employed the TUNEL method, which conclusively supported an anti-apoptotic effect of progesterone on NG2+ cells in rats killed 48 h after the lesion. These data agrees with the antiapoptotic effect of progesterone described in TBI [62], and with the decrease in TUNEL+ cells and increase in PDGFRα+ progenitors after cuprizone-induced demyelination [63]. Potential foes of OPC survival include astrocyte and microglia-derived TNFα [8] [45], neurotoxic levels of NO [64] and IL1β released from astrocytes [65]. Therefore, progesterone´s anti-apoptotic effects on OPC may depend on down-regulation of damaging factors produced by the activation of astrocytes and microglia, thus paving the way for successful remyelination. This hypothesis does not discard a direct steroid effect on OPC, although further studies are needed to elucidate this issue. In this regard, it was suggested that OPC protection from apoptosis could become a major goal for

multiple sclerosis and other demyelinating disease [64]. Results from the experiments using PRKO mice indicate that the classical PR mediates the survival of OPC. In addition, Schumacher´s group described that PR is implicated in the remyelinating effets of progesterone in organotypic slice culture [36]

4.3 Mechanism of progesterone action

The anti-inflammatory functions of progesterone and progestins in the nervous system have been described in several models [18, 23 , 30, 66]. However, the mechanisms responsible for its effects are not yet defined. The spinal cord expresses several progesterone receptors and binding molecules that could be involved in the anti-inflammatory effects. In particular, the classical PR has been detected in neurons (including rodents and humans), astrocytes and cells of the oligodendrocyte lineage [15, 31, 36]. Immunoreactive PR is not present in rodent brain microglia [67], although it was found in the BV-2 immortalized mouse microglia cell line [68]. Our study demonstrates that PR is required for the anti-inflammatory and anti-apoptotic effects of progesterone, and for the inhibition of astrocyte and microglial cell activation by the hormone.

Several hypotheses can be formulated to explain the possible mechanisms of PR actions. The lack of a responsive element for the PR on the promoters of the genes studied here (checked by Alibaba 2.1 transcription factor binding prediction program, available at http://www.gene-regulation.com/pub/programs/alibaba2/index.html) suggests the following transrepression mechanisms: (a) interaction of PR with the AP1 protein, and binding of the complex to AP-1 sites on DNA, and (b) repression of NFκB

transactivation, since PR is the only steroid receptor besides the glucocorticoid receptor (GR) able to repress NFκB mediated transcription [69]. In other tissues, progesterone-bound PR can directly repress the COX2 gene in endothelial cells [70], and regulate in a negative fashion iNOS expression by macrophages [71]. Since iNOS and COX-2 are also stimulated by IL1β and TNF-α, we cannot discard an indirect effect of progesterone due to a decrease in those cytokines [72].

An interesting possibility also emerges from the relationship between progesterone, PR and NFκB. In wild-type mice, progesterone treatment decreased the transactivation of NFκB, as shown by the inhibition of IκB-α expression. As the decrease in mRNA levels of proinflammatory factors and enzymes correlates with reduced activity of NFκB, progesterone might downregulate these factors by decreasing the transactivation of NFκB. Our results with the PRKO mice show that a functional PR is also needed for the inhibiton of NFκB transactivation and the inflammatory responses to SCI. This event has functional advantages, because inhibition of NFκB in animals with SCI enhanced locomotor behaviour [73] and increased oligodendrogenesis [11].

An intriguing question is the role of non-classical progesterone mediators during inflammation, including mPRs, the $GABA_A$ receptors, PRCM1 or sigma receptors [15, 33-36] because PRKO mice were unresponsive to progesterone. PRKO mice normally express these alternative progesterone mediators [32], but they lack progesterone immunosupression. Perhaps PR also brings a proper microenvironment for the function of “non-classical” progesterone receptors and other binding molecules in the inflamed nervous system. We conclude that PR is a key mediator of the anti-inflammatory effects of progesterone. This result

supports a critical role of PR in neuropathology [36]. Elucidation of the multiple anti-inflammatory mechanisms of progesterone remains an exciting challenge for future experiments.

