BRAIN RESEARCH 1403 (2011) 57–66

available at www.sciencedirect.com

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Treatment of experimental spinal cord injury with 3β-methoxy-pregnenolone

Yann Duchossoy[a], Sebastien David[a], Etienne Emile Baulieu[a,b,*], Paul Robel[a,b,*]

[a]MAPREG, 94276 Le Kremlin Bicêtre Cedex, France
[b]INSERM U788, 94276 Le Kremlin Bicêtre Cedex, France

ARTICLE INFO

Article history:
Accepted 31 May 2011
Available online 12 June 2011

Keywords:
Spinal cord injury
Locomotor function
Microtubule-associated-protein 2

ABSTRACT

The synthetic derivative of pregnenolone MAP4343 (3β-methoxy-pregnenolone) binds *in vitro* to microtubule-associated-protein 2 (MAP2), stimulates the polymerization of tubulin, enhances the extension of neurites and protects neurons against neurotoxic agents. Its efficacy was assessed *in vivo* with the most commonly used thoracic spinal cord compression/contusion models in rats. In the three models used, the post-traumatic subcutaneous injection of MAP4343 significantly improved the recovery of locomotor function after spinal cord injury, as shown by an earlier and more complete recovery compared to vehicle-treated rats. The first injection of MAP4343 could be delayed up to 24 h after spinal cord injury with maintained efficiency. The improvement was correlated with the preservation of both dendritic trees of motoneurons in the lumbar spinal cord caudally to the injury site, and of MAP2 at lesion site and in the lumbar spinal cord. The results obtained in three different rat models of spinal cord injury demonstrate the beneficial effects of this therapeutic strategy and identify MAP4343 as a potential treatment for acute spinal cord injury.

1. Introduction

Acute traumatic compression/contusion is the most common form of spinal cord injury (SCI) (Sekhon and Fehlings, 2001). Today, no efficient drug is available to restore spinal cord functions despite multiple therapeutic attempts (Baptiste and Fehlings, 2007). In the majority of SCI patients, a proportion of spinal cord white matter is maintained across the level of the lesion (Kakulas, 1999) and, even in the clinical setting of complete paraplegia, the cord is seldom completely severed. Spontaneous partial recovery of lost function can occur over time although axonal regeneration is extremely limited in the adult mammalian central nervous system. The mechanisms of this recovery are not fully understood, they are related at least in part to the reorganisation of connectivity between those axons which had retained their continuity and the motor neurons deprived of supra spinal control (Raineteau and Schwab, 2001).

The prevention of cytoskeletal degradation, that contributes to functional impairments after spinal cord injury, is another therapeutic target. SCI leads to cytoskeletal disruption, microtubule destabilisation and loss of microtubule-associated-protein 2 (MAP2) (Li et al., 2000; Springer et al., 1997; Yu et al., 2000; Zhang et al., 2000), due at least in part to proteolysis (e.g. by calpain). MAP2 stimulates tubulin polymerisation and is located mainly in neurons' dendrites and perikarya. It plays an important role in the maintenance of cytoskeletal integrity and in the formation and repair of dendrites (Dehmelt and Halpain 2005; Sanchez et al., 2000).

* *Corresponding authors at:* MAPREG, 94276 Le Kremlin Bicêtre Cedex, France.
E-mail addresses: etienne.baulieu@inserm.fr (E.E. Baulieu), paul.robel@inserm.fr (P. Robel).

0006-8993/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2011.05.065

Our aim is to develop a therapy to promote functional recovery by protecting the cytoskeleton and/or enhancing MAP2 activity (Gazula et al., 2004; Kalb, 2003).

We previously reported that pregnenolone *in vitro* binds to MAP2 and stimulates tubulin assembly, enhances the extension of neurites in cultured PC12 cells, protects their cytoskeleton against Nocodazole, a microtubule disrupting agent, and protects SH-SY5Y cells against the neurotoxic agent okadaic acid (Fontaine-Lenoir et al., 2006; Murakami et al., 2000).

The biosynthesis of steroid hormones involves a biochemical (oxidative) conversion of the delta5-3beta-ol group of pregnenolone into delta4-3-ketone. We attempted to block the structure of pregnenolone to prevent its conversion into metabolites endowed with progestogen, androgen, oestrogen or glucocorticoid hormonal activities, or into sulphate esters, compounds with unknown or adverse effects. After screening hundreds of natural and synthetic steroids, the synthetic derivative of pregnenolone 3β-methoxy-pregnenolone (MAP4343) has been selected to enter in preclinical development. MAP4343 has the same activity as pregnenolone *in vitro* but it cannot be converted into metabolites with hormonal activities. Inhibition of MAP2 expression by RNA interference in PC12 cells has demonstrated that the functional activity of MAP4343 is mediated by MAP2 (Fontaine-Lenoir et al., 2006).

Here, we evaluate the effects of MAP4343 on locomotor performance, lesion size, expression of MAP2 and dendrites' architecture of spinal motor neurons after SCI in rats. Since no single model replicates the entire spectrum of clinical injuries (Anderson et al., 2005; Kwon et al., 2002), the efficacy of MAP4343 has been tested in three models of SCI: two compression models and one contusion model. A compressive injury was produced by either epidural balloon inflation (Vanicky et al., 2001) or transient extradural application of a microvascular clip (Von Euler et al., 1997), whereas contusive injury was produced by a weight-drop model (Basso et al., 1996). MAP4343 was tested at two injury grades in the clip compression model, since previous reports showed that the efficacy of treatments may depend upon the severity of injury (Gruner et al., 1996; Kloos et al., 2005).

Finally, the effects of MAP4343 upon calpain activity and calpain-mediated degradation of purified high-molecular-weight microtubule-associated protein 2 (hmw-MAP2) have been examined in *in vitro* studies.

2. Results

2.1. *Effect of MAP4343 on recovery of locomotor function after spinal cord injury*

2.1.1. *Balloon model*

Spinal cord compression was induced by epidural balloon inflation (Vanicky et al., 2001) at thoracic level in rats. They displayed major motor deficits characterised by the lack of observable hindlimb movements. Motor deficits decreased partly over time. Neurological motor function of rats was evaluated by the locomotor rating scale of Basso, Beattie and Bresnahan (BBB score) (Basso et al., 1995). MAP4343-treatment (first injection, 5 min after injury, 12 mg/kg/day for 28 days) provided a significant improvement of the BBB score already 12 days after injury ($p<0.001$). The improvement of motor outcomes between MAP4343-treated and vehicle treated rats was of 3.8 points on the BBB scale at the end of the experiment (Fig. 1A: BBB score of 13.9±0.43 vs. 10.1±0.65 for vehicle-treated rats, 28 days after injury, $p<0.0001$).

MAP4343-treatment at 12 mg/kg significantly increased the number of animals able to walk with weight-supported plantar steps (BBB score ≥10) from 12 to 28 days after injury when compared to vehicle-treated animals (Fig. 1B: 71% vs. 23%, 12 days after injury and 100% vs. 53%, 28 days after injury, $p<0.01$). We also observed that more than 90% of animals treated with MAP4343 showed forelimb–hindlimb coordination (BBB score ≥12) at the end of the study, 4 weeks after surgery. On the contrary, only 35% of animals from vehicle-treated group did ($p<0.01$).

