

Neuroprotection by steroids after neurotrauma in organotypic spinal cord cultures: A key role for progesterone receptors and steroidal modulators of GABA_A receptors

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ABSTRACT

Progesterone is neuroprotective after spinal cord injury, however its mechanism of action remains unexplored. Here we used organotypic spinal cord slice cultures from 3 weeks-old mice to evaluate the mechanisms of neuroprotection by progesterone and its 5 α -reduced metabolites. In vitro spinal cord injury, using a weight drop model, induced a decrease in the number of motoneurons. This was correlated with an increase in the number of dying cells (PI⁺ cells) and in LDH release. Addition of 10 μ M of progesterone, 5 α -dihydroprogesterone (5 α -DHP) or allopregnanolone (3 α , 5 α -tetrahydroprogesterone) to the medium at the time of injury rescued the spinal cord slices from the effects of damage. Progesterone prevented membrane cell damage, motoneuron loss and cell death. These effects were not due to its bioconversion to 5 α -DHP nor to allopregnanolone, as supported by the finasteride, an inhibitor of 5 α -reductase enzymes, and by the absence of 5 α -reduced progesterone metabolites in the slices analyzed by gas chromatography–mass spectrometry. The neuroprotective effects of progesterone required PR as they could not be observed in slices from homozygous knockout PR^{-/-} mice. Allopregnanolone treatment was also neuroprotective. Its effects were not due to its bioconversion back to 5 α -DHP, which can activate gene transcription via PR, because they were still observed in slices from knockout PR^{-/-} mice. Allopregnanolone effects involved GABA_A receptors, as they were inhibited by the selective GABA_A receptor antagonist Gabazine, in both PR^{+/+} and PR^{-/-} mice. Altogether, these findings identify both PR and GABA_A receptors as important targets for neuroprotection by progestagens after spinal cord injury.

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1. Introduction

Progesterone, with its proven clinical safety (Wright et al., 2007; Xiao et al., 2008) and its efficacy as a neuroprotective factor (De Nicola et al., 2009; Gibson et al., 2009; Schumacher et al., 2007a, 2007b; Stein, 2001) may become one of the effective therapies for nervous system injuries. We have previously shown that progesterone has neuroprotective effects in the injured spinal cord (De Nicola et al., 2009; Gonzalez et al., 2004; Labombarda et al., 2011, 2009, 2006a, 2002, 2000a). Rats treated with progesterone after spinal cord contusion show better locomotor activity and less tissue

and white matter damage than control animals (Thomas et al., 1999). Spinal cord trauma leads to neuronal degeneration, astrogliosis, demyelination, and proliferation of oligodendrocyte-precursor cells. Motoneurons present several abnormalities after spinal cord injury (SCI), and we have demonstrated that some genes become stimulated by progesterone in the injured spinal cord. Thus, after complete transection of the spinal cord, progesterone treatment restores choline acetyltransferase (ChAT) levels, normalizes the expression of Na, K-ATPase and increases GAP-43 and BDNF expression (De Nicola et al., 2009; Gonzalez et al., 2004; Labombarda et al., 2002).

Progesterone has different signaling mechanisms which offer exciting possibilities for the development of specific treatments and new pharmacological trials. Indeed, progesterone may: 1 – regulate gene transcription after binding to selective intracellular progesterone receptors (PR) which belong to a super-family of zing finger transcription factors; 2 – activate signaling cascades via

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specific membrane receptors (Losel et al., 2003; Mani, 2006; Pettus et al., 2005) and 3 – modulate GABA_A receptor activity via its metabolite allopregnanolone (Belelli and Lambert, 2005; Hosie et al., 2006). We have demonstrated that the so-called classical intracellular progesterone receptors (PR), the putative membrane progesterone receptor Pgrmc1 (Labombarda et al., 2000b, 2003) and the membrane receptors mPRs (Labombarda et al., 2010) are all expressed in the spinal cord. Furthermore, progesterone can be converted within the spinal cord to its reduced metabolites 5 α -dihydroprogesterone (5 α -DHP) and allopregnanolone (Labombarda et al., 2006b; Patte-Mensah et al., 2006; Patte-Mensah and Mensah-Nyagan, 2008). The latter is a potent positive modulator of GABA_A receptor activity (Belelli and Lambert, 2005; Hosie et al., 2006). Thus, in common with other regions of the central nervous system, progesterone actions in the spinal cord may involve multiple signaling mechanisms. Elucidating the respective involvement of these different mechanisms would bring new insights into the neuroprotective effects of progesterone.

Growing experimental evidence has shown a beneficial influence of allopregnanolone on neuron viability after brain injury or in neurodegenerative conditions (Melcangi et al., 2008; Melcangi and Mensah-Nyagan, 2006; Melcangi and Garcia-Segura, 2010; Liu and Diaz Brinton, 2011). Indeed, allopregnanolone reverses neurogenic and cognitive deficits in a mouse model of Alzheimer's disease (Wang et al., 2010; Singh et al., 2012). Niemann–Pick type C disease can be blocked by appropriately timed treatment with allopregnanolone (Griffin et al., 2004). Therapy with this neurosteroid is also efficient after traumatic brain injury (Djebaili et al., 2004, 2005; He et al., 2004) after cerebral ischemia (Sayeed et al., 2006; Sayeed and Stein, 2009; Gibson et al., 2009; Liu et al., 2012) and in an experimental model of diabetic neuropathy (Leonelli et al., 2007). The potential neuroprotective effects of allopregnanolone have never been tested until now in spinal cord injury.

Due to the complexity of the *in vivo* system, the study of the signaling mechanisms by which progesterone and allopregnanolone bring neuroprotection is difficult. *In vitro* models allow precise control over the extracellular environment. Organotypic spinal cord cultures offer advantages over dissociated cultures in that they preserve the synaptic and anatomical organization of the neural circuitry and have functional characteristics similar to those found *in vivo* (Gahwiler et al., 1997). The majority of investigators previously used organotypic spinal cord cultures prepared from embryonic or newborn rats or mice. However, there are significant differences in function, neuronal connections, and regenerative capabilities between different developmental stages (Kerkut and Bagust, 1995). Therefore, we used an *in vitro* injury procedure to damage spinal cord slices in organotypic cultures derived from young post-weaning mice at a later stage of development as used in previous studies (Krassioukov et al., 2002).

The objective of this work was to study the effects of progesterone and allopregnanolone on neuronal survival and to define the role of the PR, the 5 α -reductase and the GABA_A receptors in mediating these effects using organotypic cultures of spinal cord slices.