5. Conclusions

The present results support that progesterone down regulates reactive gliosis and exerts potent anti-inflammatory effects in rats and mice with spinal cord damage. In addition, we demonstrate for the first time the role of PR in these actions. Progesterone also avoided OPC apoptosis in a PR-dependent manner, a mechanism that would enhance the commitment of progenitors to the remyelination pathway in the injured spinal cord. Thus, progesterone surges as a potential treatment for demyelinating disorders and spinal cord injury, taking into account that its anti-inflammatory actions seems to favour the remyelination process.

. **ACKNOWLEDGEMENTS**

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LEGEND TO FIGURES

Fig. 1: Progesterone down-regulated mRNA expression of inflammatory mediators, interleukin receptors and enzymes in rats killed at several times after spinal cord injury. The mRNAs of IL1β (A), IL1β R1 (B), IL1β R2 (C), IL1β Ra (D),TNFα (E), IL6 (F), iNOS (G) and COX-2 (H) showed peak expression 6 h after spinal cord injury (SCI; black columns) and declined at later times compared to controls (CTL, white columns). Progesterone treatment of rats with SCI (cross hatched columns) decreased proinflammatory cytokines and enzymes mainly at 6 h, and decreased IL1β R1 and increased IL1β Ra mRNA at 1 day after injury. Data represent the mean ± SEM for n=8 animals per group. For A-H: *p<0.05 vs CTL; **p<0.01 vs CTL; ***p<0.001 vs CTL; +p<0.05 vs SCI; ++p<0.01 vs SCI and +++p<0.001 vs SCI.

Fig. 2: Progesterone decreased proinflammatory mediators and IκB-α mRNA in wild-type but was inactive in progesterone-receptor knock-out mice. Group and column labelling as described in the legend to Figure1. A – D : SCI increased the mRNA levels of IL1β (A, *** p<0.001), TNFα (B, *** p<0.001), IL6 (C, *** p<0.001) and IκB-α (D, ** p<0.01) of wild-type (WT) mice at 6 h compared to CTL. Progesterone treatment decreased expression of these four molecules vs. SCI (+ p<0.05; ++ p<0.01). In progesterone-receptor knock-out (PRKO) mice, SCI strongly induced the mRNA for IL1β (A, *** p<0.001), TNFα (B, *** p<0.001), IL6 (C, *** p<0.001) and IκB-α (D, * p<0.05), but in PRKO mice the three cytokines and IκB mRNA did not decline following progesterone treatment. N=8 animals per group.

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Fig. 3 : Progesterone antagonized the spinal cord injury-enhanced number of GFAP+ astrocytes and OX-42+ microglia of wild-type but not progesterone-receptor knock-out mice. Group and column labelling as described in the legends to Figures 1 and 2. Significant elevations of GFAP+ astrogliosis (A, ***p<0.001) and OX-42 + microgliosis (B, ***p<0.001) occurred in both WT and PRKO mice after SCI. Progesterone-treated WT mice with SCI showed decreased astrogliosis (A,+++ p<0.001) and microgliosis (B, +++p<0.001)

compared to SCI only. Progesterone was inactive in PRKO mice. N=6 animals per group. Photomicrographs taken from the white matter of CTL (C, F), SCI (D, G) and SCI + progesterone-treated (E, H) WT mice showed increased GFAP and OX-42 staining after SCI compared to CTL and decreased staining following progesterone. PRKO mice showed enhanced staining of GFAP+ and OX-42+ cells in the SCI group (J,M) and the SCI + progesterone group (K, N) compared to CTL (I,L) . Scale bar = 20μm in C-N.

