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ABSTRACT

Keywords: Spinal cord injury (SCI) Animal model MP Glucocorticoids cDNA microarray

Spinal cord injury (SCI) is one of the most devastating medical conditions. Severe SCI often causes paraplegia or tetraplegia in victims who are usually at young and productive age, affecting quality of life and productivity for life. Methylprednisolone (MP) is still the only approven therapeutic agent for treating acute SCI. MP, like other glucocorticoids, has broad effects on transcription factors (TFs) including those affecting cell viability. The genetic mechanisms underlying MP protection in SCI remain poorly understood. The goal of this PPG aims to unveil the molecular and genetic mechanisms of MP neuroprotection in SCI. This Project serves as the animal core unit, providing SCI animals for other projects. We have acquired the expertise in inflicting SCI in both rats and mice using the well established NYU impactor. In addition to generating SCI animals for other projects in this PPG, we also investigated the gene profiling following SCI with or without MP by using the cDNA microarray technique. Adult female Long Evans rats were randomly assigned to the SCI + Vehicle group or SCI + MP group. SCI at T10 was induced with a trauma dose of 10 g x 25 mm. Vehicle or MP (30 mg/kg) was administered 10 min after SCI in SCI + Vehicle or SCI + MP, respectively. Animals were sacrificed 4 h after vehicle or MP treatment. A 5-mm section of the injured spinal cord was removed and processed for total RNA extraction. The integrity of RNA was examined by Agilent Bioanalyzer before cDNA microarray analysis was performed. Commercial Agilent rat cDNA Microarray chips containing over 14,000 rat cDNAs were used for analysis of gene expression. The 3DNA Array 350^{TM} Array Detection kit for microarrays was used for the cDNA labeling based on the provided protocol (Genisphere Inc., Hatfield, PA, USA) and Submicro^{TM} hybridization protocol provided by Genisphere was used for hybridization of Agilent cDNA Arrays. A set of genes was up-regulated by MP treatment including neuropilin (vascular endothelial growth factor receptor, VEGF receptor) and lamin B receptor that is involved in chromatin assembly. The expression of another set of genes was inhibited by MP treatment including M-CSF-induced cysteine protease (calpain 5) and dimethyladenosine transferase. Prior studies suggest that VEGF improves behavioral recovery after SCI by protection of blood vessel, inhibition of apoptosis, and potentiation of cell survival (spared tissue), suggesting that MP may protect injured spinal cords via increased expression of neuropilin. Moreover, apoptotic process may involve disassembly of chromatin. MP treatment increased the expression of lamin B receptor that is important for chromatin assembly, raising the possibility that MP treatment may protect cells in injured spinal cord against apoptosis via this mechanism. Other studies have also shown that inhibition of calpain activity promotes functional recovery following SCI. Furthermore, MP treatment decreased the number of apoptotic cells revealed by TUNEL staining and enhanced the expression of Bcl-xl, which promotes cell survival. Together, our findings strongly suggest that MP may exert its neuroprotective effect in SCI via multiple genetic mechanisms that converge on the enhancement of cell viability after SCI.

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INTRODUCTION

Spinal cord injury (SCI), usually resulting from motor vehicle accidents, sports-related activities, falls or gunshots (Stover et al., 1999; Dryden et al., 2003), is one of the most devastating medical conditions because severe SCI often leads to paraplegia or tetraplegia and drastically decreases the life quality of SCI patients and their care-taking family members . The incidence rate of SCI in western countries is approximately 28 to 50 injuries per million every year (Stover and Fine, 1987; Dryden et al., 2003). In the past several decades, although a lot of efforts have been devoted to the SCI research, the genetic mechanisms underlying the secondary damage of SCI still remains unclear. Therefore, it is pivotal for SCI researchers to first understand the cellular and molecular mechanisms following SCI before they can figure out how to restore or improve the lost functions of SCI patients.

The establishment of suitable SCI animals will facilitate the SCI research. The suitable SCI animal models include the consistency of spinal lesions, reproducibility of behavioral and histological outcomes and the comparable results between the SCI animals and human SCI. Although various techniques have been used to produce SCI in animals (Hansebout et al., 1975; Rivlin and Tator, 1978; Basso et al., 1996a; Sugawara et al., 1998; Nossin-Manor et al., 2002; Fournier et al., 2003; Purdy et al., 2003), the weight-drop technique (especially the NYU impactor) is the most common method used to induce SCI among these lesion techniques (Basso et al., 1996a&b; Liu et al., 1997; Xu et al., 1998; Yan et al., 1999; Metz et al., 2000a; Kim et al., 2001; Xu et al., 2001a&b; Yan et al., 2001; Kim et al., 2002). The use of the NYU impactor has been demonstrated to induce consistent, reproducible, and graded SCI, which closely resembles the SCI profile in humans. Thus, the NYU impactor has become the most popular spinal lesion device in SCI research (Basso et al., 1996b; Liu et al., 1997; Xu et al., 1998; Yan et al., 1999; Kim et al., 2001; Xu et al., 2001a&b; Yan et al., 2001; Kim et al., 2002)

Different species of animals such as rats, mice, rabbits, dogs, and sheep have been used to study SCI (Gruner, 1992; Basso et al., 1996a&b; Liu et al., 1997; Xu et al., 1998; Yan et al., 1999; Metz et al., 2000a&b; Bijak et al., 2001; Kanchiku et al., 2001; Purdy et al., 2003). Among experimental animals, rats and mice are the favorites of SCI researchers (Gruner, 1992; Basso et al., 1996a&b; Liu et al., 1997; Xu et al., 1998; Yan et al., 1999; Metz et al., 2000a&b; Kim et al., 2001; Xu et al., 2001a&b; Yan et al., 2001; Kim et al., 2002; Ochiai et al., 2002; Nossin-Manor et al., 2002; Gerke et al., 2003). The advantages of using rats or mice for SCI research include the shorter life span, easier handling and care-taking due to smaller sizes, more known genes for gene expression study and easier manipulation of their genotypes (production of knockout or transgenic mice) (Kim et al., 2001). Furthermore, evidence indicates that evaluation of functional, electrophysiological, and histological measures was quite comparable between the SCI rats and human SCI (Metz et al., 2000a). In this animal core, therefore, we used the rat and mice SCI models to study the genetic mechanisms of glucocorticoid neuroprotection in SCI.