2. Materials and methods

2.1. Organotypic spinal cord slice cultures

Experimental protocols were approved by the Direction départementale de la protection des populations du Val-de-Marne, France, authorization number 94-345 to R.G., accredited establishment number 94-043-13. Experiments were performed in accordance with French ethical laws (Act 87-848; Ministère de l'Agriculture et de la Forêt) and the European Communities Council Directives of November 24, 1986 (86/609/EEC) guidelines for the care and use of laboratory animals. Three weeks-old C57BL/6 male mice, PRKO (PR^{-/-}) male mice and their littermate wild-type (PR^{+/+}) for PR genes were used in this study. Genotyping was done by polymerase chain

reaction (PCR) amplification of tail DNA as previously described (Ismail et al., 2002; Labombarda et al., 2010). Mice were anaesthetized with ketamine (50 mg/kg⁻¹) and the spinal cord was aseptically extracted and placed in an ice-cold dissecting media and meninges were removed. The spinal cord was cut into 350 μ m thick slices using a McIlwain tissue chopper to generate the organotypic slice cultures. Only sections from the lumbar spinal cord were used. Spinal slices were transferred onto membranes of 30-mm Millipore culture inserts with 0.4 μ m pore size (Millicell, Millipore, Bedford, MA, USA). The inserts were placed into wells of six-well plate containing 1 ml of antibiotic-free medium containing 50% MEM with Earl's salt and glutamine, 25% Hanks balanced salt solution, and 25% horse serum supplemented with 20 mM of Hepes acid-salt and D-glucose (6 mg/ml) (Gibco-BRL). Slices were cultured on top of the membranes and were not immersed in the culture medium. To ensure the optimum viability of the cultures, 5 slices were plated per Millicell (Krassioukov et al., 2002). Slices were incubated at 35 °C in an atmosphere of humidified 5% CO₂ for seven days *in vitro* (DIV7) and media were changed three times weekly.

2.2. Assessment of structural and cellular integrity of organotypic spinal cord slice cultures

The viability of cultured slices was assessed using vital staining with the tetrazolium dye MTT (Boehringer Mannheim). This translucent yellow dye is reduced by active mitochondria and other cellular enzymes. It forms a purple precipitate that accumulates within the cytoplasm of living cells and can be visualized under light microscopy (Abe and Matsuki, 2000). Nuclear staining was assessed using DAPI (Fisher Scientific) and SYTOX13 (Molecular Probes), two cell-permeant acid stains that show a large fluorescence enhancement upon binding to nucleic acid.

2.3. Cytoarchitecture of organotypic spinal cord slice cultures

Slices were washed in phosphate buffer saline (PBS), pH 7.4 and then fixed in 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) for 1 h at room temperature. Slices were then removed from the Millicells and processed for immunocytochemistry. Slices were incubated for 1 h in PBS 0.1 M (pH 7.4) containing 0.25% Triton-X, 0.2% gelatin, 0.1% sodium azide (PBSGTA) and lysine (0.1 M), before the overnight incubation with the primary antibodies. The following primary antibodies were used: (a) monoclonal antibody against neuronal nuclei NeuN (1/1000; Chemicon) and (b) monoclonal antibody against Neurofilament 200 (1/1000; Sigma) were used to identify axons; (c) rabbit polyclonal antibody against glial fibrillary acidic protein (GFAP, 1/1000; Sigma) was used to identify astrocytes and (d) monoclonal antibody against myelin basic protein (MBP, 1/1000; Boehringer Mannheim) to visualize myelin. The following secondary antibodies were used: goat anti-rabbit and goat anti-mouse Alexa Fluor 488 (1/1000; Molecular Probes, Leiden, Netherlands). After 2 h incubation, slices were washed several times in PBS, mounted with Fluoromount (Clinisciences, France) and analyzed using a confocal Zeiss LSM 510 (Carl Zeiss Inc) image analyzing system.

2.4. *In vitro* SCI and drug treatments (Fig. 1)

SCI was elicited using a weight drop model of injury. An impactor with weight of 0.2 g was dropped from 1.7 cm onto the center of the culture slice (Krassioukov et al., 2002). Following the injury, slices were placed back in the incubator for three days until DIV10. Steroids (Sigma) were dissolved in ethanol and added to the medium at the time of the injury and maintained during three days. Based on our previous study (Ghoomari et al., 2003) progesterone (PROG), 5 α -DHP and allopregnanolone were used at the concentration of 10 μ M. The final concentration of ethanol in the medium was 0.1%. Finasteride was dissolved in DMSO and was used at 50 μ M based on a previous work (Keller et al., 2004) and was applied 15 min before SCI. Gabazine

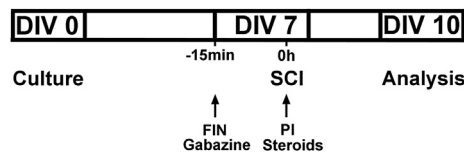


Fig. 1. Time frame of experimental procedures. Organotypic spinal cord slice cultures were generated from 3 weeks-old mice. At DIV7, trauma injury (SCI) was achieved by dropping a weight of 0.2 g from a height of 1.7 cm onto the center of the organotypic slice in the tissue culture chamber. At the time of SCI, cultures were treated or not with 10 μ M of steroids: progesterone (PROG), or 5 α -DHP, or allopregnanolone (Allo). To block the 5 α -reductase, cultures were treated with finasteride (FIN, 50 μ M) alone or in association with 10 μ M PROG. The GABA_A antagonist Gabazine (5 μ M) was used alone or in association with Allo. Finasteride and Gabazine were applied 15 min before SCI. PI was added in the same time as steroids to assess cell death. At DIV10, slices were either fixed and processed for immunofluorescence and confocal microscopy analysis or used for LDH measurement. DIV: day *in vitro*, FIN: Finasteride, SCI: spinal cord injury, PI: propidium iodide.

was dissolved in PBS and was used at 5 μM , a dose which has been shown to block GABA_A receptors (Ueno et al., 1997) and was applied 15 min before SCI. Measurements and quantifications were done in different groups of organotypic spinal cord slices: (a) without injury (CTL), (b) with an in vitro injury (SCI), (c) treated with progesterone (SCI + PROG), or (d) with 5 α -DHP (SCI + DHP), or (e) with allopregnanolone (SCI + Allo) at the time of injury. The effect of the inhibition of 5 α -reductase was evaluated in two groups of organotypic spinal cord slices treated with: (a) progesterone and finasteride (SCI + PROG + FIN), or with (b) finasteride alone (SCI + FIN). Gabazine was used in association with allopregnanolone (SCI + ALLO + Gz). CTL and SCI groups received the same amount of vehicle as treated groups.

2.5. Assessment of cell death

2.5.1. Propidium iodide (PI) uptake

Cell viability was determined by PI exclusion. When cell membranes are damaged PI enters and binds nucleic acids. At time of the injury 5 μM of PI (Molecular Probes) was added to the cultures and incubated for 3 days. Under these conditions, PI has been shown to be non toxic and has been used to ascertain the extent of cell death (Macklis and Madison, 1990). At DIV10 (Fig. 1) slices were fixed and PI fluorescence emission was captured using a Confocal Zeiss LSM 510 (Carl Zeiss Inc) image analyzing system. The bound form of PI shows increased emission at 635 nm when excited at 540 nm.