2.1.2. *Clip model*

2.1.2.1. Moderate spinal cord compression. In this experiment, a compressive injury was produced by transient extradural application of a microvascular clip (Von Euler et al., 1997). The treatment duration was reduced to six days and the first injection was delayed 24 h after surgery. The BBB scores showed that treatment with MAP4343 at 12 mg/kg/day provided significant improvement of the BBB score already 5 days after injury and until the end of experiment 56 days after surgery. Improvement in motor outcome was of 4.6 points on the BBB scale in MAP4343-treated rats (Fig. 1C: BBB score of 16.6±0.5 vs. 12±1.04, 56 days after injury, $p<0.0001$).

MAP4343-treatment significantly increased the number of rats that walked with frequent forelimb–hindlimb coordination (BBB score ≥14) 14, 21, 28 and 56 days after injury when compared to vehicle-treated ones (Fig. 1D: 67% vs. 11%, 14 days after injury and 100% vs. 39%, 28 days after injury, $p<0.01$).

2.1.2.2. Severe spinal cord compression. The motor outcome of MAP4343-treated rats, at the reduced dose of 4 mg/kg/day for six days (first injection 24 h after injury), was improved of 2 points on the BBB scale 28 days after injury (Fig. 1E: BBB score of 10.63±0.34 for MAP4343-treated rats vs. 8.76±0.25 for vehicle-treated rats, 28 days after injury, $p<0.0001$). MAP4343-treatment significantly increased the number of animals that walked with weight-supported plantar steps (BBB score ≥10) 21 and 28 days after injury when compared to vehicle-treated animals (Fig. 1F: 82% vs. 22% 21 days after injury and 87% vs. 26% 28 days after injury, $p<0.0001$). MAP4343 led to an improvement in the rats' ability to walk with weight-supported plantar steps whereas vehicle-treated animals could only stand on their hind legs, a difference of large clinical significance.

2.1.3. *Weight-drop model (spinal cord contusion)*

Contusive injury was produced by weight-drop (Basso et al., 1996). Motor outcome of the rats, treated with MAP4343 at 4 mg/kg, was improved of 2 points on the BBB scale 42 days after injury (Fig. 1G: BBB score of 13.9±1.06 for MAP4343-treated rats vs. 11±0.4 for vehicle-treated rats, 42 days after injury, $p<0.05$). MAP4343-treatment also significantly increased the number of animals that walked with frequent forelimb–hindlimb coordination (BBB score ≥14) 28, 35 and 42 days after injury when compared to vehicle-treated animals (Fig. 1H: 53% vs. 16%, $p<0.05$).

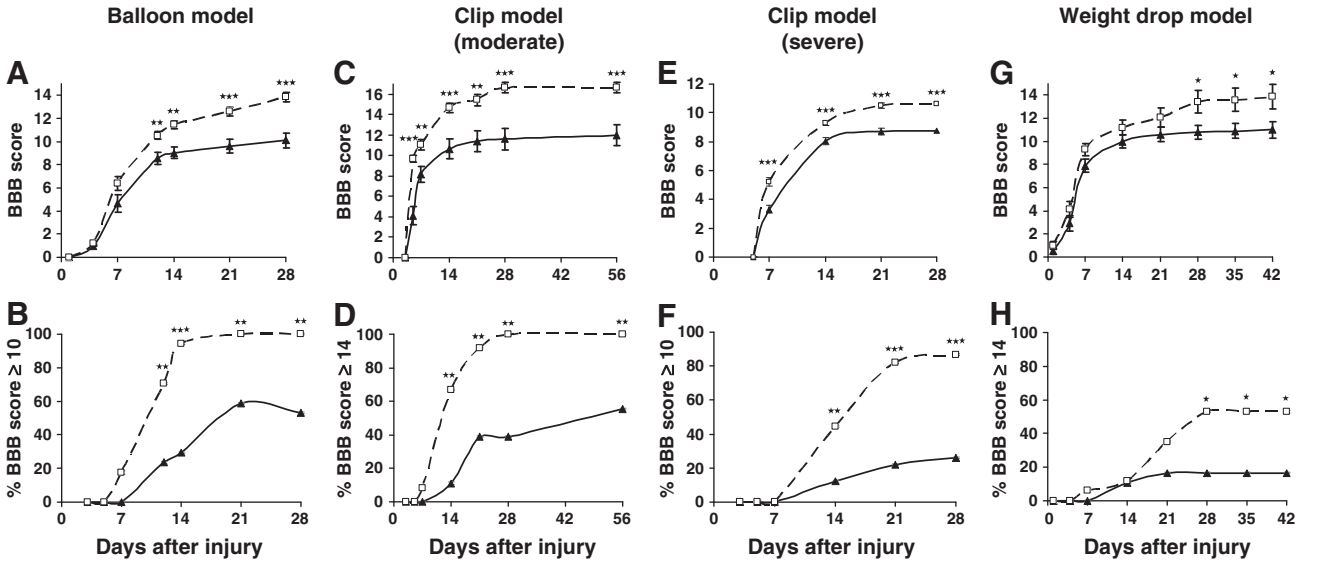

Fig. 1 – MAP4343 significantly improves functional recovery after spinal cord injury (▲: vehicle-treated rats; □: MAP4343-treated rats). Time courses of motor recovery (A) and of the proportion of animals able to walk with weight-supported plantar steps (B) (BBB score ≥10) up to 28 days post injury after balloon compression of spinal cord. Time courses of the motor recovery (C) and of the proportion of animals able to walk with frequent forelimb–hindlimb coordination (BBB score ≥14) (D) up to 56 days post injury after moderate microclip compression of spinal cord. Time courses of motor recovery (E) and of the proportion of animals able to walk with weight-supported plantar steps (BBB score ≥10) (F) up to 28 days post injury after severe microclip compression of spinal cord. Time course of the motor recovery (G) and of the proportion of animals able to walk with weight-supported plantar steps (BBB score ≥14) (H) up to 42 days post injury after spinal cord contusion (weight-drop model). (*p<0.05; **p<0.01; ***p<0.0001: MAP4343-treated vs. vehicle-treated rats).

2.2. *Lesion size*

Although locomotor activity of rats treated with MAP4343 at 4 mg/kg/day (first injection 24 h after injury) was improved, the lesion size of vehicle-treated (5.86±0.24 mm^3) and MAP4343-treated (5.47±0.30 mm^3) groups was not significantly decreased 28 days after a severe spinal cord injury (microclip model, Fig. 2), neither in the other models of SCI.