Up to now, methylprednisolone (MP) is still the only therapeutic agent that is approved by FDA

for treating acute SCI patients. The goal of this PPG is to investigate the genetic mechanisms of glucocorticoid neuroprotection in SCI. The specific aims of this animal core unit are (1) to provide SCI animals for studying genetic mechanisms of glucocorticoid neuroprotection proposed in other component projects in this PPG, (2) to evaluate the functional and morphological outcomes of SCI animals following various treatments and (3) to produce SCI animals for screening therapeutic agents. In the first year of the funding period, we have successfully established the SCI rat and mouse models at TMU and generated a lot of SCI animals to support the other component projects in this PPG. Moreover, we also investigated the gene profiling of SCI animals with or without MP treatment. Furthermore, we have performed the immunohistological assessment of lesion spinal cords receiving various treatments.

MATERIALS AND METHODS

Long Evans female rats (8 or 9 week-old) were purchased from National Laboratory Animal Center, Taipei, Taiwan. All animals were housed in a 12-h light/dark cycle and had free access to water and food all the time. All animals were randomly assigned into two groups: spinal cord injury with vehicle (SCI + V) and spinal cord injury with MP (SCI + MP), unless otherwise specified. In Project 1, Sham SCI animals and SCI animals receiving both MP plus RU486 were added as additional groups. For all surgeries, animals were anesthetized by a mixture of Zoletil 50 (25 mg/kg) (Virbac Laboratories, Carros, France) and Rompun (xylazine, 10 mg/Kg, Bayer AG, Leverkusen, Germany) via intraperitoneal injections. For animal care and surgical procedures, we followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23, revised 1978) and all surgical protocols were approved by the Animal Care and Use Committee of Taipei Medical University.

The procedures for SCI surgery, cDNA microarray analysis, DNA fragmentation, and immunocytochemistry were described in the following paragraphs.

SCI model in rats or mice: The SCI model in rats or mice has been detailed elsewhere (Xu et al., 1990; Xu et al., 1991; Xu et al., 1992; Liu et al., 1997; Xu et al., 1998). Briefly, Long Evans female rats were anesthetized by an IP injection of Zoletil 50 and Rompun. Following a T9 - T10 laminectomy, SCI was induced using a New York University (NYU) Impactor by dropping a 10 g weight at a height of 25 mm. Perioperative care was based on the MASCIS guidelines, which have been described in previous publications (Basso et al., 1996b; Liu et al., 1997; Xu et al., 1998). SCI or sham-operated animals were anesthetized and sacrificed at various time points after MP or vehicle treatment and the lesion spinal cords and the adjacent spinal tissues were collected and stored for studies proposed in other component projects and for cDNA microarray studies.

cDNA microarray analysis: Four hours after MP or vehicle treatment, SCI animals with or without MP treatment were anesthetized with a mixture of Zoletil 50 and Rompun described earlier and the 5-mm lesion spinal cord was removed, frozen immediately in liquid nitrogen and stored in

liquid nitrogen prior to RNA extraction. Total RNA was extracted by the Trizol method and the integrity of RNA was examined by Agilent Bioanalyzer (Agilent Technologies, Inc. Headquarters, Palo Alto, CA, USA) before cDNA microarray analysis started. Commercial Agilent rat cDNA Microarray chips (Agilent Technologies, Inc.) containing 18564 rat cDNAs were used for analysis of gene expression. For the cDNA labeling, we used the 3DNA Array 350^{TM} Expression Array Detection Kit for microarrays and the protocol provided by Genisphere Inc. (Genisphere Inc., Hatfield, PA, USA). For hybridization and wash, we used Submicro^{TM} protocol for Agilent cDNA Arrays provided by Genisphere Inc. The image of cDNA microarrays were scanned by Agilent Microarray Scanner and analyzed by Spotfire software (Spotfire, Somerville, MA, USA). Three replicates were performed for each cDNA microarray analysis.

DNA fragmentation:

TUNEL staining Rats were anesthetized and sacrificed at appropriate times post-injury and perfused transcardially with 50 ml of 0.9% saline followed by 500 ml of modified Zamboni fixative (Holets et al., 1987; Xu et al., 1995). After perfusion, spinal cords were removed and a 15 mm segment containing the injury epicenter was blocked and post-fixed for an additional 2 h in the same fixative. The specimens were transferred to a solution containing 30% sucrose in 0.1M phosphate buffer (PB, pH7.4) overnight. Each specimen was then blocked, from the epicenter of the injury to both rostral and caudal directions 5 mm away from the injury epicenter for horizontal sections. TUNEL reaction was conducted using TdT-FragEL DNA fragmentation kit (Cat QIA 33, Oncogene) for color reaction or Fluorescein-FragEL DNA fragmentation detection kit (Cat QIA 39, Oncogene) for fluorescence double staining.

Immunohistochemistry: Following anesthesia, rats were perfused and the spinal cords were processed for immunohistochemistry. The procedures for perfusion, post-fixation, and cryoprotection were the same as those described in TUNEL staining section. Each specimen was then blocked, from the epicenter of the injury to both rostral and caudal directions 5 mm away from the injury epicenter for horizontal sections or transverse sections. Horizontal sections of the segment were used to detect the proximo-distal extent of immunoreactivity (IR) of Bcl-x_L, PLP and caspase-3. All sections were cut at 15 μm on a cryostat and processed for immunoreactivity using the ABC (avidin-biotinylated peroxidase complex) method. Briefly, sections were incubated with the primary antibody containing 0.3% Triton X-100 and 1% normal goat serum for 24 h at 4° C. After several rinses in 0.01 M PBS, the sections were reacted according to tissue staining kit from R&D system (Cat CTS002 or CTS005) or Vectastain Elite ABC kit (Vector laboratories, Burlingame, CA) (Yan et al., 1999; Yang and Arnold, 2000a&b; Kim et al., 2001). After reactions, the sections were mounted on slides, dehydrated, cleared and cover slipped. Slides were examined with a light microscope. In control sections, the primary antibody or secondary antibody was omitted.

Immunofluorescence double labeling for confocal microscopy. In selected specimens, double

labeling of Bcl-x_L or activated caspase-3 with cell markers (PLP) were colocalized. The immunofluorescence double labeling method has been described in a previous publication (Kim et al., 2001). Briefly, spinal cord segments were embedded in tissue freezing medium, cut horizontally at 15 µm on a cryostat, and mounted on gelatin-coated slide. Before primary antibody incubation, the sections were permeabilized and blocked with 0.3% Triton X-100/ 10% normal goat serum in 0.01 M PBS for 30 min. Primary antibody, cleaved caspase 3 or Bcl-x_L (polyclonal antibody) and PLP (monoclonal antibody) were applied to the sections overnight at 4°C. On the following day, the sections were incubated with fluorescein-conjugated goat anti-rabbit (FITC) antibodies and rhodamine-conjugated rabbit anti-mouse (RITC) antibodies. Slides were coverslipped and examined with Zeiss Fluorescence microscope.