2.5.2. Lactic acid dehydrogenase (LDH) measurements

LDH is a rapidly released enzyme into the culture medium upon damage of the plasma membrane. LDH, therefore, is the most widely used marker for cytotoxicity. LDH levels were determined using a standard assay (Tox-7, Sigma Chemical, St. Louis, MO), which was performed according to the manufacturer's instructions. Cell death was expressed as a ratio of LDH release in cell culture supernatants to total LDH (Vos et al., 2000).

2.5.3. Quantitative analysis of immunocytochemistry and PI uptake

The cytoarchitectonic division of Steiner and Turner (1972) was used for laminae and cell identification in the spinal cord sections. PI⁺ cells were counted in Lamina IX. NeuN⁺ cells in lamina IX showing a clear nuclear profile with a nucleus diameter greater than 5 μm were considered motoneurons (Burke et al., 1977). PI, and NeuN fluorescence was observed using a confocal Zeiss 510 (Carl Zeiss Inc) image analysis system. Images were acquired and the number of positive cells per 90,000 μm^2 was counted using NIH image software.

2.5.4. Steroid measurements

Steroids were extracted from the medium and from the organotypic slices and measurements of progesterone and its reduced metabolites were performed using a validated gas chromatography–mass spectrometry (GC/MS) (Liere et al., 2000) which was already used for steroid measurements after in vivo spinal cord injury (Labombarda et al., 2006b). Briefly, steroids were extracted overnight from 10 organotypic spinal cord slices from male mice (weight of 1 slice = 1.173 \pm 0.01 mg) or from the medium by adding MeOH. The internal standards (19 nor-PROG and ²H₆-5 α -DHP for PROG, 3 α / β 5 α -THP and 5 α -DHP, respectively) were added into the extracts for quantification. After centrifugation and washing, the samples were purified and fractionated by Solid Phase Extraction (SPE) on C18 columns. Samples are submitted to a recycling/elution SPE protocol in order to separate sulfated, free and lipoidal steroids. The progestagens-containing free fraction were filtered through a PVDF membrane and submitted to High Performance Liquid Chromatography (HPLC) (Thermo Fisher Scientific, San Jose, USA) with a Lichrosorb Diol column and collected in a fraction collector. Two fractions are collected and derivatized before GC–MS analysis. The first one (containing 5 α -DHP) was derivatized with a mixture of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide (NH₄I), and dithioerythritol (DTE) (1000:2:5, v/w/w). The other fraction, containing PROG and 3 α / β 5 α -THP were derivatized with heptafluorobutyric anhydride (HFBA). Samples were analyzed by GC/MS using a fused-silica capillary column and GC was performed in the splitless mode. The mass spectrometer was operated in the electron impact mode. Analyses were performed in Single Ion Monitoring (SIM) mode. Identification of each steroid was supported by its GC retention time and its two diagnostic ions and quantification was achieved according to the major diagnostic ion and by using a previously established calibration curve for each steroid (Labombarda et al., 2006b; Liere et al., 2000; Liu et al., 2012). The detection limits for PROG, 5 α -DHP, 3 α 5 α -THP and 3 β 5 α -THP are 1, 2, 4 and 2 ng/g from 10 mg of tissue and 0.01, 0.02, 0.05 and 0.02 ng/ml from 1 ml of medium, respectively.

Data (mean \pm SEM) were expressed as ng/mg in the tissue and as ng/ml in the medium (Weight of 1 spinal cord slice = 1173 \pm 0.01 mg).

2.5.5. Statistical analysis

The statistical analysis was performed with GraphPad Prism 4.0. Data are expressed as the mean \pm standard error of the mean (SEM) and *n* refers to the number of animals studied. *n* = 6 for each experimental group. Results were analyzed by one-way ANOVA followed by post-hoc Newman–Keuls test. Results of measurements of steroids by GCMS were analyzed by Student's *t*-test for comparing

levels between SCI + PROG and SCI + PROG + FIN groups. Statistical significance was established at *p* < 0.05.

3. Results

3.1. Characterization and viability of the organotypic slices cultures of spinal cords from young mice

Organotypic spinal slice cultures were successfully set up using the interface method developed by Stoppini et al. (1991) with culture medium modifications that increase viability of the spinal cord. Spinal cords from 3 to 10-weeks-old mice (C57BL/6) were tested for their suitability to generate the organotypic slices cultures. Spinal cords from 3 weeks-old mice gave the best results: ease in the meningeal removal, spinal cord sectioning, culture viability and well-preserved gross organotypic morphology, including identifiable dorsal and ventral grey regions and central canal. Thus, for all the subsequent experiments, 3 weeks-old mice were used. Spinal cord organotypic slices were successfully cultured for 10 days, explants were observed in a daily basis using phase contrast microscopy. Spinal cord cultures prepared in this way maintain an excellent morphology, facilitating the identification of neuronal populations using conventional light microscopy. Slices spontaneously attach to the filter supports and flatten to less than 100 μm in thickness from the original starting thickness of 350 μm . Assessment of cell death was performed using LDH activity at different times after culturing (DIV1, DIV3, DIV7, DIV10). The ratio of LDH release/Total LDH is an index which reflects the cell death. This ratio was high at DIV1 and DIV3 and decreased at DIV7 and DIV10 (Fig. 2A, *p* < 0.001 DIV7 versus DIV1 and DIV10 versus DIV1). This death during the first days of the culture is a typical response to axotomy and tissue slicing. Therefore, organotypic spinal cord cultures were allowed a 7-days recovery period after preparation, before performing SCI and pharmacological treatments. Assessment of cellular viability of cultured slices was performed at DIV7 using vital staining with MTT. DAPI and Sytox-green 13, were used for quick observation of cell nuclei in the cultured slices. Intense nuclear staining and the absence of apoptotic features were observed with these two nuclear markers (Data not shown). The identification of neurons, axons, astrocytes and myelin in organotypic spinal cord slices at DIV7 was done by immunofluorescence (Fig. 2B). NeuN immunoreactivity was observed in a typical butterfly-shaped grey matter with clearly labeled neurons. NF 200 immunoreactivity was observed in several neurites. GFAP⁺ cells showing multiple processes typical of astrocyte morphology was observed in the white and grey matters. MBP immunoreactivity was observed in oligodendrocyte cells bodies and processes.

3.2. Progesterone had no effects on PI⁺ and NeuN⁺ cell number in control (non-injured) organotypic spinal cord slice cultures

Slices were treated during 3 days between DIV7 and DIV10, and the effects of progesterone and vehicle (EtOH) were examined on: 1) cell death assessed by counting the number of PI⁺ cells in lamina IX, and 2) the number of motoneurons by counting NeuN⁺ cells in lamina IX with a nucleus diameter greater than 5 μm . There was neither a toxic nor a protective effect of progesterone or EtOH, as the number of PI⁺ cells, and motoneurons (NEUN⁺ cells) were similar in the control untreated, EtOH and progesterone-treated groups (Table 1).

