

HIGH-DOSE CORTICOSTEROIDS AFTER SPINAL CORD INJURY REDUCE NEURAL PROGENITOR CELL PROLIFERATION

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Abstract—We assessed whether a clinical dose of the anti-inflammatory drug methylprednisolone (MP) given to adult mice acutely after spinal cord injury (SCI) influences spinal cord or hippocampal progenitor cells. Mice underwent a thoracic dorsal hemisection of the spinal cord and received 30 mg/kg MP immediately and 24 h post-lesion. 5-Bromo-2-deoxyuridine (BrdU) was administered after lesion either acutely (1–6 days) or late (22–27 days) to label proliferating cells. Reaction of microglia/macrophages was quantified 7 days post-lesion and proliferation as well as differentiation of neural progenitor cells (NPCs) was analyzed after two survival times (7 days and 28 days). We also tested the influence of MP on microglia and adult NPCs *in vitro*. MP treatment reduced the number of cells proliferating acutely after SCI in the spinal cord and hippocampus. Besides reducing activation and proliferation of microglia/macrophages in the spinal cord, MP also decreased the number of oligodendrocyte progenitor cells (OPCs). Analysis of acutely BrdU-labeled cells at 28 days post-lesion suggests that proliferation and number of OPCs were changed chronically. Late proliferating cells were no longer influenced by the glucocorticoid regimen. *In vitro* experiments showed an inhibitory effect of MP on adult spinal cord and hippocampal progenitor cell proliferation. Both cell types express the glucocorticoid and mineralocorticoid receptors allowing a direct effect of MP. Our results show that MP reduces OPC proliferation after SCI either by affecting progenitor cells directly or via its anti-inflammatory effects. These findings open the question to which extent MP treatment limits the repair capacity of endogenous progenitor cells after CNS injury. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: methylprednisolone, spinal cord progenitor cells, oligodendrocyte progenitor cells, inflammation, microglia, NG2.

Neural progenitor cells (NPCs) are present in the entire CNS. NPCs are multipotent cells able to differentiate into neurons, astrocytes and oligodendrocytes. Whereas the

hippocampal dentate gyrus and the subventricular zone of the forebrain are considered neurogenic regions, the adult spinal cord harbors progenitor cells that *in vivo* give rise to glial cells (Horner et al., 2000). In the injured spinal cord NPCs show increased proliferation, and are believed to contribute to the repair processes (Horky et al., 2006; Lytle and Wrathall, 2007).

Injury to the spinal cord has devastating consequences. The primary injury disrupts ascending and descending pathways, and results in local ischemia, edema formation and inflammation. This complex cascade of events leads to secondary damage including neuronal and glial cell death and demyelination of spared fibers (Schwab and Bartholdi, 1996). Proliferation of oligodendrocyte progenitor cells (OPCs) and remyelination in the weeks after spinal cord injury (SCI) takes place to some extent partially restoring conduction velocity (Ishii et al., 2001; McTigue et al., 2001; Rabchevsky et al., 2007). Many experimental and clinical treatment strategies aim at intervening in the acute phase after SCI to limit secondary tissue damage. Acute clinical treatments of spinal cord-injured patients involve the use of methylprednisolone (MP) given within the first few hours up to 24 h after injury. MP is clinically used in a mega-dose as an anti-inflammatory agent and radical scavenger to minimize the extent of secondary injury and neuronal damage (Hall and Springer, 2004). However, the experimental (Behrmann et al., 1994; Rabchevsky et al., 2002) as well as the clinical data (George et al., 1995; Hurlbert, 2000; Sayer et al., 2006) using MP after SCI remain largely inconclusive and controversial with regard to improved functional outcome. As a glucocorticoid, MP has several immunosuppressive effects such as reducing macrophage-microglial proliferation and decreasing cytokine secretion (Saunders et al., 1987; Hall and Springer, 2004). Besides, MP may also act directly and indirectly on adult NPCs (Yan et al., 1999; Garcia et al., 2004). It is well established that corticosteroids decrease the proliferation of adult progenitor cells in the hippocampal dentate gyrus (Gould et al., 1992; Cameron and Gould, 1994) as well as the proliferation of NG2+ OPCs in the gray and white matter of the brain (Alonso, 2000).

However, little is known about the effects of corticosteroids on cells in other CNS regions, such as the spinal cord. Rather conflicting data have been published on their effects on remyelination after spinal cord demyelinating lesions (Triarhou and Herndon, 1986; Pavelko et al., 1998). Interestingly, a recent paper (Chari et al., 2006) shows delayed oligodendrocyte-mediated repair after toxin-induced demyelination lesions in the adult rat spinal cord attributed to the inhibition of OPC differentiation by corticosteroid treat-

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Abbreviations: BrdU, 5-bromo-2-deoxyuridine; GM, gray matter; GR, glucocorticoid receptor; LPS, lipopolysaccharide; IWM, lateral white matter; MP, methylprednisolone; MR, mineralocorticoid receptor; NPC, neural progenitor cell; OPC, oligodendrocyte progenitor cell; PFA, paraformaldehyde; SCI, spinal cord injury; TUNEL, dUTP-nick end labeling; vWM, ventral white matter.

ment. In our study we assessed the effects of a treatment with the anti-inflammatory drug MP on spinal cord as well as on hippocampal progenitor and on microglial cells *in vitro* and *in vivo* following SCI in adult mice. We treated isolated adult neural progenitor and microglial cells with different concentrations of MP and found dose-dependent effects on proliferation and cell death. Spinal cord-injured mice received a clinical high-dose MP treatment and were injected with 5-bromo-2-deoxyuridine (BrdU), a thymidine analogue, either acutely (1st week) or late (4th week) after lesion to assess proliferation and to label progenitor cells for lineage tracing. We found changes in the proliferation of not only spinal cord OPCs but also hippocampal NPCs acutely after lesion and MP treatment. Our results suggest that treatment with MP after SCI reduces microglia/macrophage activation and may directly or indirectly limit the repair capacity of endogenous spinal cord progenitor cells.

EXPERIMENTAL PROCEDURES

Animals and SCI

All experiments were performed with adult female C57BL/6 mice (2 months; Harlan, Boxmeer, The Netherlands) and were conducted in compliance with the Principles of Laboratory Animal Care (National Institutes of Health Publication no. 86-23, revised 1985) and were approved by the veterinary department of the canton of Zurich. Mice were deeply anesthetized and a dorsal hemisection was performed at the level T8/9 of the spinal cord as described previously (Dimou et al., 2006).