RESULTS AND DISCUSSION

Setup of SCI animal core at Taipei Medical University

We have ordered an NYU impactor and modified its spinal clamps for rats (Figure 1A) and mice (Figure 1B). Our modified spinal clamps for rats and mice have improved the clamping ability of original NYU clamps and allowed the use of the NYU impactor to produce SCI in mice. Up to now, we have performed SCI in over 100 rats and provided these SCI rats to component projects 1, 2, and 3. We are currently evaluating functional recovery of SCI animals following different drug delivery strategies.

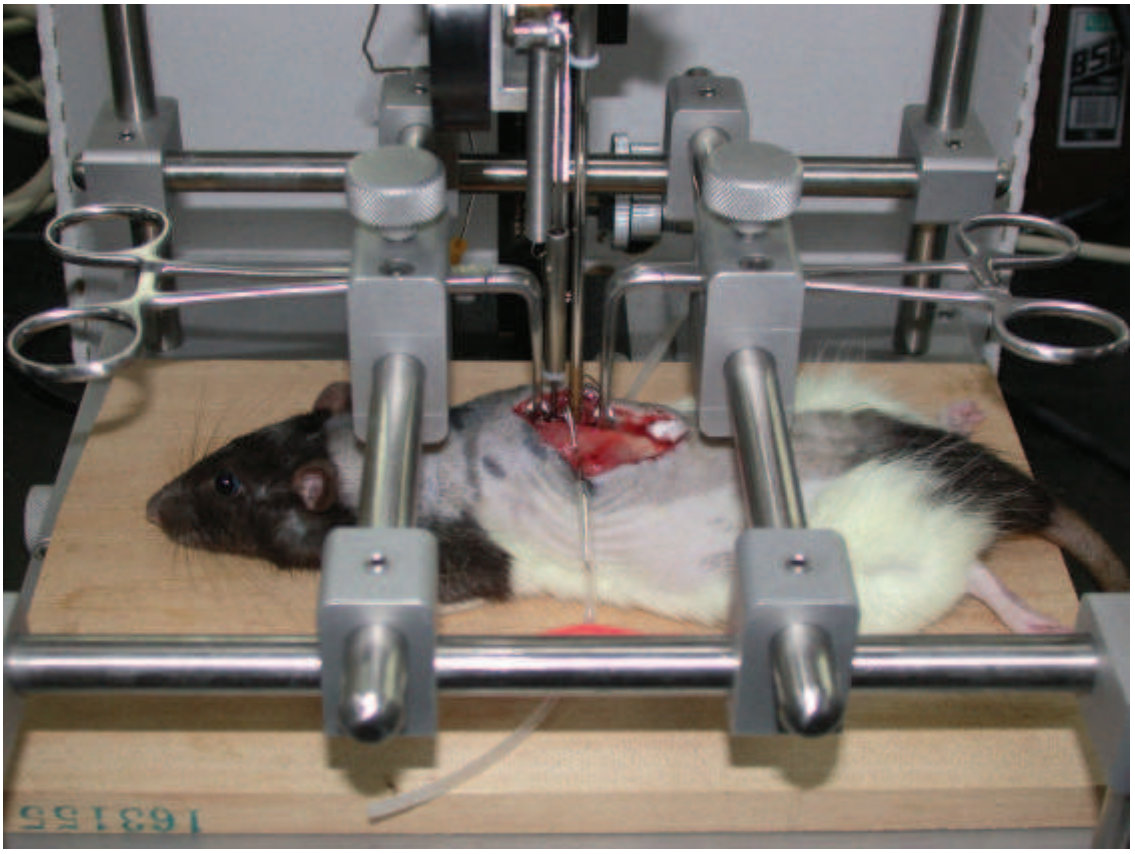

Figure 1A The NYU impactor with modified spinal clamps for rats.

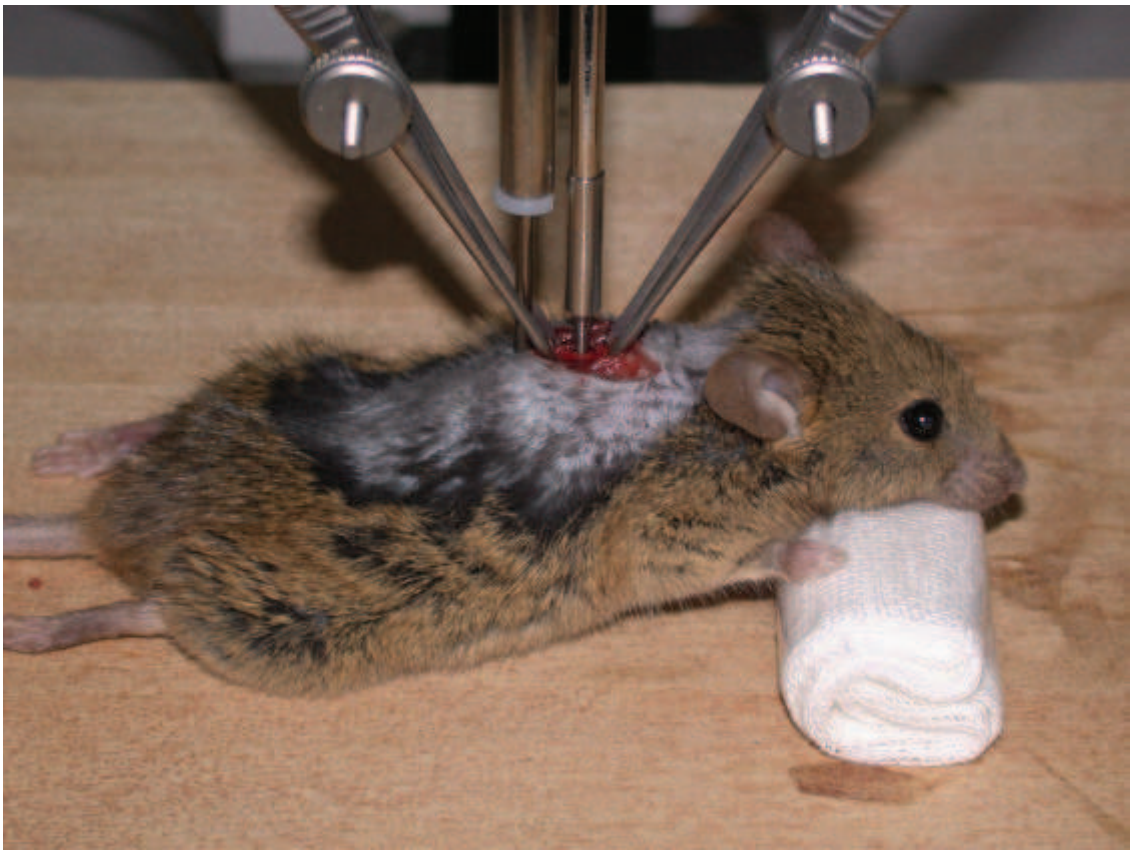

Figure 1B. The NYU impactor with modified impactor head and spinal clamps for mice.