3.3. Progesterone and its 5 α -reduced metabolites decreased membrane damage induced by SCI

To study the effects of SCI and treatment by progesterone and its reduced metabolites, organotypic spinal cord cultures were injured

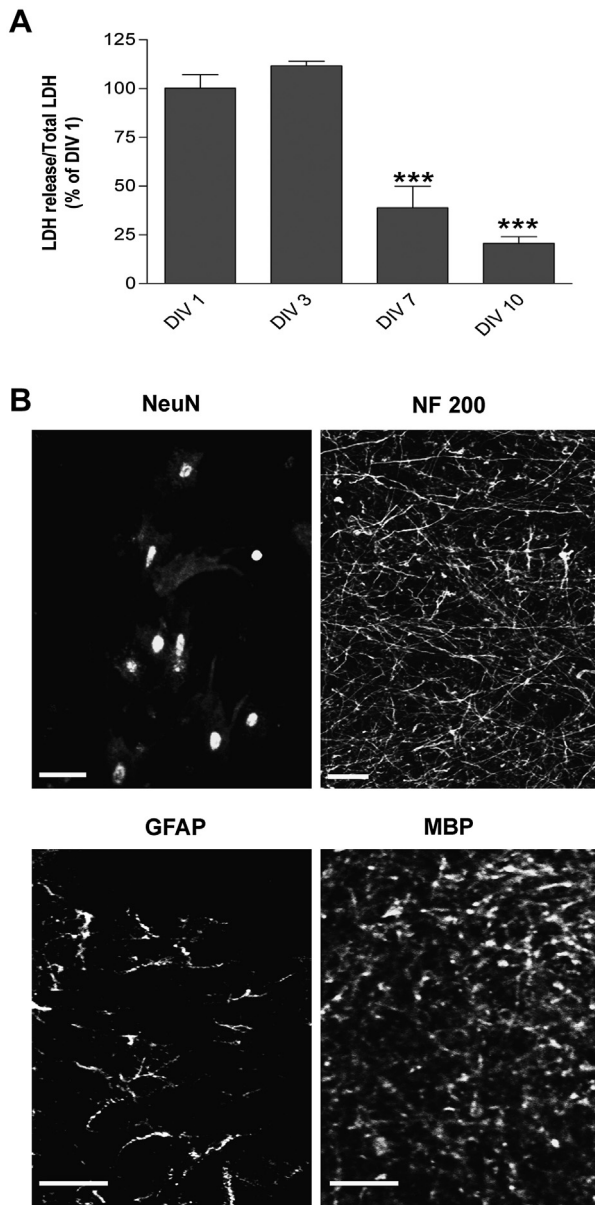


Fig. 2. Organotypic spinal cord slices characterization: viability and cell identification. A) Index of cell death (LDH release/Total LDH) from DIV1 to DIV10. Significant decrease in cell death was observed at DIV7 and DIV10 (***) $p < 0.001$ DIV7 versus DIV1; (***) $p < 0.001$ DIV10 versus DIV1) Data represent means \pm s.e.m., $n = 6$ per group. B) Cytoarchitecture of organotypic spinal cord slices as assessed by immunofluorescence and confocal microscopy. The immunostaining showed the preservation of neurones (NeuN), neurites (NF 200), astrocytes (GFAP) and myelin (MBP) in the organotypic spinal cord slice cultures. Scale bars: 20 μ m.

and treated by one of the steroids (10 μ M) at DIV7 and the effects were analyzed at DIV10. SCI induced membrane damage and cell death as shown by the increase in the ratio of LDH release/Total LDH (Fig. 3A, $p < 0.01$ SCI versus CTL). Progesterone treatment decreased the ratio of LDH release/Total LDH (Fig. 3A, $p < 0.01$

Table 1

Progesterone had no effects on PI⁺ and NeuN⁺ cell number in control (non-injured) organotypic spinal cord slice cultures.

Cell number	CTL	EtOH	PROG
PI ⁺	28.50 \pm 1.55	27.33 \pm 5.20	22.75 \pm 3.63
NeuN ⁺	22.02 \pm 1.16	21.56 \pm 2.21	24.33 \pm 5.20

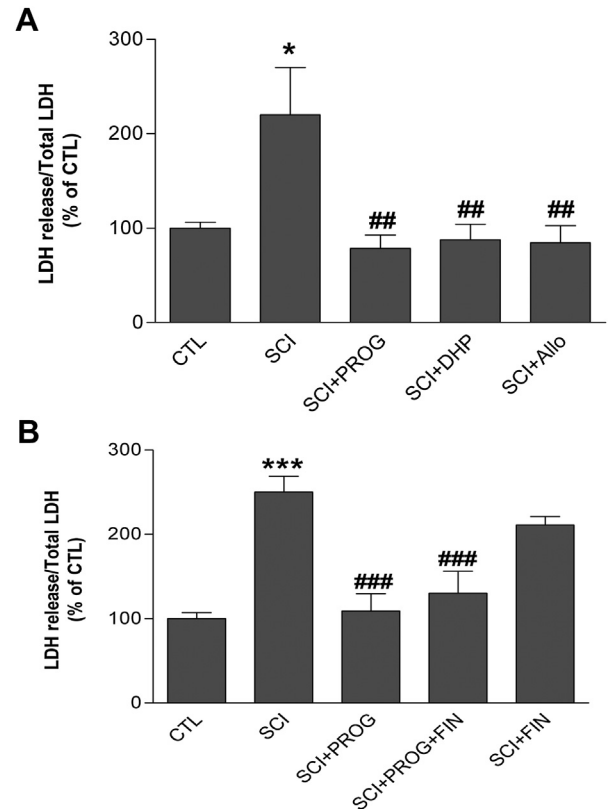


Fig. 3. Role of neurosteroid pathways in neuroprotection. A) Progesterone and its 5 α -reduced metabolites prevented membrane damage induced by SCI as shown by LDH measurements. SCI induced an increase in LDH/Total LDH ratio (* $p < 0.05$ SCI versus control), whereas PROG, 5 α -DHP, or Allo (10 μ M) treatments dumped this increase (** $p < 0.01$ SCI + PROG versus SCI, ** $p < 0.01$ SCI + 5 α -DHP versus SCI, ** $p < 0.01$ SCI + Allo versus SCI). B) Finasteride did not block the progesterone effect. Progesterone alone decreased the LDH/Total LDH ratio (***) $p < 0.001$ SCI + PROG versus SCI). Treatment with Finasteride (FIN), in association with progesterone also decreased the LDH/Total LDH ratio (***) $p < 0.001$ SCI + PROG + FIN versus SCI). FIN alone was without effect. Data represent means \pm s.e.m., $n = 6$ per group.