2.3. *Effect of MAP4343 treatment on MAP2 after severe spinal cord compression (clip model)*

2.3.1. *Western blot analysis*

Densitometric analyses of MAP2 expression showed that SCI had decreased MAP2 amounts at all time intervals examined. Twenty four hours after injury, before treatment onset, the amount of MAP2 was 40.5±3% of control (uninjured rats) in the thoracic segment of spinal cord encompassing the lesion site. In vehicle-treated rats, decrease was still more prominent 7, 14 and 28 days after injury with values of 8±3, 4±2 and 16±4% of control, respectively. MAP4343 treatment maintained significantly larger levels of MAP2 at the lesion site (respectively, 47±8, 59±7 and 58±7% of control, 7, 14 and 28 days after injury, $p<0.01$) and in the lumbar spinal cord (87±12% in MAP4343-treated vs. 28±5% in vehicle-treated rats, 28 days after injury, $p<0.01$) (Fig. 3). Analyses of actin expression showed no significant difference between groups. Effect of MAP4343 on MAP2 in uninjured rats was analysed in a separate set of experiments: no significant change in the expression of MAP2 was observed (data not shown).

2.3.2. *Immunohistology*

Analyses of MAP2 staining showed that MAP2 staining intensity was significantly decreased in the lumbar spinal cord of vehicle-treated injured rats compared to vehicle-

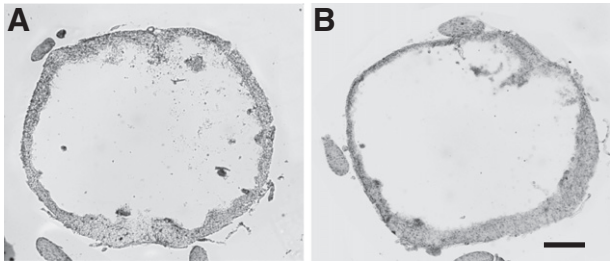

Fig. 2 – Photomicrographs of representative spinal cord transverse sections at the lesion epicentre after severe spinal cord injury (clip model). The spinal cord sections were made 28 days after injury and stained with neutral red. In the injury epicentre (T9), a large hollow is surrounded by a rim of spared white matter. No significant differences in lesion sizes were observed between vehicle-treated (A) and MAP4343-treated (B) rats. Scale bar=1 mm.

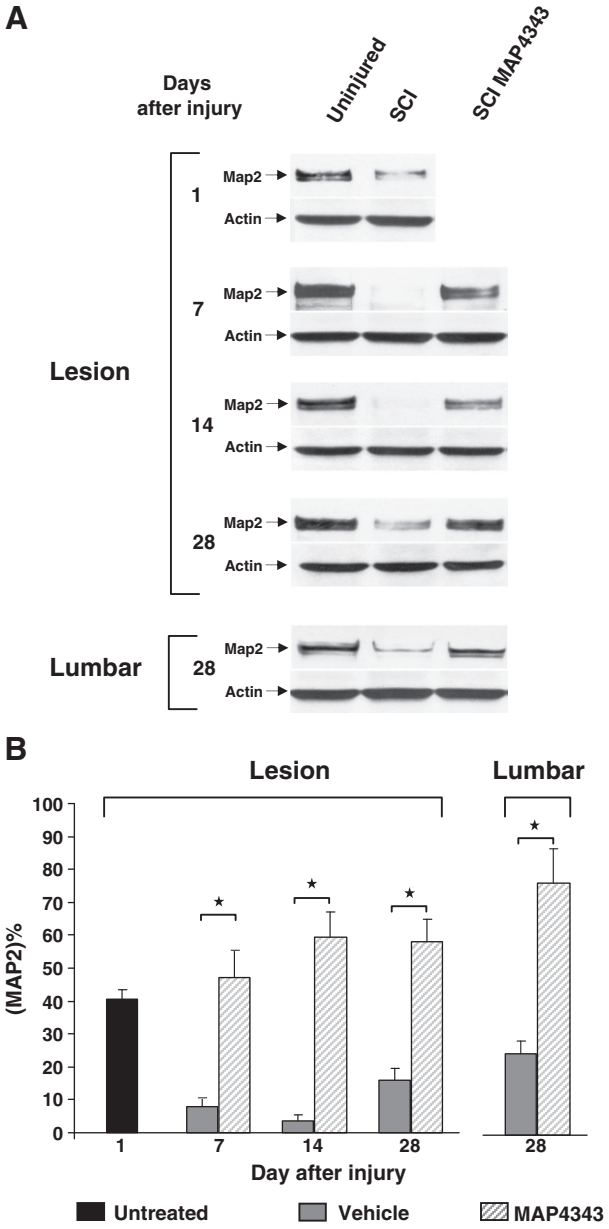

Fig. 3 – Effect of MAP4343 treatment on MAP2 levels after severe spinal cord compression (clip model): Western-blots. Representative Western-blots (A) and bar graphs (B) of hmw-MAP2 and actin levels in the thoracic segments of spinal cord encompassing the injury site (lesion site) 1, 7, 14 and 28 days after SCI and in the lumbar spinal cord of vehicle-treated uninjured rats (uninjured) and vehicle (SCI) or MAP4343-treated (SCI MAP4343) injured rats. Results are expressed as percent of uninjured control rats' level. (A) Western-blots showed that SCI results in a dramatic decrease of hmw-MAP2 amounts at lesion site 24 h after surgery (black column, untreated). Decrease was larger 7 days after injury and persisted until the end of experiment (28 days after surgery) in vehicle-treated rats. In MAP4343-treated rats (4 mg/kg/day), the amount of hmw-MAP2 was significantly larger than in vehicle-treated rats 7, 14 and 28 days after injury. In the lumbar spinal cord, the amount of hmw-MAP2 was also significantly larger in SCI MAP4343-treated rats than in SCI vehicle-alone ones, 28 days after injury (*$p<0.01$ SCI-MAP4343 compared with SCI-vehicle). No significant differences in the expression of actin were observed (quantification not shown).

treated uninjured rats 28 days after injury (47±6.3%, $p<0.05$) (Fig. 4). MAP4343-treatment (4 mg/kg/day during 6 days) significantly increased MAP2 staining in SCI rats (77±4.7% in MAP4343-treated vs. 47±6.3% in vehicle-treated rats, $p<0.05$) but had no significant effect in uninjured rats.