MP and BrdU treatment *in vivo*

Animals of the MP treatment group ($n=6$) received an i.p. injection of MP (Solu-Medrol, 30 mg/kg; Pfizer AG, Zurich, Switzerland) immediately and 24 h after SCI. Lesioned control mice ($n=6$) were injected with 0.9% NaCl. BrdU (50 mg/kg; Sigma-Aldrich, Buchs, Switzerland) was injected intraperitoneally twice daily either from days 1–6 (acute BrdU) or from days 22–27 (late BrdU) post-lesion. Mice were perfused 1 day (acute BrdU—acute group, Fig. 1A; late BrdU—chronic group, Fig. 1C) or 3 weeks after the last BrdU injection (acute BrdU—chronic group; Fig. 1B). An intact control group of mice ($n=3$) underwent the same MP/BrdU protocol.

Tissue collection and immunohistochemistry

One or 4 weeks after injury, mice were anesthetized and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains and spinal cords were removed and postfixed overnight at 4 °C followed by cryopreservation in 30% sucrose in PB. Spinal cord and brain tissue was cut into 30 μm thick cross-sections.

All immunohistochemical stainings were performed as described previously (Thallmair et al., 2006). For double-labeling, we combined lineage-specific markers with anti-BrdU staining. Primary antibodies were rat anti-BrdU IgG antibody (1:500, Biozol, Eching, Germany) for proliferating cells, rabbit anti-Iba1 (1:1000, Wako, Japan) for microglia/macrophages, rabbit anti-GFAP (1:2000, Dako, Glostrup, Denmark) for astrocytes and rabbit anti-NG2 (1:200, Chemicon (Millipore), Billerica, MA, USA) for OPCs. Secondary antibodies (each 1:500; Jackson ImmunoResearch Europe Ltd., Suffolk, UK): biotin-conjugated donkey anti-rat antibody followed by incubation with streptavidin-DTAF (1:500) and Cy3-conjugated donkey anti-rabbit antibody. Controls omitting the primary antibodies were performed alongside all immunostaining procedures. All stainings were optimized for cell-specific labeling and to reduce background such as non-specific staining.

Quantification of *in vivo* experiments

We quantified the immunostainings in the medial gray matter (GM), lateral white matter (IWM) and ventral white matter (vWM), 4 and 2 mm rostral (r) and caudal (c) from the lesion site (Fig. 1D). These regions were chosen since they were not affected directly by the mechanical lesion, however, inflammatory cells were present in these areas at all distances analyzed. For semi-quantitative measurements of the Iba1—as well as GFAP—density within the spinal cord sections, fluorescent images were taken at 5 \times using a fluorescence microscope (Axioskop 2 plus; AxioVision software, Carl Zeiss AG, Feldbach, Switzerland) and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). The density of Iba1 staining was measured in GM, IWM and vWM 4 and 2 mm rostral (r) and caudal (c) from the lesion site (Fig. 1D). For each distance three sections per animal were analyzed. The mean of the values of each area at a specific distance was set to 1 for lesioned control mice. All other values were calculated as the ratio according to the corresponding mean of the control animals.

Quantification of BrdU+ cells that were co-immunostained with lineage-specific markers was performed at a confocal microscope (Leica DMRE; Leica GmbH, Wetzlar, Germany) under 40 \times magnification in association with the Imaris 5.0.1 software (Bitplane AG, Zurich, Switzerland). We analyzed cells for double-labeling in the GM, IWM and vWM (Fig. 1D). For each distance three sections per animal were analyzed. For image analysis, z-stacks were imported to Imaris, 3D-reconstructions were done and a square area of 120 μm side length was quantified.

Photomicrographs of BrdU+ cells in the spinal cord and hippocampus at different time points were taken at 5 \times and 63 \times and quantification in the hippocampal dentate gyrus was performed under 40 \times magnification using a fluorescence microscope (Axioskop 2 plus; AxioVision software, Zeiss). NeuroLucida system (MicroBrightField, Colchester, VT, USA) was used for measurement of the analyzed hippocampal area.

Isolation, MP and BrdU treatment of murine microglial cells and NPCs *in vitro*

Murine microglial cells were isolated from primary mixed glial cell cultures prepared from newborn C57BL/6 mouse cerebella as described previously (Song et al., 2002). Microglial cells were activated by addition of lipopolysaccharide (LPS; 1 mg/ml) to the medium overnight. MP (0.5, 1, 5, 10 ng/ml) was added to the medium and replaced with each medium change. After 5 days, BrdU (5 μM) was added to the medium overnight and cells were fixed with 4% PFA the next day and immunostained.

Adult mouse hippocampi and spinal cords ($n=20$) were freshly dissected and NPCs were isolated as described previously (Thallmair et al., 2006). Briefly, mouse hippocampi and spinal cords were minced with scalpels and digested in a papain (Worthington Biochemical Corp., Lakewood, NJ, USA), DNase I (Worthington), dispase II (Roche, Indianapolis, IN, USA) mixture in Ham's/DMEM F12 (Invitrogen) medium at 37 °C. Digestion was stopped by washing with Ham's/DMEM F12 + 10% fetal bovine serum (FBS). A Percoll density gradient (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was used to enrich for progenitor cells (Ray and Gage, 2006). The myelin debris at the top of the gradient was aspirated and 2/3 of the fraction between the debris and the red blood cell layer was collected. Cells were plated in Ham's/DMEM F12 containing 2 mM glutamine (Invitrogen), N2 supplement (Invitrogen), FGF-2 (20 ng/ml; Pepro Tech, Rocky Hill, NJ, USA), EGF (20 ng/ml; Pepro Tech) and heparin (5 $\mu\text{g}/\text{ml}$; Sigma) on plastic tissue culture dishes. Cells were maintained at 37 °C in 5% CO₂, 3% O₂ and half of the medium was changed every third day. Cultured hippocampal or spinal cord progenitor cells were plated on four well chamber slides (15,000 cells/well) and MP (0.5, 1, 5 ng/ml) was added to the proliferation medium