MP treatment significantly enhanced the expression of neuropilin (VEGF receptor)

MP treatment following SCI significantly induced over 2-fold (mean of three replicates) expression of 748 genes in three replicate cDNA microarrays. Among these 748 genes, there were 18 genes with 2-fold increase following MP treatment in all three replicates, including neuropilin (Figure 2A) and NBP60 (Figure 2B). Neuropilin is the receptor for vascular endothelial growth factor (VEGF), which has been demonstrated to improve the functional recovery in SCI animals, to increase the spared spinal tissue and to decrease the amount of apoptotic cells (Widenfalk et al., 2003). Moreover, disassembly of chromatin is a sign of apoptosis and NBP60 (the lamin B receptor), which is a nuclear localization signal binding protein, is important for chromatin assembly. MP-induced increase of neuropilin and NBP60 and the improved functional recovery in SCI animals receiving VEGF together strongly suggest that MP may exert its neuroprotective effect in SCI animals in part via potentiation of VEGF effect and prevention of apoptosis.

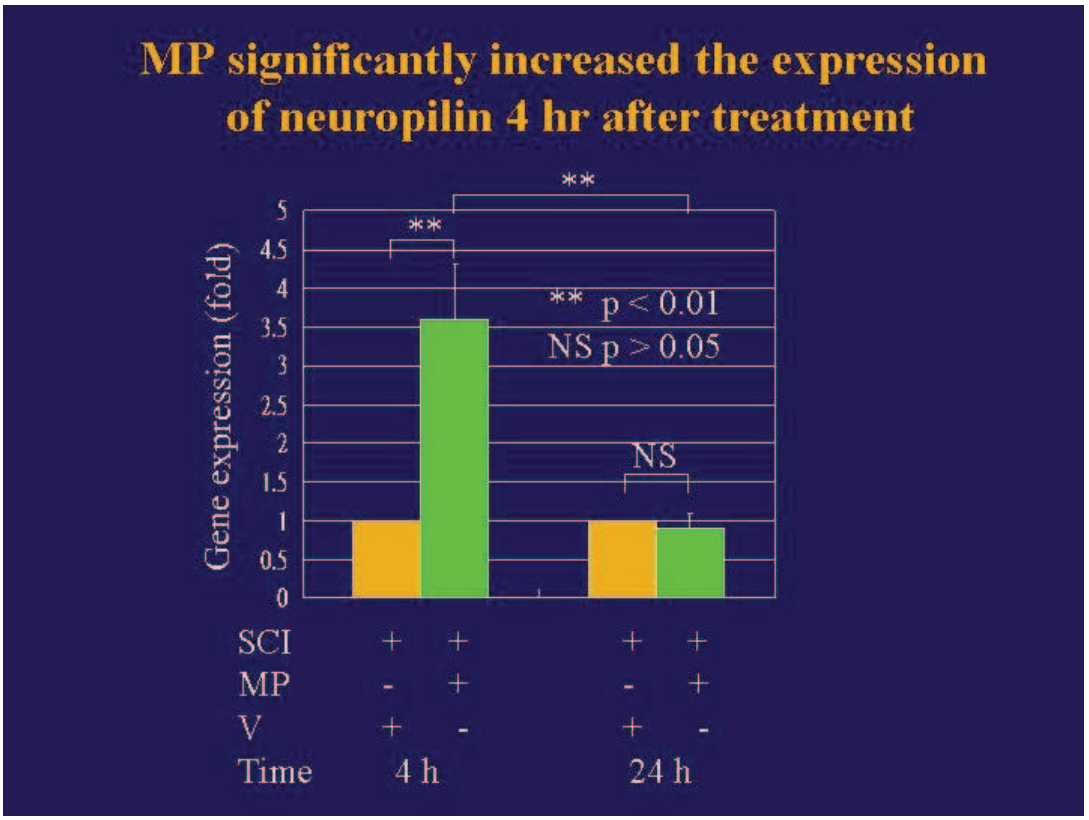

Figure 2A. Application of methylprednisolone significantly increases the expression of neuropilin in SCI animals 4 h after treatment.