2.4. *Effect of MAP4343 on motor neurons' dendrite tree after SCI*

We have studied the changes in motor dendrites' architecture that occur four weeks after SCI and the results of MAP4343-

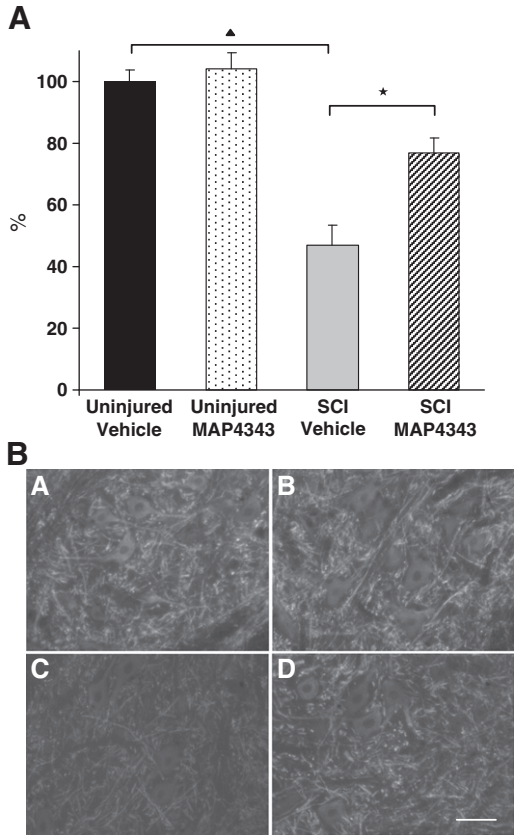

Fig. 4 – Effect of MAP4343 treatment on MAP2 after severe spinal cord compression (clip model): MAP2 immunostaining 28 days after injury. (A) Staining intensity was significantly decreased in the lumbar spinal cord of vehicle-treated injured rats compared to uninjured rats. MAP4343-treatment (4 mg/kg/day during 6 days) significantly increased MAP2 staining in SCI rats. On the contrary, no significant differences in MAP2 immunostaining were observed between vehicle-treated and MAP4343-treated uninjured groups. Results are expressed in percentage of staining obtained in the vehicle-treated uninjured rats. (▲$p<0.05$ vehicle-treated injured rats vs. vehicle-treated uninjured rats, *$p<0.05$ MAP4343-treated injured rats vs. vehicle-treated injured rats). (B) Examples of MAP2-immunostaining in the ventral horn of the lumbar spinal cord of vehicle-treated uninjured rats (A), MAP4343-treated uninjured rats (B), vehicle-treated injured rats (C) and MAP4343-treated injured rats (D).

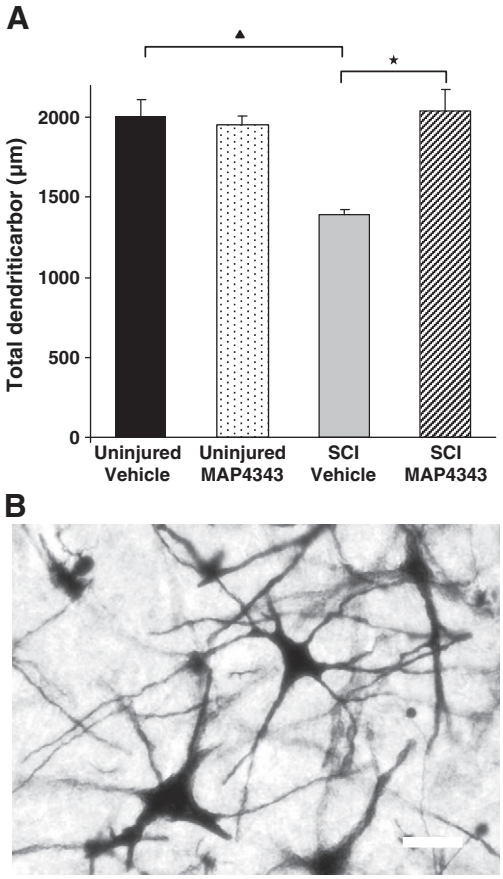

Fig. 5 – Effect of MAP4343 treatment on total dendritic arbours of lumbar spinal cord motoneurons in uninjured and injured rats 28 days after SCI. (A) Spinal cord injury significantly decreased dendritic arbour length of motoneurons by approximately 30% in the lumbar spinal cord of vehicle-treated rats. The motoneuron dendrites from the MAP4343-treated SCI group were significantly larger than in the vehicle-treated SCI group and indistinguishable from the dendrites in the vehicle-treated uninjured group (▲$p<0.0001$ vehicle-treated injured rats vs, vehicle-treated uninjured rats, *$p<0.0001$ MAP4343-treated injured rats vs, vehicle-treated injured rats). (B) Golgi-impregnated neurons in the ventral horn of the lumbar spinal cord (vehicle-treated uninjured rats).

treatment (6 daily subcutaneous injections of MAP4343 4 mg/kg, in sesame oil solution, first injection 24 h after injury).

One month after SCI (clip model, severe injury), the total dendrite arbour size of motoneurons in the lumbar spinal cord of vehicle-treated animals was significantly decreased (more than 30%) when compared with lumbar motoneurons of vehicle-treated uninjured animals (1396±27 vs. 2007±107 μm, $p<0.0001$) (Fig. 5). The motoneurons' dendrites from the MAP4343-treated SCI rats were significantly larger than in the vehicle-treated SCI ones (2044±135 μm vs. 1396±27 μm, $p<0.0001$) and alike the dendrites of vehicle-treated uninjured rats (2007±107 μm) and of MAP4343-treated uninjured group (1956±56 μm). No statistically significant difference in the number of primary dendrites by neuron was found between vehicle-treated uninjured, MAP4343-treated uninjured and vehicle-treated injured groups (5.72±0.2, 5.85±0.15 and 5.63±0.06 respectively). The difference between the numbers of primary dendrites in vehicle- and MAP4343-treated SCI rats (5.63±0.06 and 6.03±0.16 respectively) was quite small (less than 7%), although statistically significant ($p<0.05$).

2.5. *Effect of MAP4343 on calpain 1-mediated MAP2 degradation in vitro*

In the absence of calpain, the extent of the high molecular weight (hmw) MAP2s (MAP2a and MAP2b, with molecular

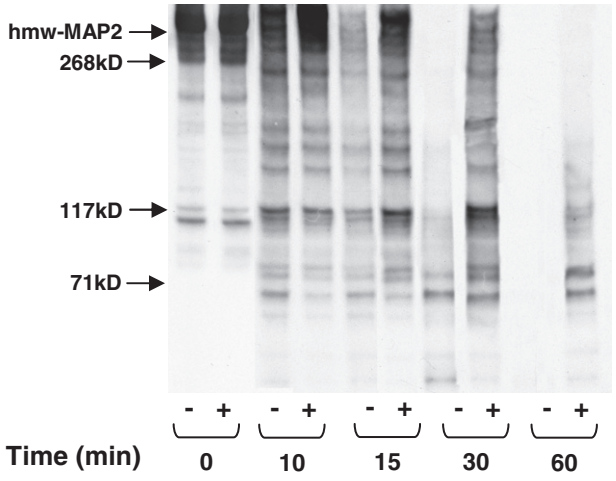

Fig. 6 – Representative Western blots of hmw-MAP2 degradation by calpain I in the presence (+) or absence (–) of MAP4343 (40 μM). Overexposure of the film was necessary to detect degradation products of hmw-MAP2. MAP4343 decreased the rate and extent of MAP2 cleavage by calpain 1 after 10, 15, 30 and 60 min of incubation.

weights >280 kD) degradation was minimal up to the end of experiment (60 min after beginning of incubation, data not shown). The incubation of MAP2 with calpain 1 resulted in the complete degradation of hmw-MAP2 into several peptides after 30 min of incubation (Fig. 6). This breakdown of MAP2 was partially prevented by MAP4343 (40 μM). MAP4343 had no significant effect on calpain-mediated degradation of tau (results not shown).