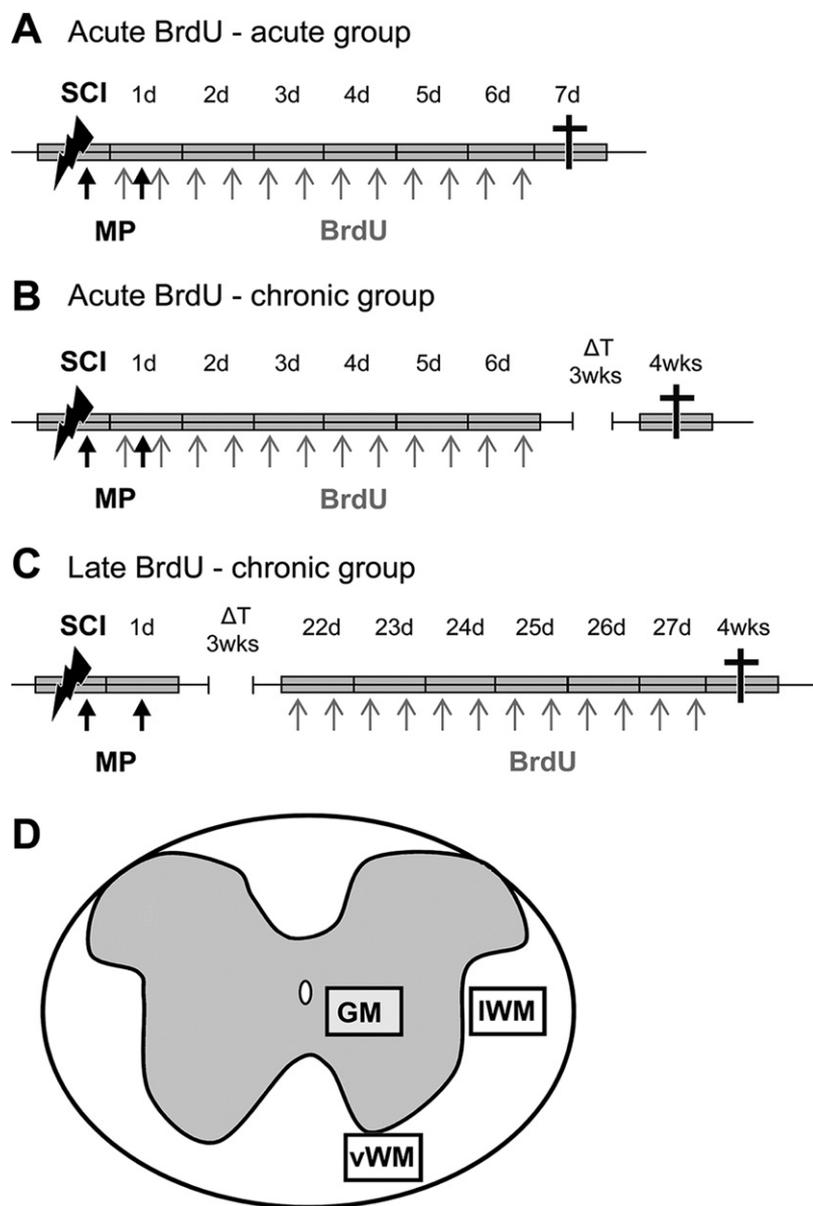


Fig. 1. Experimental design. (A–C) Mice were injected with MP (30 mg/kg) or vehicle (saline) immediately and 24 h after SCI. All mice received an i.p. BrdU injection twice daily for 6 consecutive days either immediately after lesion to label acutely proliferating cells (acute BrdU; A, B) or during the 4th week (late BrdU; C). Mice were sacrificed either 1 day (acute BrdU–acute group, A; late BrdU–chronic group, C) or 3 weeks (acute BrdU–chronic group, B) after the last BrdU injection to evaluate cell proliferation, survival and differentiation. (D) Schematic drawing of a mouse spinal cord cross section. Cell countings were performed using analyses squares in the medial GM, IWM and vWM, 4 and 2 mm rostral (r) and caudal (c) from the lesion site.

(DMEM/F12, N2, FGF2, EGF, heparin). Fresh MP was added after 24 h. One day later, BrdU (5 μ M) was added to the medium overnight and cells were fixed with 4% PFA the next day and immunostained.

Immunocytochemistry and quantification of *in vitro* experiments

Immunocytochemical staining was performed as described previously (Thallmair et al., 2006). Primary antibody was rat anti-BrdU IgG antibody (1:500, Biozol) for proliferating cells. Secondary antibody was biotin-conjugated donkey anti-rat antibody (1:500; Jackson ImmunoResearch) followed by incubation with streptavidin-DTAF (1:500). Staining for apoptotic cell death was performed

using dUTP-nick end labeling (TUNEL, *in situ* cell death detection kit, Roche, Mannheim, Germany) according to manufacturer's instructions.

All fluorescent cell stainings were analyzed using a fluorescence microscope (Axioskop 2 plus; AxioVision software, Zeiss). For quantification, four to six fields in each chamber of the chamber slide were randomly selected and at least 200 DAPI+ cells were analyzed for their BrdU-labeling. The morphological analysis of microglia based on the following characteristics (Streit and Kreutzberg, 1988; Zhang et al., 1997): Cells with a small cell body and long, branched processes were identified as ramified microglia, whereas cells with retracted processes and enlarged cell bodies were determined as activated microglia.

qRT-PCR

Two-step quantitative RT-PCR was performed with Applied Biosystems (AB, Foster City, CA, USA) 7500 Real Time System and AB SYBR green PCR master mix. The following primer sequences were used: glucocorticoid receptor (GR) (Nr3c1) sense primer (SP) CCTGGTGTGCTCCGATGAAGCT, antisense primer (ASP) GGTAATTGTGCTGCCTTCCACTGC, mineralocorticoid receptor (MR) (Nr3c2) SP GTGTGGAGATGAGGCTTCTGGGTG, ASP AGTTGTGTTGCTTCCACGGCTC. Nr3c1 and Nr3c2 expression was normalized to elongation factor 1, TATA box binding protein and tubulin- β expression.

Statistical analysis

All data are shown as mean values \pm standard error of the mean (SEM). Graphs were generated using GraphPad Prism (GraphPad Software Inc., USA). Statistical analyses were performed using analysis of variance tests (ANOVA) of the appropriate design (one-way, two-way; SPSS, Chicago, IL, USA). Significant differences were assumed at a level of $P < 0.05$.

RESULTS

MP treatment reduces the amount of activated microglia/macrophages after SCI

It was shown previously that MP reduces the inflammatory response after SCI and that microglial cells express the GR and MR (Bartholdi and Schwab, 1995; Tanaka et al., 1997). Thus, we assessed the activation state of microglia after MP or control treatment *in vitro* and *in vivo*. Cultures of LPS-activated microglial cells were treated with various concentrations of MP (0.5, 1, 5, 10 ng/ml) and analyzed for proliferation and activation. With increasing MP concentrations the amount of proliferative microglia was reduced as shown by a decreased BrdU incorporation (Fig. 2A; 1 ng/ml, $P = 0.053$; 5 ng/ml, $P = 0.002$; 10 ng/ml, $P = 0.004$). Quantification of the activation state—characterized by cell morphology—showed a clear dose-dependent effect of MP on microglial cells. A low dose of MP (1 ng/ml) showed a 13% reduction of activated microglial cells compared to control cultures, whereas the highest MP dose (10 ng/ml) reduced activated microglia by 72% (1 ng/ml, $P = 0.004$; 5 ng/ml, $P = 0.025$; 10 ng/ml, $P < 0.0001$; Fig. 2B). While the percentage of activated microglia decreased, the number of ramified microglia increased in a concentration-dependent manner when treated with MP (Fig. 2B). Higher concentrations of MP (5 and 10 ng/ml) induced apoptotic cell death (Fig. 2B).