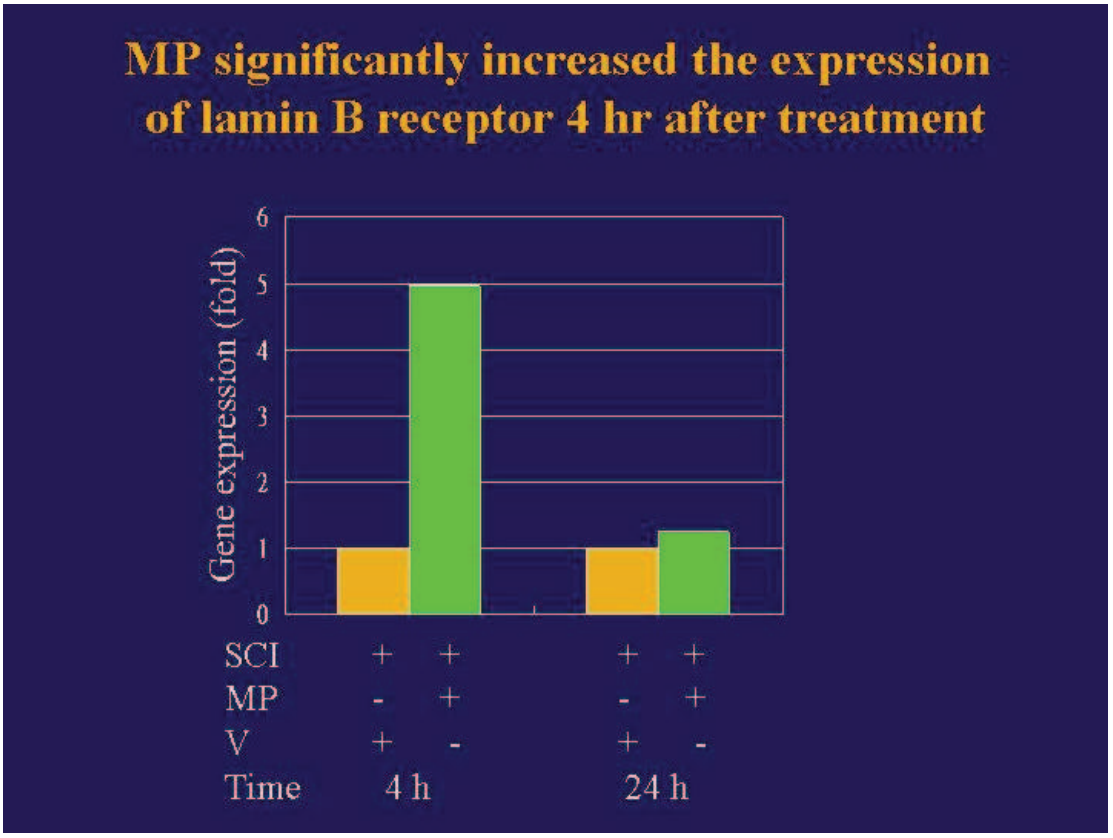

Figure 2B. The expression of lamin B receptor in SCI animals increases dramatically 4 h after methylprednisolone treatment.

MP treatment drastically suppressed the expression of calpain 5

MP treatment following SCI suppressed the expression (the mean expression is lower than 0.5 fold) of 118 genes in three replicate cDNA microarrays. Among these 118 genes, MP treatment induced a lower than 0.57-fold expression in 12 genes when compared with the control group in all three replicates, including calpain 5 (Figure 2C) and dimethyladenosine transferase (Figure 2D). Calpain 5 is a cysteine-type peptidase and endopeptidase and contributes to apoptosis and necrosis of cells after activation (Ray et al., 1999; Bianchi et al., 2004). Previous findings indicate that spinal cord injury increases the expression of calpain in the lesioned spinal cord (Banik et al., 1997; Ray et al., 1999). Evidence also shows that inhibition of calpain activity decreases the amount of apoptosis in the lesioned spinal cord of SCI animals (Ray et al., 1999). MP-induced suppression of calpain 5 and the decreased apoptosis by inhibition of calpain activity together strongly suggest that MP may exert its neuroprotective effect in SCI animals via inhibition of calpain activity.

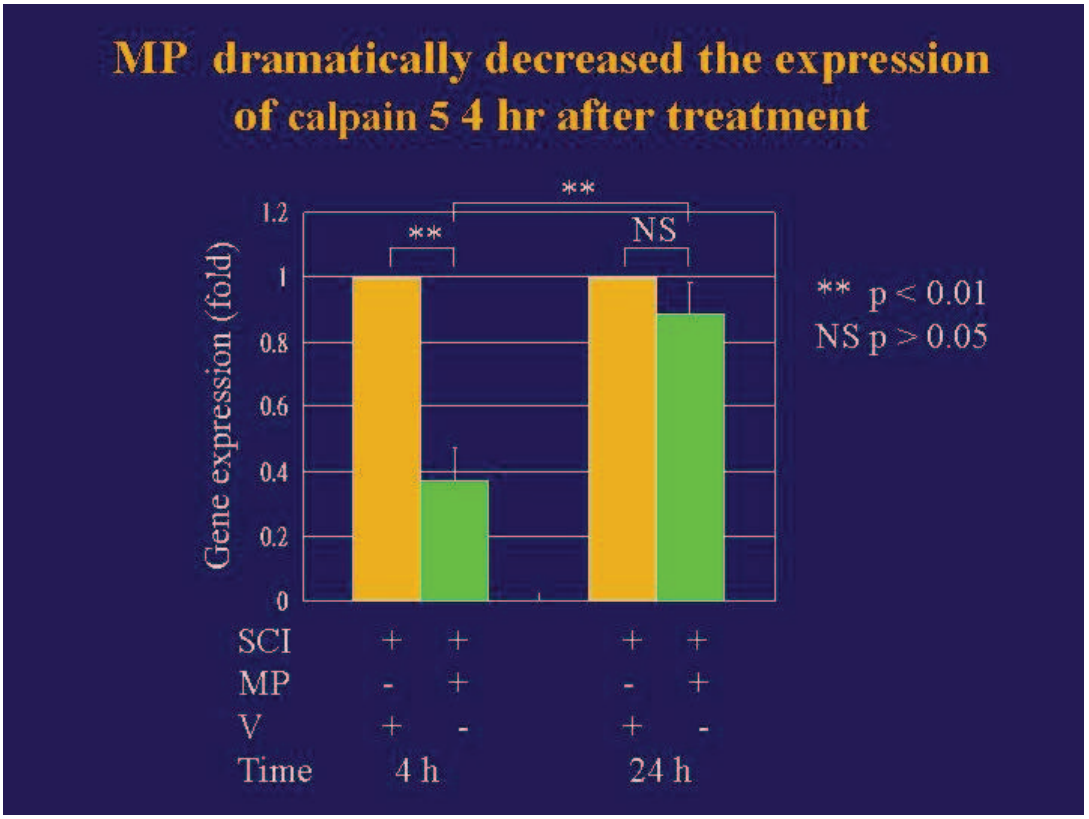

Figure 2C. Application of methylprednisolone dramatically suppresses the expression of calpain 5 in the injured spinal cords 4 h after treatment.