Fig. 4 Progesterone increased oligodendrocyte progenitors showing NG2+ staining from wild-type but not progesterone-receptor knock-out mice with spinal cord injury. Group and column labelling as described in the legends to Figures 1 and 2. A: In WT mice, NG2+ staining of OPC increased at 24 (***p<0.001) and 48 h (***p<0.001) after SCI vs. CTL. Progesterone further enhanced NG2+ cells at 48 h (+++p<0.001). B: In PRKO mice, NG2+ cells increased 48 h after SCI. The increase was similar without or with progesterone treatment (*** and +++ p<0.001) vs CTL. Data represent n= 6 mice per group. C – H : Photomicrograhs showing differences of NG2+ cell number at 48 h in WT and PRKO (KO) mice with SCI (D, G) vs. their CTL groups (C, F). Progesterone treatment increased NG2+ cells in WT mice only (E) . Scale bar = 10μm in C-H.

Fig. 5: Progesterone reduced apoptosis of cells showing NG2+ staining in wild-type but not progesterone-receptor knock-out mice following spinal cord injury. Group and column labelling as described in the legends to Figures 1 and 2. A: Double-labelled TUNEL+ / NG2+ apoptotic progenitor cells were reduced after progesterone treatment of WT mice (+p<0.05) with SCI, but not in PRKO (KO) mice with similar SCI. B: Data plotted as % TUNEL+ NG2+ cells / total NG2+ cells. Progesterone treatment following SCI reduced by half TUNEL+ progenitors of wild-type mice but not PRKO mice (+p<0.05). C: SCI group, 12 μm confocal stack with orthogonal views taken at the center of one cell; C1,C2: 0.3 μm single channel confocal images of TUNEL (C1, green) and NG2+ cell (C2, red) channel. D: Confocal image of SCI group showed several apoptotic NG2+ cells (arrows). D1, D2: single channel for TUNEL + cell (D1, green) and NG2+ cell (D2, red). E: representative confocal image of SCI+progesterone

mouse showed an apoptotic cell lacking NG2+ staining (arrowhead). E1, E2: single channel for TUNEL+ cell (E1, green) and NG2+ cell (E2, red). Scale bar: 20 μm in C, D, E : 10 μm in C1, C2, D1, D2, E1, E2.

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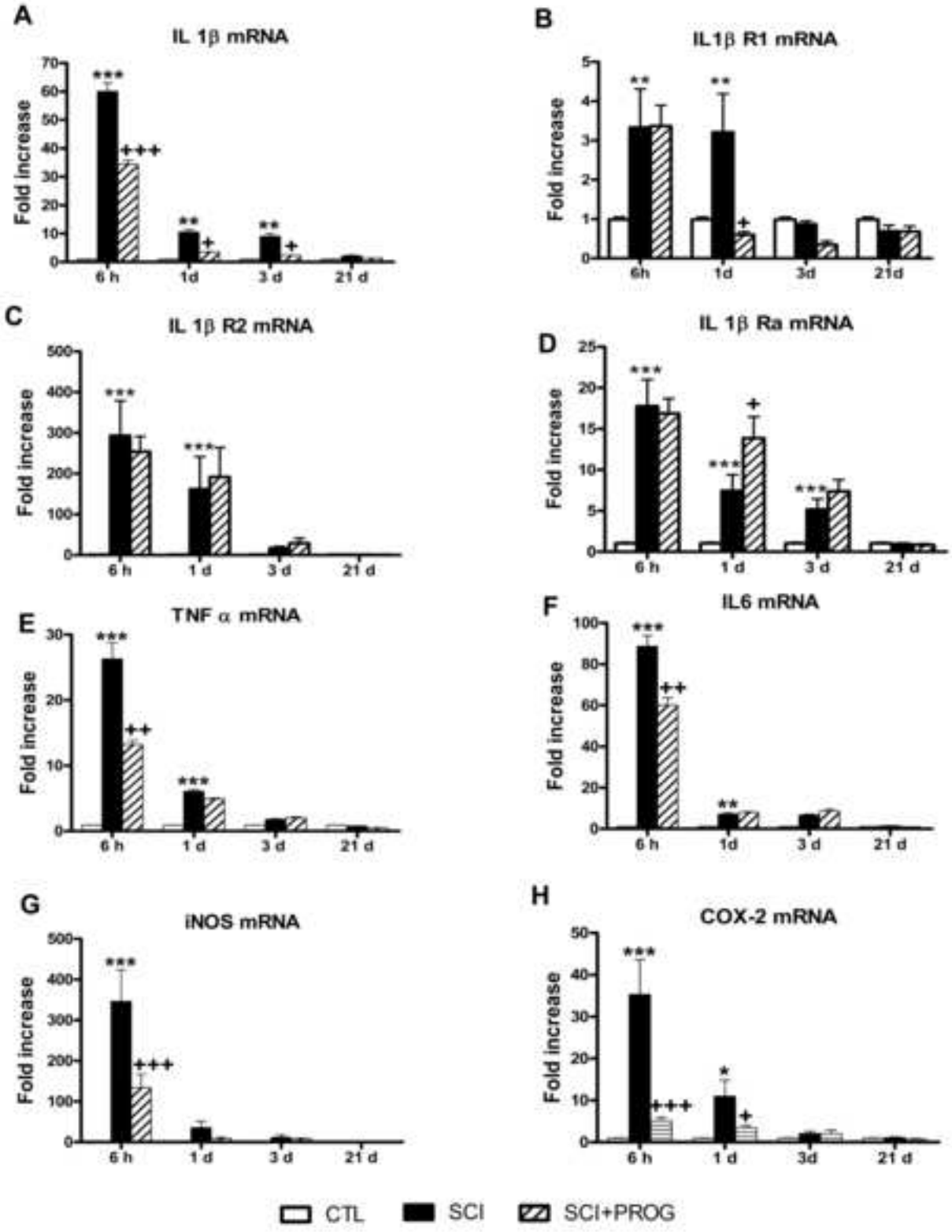