To test whether MP also reduces microglia/macrophage activation *in vivo* after SCI a clinically relevant high-dose of MP (30 mg/kg) was given to lesioned mice twice, immediately and 24 h after SCI, and the microglia/macrophage response was quantified at 7 days post-lesion (Popovich et al., 1997) using Iba1 immunohistochemistry and density measurements. In all regions analyzed (GM, IWM, vWM) MP reduced the Iba1 staining density by 25%–50% (Fig. 2C, D) and showed a significant effect of treatment ($P < 0.001$). These findings are in agreement with our *in vitro* results and corroborate previous publications (Bartholdi and Schwab, 1995; Taoka et al., 2001) demonstrating an anti-inflammatory effect of MP in the injured spinal cord.

Next, we assessed whether the reduced microglia/macrophage response as represented by the decreased Iba1 density *in vivo* after SCI and MP treatment is related to diminished cell proliferation. We injected MP-treated and control mice with BrdU over 6 days after the injury and quantified BrdU+ cells at 7 days post-lesion (acute BrdU–acute group; Fig. 1A). The analysis showed that MP treatment led to a 30% reduction of BrdU-labeled cells in the medial GM, IWM and vWM, 4 and 2 mm rostral and caudal from the lesion site (Fig. 3A, B). We found regional differences in the amount of proliferation with the IWM showing the highest amount of BrdU+ cells (Fig. 3B). This difference may be related to the extent of damage and Wallerian degeneration due to the dorsal hemisection. Thus, in the figures we show the results obtained in the IWM. Using double-immunofluorescence and confocal microscopy we assessed BrdU+ cells for co-labeling with the microglia/macrophage marker Iba1. The percentage of proliferating microglia/macrophages (BrdU+/Iba1+) representing 40%–50% of all BrdU+ cells was not changed by acute MP treatment compared to control treatment in any of the regions assessed (Fig. 3C). Thus, the reduced Iba1 density correlates with a decrease in microglia/macrophage proliferation.

MP treatment affects adult NPCs *in vitro* and after SCI

Since only approximately half of the BrdU+ cells could be attributed to the microglia/macrophage lineage, we assumed that other proliferative cells in the spinal cord, e.g. NPCs, were influenced by MP. We performed immunostainings for oligodendroglial progenitor cells (NG2) and astrocytes (GFAP). The latter have also been suggested to represent neural stem cells in injury models (Lang et al., 2004; Buffo et al., 2008). MP reduced the number of proliferating NG2+ cells in all regions analyzed (GM, IWM, vWM) by 20%–40% (Fig. 3D). In contrast, we did not detect a significant change in the BrdU+/GFAP+ cell population (Fig. 3E) or the astrocytic response as assessed by GFAP densitometry (data not shown).

Adult NPCs may be influenced by MP directly or indirectly via MP's effect on inflammation, or by a combination of both. Since it has not been known until now whether spinal cord progenitor cells express receptors for corticosteroids, we assessed isolated adult spinal cord progenitor cells for the presence of GR and MR by immunoblots (data not shown) and qRT-PCR. Hippocampal NPCs served as a positive control (Garcia et al., 2004). As shown in Fig. 4A both receptors are expressed in adult spinal cord progenitor cells suggesting that MP could act directly on NPCs.

To study a possible direct effect of MP on NPCs in more detail, adult spinal cord and hippocampal progenitor cells were cultured in the presence of different concentrations of MP (0.5, 1, 5 ng/ml) and BrdU incorporation was assessed. Hippocampal progenitor cells served as a control in this assay since it was shown that GR agonists can affect their proliferation directly (Yu et al., 2004). The *in vitro* experiments revealed that MP reduces the proliferation of adult mouse spinal cord progenitor cells, although to a much lesser extent than that of hippocampal progenitor cells (Fig. 4B). Whereas the proliferation of hippocampal