SCI + PROG versus SCI). Treatments with 5 α -DHP or with allopregnanolone also decreased the ratio of LDH release/Total LDH (Fig. 3A, $p < 0.01$ SCI + DHP versus SCI, $p < 0.01$ SCI + Allo versus SCI) and were as efficient as the treatment with progesterone (no significant differences between SCI + PROG, SCI + DHP and SCI + Allo (Fig. 3A)).

3.4. The neuroprotective effect of progesterone does not require its 5 α -reduction

As 5 α -DHP and allopregnanolone were neuroprotective when added to the culture medium, we wondered if the neuroprotective effect of progesterone treatment was due to its bioconversion to 5 α -DHP and allopregnanolone. To test this hypothesis, we used finasteride, a 5 α -reductase inhibitor. Similar ratios of LDH release/Total LDH were observed after treatment of the injured slices with progesterone alone or in association with finasteride (Fig. 3B, $p < 0.01$ SCI + PROG and SCI + PROG + FIN versus SCI). We then measured steroid levels by GC/MS and showed that progesterone was not bioconverted to its 5 α -reduced metabolites in this in vitro system. Indeed, treatment with progesterone produced high levels of progesterone in the spinal cord slices, but levels of 5 α -DHP, allopregnanolone and isoallopregnanolone (3 β ,5 α -tetrahydroprogesterone) were below the detection limit of the GC/MS

Table 2

Levels of progesterone and its 5 α -reduced derivatives in the injured organotypic spinal cord slices treated with progesterone alone or in association with finasteride: analysis by GC/MS.

Treatments		PROG	5 α -DHP	Allo	3 β ,5 α -THP
SCI + PROG	Slices (ng/mg)	13.83 \pm 1.11	nd	nd	nd
	Medium (ng/ml)	578.03 \pm 32.87	nd	nd	nd
SCI + PROG + FIN	Slice (ng/mg)	13.64 \pm 1.16	nd	nd	nd
	Medium (ng/ml)	575.18 \pm 44.35	nd	nd	nd

5 α -DHP: 5 α -dihydroprogesterone.

Allo: allopregnanolone (3 α ,5 α -tetrahydroprogesterone).

3 β ,5 α -THP: 3 β ,5 α -tetrahydroprogesterone.

FIN: Finasteride.

nd: not detected.

analysis. In addition, treatment with progesterone plus finasteride achieved similar levels of progesterone in the spinal cord as with progesterone alone, but as expected, 5 α -DHP or allopregnanolone or isoallopregnanolone were not detected in the slices or in the medium in the SCI + PROG + finasteride group (Table 2).

3.5. The neuroprotective effects of progesterone require the intracellular PR

To study the role of nuclear progesterone receptors (PR) in mediating the neuroprotective effects of progesterone, we prepared organotypic spinal cord cultures from wild type PR^{+/+} mice and from knockout PR^{-/-} mice. In slice cultures from wild type PR^{+/+}, SCI increased the number of dying cells as shown by counting the number of PI⁺ cells (Fig. 4A, $p < 0.05$ SCI versus CTL) and progesterone treatment decreased the number of PI⁺ cells (Fig. 4A, $p < 0.01$ SCI + PROG versus SCI). The ratio of LDH release/Total LDH increased after SCI (Fig. 4C, $p < 0.001$ SCI versus CTL) and progesterone treatment decreased it (Fig. 4C, $p < 0.001$ SCI + PROG versus SCI). SCI induced a reduction of NeuN⁺ motoneurons (Fig. 4E, $p < 0.05$ SCI versus CTL). In the presence of progesterone NeuN⁺ cells were significantly replenished (Fig. 4E and G, $p < 0.01$ SCI + PROG versus SCI). The neuroprotective effects of progesterone treatment observed in wild type PR^{+/+} (Fig. 4A, C, E and G) could not be observed in PR^{-/-} mice (Fig. 4B, D, F and H) as shown by counting of PI⁺ cells (Fig. 4B, $p < 0.05$ SCI versus CTL and $p < 0.01$ SCI + PROG versus CTL), measurement of LDH release (Fig. 4D, $p < 0.01$ SCI versus CTL and SCI + PROG versus CTL), and NeuN⁺ cell counting (Fig. 4F and H, $p < 0.01$ SCI versus CTL and SCI + PROG versus CTL). No statistically significant differences were observed between SCI and SCI + PROG-treated slices for the 3 parameters studied in PR^{-/-} mice.

3.6. The neuroprotective effects of allopregnanolone involve GABA_A receptors

Allopregnanolone does not bind to PR, but it is a well known positive modulator of GABA_A receptors. To determine whether the

effect of allopregnanolone on LDH release are dependent on GABA_A receptors, we incubated spinal cord slices from wild type PR^{+/+} mice with 10 μ M of the steroid in the presence or absence of the selective GABA_A receptor antagonist Gabazine (5 μ M). Treatment with Gabazine alone had no effect on the ratio of LDH release/Total LDH (CTL 100% \pm 15; SCI 230% \pm 19; SCI + Gz 283% \pm 42). No statistically significant difference was observed between SCI and SCI + Gz treated slices. However, the association of Gabazine with Allo, prevented the allopregnanolone protective effect as shown by the significant differences in the ratios of LDH release/Total LDH (SCI 230% \pm 19; SCI + Allo 115.9% \pm 18; SCI + Allo + Gz 278% \pm 48; $p < 0.05$ SCI versus SCI + ALLO and SCI + Allo versus SCI + Allo + Gz). As allopregnanolone can be converted back to 5 α -DHP, which then interacts with PR and activates gene transcription and regulates neuronal functions (Rupprecht et al., 1993, 1996) we checked whether there may be a contributory role of PR in mediating the effects of allopregnanolone. If the protective effects of allopregnanolone are mediated via its conversion to PR-active 5 α -DHP, they should no longer be observed in PR^{-/-} mice. To test this possibility, we use organotypic slices from PR^{-/-} mice. Fig. 5 shows that allopregnanolone is neuroprotective in PR^{-/-} mice. Indeed, allopregnanolone decreased the ratio of LDH release to Total LDH, and Gabazine inhibited the effect of allopregnanolone (Fig. 5A, $p < 0.01$ SCI + Allo versus SCI and $p < 0.01$ SCI + Allo + GZ versus SCI + Allo). The effects of allopregnanolone on cell death were also analyzed by counting the number of PI⁺ cells and NeuN⁺ cells in PR^{-/-} mice. Allopregnanolone decreased the number of PI⁺ cells and Gabazine inhibited the allopregnanolone effect (Fig. 5B, $p < 0.001$ SCI + Allo versus SCI and $p < 0.001$ SCI + Allo + GZ versus SCI + Allo). Gabazine inhibited also the increased number of NeuN⁺ cells induced by allopregnanolone (Fig. 5C–D, $p < 0.001$ SCI + Allo versus SCI and $p < 0.001$ SCI + Allo + GZ versus SCI + Allo).