Figure 1 two column fitting image

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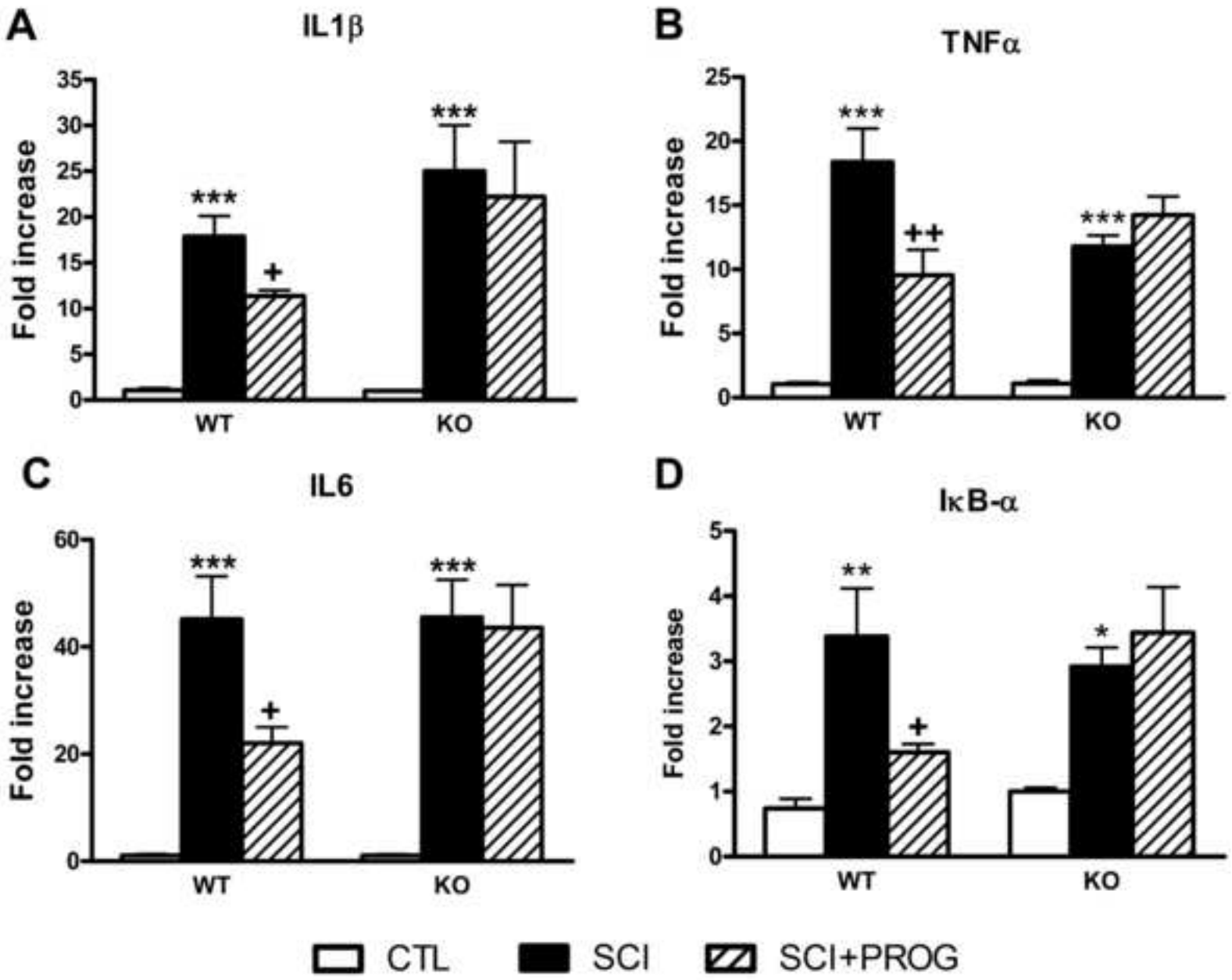