2.6. *Effect of MAP4343 on calpain 1 activity in vitro*

MAP4343 up to 400 μM did not affect calpain 1 activity in the bioluminescent assay for calpain activity developed by Promega (data not shown).

3. Discussion

In three models of spinal cord injury, MAP4343 treatment resulted in a markedly better recovery of motor function. The first model, the balloon model, used MAP4343 at 12 mg/kg/day for 28 days. We obtained good results with this treatment modality. However, during preclinical development, it appeared that long-term injections with sesame oil (28 days) provoked pulmonary embolism in some animals. Hence we decided to reduce treatment duration to 6 injections using the moderate clip injury model at the dose of 12 mg/kg/day. In the third step of development, we were limited by the solubility of our compound in sesame oil and (potentially) by the limited injection volume of oil allowed in human. Therefore, we decided to decrease the amount of MAP4343 to 4 mg/kg/day for both the severe clip model and the weight drop model and we performed most our analyses (histology, immunohistochemistry, Golgi staining and biochemistry) with this treatment modality.

MAP4343 improved recovery even when the first injection was delayed 24 h after SCI. This result is important because most drugs reported to improve the recovery of neurological functions in animal models of SCI were only efficient when given before or immediately after insult.

Despite the improvement of locomotor function of rats treated with MAP4343, tissue loss was not significantly decreased. However, it was previously reported that treatments stimulating neuronal plasticity could improve motor function without decreased lesion size after SCI (Lankhorst et al., 2001; Rabchevsky et al., 2000). This histological analysis suggests that mechanisms other than tissue sparing underlie the observed improvement, but it cannot be excluded that a small difference in the amount and/or quality of remaining white matter would account for the motor improvement observed.

In all experiments, spinal cord injury decreased MAP2 levels at all time intervals examined at the lesion site and in the lumbar spinal cord. It was previously reported that the SCI-induced increase of calpain activity leads to decreased MAP2 in the spinal cord (Springer et al., 1997). Microtubule destabilisation and loss of MAP2 may contribute to cytoskeleton degradation, neuronal dysfunction, and ultimately to functional impairment after spinal cord injury (Li et al., 2000; Springer et al., 1997; Yu et al., 2000; Zhang et al., 2000). Delayed MAP4343 treatment (first injection 24 h after lesion) prevented significantly the loss of MAP2 which normally occurs at lesion site and in the lumbar spinal cord after injury. Increased MAP2 amount and stabilisation of cytoskeleton play a role in improved functional outcomes of SCI (Perez-Espejo et al., 1996).

MAP2 is an excellent substrate for calpain and the increase of calpain activity in spinal cord and brain lesions supports a pivotal role for calpain in CNS trauma (Springer et al., 1997). The inhibition of calpain activity by MAP4343 was investigated. Activity assays showed that MAP4343 has no inhibitory activity up to 400 μM. This suggests that inhibition of calpain 1-mediated purified-MAP2 degradation is due to MAP4343 binding to MAP2, thus preventing access of calpain to its MAP2 digestion sites. Otherwise MAP4343 may increase the binding of MAP2 to the microtubule lattice and render it physically inaccessible to intracellular proteases, and/or may affect phosphorylation of MAP2 and thereby its degradation (Quinlan and Halpain, 1996; Schumacher et al., 1999). MAP4343 could also protect and/or restore dendritic microtubule structures (Fontaine-Lenoir et al., 2006), prevent MAP2 disorganisation and subsequent loss as suggested by a previous report that calpain-activated MAP2 proteolysis does not occur when microtubules are stabilised (Hoskison and Shuttleworth, 2006).

We also studied the changes in motor neurons' dendritic architecture after severe SCI in rats. Anatomical investigations were focussed on the motor neurons' dendrite trees in the lumbar spinal cord. As final common pathway for motor output, these cells play a critical role below the lesion site in the control of motor function. The size and complexity of the dendrite tree relate directly to the number of synaptic inputs received by the neurons and the formation of new synapses constitutes a crucial element of rehabilitation in humans (Bareyre et al., 2004; Raineteau and Schwab, 2001).

When compared with animals with an intact spinal cord (vehicle-treated uninjured rats), the dendrite trees of lumbar motor neurons from animals with spinal cord compression

displayed marked atrophy. Such decreased size of the total dendritic arbour of motoneurons in the lumbar spinal cord was previously described after a complete spinal cord transection on postnatal day 23 (Gazula et al., 2004). Moreover, after hemisection of the spinal cord, the dendrites' length of motoneurons ipsilateral to the lesion is also decreased (Hirakawa and Kawata, 1992). Such atrophy did not occur 28 days after spinal cord compression injury in the motoneurons of MAP4343-treated rats. Indeed, the subcutaneous delivery of MAP4343, starting 24 h after injury and continued for 6 days, increased the size of lumbar spinal motoneurons' dendritic arbours compared to vehicle-treated rats after severe injury. MAP4343 per se, did not modify significantly the dendrite arbours of uninjured animals. The dendrites' lengths in this study were underestimates, not only because segments were cut, but also because they were likely reduced by the shrinkage of tissue due to histological processing. The extent of shrinkage probably differs according to procedure, thus the results of this study cannot be directly compared to other types of Golgi staining or to other methods of dendrites' arbour analyses (Kitzman, 2005; Gazula et al., 2004). Nevertheless the differences between injured and uninjured animals and the effects of MAP4343-treatment are reliable. The effects of MAP4343 on dendrites' size may result from an action on dendrite growth or stabilisation (Fontaine-Lenoir et al., 2006) but also from the increased use of hindlimbs by MAP4343-treated rats (Gazula et al., 2004). Our data suggest that dendrites' growth in lumbar spinal cord neurons may contribute to functional improvement in MAP4343-treated rats. However, MAP4343-treatment could affect other regions of the central nervous system implicated in spontaneous reorganisation of the central nervous system after spinal cord lesions around the lesion, rostral to injury, and in supraspinal structures (Lynskey et al., 2008).

4. Conclusion

In two models of spinal cord compression and one model of spinal cord contusion, administration of MAP4343 produces locomotor recovery. This improvement is correlated with the maintenance of the dendritic trees of motoneurons in the lumbar spinal cord caudally to the injury site and of MAP2 levels at the lesion site and in the lumbar spinal cord.

5. Experimental procedure

5.1. *Models of spinal cord injury and surgical procedures*

Experimental procedures were in full accordance with the recommendations of EU (84/609/RRC). Animal care followed the European Communities Council Directive of November 24, 1986 (86/609/EEC).

Adult Sprague–Dawley male rats were maintained in the animal facility under standard housing conditions. Prior to surgery, rats were deeply anaesthetized by intra peritoneal injection of 3.5% chloral hydrate solution (1.2 ml per 100 g body weight). Surgery was performed under sterile conditions and body temperature was maintained at ~37 C.