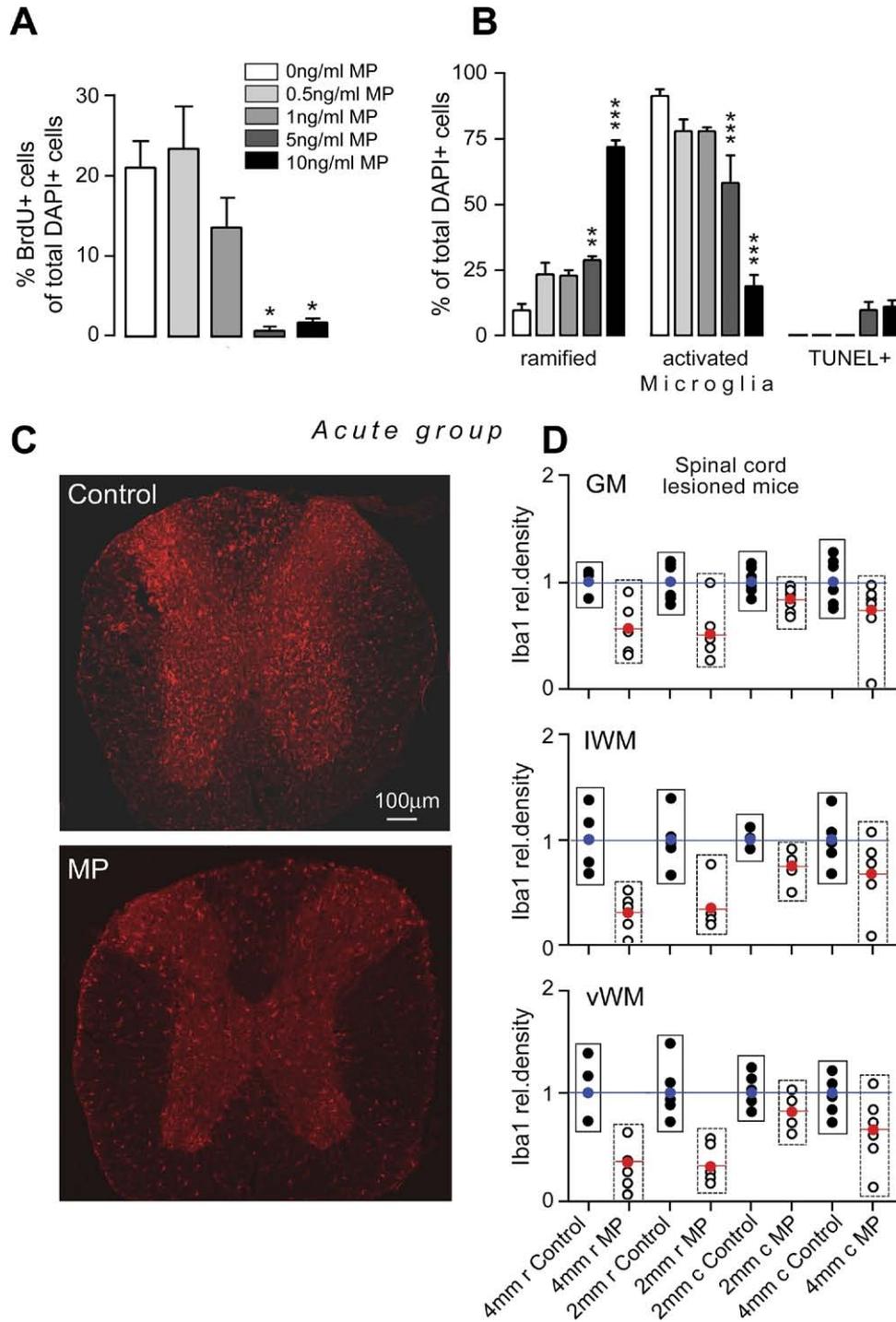


Fig. 2. MP treatment decreases proliferation, activation and in high doses survival of microglia *in vitro* and has an anti-inflammatory effect in the spinal cord at 7 days after SCI. (A, B) *In vitro* assays studying the effect of MP on microglia. (A) MP reduces the number of BrdU+ microglial cells in a concentration-dependent manner and (B) increases the number of ramified, resting microglia while decreasing the amount of activated microglial cells. High doses of MP enhance the number of TUNEL+ microglial cells. (C) Spinal cord cross-sections (2 mm rostral from the lesion site) 7 days after lesion stained for microglia/macrophages (Iba1). Control mice show a higher Iba1+ cell density than MP-treated. (D) Iba1 staining is reduced in MP-treated mice in all regions analyzed (see Fig. 1D and Exp. Procedures). Open circles represent individual animals as relative values compared to the control animals (black circles, set to 1, blue line). The red circle and line represent the average density for the MP-treated animals (open circles) at the given distance. Bars represent mean±SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to 0 ng/ml MP.

progenitor cells was significantly decreased in a dose-dependent manner showing a reduction by 40% with the

lowest and 75% with the highest MP concentration, the proliferation of spinal cord progenitor cells was significantly

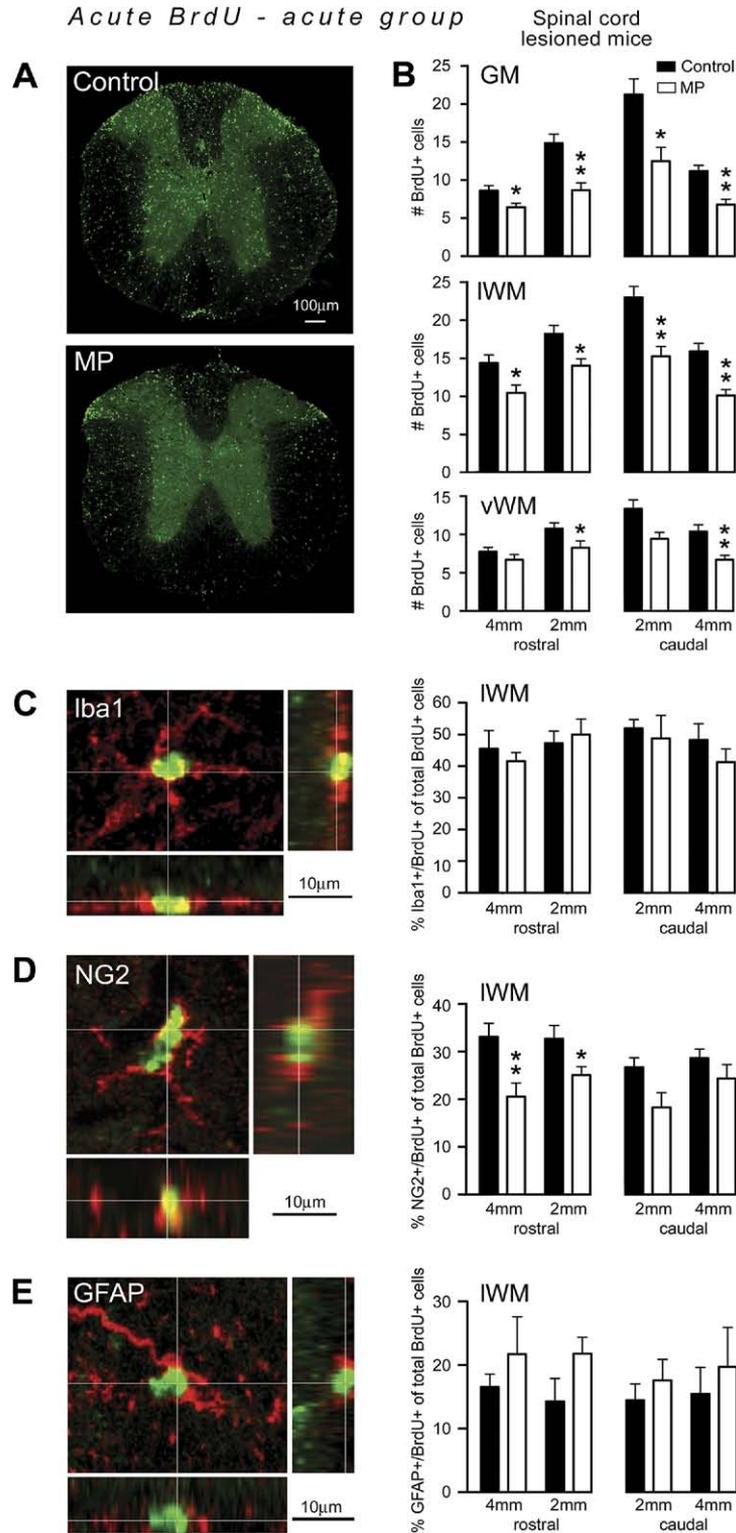


Fig. 3. MP treatment decreases proliferation of acutely BrdU-labeled inflammatory and non-inflammatory cells in the spinal cord at 7 days after SCI (acute BrdU-acute group). (A) Spinal cord cross-sections (2 mm rostral from the lesion site) 7 days after lesion stained for proliferating cells that were labeled with BrdU within the 1st week after lesion. Control mice show a higher BrdU+ cell density than MP-treated mice. (B) The number of BrdU+ cells is significantly reduced in MP-treated mice in all regions analyzed (see Fig. 1D and Exp. Procedures). (C) Confocal image stack of a BrdU (green) and Iba1 (red) double-stained cell and the percentage of BrdU+/Iba1+ cells within the BrdU+ cell population. The proportion of proliferating microglia/macrophages ranges from 40% to 50% and is not dependent on area, distance to the lesions site or treatment. (D) Confocal image stack of a BrdU (green) and NG2 (red) double-stained cell and the percentage of BrdU+/NG2+ cells within the BrdU+ cell population. MP treatment leads to a reduced proportion of BrdU+/NG2+ cells. (E) Confocal image stack of a BrdU (green) and GFAP (red) double-stained cell and the percentage of BrdU+/GFAP+ cells within the BrdU+ cell population. The proportion of proliferating GFAP+ cells is not significantly changed by MP treatment. Bars represent mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