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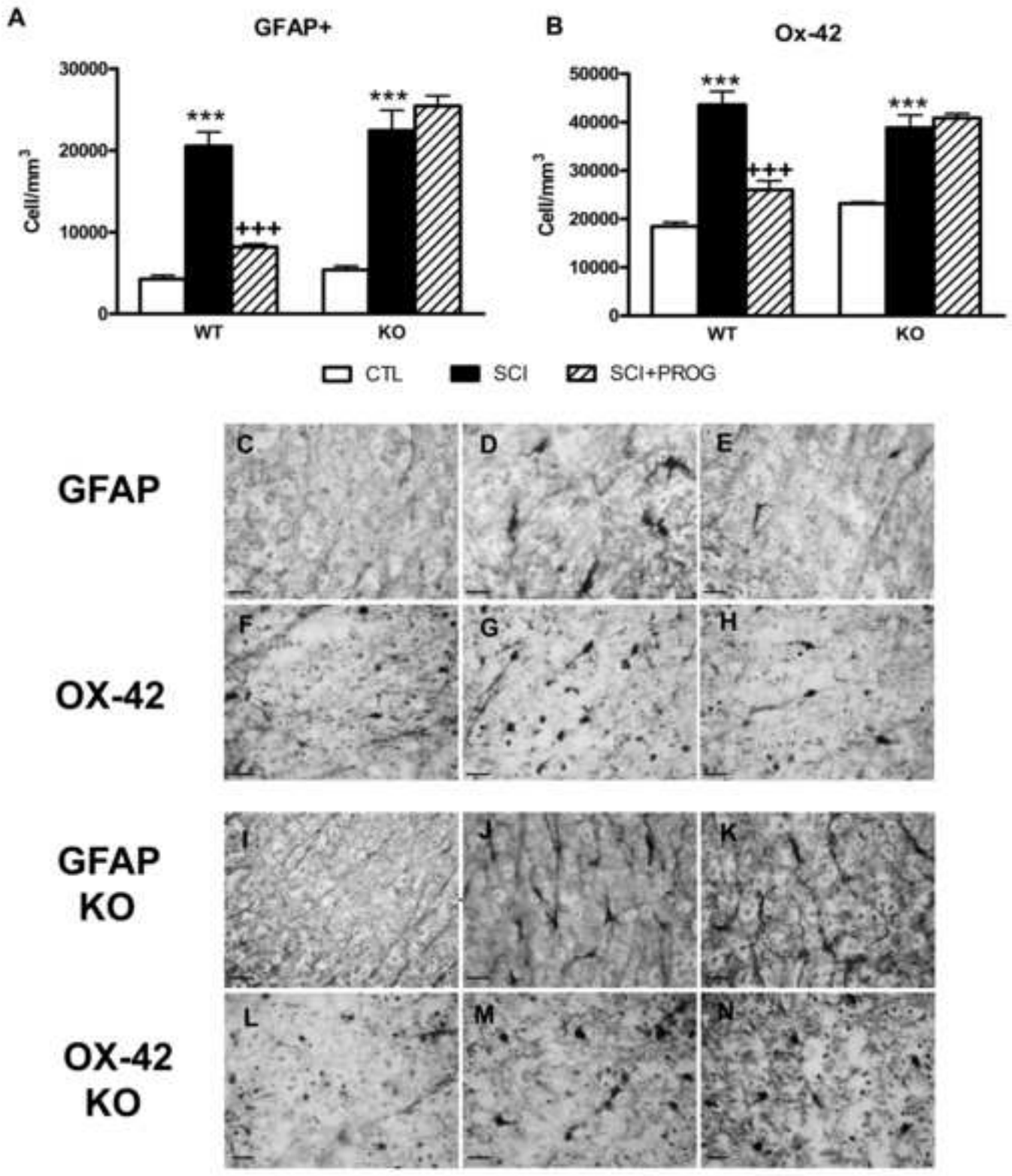