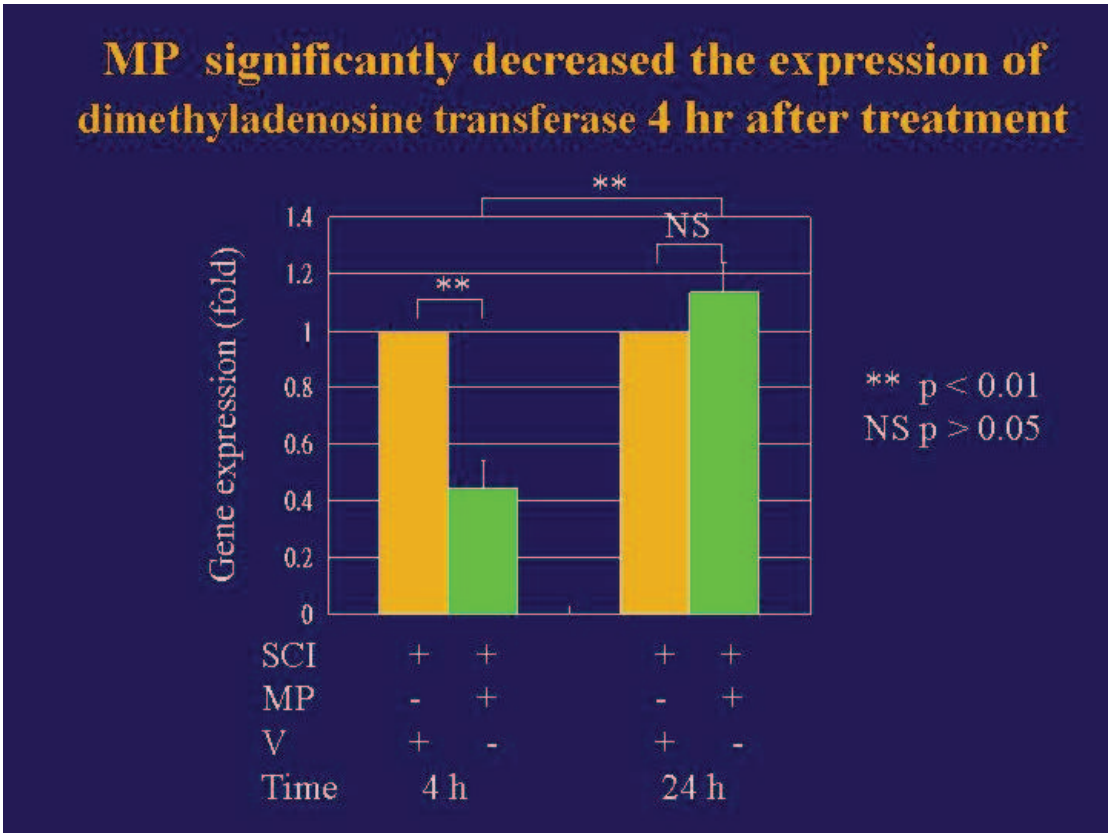

Figure 2D. Methylprednisolone inhibits the expression of dimethyladenosine transferase in SCI animals 4 h after application.

SCI decreased the Bcl-x_L expression, but increased apoptosis of oligodendrocytes (OLG)

The recent findings of our research group indicated that SCI reduced Bcl-x_L expression for up to 14 days after injury (Fig 3A). The immunocytochemistry data also showed the decrease of Bcl-x_L expression in both gray matter (GM) and white matter (WM) surrounding the impact site (Fig 3B). Furthermore, SCI induced DNA fragmentation, a biochemical feature of apoptosis (Fig 3C). Prominent among cells undergoing apoptosis in WM were oligodendrocytes (OLG), which immunostained for both activated caspase-3 and the OLG marker PLP (Fig 3D).

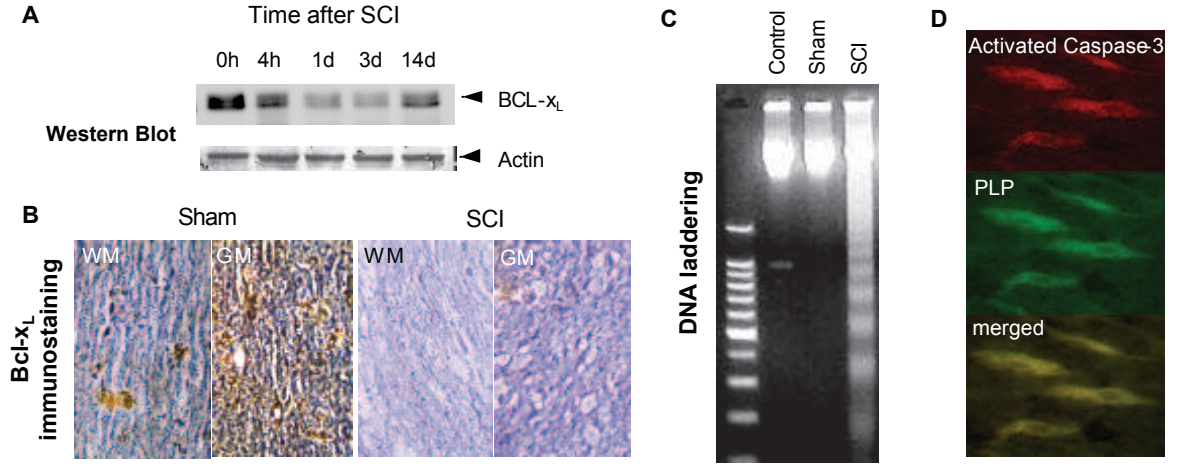

Figure 3. Following thoracic laminectomy, rats were subjected to SCI or sham surgery. At the times indicated, rats were sacrificed, and the lesioned spinal cords including the adjacent area or sham-operated spinal cords were removed. Tissues were processed for Western blotting (0 h to 14 days after SCI), immunohistochemistry (24 h after SCI), or DNA laddering (24 h after SCI). (A) Western blotting results showed that SCI decreased the Bcl-x_L expression for up to 14 days. (B) Immunohistochemical data demonstrated that SCI dramatically reduced Bcl-x_L immunoreactivity (brown) in both gray and white matters. (C) SCI also resulted in DNA laddering 24 h after injury. (D) SCI resulted in apoptosis of oligodendrocytes manifested by double labeling of activated-caspase-3 (Texas Red) and PLP (FITC) (oligodendrocyte marker) in the white matter of lesioned spinal cords.

MP reduced internucleosomal DNA fragmentation and TUNEL staining

Recently, three groups have reported preliminary evidence that GCs may inhibit apoptosis following SCI (Ray et al., 1999; Brandoli et al., 2001; Zurita et al., 2002). Our findings are in agreement. Rats treated with MP (30 mg/kg) 10 min after spinal cord contusion resulted in a decrease in DNA laddering (Fig 4A), and a decrease in TUNEL-positive cells (Fig 4B), quantified by counting cells in 5 sections separated by 30 μm at the epicenter (Fig 4C). Co-treatment with RU486 reversed the protective effects of MP.