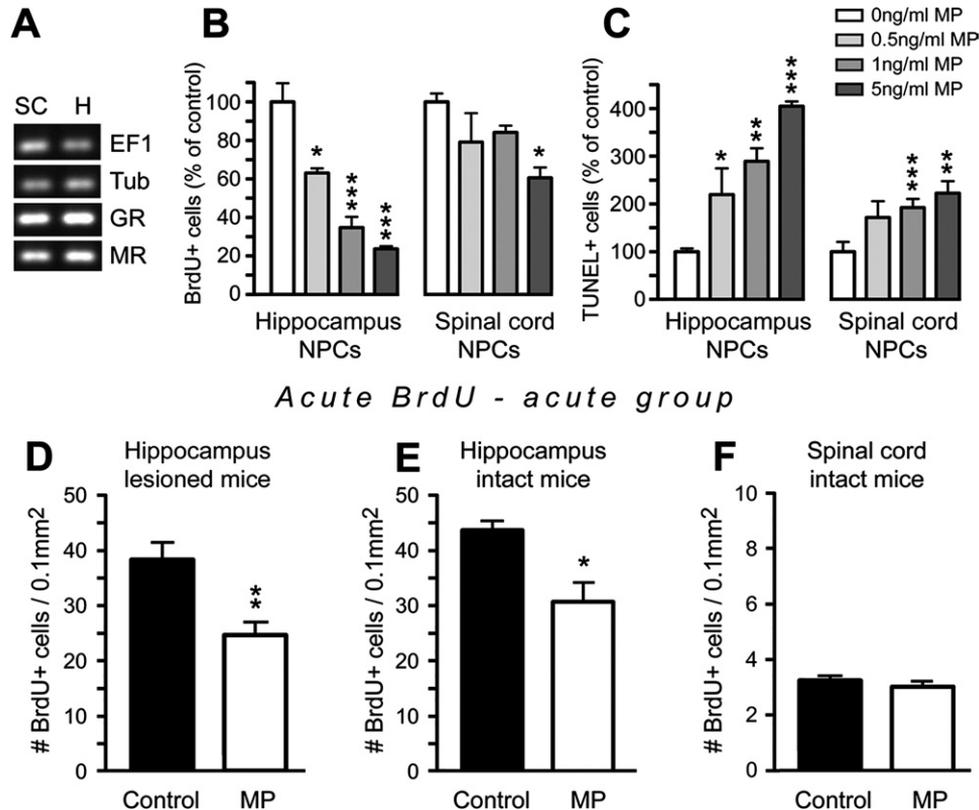


Fig. 4. MP treatment has a direct anti-proliferative effect on spinal cord and hippocampal progenitor cells mediated by the expression of the glucocorticoid and the mineralocorticoid receptors. (A) RT-PCR demonstrates that spinal cord progenitor cells (SC) express the GR and the MR. Elongation factor (EF) and tubulin (Tub) expression was used to normalize the data. Hippocampal progenitor cells (H) served as a positive control. (B, C) Direct effect of MP on adult spinal cord and hippocampal progenitor cells *in vitro*. (B) MP reduces the number of BrdU+ spinal cord and hippocampal progenitor cells in a concentration-dependent manner, however, hippocampal progenitor cells are more sensitive to MP. (C) High doses of MP increase the number of TUNEL+ progenitor cells. (D, E) Direct effect of MP on hippocampal progenitor cells in lesioned and intact mice. The number of BrdU+ cells in the hippocampal dentate gyrus of lesioned (D) and intact (E) MP-treated mice is significantly reduced at 7 days after SCI and MP treatment or MP treatment alone. (F) In contrast, MP does not have a significant effect on spinal cord progenitor cells in intact mice at 7 days after MP treatment. Bars represent mean \pm SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

decreased (40%) only by the highest MP dose (Fig. 4B). Interestingly, TUNEL staining revealed that MP also increases apoptosis in cultured adult NPCs (Fig. 4C). Again, hippocampal progenitor cells were more sensitive to MP than spinal cord progenitor cells: 5 ng/ml MP induced a fourfold increase of TUNEL+ hippocampal progenitor cells, whereas this MP dose led only to a twofold increase of TUNEL+ spinal cord progenitor cells. TUNEL staining of injured spinal cord tissue 1 week after SCI, however, did not reveal any differences in TUNEL+ cells in any of the regions analyzed. Also, MP did not change the differentiation of adult NPCs *in vitro* (data not shown).

To study the consequences of MP treatment on NPCs independent of its effects on inflammation *in vivo*, we analyzed NPC proliferation in the hippocampal dentate gyrus, a region that is not affected by SCI, in the same group of lesioned mice. In addition, we assessed BrdU+ cells in the spinal cord and hippocampus of intact animals.

Analysis of the hippocampal dentate gyrus in the lesioned mice showed that the number of BrdU+ cells was significantly reduced in MP-treated mice at 7 days post-lesion (Fig. 4D; $P = 0.005$) compared to control mice. Similarly, the

amount of BrdU+ cells was decreased in the hippocampal dentate gyrus of intact animals that had undergone the same MP and BrdU regimen (Fig. 4E; $P = 0.029$), indicating also that NPC proliferation in the hippocampus is not changed by SCI. Proliferating cells in the spinal cord of intact MP-treated mice were rare, and their number did not significantly differ from control mice (Fig. 4F; $P = 2.75$).

These results show that MP can directly regulate adult NPC proliferation, and that progenitor cells derived from different CNS regions do not react to the same extent to MP.

MP treatment affects the number of acutely proliferating OPCs after SCI chronically while it does not affect late proliferating OPCs

Our next question was whether the clinically relevant treatment with MP following SCI has long-term effects on adult NPCs. In chronic animals BrdU was injected over 6 days in the 1st week (acute BrdU–chronic group; Fig. 1B) or in the 4th week (late BrdU–chronic group; Fig. 1C) post-lesion. All animals received MP or saline acutely after injury and were analyzed 4 weeks after injury.