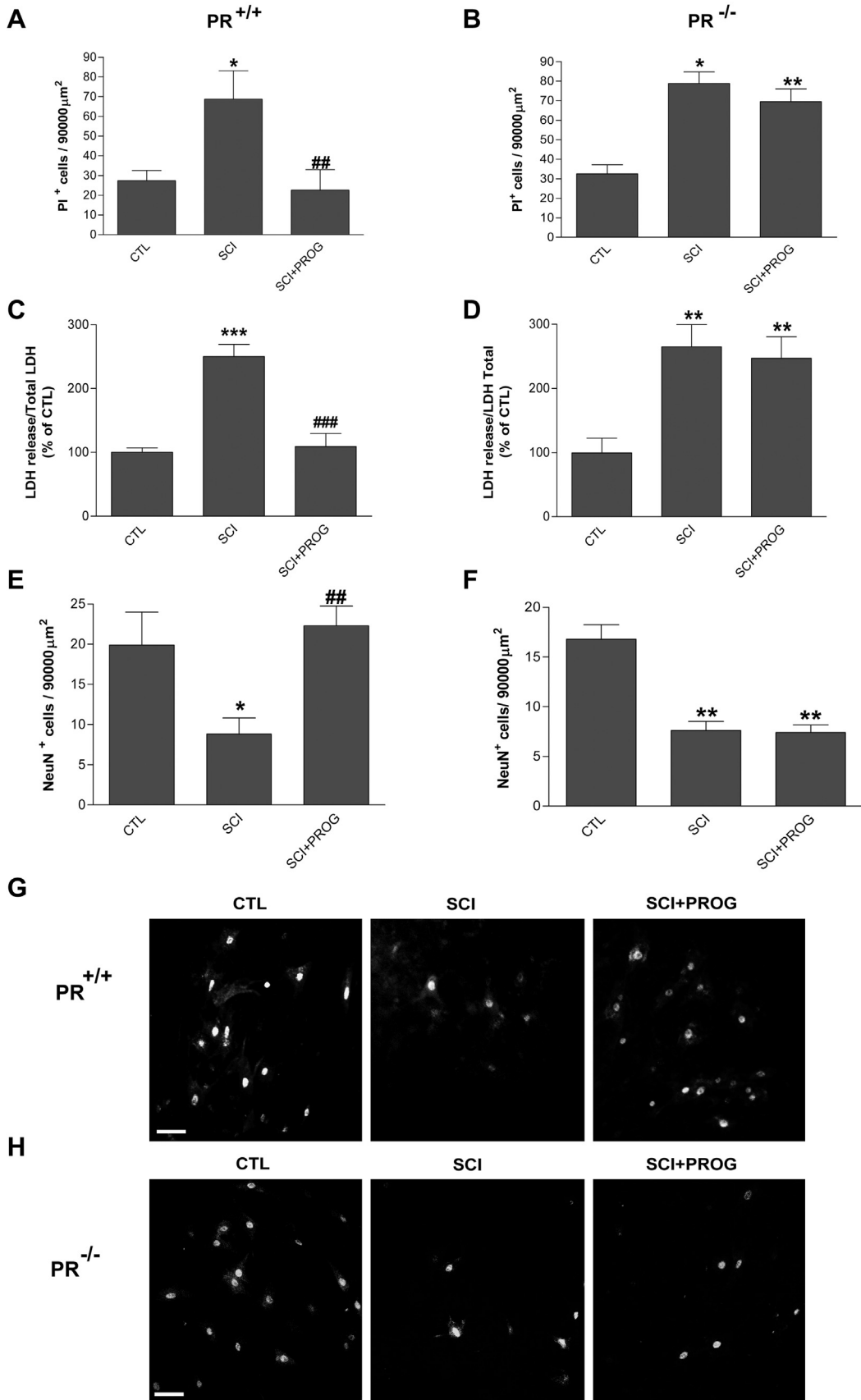
4. Discussion

We show in this study that treatment with progesterone or its derivatives, 5 α -DHP and allopregnanolone, protects against the loss of membrane integrity and against motoneuron death in injured organotypic spinal cord cultures from young mice. The neuroprotective effects of progesterone required PR and did not involve the bioconversion of progesterone to its 5 α -reduced metabolites within the spinal slices. The neuroprotective effect of allopregnanolone involved GABA_A receptors.

4.1. Advantages of the organotypic spinal cord slices cultures from young mice for studying neuroprotective mechanisms

Organotypic spinal cord cultures offer advantages over dissociated cultures in that they preserve the synaptic and anatomical organization of the neuronal circuitry and have functional characteristics similar to those found in vivo (Gahwiler et al., 1997). We examined the effects of an in vitro injury and steroid treatment on organotypic slices derived from young mice. We tested the

Fig. 4. Progesterone neuroprotective effect is PR dependent: Progesterone is neuroprotective in PR^{+/+} mice (A, C, E, G, A) Quantification of PI⁺ cells density in lamina IX. SCI induced an increase in the number of PI⁺ cells (* $p < 0.05$ SCI versus CTL), whereas PROG (10 μ M) prevented this increase (### $p < 0.01$ SCI + PROG versus SCI). C) LDH measurements. SCI induced an increase in LDH/Total LDH ratio (*** $p < 0.001$ SCI versus control), whereas PROG dumped this increase (### $p < 0.001$ SCI + PROG versus SCI). E) Quantification of NeuN⁺ cells density in lamina IX. SCI induced a decrease in NeuN⁺ cell density (* $p < 0.05$ SCI versus CTL) whereas PROG induced an increase in NeuN⁺ cell density (## $p < 0.01$ SCI + PROG versus SCI). G) Representative confocal microscopic views of NeuN immunofluorescence staining in lamina IX showing a decrease in NeuN⁺ cells density after SCI and an increase after PROG treatment. Scale bars: 20 μ m. Data represent means \pm s.e.m., $n = 6$ per group. Progesterone is not neuroprotective in knockout PR^{-/-} mice (B, D, F, H). B) Quantification of PI⁺ cells density in lamina IX. SCI induced an increase in the number of PI⁺ cells (* $p < 0.05$ SCI versus CTL) and PROG (10 μ M) did not prevent this increase (** $p < 0.01$ SCI + PROG versus CTL). D) SCI induced an increase in LDH/Total LDH ratio (** $p < 0.01$ SCI versus CTL), and PROG (10 μ M) treatment did not prevent this increase (** $p < 0.01$ SCI + PROG versus CTL). F) Quantification of NeuN⁺ cells density in lamina IX. SCI induced a decrease in NeuN⁺ cell density (** $p < 0.01$ SCI versus CTL) and PROG (10 μ M) did not prevent this decrease (** $p < 0.01$ SCI + PROG versus CTL). H) Representative confocal microscopic views of NeuN immunofluorescence staining in lamina IX showing a decrease in NeuN⁺ cells density after SCI. PROG (10 μ M) did not prevent this decrease. Scale bars: 20 μ m Data represent means \pm s.e.m., $n = 6$ per group.



feasibility of the cultures using mice of different ages between 3 and 10 postnatal weeks. 3 weeks old mice gave the best results. The *in vitro* injury model by weight-dropping technique presents a system for identification of major cellular mechanisms of SCI and is well suited to address the mechanisms by which progesterone and allopregnanolone bring neuroprotection. Thus, such a model system can be exploited to investigate the effects of different drugs that may be interesting for developing treatment strategies for SCI.