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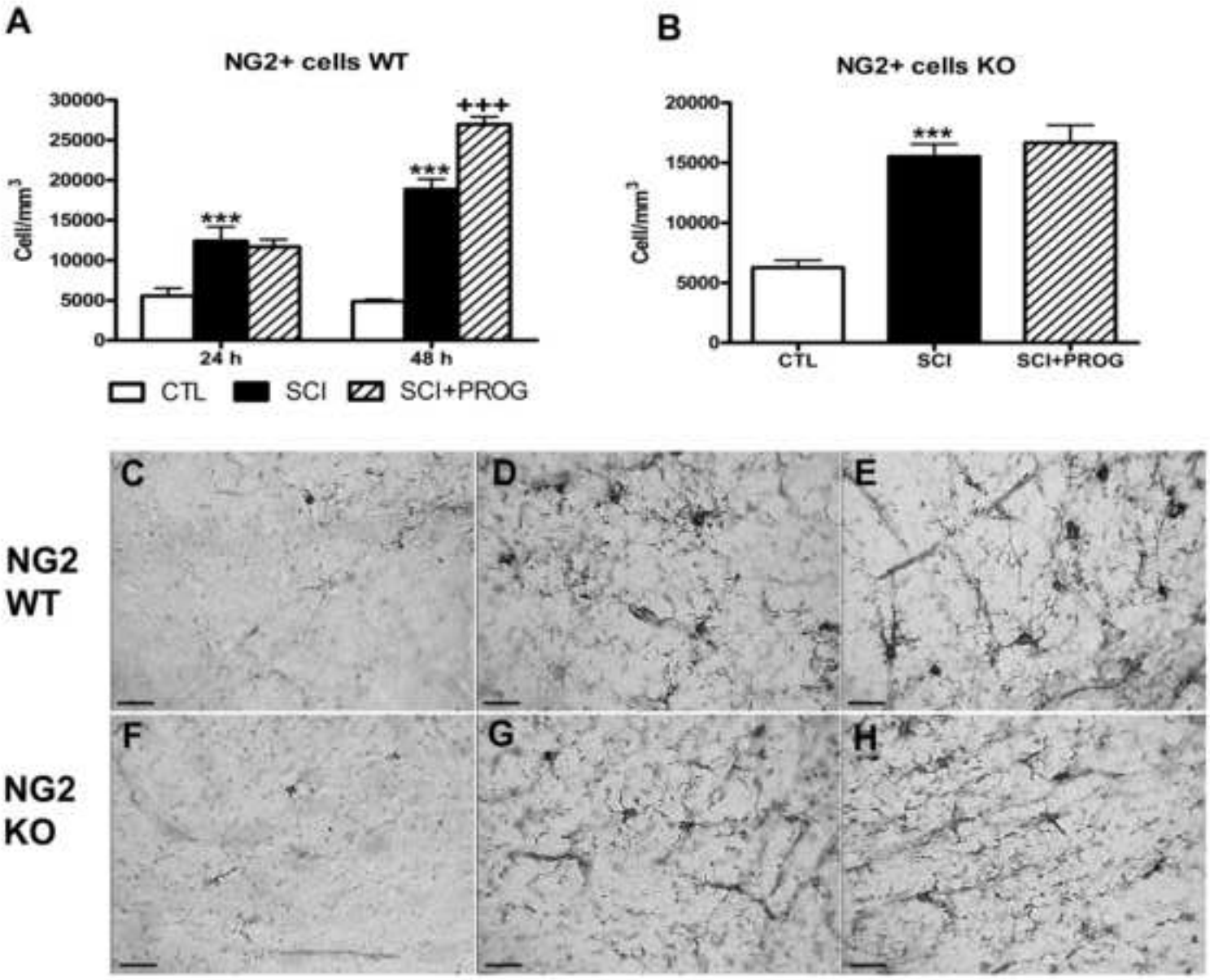