When acutely proliferating cells in the spinal cord were BrdU-labeled and analyzed 4 weeks post-lesion in MP-treated or control mice (acute BrdU–chronic group) we found no difference in terms of BrdU+ cells (Fig. 5A, B). The number of BrdU+/NG2+ cells was reduced in the control group, but the percentage of BrdU+/NG2+ in relation to the total BrdU+ cell number was not significantly changed (Fig. 5C). It was obvious, however, that cell nuclei showed a weaker and more punctate BrdU staining in the acute BrdU–chronic group compared to the other groups in which the survival time after BrdU application was shorter. Longer and higher proliferation activity leads to a faster dilution of the BrdU signal (Fig. 5D). When comparing the BrdU signal of hippocampal NPCs that had acutely proliferated after SCI 4 weeks post-lesion in MP-treated and control mice we found that the BrdU staining was weaker in the control group. Accordingly, the quantification of BrdU-labeled cells in the hippocampal dentate gyrus of lesioned MP-treated and control mice showed that saline-treated animals had fewer hippocampal BrdU+ cells (Fig. 5E; $P=0.009$). This observation indicates that the BrdU signal may have been more diluted in cells of control mice, presumably due to higher cell proliferation, and therefore may have been below the detection level used for quantification.

When we applied BrdU in the 4th week (days 22–27) after SCI to label proliferative cells late after lesion (late BrdU–chronic group) we did not detect any differences in the number (Fig. 5F) or fate choice (data not shown) of BrdU+ cells in the spinal cord of MP-treated or control mice. Similarly, the number of BrdU+ cells in the hippocampal dentate gyrus was not changed in the late BrdU–chronic group (Fig. 5G) by MP treatment after lesion.

These results indicate that MP treatment after SCI leads to transient impairment of progenitor cell proliferation.

DISCUSSION

In the present study the effects of the anti-inflammatory synthetic glucocorticoid MP on proliferative cells of the lesioned mouse spinal cord, as well as of the intact spinal cord and hippocampus were investigated. MP is widely used in acute care management of spinal cord–injured patients, although its beneficial effects are debated (Behrman et al., 1994; Taoka et al., 2001). Our data show that microglia/macrophages are less activated, less proliferative and may undergo apoptosis after MP treatment. These observations corroborate earlier findings (Oudega et al., 1999; Chan et al., 2003). We also observed that adult mouse spinal cord as well as hippocampal progenitor cells, were affected by the MP treatment *in vitro*. Proliferation was reduced and a tendency for increased cell death when treated with high concentrations of MP in culture was observed. Our *in vivo* data parallel the *in vitro* findings by revealing a 50% decrease in acutely proliferating cells after SCI and MP application, however, we did not detect an increased cell death *in vivo*. Analysis of proliferative NG2+ cells early after injury demonstrated a reduced number of

OPCs. BrdU incorporation was changed—even in regions not affected by the spinal cord lesion and subsequent inflammation, such as the hippocampus—suggesting that MP acts, at least partially, directly on progenitor cell proliferation. In the acute group we found a decline of BrdU+ cells in the hippocampal dentate gyrus of lesioned and intact animals corroborating earlier findings showing that glucocorticoids decrease the proliferation of hippocampal progenitor cells (Alonso, 2000; Wong and Herbert, 2006). The presence of GRs on progenitor cells was described for adult mouse hippocampal progenitor cells (Garcia et al., 2004). Our study suggests that adult mouse spinal cord progenitor cells may belong to the group of NPCs that can be directly influenced by glucocorticoids, since we found both receptors (GR and MR) to be expressed *in vitro*. Interestingly, spinal cord progenitor cells were less sensitive to MP. This may be due to the lower expression level of GR and MR in spinal cord progenitor cells compared to hippocampal progenitor cells. Thus, progenitor cells derived from different CNS regions do not react to the same extent to MP.

It is known that adult progenitor cells are influenced by the inflammatory response after injury (Foote and Blake-more, 2005) and that microglia is able to influence migration and differentiation of NPCs (Aarum et al., 2003). In our study we do not exclude that MP affects adult NPCs in the spinal cord also via its anti-inflammatory effects. The number of both, proliferative microglia/macrophages and proliferative OPCs was reduced in all of the spinal cord regions analyzed. This suggests that inflammation affects the activity of OPCs after SCI. Kotter et al. (2005) showed that macrophage-depletion in an experimental rat spinal cord demyelination model corresponds with delayed recruitment of OPCs. It was also demonstrated that inhibition of microglia/macrophage activation impairs remyelination (Li et al., 2005). Demyelination and sparse remyelination after SCI are thought to play an important role in the functional deficits after SCI. There are different ways in which microglia/macrophages might benefit remyelination. They can be involved in the process of remyelination by phagocytotic clearance of myelin debris (Copelman et al., 2001), but also by secretion of different factors including cytokines and growth factors (Nathan, 1987; Hinks and Franklin, 1999; Arnett et al., 2001). Our finding of a reduced proliferative activity of NG2+ cells suggests that MP treatment after SCI may negatively affect myelin repair by minimizing the beneficial effects of microglia/macrophages due to reduction of their activity. Thus, MP treatment could affect remyelination after SCI either directly or indirectly via its anti-inflammatory effects.

Here we demonstrate that the proliferation of spinal cord progenitor cells is negatively affected by MP application and concerned the oligodendrocyte lineage. Chari et al. (2006) attributed the delay in oligodendrocyte-mediated repair after toxin-induced demyelination to the inhibition of differentiation of OPCs into mature oligodendrocytes, with no effect observed on recruitment of OPCs to the lesion site. In our *in vitro* experiments MP did not influence the