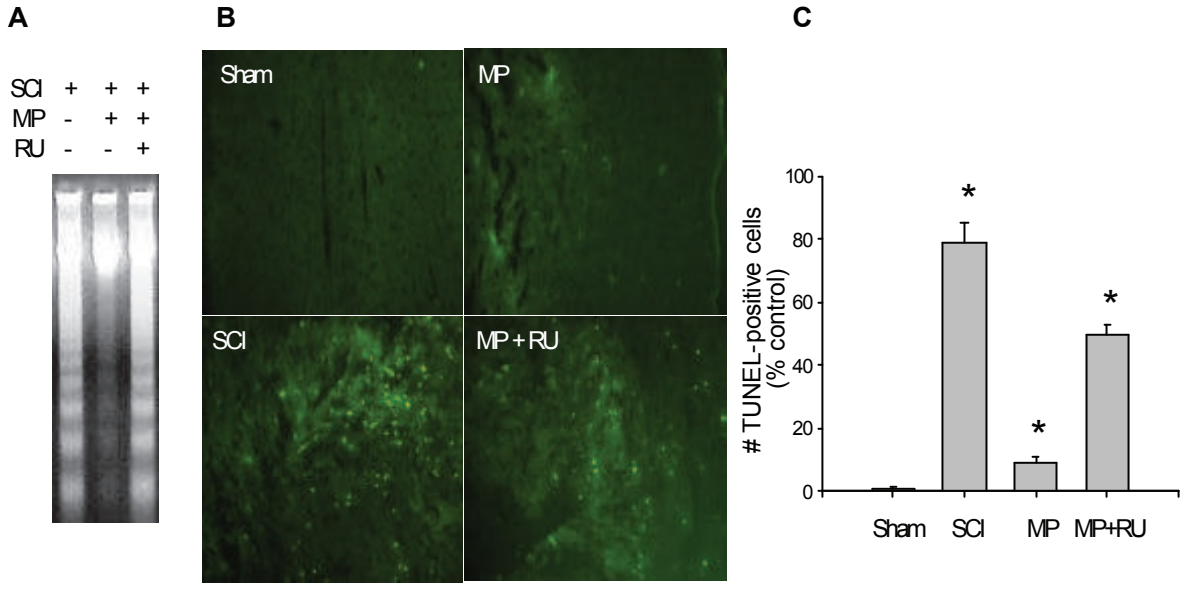

Figure 4. Spinal cords from sham-operated rats or SCI rats treated with vehicle, MP, or MP+RU486 were processed for DNA laddering and TUNEL immunohistochemistry 24 h after SCI or sham operation. DNA was extracted from spinal cords and electrophoresed. (A) DNA laddering experiments indicated that MP decreased DNA fragmentation induced by SCI, but RU486, a glucocorticoid receptor antagonist, counteracted the MP's effect. (B) Fifteen μm sections were obtained at the epicenter of sham-lesioned or lesioned spinal cords and processed for TUNEL immunohistochemistry. SCI increased the TUNEL (apoptotic marker) staining but MP treatment

dramatically decreased the TUNEL immunoreactivity. However, RU486 significantly attenuated the MP's effect on reducing TUNEL staining. (C) SCI significantly increased the percentage of TUNEL-positive cells. MP treatment drastically reduced the number of TUNEL-positive cells induced by SCI, however, RU486 significantly attenuated the MP's effect. *p < 0.01 ANOVA, with post-hoc Bonferroni test, compared to sham.

MP enhanced Bcl-x_L expression after SCI

To determine the effect of MP treatment on Bcl-x_L expression after SCI, we performed RT-PCR, Western blotting and immunohistochemistry on spinal cord samples from rats treated with MP with or without GR blockade by RU486. SCI decreased Bcl-x_L (mRNA and protein), but increased Bcl-x_S mRNA (Fig 5A, B). Treatment with MP increased Bcl-x_L and reduced Bcl-x_S mRNA; and cotreatment with RU486 reversed MP's actions (Fig 5A, B). Immunohistochemical staining revealed that MP increased Bcl-x_L in both gray matter and white matter (Fig 5C). These data are consistent with the contention that MP regulates Bcl-x_L expression, and may contribute to its neuroprotective effects after SCI.

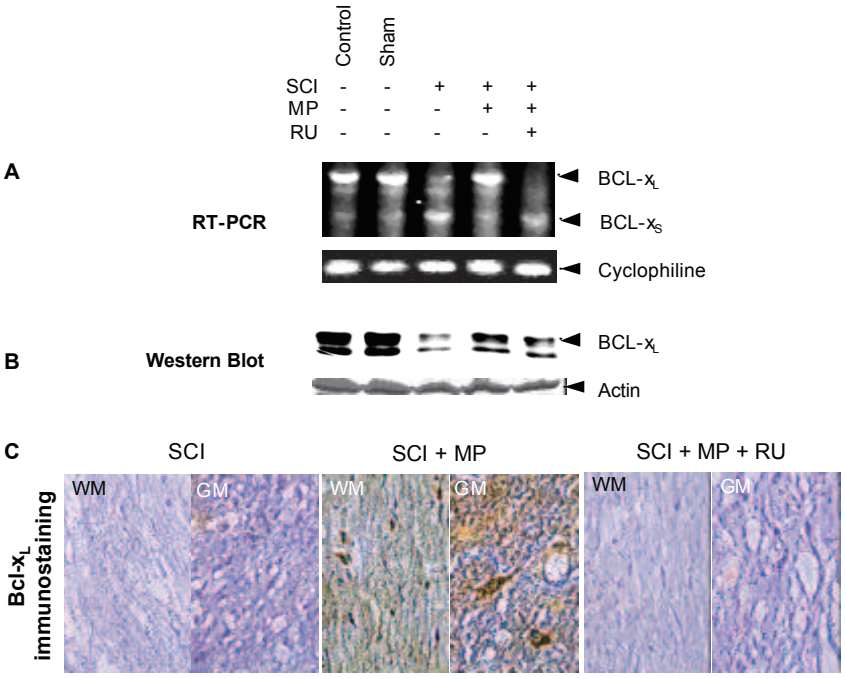

Figure 5. Spinal cords from sham-operated rats or SCI rats with vehicles, MP or MP+ RU486 were removed and processed for RT-PCR, Western blotting and immunohistochemistry 24 h after surgery. (A) RT-PCR data showed that SCI decreased the expression of Bcl-$_{XL}$ mRNA, but increased the expression of Bcl-$_{XS}$ mRNA. MP treatment after SCI significantly increased the Bcl-$_{XL}$ expression, but decreased Bcl-$_{XS}$ expression. Nonetheless, the MP's effect on Bcl-$_{XL}$ and Bcl-$_{XS}$ expression was drastically decreased by cotreatment of RU486. (B) Western blot data showed the expression of Bcl-$_{XL}$ protein was decreased by SCI. MP treatment after SCI increased the expression of Bcl-$_{XL}$ protein. Cotreatment of RU486 with MP attenuated the MP's effect on Bcl-$_{XL}$ protein expression. (C) Immunohistochemistry data showed the similar results.