5.1.1. *Balloon model*

Laminectomy was performed at thoracic T10 level. The balloon (Goldvalve GVB15, Nycomed), mounted on a catheter connected to a 50 μl Hamilton syringe, was inserted into the epidural space and moved rostrally up to the vertebra above the laminectomy site. The balloon was rapidly inflated with 13 μl of saline for 5 min. After compression, the balloon was deflated and removed. Then the vertebral muscles were sutured and the skin closed with wound clips. A total of 40 male Sprague Dawley rats (300–350 g; Janvier, France) were used to perform this experiment. The rats were divided in 2 groups treated with daily subcutaneous injections of either vehicle (sesame oil) or MAP4343 at 12 mg/kg, during 28 days (first injection 5 min after injury). Three rats were excluded from the study because the balloon was deflated during the surgical procedure. Three rats were euthanized for persistent hematuria and weight loss before the end of experiment (two in the vehicle-treated group and one in the MAP4343-treated group). At the end of the experiment, 1 month after surgery, the numbers of rats were 17 both in vehicle-treated group and in MAP4343-treated group.

5.1.2. *Clip model*

Laminectomy was performed at T9 to expose the cord, leaving the dura intact. A microclip (microvascular clip 10-b Zen Temporary Clip, Ohwa Tsusho CO. LTD, Tokyo/Japan) with arms 13 mm long × 0.4 mm thick was used to pinch the cord at a width of 0.5 mm (moderate injury) or 0.05 mm (severe injury). Compression was produced by inserting the arms of the clip between the lateral sides of the cord and the vertebral walls. A calibrated gauge was used to set the desired level of cord compression (0.5 mm wide for moderate injury or 0.05 mm wide for severe injury). The gauge was inserted between the arms of the clip just above the cord surface, and the clip closed slowly until both arms contacted the gauge (1–2 s). The clip was held closed for 60 s (severe injury) or for 30 s (moderate injury) then removed. The cord was rinsed with saline at room temperature and any blood removed.

5.1.2.1. *Moderate compression (clip model).* A total of 30 male Sprague Dawley rats (280–300 g; Janvier, France) were used. They were divided into two groups: spinal cord injured rats treated with vehicle and spinal cord injured ones treated with MAP4343. Daily subcutaneous injections of either sesame oil or 12 mg/kg MAP4343 in sesame oil were done for 6 days, first injection 24 h after spinal cord compression. After surgery, no animal was excluded or died up to the end of the experiment, 2 months after injury.

5.1.2.2. *Severe compression (clip model).* A total of 95 male Sprague Dawley rats (280–300 g; Janvier, France) were used. They were divided into two groups: spinal cord injured rats treated with vehicle and spinal cord injured ones treated with MAP4343. Daily subcutaneous injections of either sesame oil or 4 mg/kg MAP4343 in sesame oil were done for 6 days. The first injection was 24 h after spinal cord compression. Seven rats were euthanized for persistent hematuria and weight loss before the end of experiment (four in the vehicle-treated group and three in the MAP4343-treated group). Uninjured rats were treated in the same conditions with vehicle or MAP4343 to

serve as control for Western-blot, immunohistochemistry and dendrites' size evaluation.

5.1.3. *Weight drop model*

The efficacy of MAP4343 has been confirmed in a model of spinal cord contusion. This experiment was performed by a specialised service company (Pharmaxon, France). Contusion was induced by the weight-drop device developed at New York University (Basso et al., 1996). This system produces standardised injuries of the spinal cord through a rapid single-impact and force-controlled contusion. A total of 37 male Sprague Dawley rats (280–300 g; Janvier, France) were used to perform this experiment. Treatment modalities were the same as for severe clip model. Two rats died before the end of experiment (one in each group).

5.1.4. *Post operative care*

After surgery, body temperature was maintained at approximately 37 °C until complete recovery from anaesthesia. Until spontaneous voiding, bladders were manually emptied 3 times per day. Most rats showed no sign of overt pain or distress. Postoperative urinary complications occurred in some of them (infection, hematuria or persistent retention). Those with symptoms of clinical deterioration or suffering were euthanized.

5.2. *Drug administration*

MAP4343 was dissolved in sesame oil and injected under the back skin. Vials with vehicle or MAP4343 were prepared and coded by a person neither in charge for injections nor for experiments.

5.3. *Assessment of motor function*

Motor function of the rats was evaluated with the locomotor rating scale of Basso, Beattie and Bresnahan (BBB score) (Basso et al., 1995). In this scale, rats are assigned a score ranging from 0 (no observable hindlimb movement) to 21 (normal gait). The rats were tested for functional deficits by two examiners blinded to the treatments received.

5.4. *Quantification of lesion volume*

Lesion sizes were determined on horizontal sections (20 μm). On each section analysed, intraspinal lesion cavity and tissue narrowing were outlined and measured, according to Peng et al. (2009). The resulting areas were summed and multiplied by the spacing between sections (200 μm) to provide a mean volume (mm^3) according to Cavalieri's principle (Michel and Cruz-Orive, 1988).

5.5. *Biochemical and immunohistological analyses*

Rats received daily doses of either MAP4343 (4 mg/kg/day, first injection 24 h after injury, 6 injections) or vehicle and were killed at different post-lesion times (7, 14 and 28 days). Severe injury was produced with a clip (clip model). At the end of the experiment, rats were sacrificed by decapitation, their spinal cords and those of control uninjured rats were rapidly removed. The cords were dissected and the injury sites and caudal segments were frozen in cold isopentane (−80 °C) (6 mm long spinal cord segments centred at lesion epicentre and 12 mm long lumbar spinal cord segments taken from 12 mm to 24 mm caudally to the lesion epicentre).

5.5.1. *Western blots*

At least six animals from each group at each post-lesion time were used for Western blot analyses. Six more rats were sacrificed 24 h after surgery to study the short term effects of SCI on MAP2 before treatment onset. Proteins were prepared from spinal cord tissue and Western blots (anti-MAP2, Sigma, clone AP20: 1:1000; anti-actin, Sigma, A5060: 1:250) were performed as previously described (Fontaine-Lenoir et al., 2006). A semi-quantitative evaluation of protein amounts on immunoblots was performed (NIH Image J densitometry software).

5.5.2. *Immunohistochemistry*

At least six animals from each group at each post-lesion interval were used. Coronal sections were cut at 20 μm width with a cryostat and mounted on superfrost slides. Sections were dried for 90 min at room temperature and stored at −80 °C until use. Before processing for immunohistochemistry, sections were post-fixed with PFA (4% solution in PBS) for 30 min, then treated with methanol at −20 °C for 15 min to enhance antibodies' penetration.

Immediately after pre-treatment, sections were washed in PBS and incubated overnight at 21 °C with anti-MAP2 antibodies (Sigma, clone AP20, 1:2500) in PBS containing 1% Triton X-100 and 10% normal goat serum (NGS). Then, after three washes in PBS, the sections were incubated at room temperature with the appropriate secondary antibody (goat anti mouse FP-SA4010, Interchim) at a concentration of 1:400 in PBS with 2% NGS and 0.3% triton X-100 for 4 h. Finally, after three washes in PBS, sections were coverslipped with Gel Mount (Sigma). Spinal cord sections were incubated in the same solutions without primary antibody to ensure that immunostaining was specific. These control sections were also used to calculate background (see below).