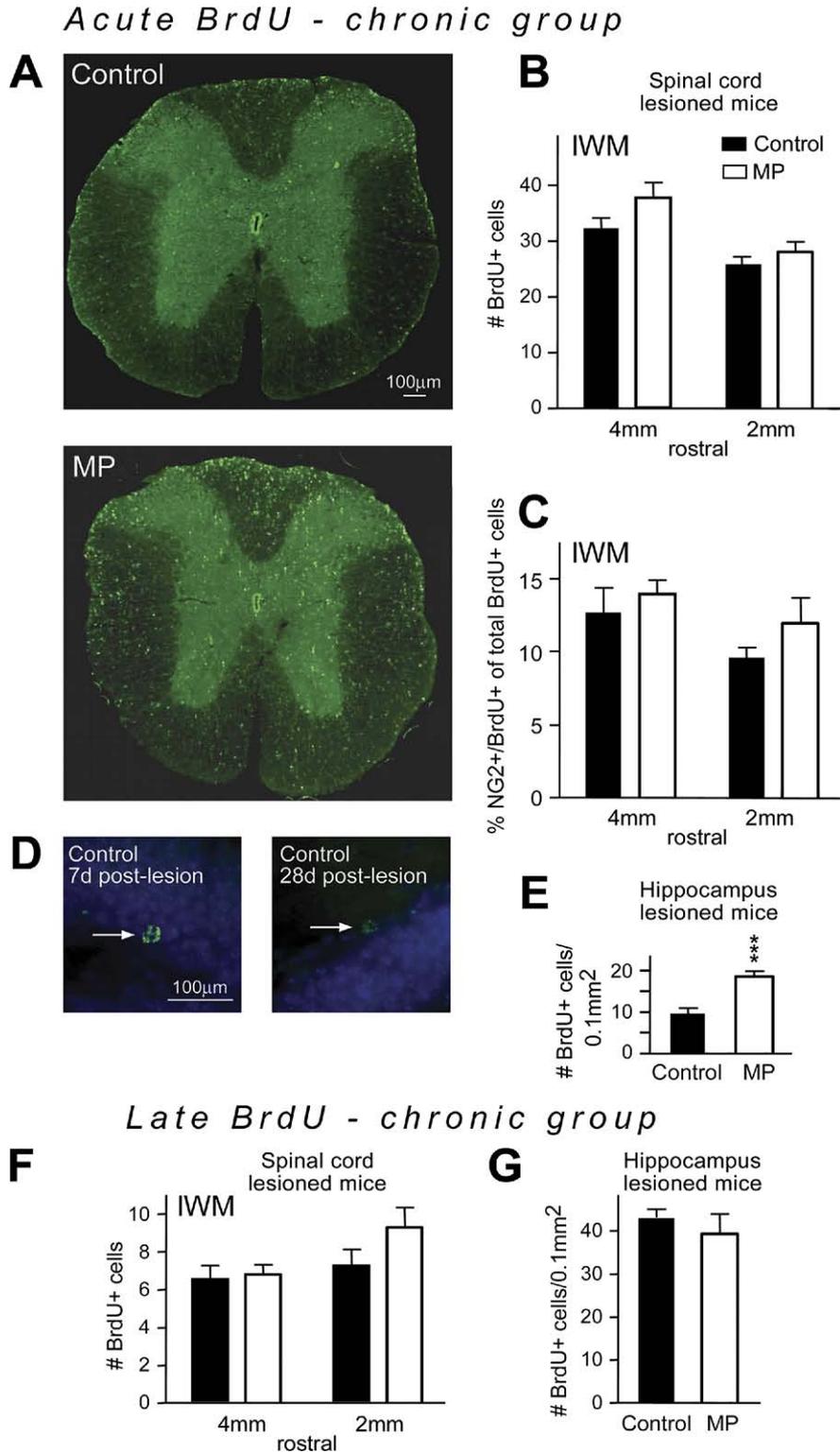


Fig. 5. MP treatment affects the number of acutely proliferating cells after SCI chronically (acute BrdU–chronic group) while it does not affect late proliferating cells (late BrdU–chronic group). (A–E) Acute BrdU–chronic group. (A) Spinal cord cross-sections (2 mm rostral from the lesion site) of mice at 28 days after lesion stained for proliferating cells (BrdU) that were labeled with BrdU within the 1st week after lesion. Control mice show a weaker BrdU staining than MP-treated, indicating a dilution of the BrdU signal, presumably due to higher cell proliferation activity in control mice. (B) The number of BrdU+ cells is not significantly different between control mice and MP-treated mice in any of the spinal cord regions analyzed (see Fig. 1D and Exp. Procedures). (C) The proportion of BrdU+/NG2+ cells is not significantly different between control mice and MP-treated mice in any of the regions. (D) Hippocampal sections of control mice at 7 and 28 days after lesion stained for proliferating cells that were labeled with BrdU within the 1st week after lesion. Proliferating cells in the dentate gyrus (arrows) show a weaker and more punctate BrdU staining when the last BrdU injection was given 3 weeks earlier, indicating a dilution of the BrdU signal due to proliferation activity over time. (E) The number of BrdU+ cells in the hippocampal dentate gyrus is significantly lower in control mice compared to MP-treated mice, however, the BrdU signal was weaker and more punctate in the control mice. (F, G) Late BrdU–chronic group. The number of BrdU+ cells labeled in the 4th week after lesion is not significantly changed in the spinal cord (F) and hippocampus (G) of MP-treated mice 28 days after lesion. Bars represent mean±SEM; *** $P < 0.001$.

differentiation of adult NPCs suggesting that MP does not delay or change the lineage choice directly via GR/MR.

This study shows that the number of proliferating OPCs is reduced early after SCI and MP treatment, and according to the chronic results there is no compensation for this reduction. When studying the acute BrdU–chronic group we found that the BrdU-staining in the control animals was weaker and more punctate than in the MP-group. We conclude that the progenitor cells in the control group proliferated more over the course of 4 weeks leading to a dilution of the BrdU. Some cells may even have escaped the quantification since their BrdU content was below the sensitivity of the detection method. In contrast, NPCs in the MP-treated mice underwent fewer mitoses.

In the late BrdU–chronic group we analyzed cells proliferating in the 4th week after SCI and did not detect any difference in BrdU incorporation between MP-treated and control mice demonstrating that cells proliferating late after injury were no longer affected by MP. This result was expected since MP was applied acutely after SCI and is metabolized rather quickly (Ramakrishnan et al., 2002). The observation corresponds to previously reported data (Chari et al., 2006) showing that corticosteroid treatment does not cause permanent impairment of remyelination in the spinal cord. However, after the proliferation had been suppressed acutely after the injury due to MP treatment we did not detect any compensatory increase in NPC proliferation in the MP-treated group at the 4 week time point. Our results suggest that after the initial decrease in NPC proliferation, MP-treated animals are not able to restore an equal total number of NPCs as control animals. The relevance of the persisting initial difference in NPC proliferation for the extent of remyelination remains to be analyzed. Another recent finding showing that MP treatment may inhibit oligodendrocyte cell death after SCI (Lee et al., 2008) points to complex effects of the glucocorticoid. The complexity of MP's effects is well in line with the contradictory clinical literature over the last 15 years of MP use in SCI acute care management. Our data confirm that acute high-dose MP treatment after SCI reduces inflammation and suggest that OPCs may be negatively influenced by MP. Whether the sum of all effects of MP is beneficial, neutral or counterproductive for the acutely injured spinal cord remains to be analyzed in detail.

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