4.2. Neuroprotective effects of progesterone require the expression of the nuclear progesterone receptors (PR)

Progesterone has no effects in control spinal cords without injury, and was robustly neuroprotective after injury in this *in vitro* model, as it has been shown previously *in vivo* after spinal cord transection (De Nicola et al., 2009). In the injured spinal cord slice cultures, progesterone decreased LDH release, reflecting a decreased membrane damage and increased membrane integrity. Membrane damage due to the initial traumatic event can be responsible for the initiation of the delayed pathological events and cell death after traumatic injury. The loss of membrane integrity triggers many downstream events resulting in secondary cellular damage. Thus, cells that do not die acutely may nevertheless go on to enter apoptosis. Repairing the initial membrane damage caused by trauma not only rescues the cells from acute cellular death but could also block the secondary cascades leading to delayed cell death. Direct count of motoneurons showed that treatment by progesterone of the injured spinal cord cultures which had lost 60% of their motoneurons by SCI increased the number of large NeuN⁺ cells. These data demonstrated that the neuroprotective effect of progesterone reflects significantly enhanced motoneuron survival.

Progesterone may act through several mechanisms. In rat Purkinje cell cerebellar cultures progesterone has been shown to be protective against oxygen–glucose deprivation through potentiation of GABA_A receptor activity indirectly by its metabolites, such as allopregnanolone (Ardeshiri et al., 2006). Our data concerning the measurements of progesterone and its derivatives in both the spinal cord slices and the medium after treatment with progesterone and progesterone + finasteride had shown that large amounts of progesterone were achieved in the tissue (≈ 14 ng/mg) but progesterone was not bioconverted to 5 α -DHP nor to allopregnanolone demonstrating that the neuroprotective effects of progesterone in spinal cord cultures were not due to its bioconverted metabolites but rather to progesterone itself.

Progesterone can activate nuclear progesterone receptors or membrane progesterone receptors. Our results using knockout PR^{-/-} mice, clearly demonstrate that the nuclear progesterone receptors play a pivotal role in the mediation of the neuroprotective effects of progesterone at least concerning membrane damage and motoneurons survival. Our data support a relevant role of progesterone in neuroprotection independently from its bioconversion to allopregnanolone and point to the intracellular receptors PR as a target for pharmacological treatments after spinal cord injury. PR seem to be important mediators of neuroprotection after the nervous system injuries, indeed, we have recently shown that PR are direct key targets for both endogenous neuroprotection and for therapeutic strategies after stroke (Liu et al., 2012). However, in other models of neurodegeneration, progesterone metabolism participates to the neuroprotective effect of the hormone. Thus, in an *in vivo* experimental model of excitotoxic cell death, progesterone administration increased the levels of 5 α -DHP and allopregnanolone in plasma and hippocampus and prevented kainic-acid-induced neuronal loss. The administration of the 5 α -reductase inhibitor finasteride abolished the neuroprotective effect of progesterone suggesting that progesterone metabolism to its reduced derivatives is necessary for the

neuroprotective mechanism of progesterone against excitotoxic neuronal death (Ciriza et al., 2006). More recently, Ishihara et al. have shown that allopregnanolone converted from progesterone in hippocampal slices could protect neurones from tributyltin-induced neurotoxicity by a GABA_A receptor-dependent mechanism (Ishihara et al., 2012).

4.3. Neuroprotective effects of allopregnanolone involve the modulation of GABA_A receptors

Allopregnanolone has been shown to be neuroprotective in different animal models such as traumatic brain injury, ischemia, and neurodegenerative diseases (Djebaili et al., 2004; Sayeed et al., 2006; Sayeed and Stein, 2009; Griffin et al., 2004; Wang et al., 2010; Singh et al., 2012; Melcangi and Mensah-Nyagan, 2006; Liu and Diaz Brinton, 2011).

We show here for the first time that 5 α -DHP and allopregnanolone are neuroprotective in the injured spinal cord. At the dose tested, 5 α -DHP and allopregnanolone were as efficient as progesterone in decreasing membrane damage and preventing neuronal death, and they may thus represent potential therapeutic agents for treatments of SCI. Our results showed that allopregnanolone effects are mediated by GABA_A receptors, thus drugs targeting GABA_A receptors should also be evaluated for their neuroprotective effects in spinal cord injury. The treatment with GABA_A receptor modulators such as the neuroactive steroids may be useful for preventing neurodegeneration after spinal cord injury and may represent a novel neuroprotective strategy. Coordinated balance of inhibitory and excitatory neurotransmission, mediated respectively by GABA_A and glutamate receptors, controls neuronal communication for the normal nervous system function (Dingledine et al., 1999; Mehta and Ticku, 1999). However, after spinal cord injury there is a profound shift of the balance in the direction of overexcitation due to the excess release of excitatory amino acids and hypofunction of GABAergic tone (Gwak and Hulsebosch, 2011). Our data provide evidence suggesting that allopregnanolone by potentiating GABA_A receptors may lead to an anti-excitotoxic effect which protects neurons from death after spinal cord injury.

4.4. Translational perspectives/therapeutic options

Studies using progesterone are more advanced than those using allopregnanolone. Indeed, we have shown using an *in vivo* experimental spinal cord injury, that molecular markers of functional motoneurons become impaired, including brain-derived neurotrophic factor mRNA, Na, K-ATPase mRNA, microtubule-associated protein 2 and choline acetyltransferase. Progesterone treatment restores the expression of these molecules. Spinal cord injury also causes oligodendrocyte loss and demyelination. A short progesterone treatment enhances proliferation and differentiation of oligodendrocyte progenitors into mature myelin-producing cells, whereas prolonged treatment increases a transcription factor (Olig1) needed to repair the injury-induced demyelination (De Nicola et al., 2009). Similar data are still needed to screen the *in vivo* neuroprotective potency of allopregnanolone after spinal cord injury.