After immunohistochemistry, sections were digitised as greyscale images. Images were captured. The intensities of MAP2 immunostaining were quantitatively assessed by making optical density measurements directly from the sections' images. Background values obtained in the same spinal cord region from control sections, incubated without primary antibody, have been subtracted.

5.6. *Dendrites' sizes*

The changes in motor neurons' dendrites architecture that occur after spinal compression in rats without or with MAP4343 treatment were studied. This study was performed on 16 spinal cord injured animals (clip model, severe injury) treated with either vehicle or MAP4343 at 4 mg/kg/day, 6 injections, first injection 24 h after injury and on six uninjured rats treated with vehicle and 6 uninjured animals treated with MAP4343 (4 mg/kg/day, 6 injections). Twenty days after the last injection, the animals were given an overdose of sodium pentobarbital and intracardially perfused with 0.9% saline. The spinal cords were rapidly removed then placed in Golgi–Cox solution (Glaser and Van der Loos, 1981). The spinal cords were stored for 14 days in the dark then dehydrated as follows

50% ethanol for 1 h, 80% ethanol for 1 h, two changes of 95% ethanol each for 6 h, and two changes of 100% ethanol each for 12 h. Thereafter spinal cords were processed for paraffin embedding and lumbar enlargements, taken from 12 mm to 24 mm caudally to the lesion epicentre, were cut coronally on a microtome at 80 μm (Model 840, American optical Corp, Buffalo, New York, USA). A technical maneuver allowed us to double the maximum thickness feasible with this microtome (stop, back, and forth just before slicing).

Immediately before use, sections were rehydrated and developed using a procedure described by Gibb and Kolb (1998), then placed on slides and mounted in Eukitt.

The sections were examined with a Zeiss microscope and the dendritic processes of well impregnated spinal neurons, localised to the Rexed lamina IX of the ventral horn, were drawn. According to their morphology and location, these neurons were classified as motoneurons (Molander et al., 1984). The camera *lucida* drawings were scanned and analysed with Scion Image. The total length of the dendritic arbour and the number of primary dendrites were both measured. One hundred eighteen, 142, 271, and 225 neurons were analysed in non-operated vehicle-treated, non-operated MAP4343-treated, SCI vehicle-treated and SCI MAP4343-treated rats, respectively.

5.7. *Effect of MAP4343 upon calpain-mediated degradation of purified MAP2-preparation of microtubule proteins*

5.7.1. *Preparation of microtubule proteins*

Rat brains were washed twice in buffer L (0.1 M Mes/1 mM EGTA/0.1 mM EDTA/1 mM $MgCl_2$/1 mM DTT/1 mM PMSF, pH 6.4). MAP2 was purified by the microtubule heat-denaturation and column gel-filtration protocols of Fellous et al. (1977), except that Sephacryl 300 was used instead of Ultrogel ACA34. Tau (hT43) was a generous gift of Dr M. Goedert.

5.7.2. *Incubation of MAP2 with calpain*

Purified MAP2, (0.2 mg/ml) was incubated with calpain 1 (2 μg/ml) (Sigma, C6108) with and without MAP4343 (40 μM).

After incubation at 30 °C for 10, 15, 30 or 60 min, proteolysis was stopped by adding Laemmli gel buffer containing SDS and boiled for 5 min. The extent of degradation of hmw-MAP2 was analysed by SDS-PAGE (Coomasie Blue staining) and subsequently by Western-blots (anti-Map2, AP20).

5.8. *Effect of MAP4343 on calpain activity*

A bioluminescent assay for calpain activity developed by Promega (Calpain-Glo™ Protease assay) has been used to determine if MAP4343 is a calpain inhibitor. This assay provides a succinyl proluminescent calpain substrate, Suc-LLVY-aminoluciferin, in a buffer system optimised for calpain activity and luciferase activity. Following calpain cleavage, the substrate for luciferase (aminoluciferin) is released, allowing the luciferase reaction to occur and produce light. The signal is proportional to the amount of calpain activity present. The assay was performed according to the manufacturer's instructions. MAP4343 was dissolved in DMSO, serially diluted and combined with human calpain I (20 nM) (Sigma, C6108). The final DMSO concentration was 0.1%. Luminescence was recorded 10 min after reagent addition.

5.9. *Statistics*

All data were expressed as mean ± s.e.m. and analysed using Statistica 7.1 (StatSoft). For comparison of Western blots, immunostaining and dendrites' lengths, one-way analysis of variance was used to test for an overall difference between groups and the Bonferroni post-hoc test was used to determine individual group differences. Comparison of lesion volumes was made using unpaired t tests. Data from BBB scales were analysed using parametric statistics as discussed by Scheff et al. (2002): mixed factorial (repeated measures) ANOVA was used for comparison of BBB scores. Contingence tables as function of motor recovery thresholds were analysed with the Fisher non-parametric test.

Disclosure

All authors are MAPREG employees. MAPREG has a financial stake in the outcome of this research, done under company auspices. 3β-Methoxy-pregnenolone was patented by MAPREG Cy (17.01.03/FRA 0300507 and 03.07.08 EU 047027222) for its effectiveness in CNS diseases in which neuronal microtubules are disorganised, in particular spinal cord injury.

REFERENCES

Anderson, D.K., Beattie, M., Blesch, A., Bresnahan, J., Bunge, M., Dietrich, D., Dietz, V., Dobkin, B., Fawcett, J., Fehlings, M., Fischer, I., Grossman, R., Guest, J., Hagg, T., Hall, E.D., Houle, J., Kleitman, N., McDonald, J., Murray, M., Privat, A., Reier, P., Steeves, J., Steward, O., Tetzlaff, W., Tuszynski, M.H., Waxman, S.G., Whittemore, S., Wolpaw, J., Young, W., Zheng, B., 2005. Recommended guidelines for studies of human subjects with spinal cord injury. Spinal Cord 43, 453–458.

Baptiste, D.C., Fehlings, M.G., 2007. Update on the treatment of spinal cord injury. Prog. Brain Res. 161, 217–233.

Bareyre, F.M., Kerschensteiner, M., Raineteau, O., Mettenleiter, T.C., Weinmann, O., Schwab, M.E., 2004. The injured spinal cord spontaneously forms a new intraspinal circuit in adult rats. Nat. Neurosci. 7, 269–277.

Basso, D.M., Beattie, M.S., Bresnahan, J.C., 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. J. Neurotrauma 12, 1–21.

Basso, D.M., Beattie, M.S., Bresnahan, J.C., 1996. Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transection. Exp. Neurol. 139, 244–256.

Dehmelt, L., Halpain, S., 2005. The MAP2/Tau family of microtubule-associated proteins. Genome Biol. 6, 204.

Fellous, A., Francon, J., Lennon, A.M., Nunez, J., 1977. Microtubule assembly in vitro. Purification of assembly-promoting factors. Eur. J. Biochem. 78, 167–174.