CONCLUSION

Our cDNA microarray data show that MP treatment significantly increases the expression of neuropilin (VEGF receptor) and inhibits the expression of calpain 5 four h after application. Previous findings have demonstrated that application of VEGF or inhibition of calpain activity significantly promotes the functional recovery in SCI animals. Moreover, our immunohistochemical studies reveal that MP treatment decreases the amount of apoptotic cells and enhances the expression of cell survival-promoting gene – Bcl-xl in lesioned spinal cords. These findings together lead us to propose the hypothesis that MP exerts its neuroprotective effect on SCI animals via multiple mechanisms that converge on promoting cell survival in the injured spinal cord (Figure 6).

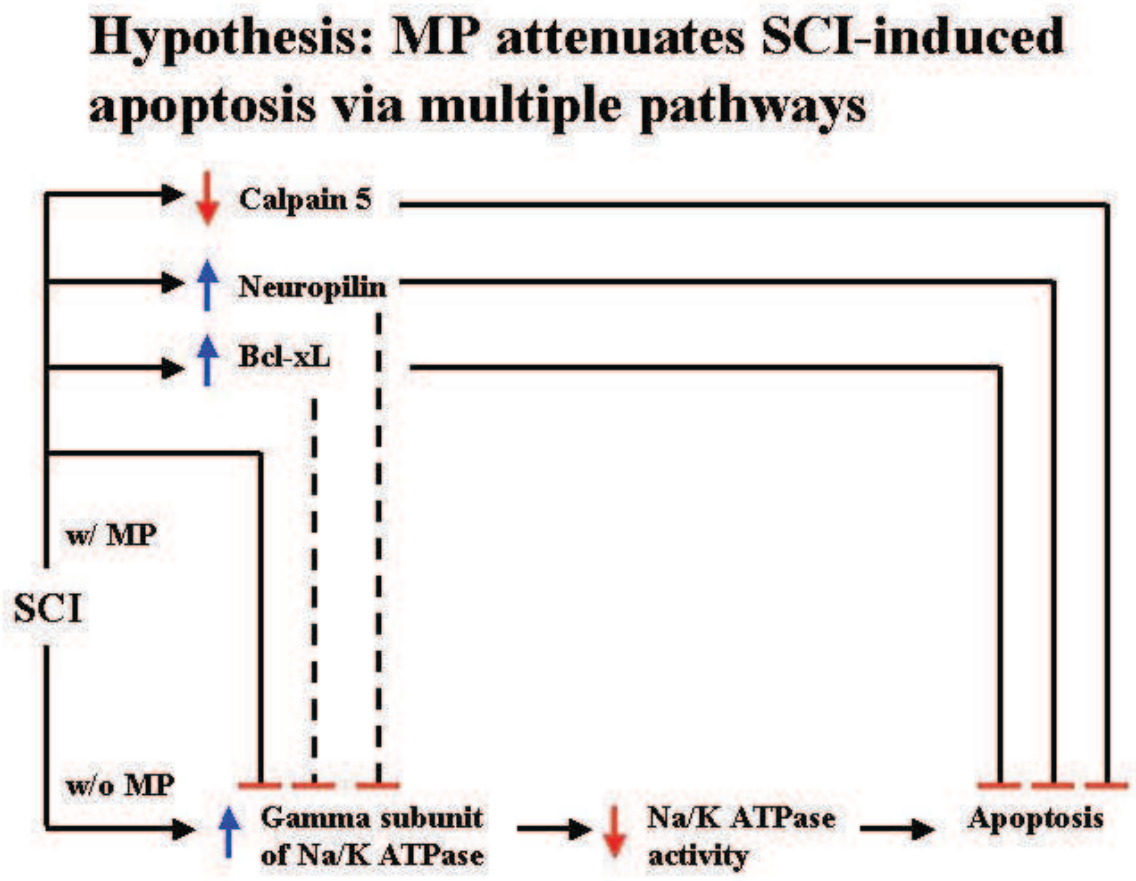

Figure 6. MP may attenuate the SCI-induced apoptosis via multiple mechanisms.

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SELF-EVALUATION OF GRANT PROGRESS REPORT

Up to now, we have carried out the goals proposed in this animal core in a satisfactory way: (1) to establish an SCI animal core at Taipei Medical University and (2) to provide SCI animals for studying genetic mechanisms of glucocorticoid neuroprotection proposed in component projects 1, 2 and 3. We have successfully set up an SCI animal core at Taipei Medical University and have generated sets of modified spinal clamps for rats and mice. The modifications of an NYU impactor clamps have facilitated the SCI surgery at this animal core. Moreover, we have provided the component projects in this PPG with the SCI animals they needed. We also performed immunohistological evaluation of lesioned spinal cords to support component project 1. Furthermore, we have assessed the gene profiling in SCI animals with or without MP treatment by using cDNA microarray technique. We are currently verifying the expression of MP up-regulated or down-regulated genes by using real-time RT-PCR. Our results strongly suggest that MP protects SCI animals via multiple pathways leading to the enhancement of cell survival following SCI. The discovery of genes activated or suppressed by MP may lead to the breakthrough in the SCI research and facilitates the development of an innovative therapeutic strategy for treating SCI patients.

During the grant funding period, we have published one manuscript in Annals of the New York Academy of Sciences (In press, 2005) and one manuscript is under review of Journal of Cellular Biochemistry. Moreover, we presented two posters at the Annual meeting of Society of Neuroscience last October. We acknowledged the NSC support in both manuscripts and both posters.

Liang-Yo Yang, Wun-Chang Ko, Chun-Mao Lin, Jia-Wei Lin, Jen-Chine Wu, Chien-Ju Lin**,** Huey-Hwa Cheng, and Chwen-Ming Shih. Antioxidant N-acetylcysteine blocks nerve growth factor-induced H_2O_2/ERK signaling in PC12 cells. Annals of the New York Academy of Sciences (2005) (SCI).

Liang-Yo Yang, Jia-Wei Lin, Shu-Fen Lee,Tsung I Hung, Yau-Huei Wei**,** Chwen-Ming Shih. Antioxidant enzymes as differentiation markers of hepatocellular carcinoma (Submitted).

Conference presentation

Yang, L. Y., Zhang, Y., & Chang C. Evaluation of CNS sexual dimorphisms in androgen receptor knockout mice. Society for Neuroscience, 34th Annual Meeting, San Diego, California, October 23-27, 2004, Abstract No 72.3.

Lin, J. W., Wei, L., Chiu, W. T., & ***Yang, L. Y.** Early detection of dopaminergic neuronal loss by biochemical changes in cerebrospinal fluid. Society for Neuroscience, 34th Annual Meeting, San Diego, California, October 23-27, 2004, Abstract No 562.20.