Progesterone is used in a number of clinical applications. Extensive studies have been conducted to evaluate its safety for clinical use (Goletiani et al., 2007). Progesterone is receiving much attention as a neuroprotective agent and is making its way into neurological practice. Indeed, two phase II trials have already assessed the safety and beneficial effects of progesterone after traumatic brain injury (Stein, 2013), and their encouraging outcomes have spurred the launching of two large phase III multicenter trials (Protect III, 2011, at <http://www.clinicaltrials.gov/>

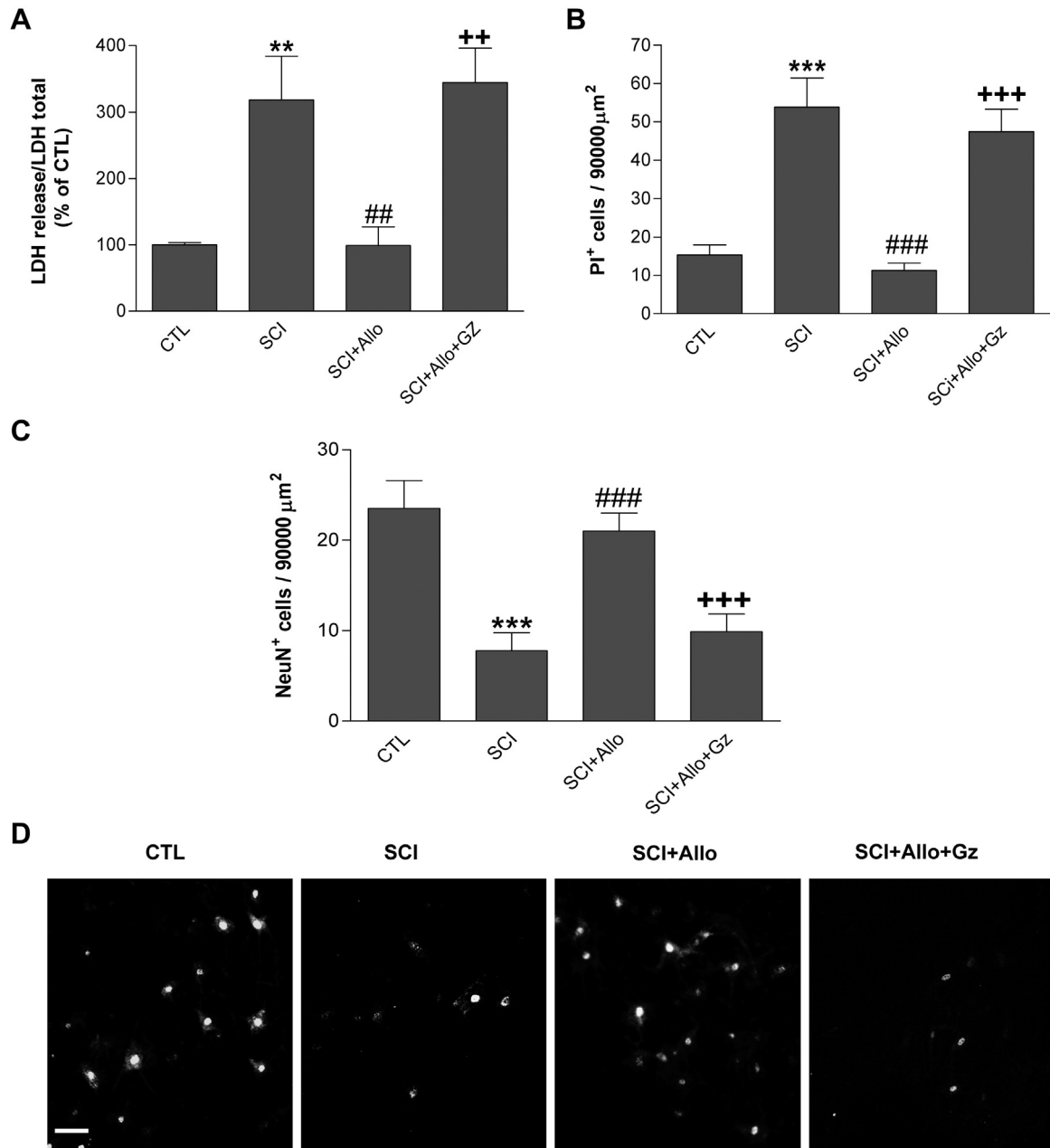


Fig. 5. Allopregnanolone is neuroprotective in $PR^{-/-}$ mice and its effects involve $GABA_A$ receptors. A) SCI increased the LDH/Total LDH ratio, allopregnanolone decreased it and Gabazine inhibited the effect of allopregnanolone. ** $p < 0.01$ vs CTL; ## $p < 0.01$ vs SCI; ++ $p < 0.01$ vs SCI + Allo. B) Quantification of PI⁺ cell density in lamina IX. SCI increased the PI⁺ cells density, allopregnanolone decreased it and Gabazine inhibited the effect of allopregnanolone. *** $p < 0.001$ vs CTL; ### $p < 0.001$ vs SCI; +++ $p < 0.001$ vs SCI + Allo. C) Quantification of NeuN⁺ cells density in lamina IX. SCI decreased NeuN⁺ cell density, allopregnanolone increased it and Gabazine inhibited the effect of allopregnanolone. *** $p < 0.001$ vs CTL; ### $p < 0.001$ vs SCI; +++ $p < 0.001$ vs SCI + Allo. D) Representative confocal microscopic views of NeuN immunofluorescence staining in lamina IX, showing decreased NeuN⁺ cells after SCI and increased NeuN⁺ cells following addition of allopregnanolone (SCI + Allo). This effect was blocked in the presence of Gabazine (SCI + Allo + Gz). Data represent means \pm s.e.m., $n = 6$ per group.

NCT00822900 and SynAPSe, 2011, at <http://www.synapse-trial.com>). The evaluation of safety and efficacy of allopregnanolone in humans is still lacking.

Multiple therapeutic approaches remain to be further explored for promoting spinal cord repair with progestagens. The administration of progesterone or allopregnanolone is an interesting option. Important issues should be addressed: 1 – the best formulations (natural, micronized natural and synthetic). 2 – the choice of the mode of administration which gives a great bioavailability, a long half-life and which allows these neuroactive steroids to reach the spinal cord prior to their metabolism by the

liver. 3 – dosing regimens. Stimulating the biosynthesis of endogenous progesterone and/or allopregnanolone in the spinal cord offers another therapeutic option which deserves particular attention.

Spinal cord injury is an extremely important issue for its devastating consequences and for the limited capacity of regeneration exhibited by the adult central nervous system. Finding and designing drugs that may help restoring the missing functions is a crucial objective of neuropharmacological research. For spinal cord injury, human studies are still lacking for both progesterone and allopregnanolone. Progesterone may be the pleiotropic candidate

drug that can markedly attenuate the complex injury cascade after spinal cord injury. Allopregnanolone may represent an alternative strategy which target specifically GABA_A receptors. A careful evaluation of the potential of these two neurosteroids as neuroprotective agent after spinal cord injury is needed.

In conclusion, our study shows that both steroids, progesterone and allopregnanolone, are neuroprotective after spinal cord injury. The intracellular progesterone receptors (PR) are key targets for neuroprotection by progesterone. In addition, the modulation of GABA_A receptors by the neuroactive steroid allopregnanolone might represent an alternative therapeutic approach. Therefore, molecules targeting progesterone receptors or modulating GABA_A receptors should be considered for the promotion of recovery after spinal cord injury.

Competing interests

FL, AG, PL, AFDN, MS and RG have nothing to disclose.

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