Fontaine-Lenoir, V., Chambraud, B., Fellous, A., David, S., Duchossoy, Y., Baulieu, E.E., Robel, P., 2006. Microtubule-associated protein 2 (MAP2) is a neurosteroid receptor. Proc. Natl. Acad. Sci. U. S. A. 103, 4711–4716.

Gazula, V.R., Roberts, M., Luzzio, C., Jawad, A.F., Kalb, R.G., 2004. Effects of limb exercise after spinal cord injury on motor neuron dendrite structure. J. Comp. Neurol. 476, 130–145.

Gibb, R., Kolb, B., 1998. A method for vibratome sectioning of Golgi–Cox stained whole rat brain. J. Neurosci. Methods 79, 1–4.

Glaser, E.M., Van der Loos, H., 1981. Analysis of thick brain sections by obverse-reverse computer microscopy: application of a new, high clarity Golgi-Nissl stain. J. Neurosci. Methods 4, 117–125.
Gruner, J.A., Yee, A.K., Blight, A.R., 1996. Histological and functional evaluation of experimental spinal cord injury: evidence of a stepwise response to graded compression. Brain Res. 729, 90–101.
Hirakawa, M., Kawata, M., 1992. Influence of spinal cord hemisection on the configurational changes in motor and primary afferent neurons and the chemical messenger alterations in the rat lumbar segments. J. Hirnforsch. 33, 419–428.
Hoskison, M.M., Shuttleworth, C.W., 2006. Microtubule disruption, not calpain-dependent loss of MAP2, contributes to enduring NMDA-induced dendritic dysfunction in acute hippocampal slices. Exp. Neurol. 202, 302–312.
Kakulas, B.A., 1999. A review of the neuropathology of human spinal cord injury with emphasis on special features. J. Spinal Cord Med. 22, 119–124.
Kalb, R.G., 2003. Getting the spinal cord to think for itself. Arch. Neurol. 60, 805–808.
Kitzman P., 2005. Alteration in axial motoneuronal morphology in the spinal cord injured spastic rat. Exp. Neurol. 192, 100–108.
Kloos, A.D., Fisher, L.C., Detloff, M.R., Hassenzahl, D.L., Basso, D.M., 2005. Stepwise motor and all-or-none sensory recovery is associated with nonlinear sparing after incremental spinal cord injury in rats. Exp. Neurol. 191, 251–265.
Kwon, B.K., Oxland, T.R., Tetzlaff, W., 2002. Animal models used in spinal cord regeneration research. Spine 27, 1504–1510.
Lankhorst, A.J., ter Laak, M.P., van Laar, T.J., van Meeteren, N.L., de Groot, J.C., Schrama, L.H., Hamers, F.P., Gispen, W.H., 2001. Effects of enriched housing on functional recovery after spinal cord contusive injury in the adult rat. J. Neurotrauma 18, 203–215.
Li, G.L., Farooque, M., Lewen, A., Lennmyr, F., Holtz, A., Olsson, Y., 2000. MAP2 and neurogranin as markers for dendritic lesions in CNS injury. An immunohistochemical study in the rat. A.P.M.I.S. 108, 98–106.
Lynskey, J.V., Belanger, A., Jung, R., 2008. Activity-dependent plasticity in spinal cord injury. J. Rehabil. Res. Dev. 45, 229–240.
Michel, R.P., Cruz-Orive, L.M., 1988. Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J. Microsc. 150, 117–136.
Molander, C., Xu, Q., Grant, G., 1984. The cytoarchitectonic organization of the spinal cord in the rat. I. The lower thoracic and lumbosacral cord. J. Comp. Neurol. 230, 133–141.
Murakami, K., Fellous, A., Baulieu, E.E., Robel, P., 2000. Pregnenolone binds to microtubule-associated protein 2 and stimulates microtubule assembly. Proc. Natl. Acad. Sci. U. S. A. 97, 3579–3584.
Peng, W., Cotrina, M.L., Han, X., Yu, H., Bekar, L., Blum, L., Takano, T., Tian, G.F., Goldman, S.A., Nedergaard, M., 2009. Systemic administration of an antagonist of the ATP-sensitive receptor P2X7 improves recovery after spinal cord injury. Proc. Natl. Acad. Sci. U. S. A. 106, 12489–12493.
Perez-Espejo, M.A., Haghighi, S.S., Adelstein, E.H., Madsen, R., 1996. The effects of taxol, methylprednisolone, and 4-aminopyridine in compressive spinal cord injury: a qualitative experimental study. Surg. Neurol. 46, 350–357.
Quinlan, E.M., Halpain, S., 1996. Postsynaptic mechanisms for bidirectional control of MAP2 phosphorylation by glutamate receptors. Neuron 16, 357–368.
Rabchevsky, A.G., Fugaccia, I., Turner, A.F., Blades, D.A., Mattson, M.P., Scheff, S.W., 2000. Basic fibroblast growth factor (bFGF) enhances functional recovery following severe spinal cord injury to the rat. Exp. Neurol. 164, 280–291.
Raineteau, O., Schwab, M.E., 2001. Plasticity of motor systems after incomplete spinal cord injury. Nat. Rev. Neurosci. 4, 263–273.
Sanchez, C., Diaz-Nido, J., Avila, J., 2000. Phosphorylation of microtubule-associated protein 2 (MAP2) and its relevance for the regulation of the neuronal cytoskeleton function. Prog. Neurobiol. 61, 133–168.
Scheff S.W., Saucier D.A., Cain M.E., 2002. A statistical method for analyzing rating scale data: the BBB locomotor score. J. Neurotrauma. 10, 1251–1260.
Schumacher, P.A., Eubanks, J.H., Fehlings, M.G., 1999. Increased calpain I-mediated proteolysis, and preferential loss of dephosphorylated NF200, following traumatic spinal cord injury. Neuroscience 91, 733–744.
Sekhon, L.H., Fehlings, M.G., 2001. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. Spine 26 (24 Suppl), S2–S12.
Springer, J.E., Azbill, R.D., Kennedy, S.E., George, J., Geddes, J.W., 1997. Rapid calpain I activation and cytoskeletal protein degradation following traumatic spinal cord injury: attenuation with riluzole pretreatment. J. Neurochem. 4, 1592–1600.
Vanicky, I., Urdzikova, L., Saganova, K., Cizkova, D., Galik, J., 2001. A simple and reproducible model of spinal cord injury induced by epidural balloon inflation in the rat. J. Neurotrauma 18, 1399–1407.
Von Euler, M., Seiger, A., Sundstrom, E., 1997. Clip compression injury in the spinal cord: a correlative study of neurological and morphological alterations. Exp. Neurol. 145, 502–510.
Yu, W.R., Westergren, H., Farooque, M., Holtz, A., Olsson, Y., 2000. Systemic hypothermia following spinal cord compression injury in the rat: an immunohistochemical study on MAP 2 with special reference to dendrite changes. Acta Neuropathol. (Berl) 100, 546–552.
Zhang, S.X., Underwood, M., Landfield, A., Huang, F.F., Gison, S., Geddes, J.W., 2000. Cytoskeletal disruption following contusion injury to the rat spinal cord. J. Neuropathol. Exp. Neurol. 59, 287–296.