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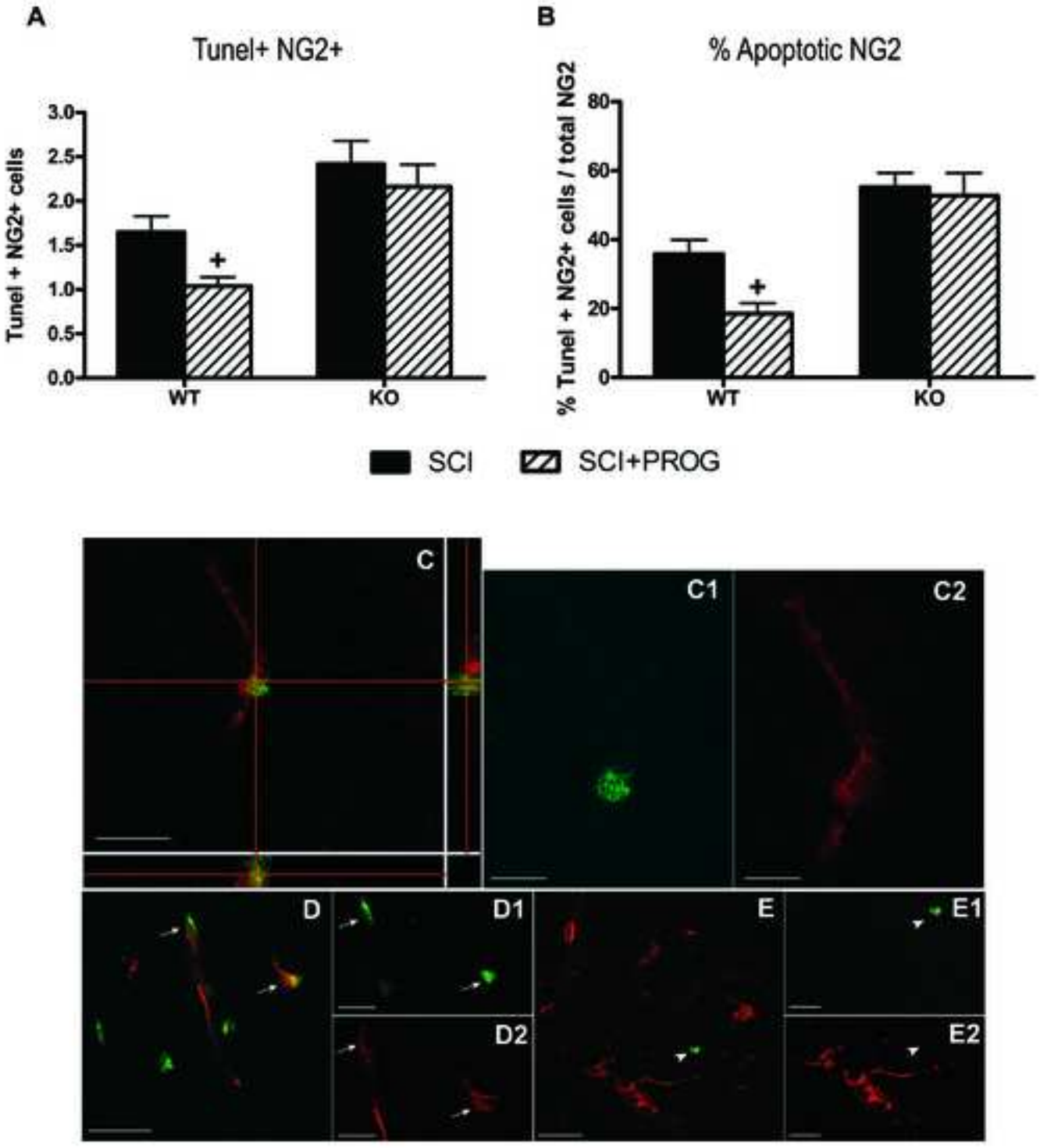

Figure 5 two column fitting image

Table 1

Table 1: Rat Foward and Reverse primers sequences

Gene	Primer sequence	Reference
IL 1 β	F: 5´CACCTCTCAAGCAGAGCACAG 3´ R: 5´GGGTTCCATGGTGAAGTCAAC 3´	NM_031512.2
IL 1β R1	F: 5´GTTTTTGGAACACCCTTCAGCC 3´ R: 5´ACGAAGCAGATGAACGGATAGC 3´	XM_006244754.2
IL 1 β R2	F: 5´CATTCAGACACCTCCAGCAGTTC 3´ R: 5´ACCCAGAGCGTATCATCCTTCAC 3´	XM_008766984.1
IL1 ra	F: 5´AAGACCTTCTACCTGAGGAACAACC 3´ R: 5´GCCCAAGAACACATTCCGAAAGTC 3´	XM_006233638.2
TNF α	F: 5´TCGTAGCAAACCACCAAGCA 3´ R: 5´CCCTTGAAGAGAACCTGGGAGTA 3´	X66539.1
IL 6	F: 5´AAGTCGGAGGCTTAATTACATATGTTC 3´ R: 5´TGCCATTGCACAACTCTTTTCT 3 ´	NM_012589.2
Cox-2	F: 5´TTTGTTGAGTCATTCACCAGACAGAT 3´ R: 5´ACGATGTGTAAGGTTTCAGGGAGAAG 3´	S67722.1
iNOS	F: 5´CCAGAGCAGTACAAGCTCAC 3´ R: 5´CCACAACTCGCTCCAAGATC 3´	AY211532.1

Table 2

Table 2: Mouse Foward and Reverse primers sequences

Gene	Primer sequence	Gen Bank Accession Number
IL1β	F: 5´ CAACCAACAAGTGATATTCTCCATG 3´ R: 5´GATCCACACTCTCCAGCTGCA 3´	NM_008361
TNF α	F: 5´GAAAAGCAAGCAGCCAACCA 3´ R: 5´CGGATCATGCTTTCTGTGCTC 3´	NM_013693
IL 6	F: 5´GAG GATACCACTCCCAACAGACC 3´ R: 5´AAGTGCATCATCGTTGTTCATACA 3´	NM_031168