Electromagnetic Biology and Medicine, 29: 113–121, 2010

ISSN: 1536-8378 print/1536-8386 online
DOI: 10.3109/15368378.2010.500568

informa
healthcare

Magnetic Stimulation Influences Injury-Induced Migration of White Matter Astrocytes

ZHENG-YU FANG,[1] ZHE LI,[1] LIANG XIONG,[2] JIE HUANG,[1] AND XIAO-LIN HUANG[1]

[1]*Department of Rehabilitation Medicine, WHO Collaborating Centre for Training and Research in Rehabilitation (CHN-60), Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*
[2]*Department of Respiratory Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China*

This study investigates the effects and underlying mechanism of magnetic stimulation on injury-induced migration of white matter astrocytes. Twenty-four adult healthy SD rats were selected to inject 0.5 ml of 1% ethidium bromide (EB) in PBS into the dorsal spinal cord funiculus on the left side at the T10-11 level to make located spinal cord injury models. Then they were randomly divided into four groups (A, B, C, and D). Groups A, B, C, and D were exposed to 1 Hz pulsed magnetic stimulation underwent 5-min sessions on 14 consecutive days at the following levels: 0T (Group A) 1.9 × 40% T (Group B); 1.9 × 80% T (Group C); 1.9 × 100% T (Group D). On day 14 after stimulation, the rats were killed and the expression of glial fibrillary acidic protein (GFAP), microtubule associated protein-2 (MAP-2), extracellular signal-regulated kinase1/2 (ERK1/2), and the volume of holes were detected with immunohistochemistry. Quantitative analysis of the expression of GFAP, MAP-2, and ERK1/2 were performed with the image analysis system. With the increase of magnetic stimulation intensity, the volume of hole decreased at day 14 ($P < 0.05$). In lesion areas, the expression of GFAP and ERK1/2 could be seen, while that of MAP-2 did not change before and after magnetic stimulation. Significant difference was revealed in the expression of GFAP, ERK1/2 among the four groups. It was significantly higher in the magnetic stimulation groups than that in the control group ($P < 0.05$). After magnetic stimulation, astrocytes migrated into the hole. U0126, a potent and selective MEK1/2 inhibitor, inhibited up-regulation of pERK1/2 which was stimulated by magnetic stimulation. These data indicate that magnetic stimulation increases the migratory capacity of reactive white matter astrocytes in the injured center nervous system, which may be associated with activation of MEK1,2/ERK mitogenic pathway.

Keywords Magnetic stimulation; Astrocyte; Migration.

*Zhe Li is Co-First author.

Address correspondence to Xiao-Lin Huang, Department of Rehabilitation Medicine, WHO Collaborating Centre for Training and Research in Rehabilitation (CHN-60), Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China; E-mail: xiaolinh2006@yahoo.com.cn

Introduction

Astrocytes play a crucial role in CNS injury and disease, e.g., in the formation of a glial scar, and restoration of a disrupted glial limitans and blood-brain barrier. Reactive white matter astrocytes are key players in the development of a non permissive environment to neurite growth, in the development of a glial scar following injury, and appear to be critical for efficient remyelination by oligodendrocytes following demyelination (Silver et al., 2004). After spinal cord injury, glial scar because of reactive gumnosis forms mechanical barrier of neural regeneration, which become an obstruction of axonal regrowth and nerve grafts (Pekny et al., 2005). Glial scar occurs mainly by astrocyte migration. If we can regulate the migration of astrocytes, and then adjust the formation of glial scar, we maybe ameliorate the environment of nerve regeneration and improve nerve regeneration.

Since the 1990s, people have paid close attention to the application of magnetic stimulation technology in central nervous system diseases (Koch et al., 2009; Nardone et al., 2008). In recent years, the effects of magnetic stimulation on nerve regeneration have become the hot point of research. Magnetic stimulation improves microenvironment of nerve regeneration by adjusting ionic equilibrium, neurotrophic factors, c–fos genes, glial cell, and cell apoptosis after neural injury. After spinal cord injury, water in spinal cord tissues increases, calcium ion steps up, and magnesium ion descends in the zone of injury, while magnetic stimulation could lessen ion disequilibrium post spinal cord injury and have protective effect to secondary spinal cord injury (Crowe et al., 2003; Krause et al., 2003; Philip et al., 1999; Tsai et al., 2009).

Some studies suggest that magnetic stimulation may influence the formation of glial scar. However, the effect is indefinite. The reason perhaps is that stimulation intensity, frequency, interval, and stimulation volume dose impact the effect of magnetic stimulation. Our research is going to select specified spinal cord injury animal model and adopt fixed stimulation frequency and stimulation dose to investigate the effect of different stimulation intensity on injury-induced migration of white matter astrocytes.

Material and Methods

Animals

The experiments were carried out in accordance with the Declaration of Helsinki. The ethical approval was granted by the Animal Care Committee of Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Adult (220–300 g) male Sprague–Dawley rats were used in the experiments. The rats were housed in a temperature- and humidity-controlled environment and were maintained on a 12 h light/dark cycle. Food and water were available ad libitum.

Ethidium Bromide Injury

Twenty-four SD rats were used for this study (0 T ($n = 6$), $1.9 \times 40\%$ T ($n = 6$), $1.9 \times 80\%$ T ($n = 6$) and $1.9 \times 100\%$T ($n = 6$)). A partial laminectomy was made over the dorsal spinal cord at the T10-11 level, and 0.5 µl of 1% ethidium bromide (EB) in PBS was injected over a period of 5 ms with a glass micropipette (outer

diameter 20 μm) in the dorsal funiculus on the left side. Care was taken to keep the same angle and penetrating distance from the spinal cord surface with the micropipette (Fang et al., 2006). Animals were allowed to survive for 14 days.

Magnetic Stimulation

The electromagnetic data of the magnetic stimulator (Dantec magnetic Stimulator-Maglite, Denmark) were set as: stimulation wave form; biphasic; frequency, 1 Hz. We used magnetic coil transducer MC 125(number of winding: 13; inner radius: 10 mm; outer radius: 60 mm; winding height: 6 mm; encapsulation overall 2 mm PVC minimum, Dante code: 16 ess). The animals were exposed to 1 Hz (0 T, 0.76 T, 1.52 T, or 1.9 T, respectively) magnetic stimulation. Continuous stimulation of 5 min was applied once daily for 14 days. The animals were placed below the center of the magnetic coil and stimulated at room temperature at the following intensities: Group A: 0 tesla; Group B: 0.76 tesla; Group C: 1.52 T; and Group D: 1.9 T. In Group E, mice were pre-treated with 10 μM U0126, then exposed to 1 Hz, 1.9 T magnetic stimulation. A control group without exposure to stimulation was subjected to the same environmental influences and analyzed in an identical manner as the experimental groups.

Preparation of Tissue from Ethidium Bromide Experiments

After EB injection, A, B, C, and D group were exposed to 0 T (1 Hz), 0.76 T (1 Hz), 1.52 T (1 Hz), or 1.9 T(1 Hz) magnetic stimulation, respectively. Fourteen days after magnetic stimulation, the animals were anesthetized and perfused via the left ventricle, first with saline (37°C), followed by a solution of 4% formaldehyde (w/v) and 14% saturated picric acid solution (v/v) in a 0.15 M phosphate-buffered saline (PBS; pH 7.4, 4°C). Intact control animals were perfused in the same way. Spinal cord segments Th 10–11 were removed, tissue blocks post-fixed for 4 h, and cryoprotected overnight in a solution of 10% sucrose in PBS (4°C). Paraffin sections (5 μm) were made in the horizontal plane from the spinal cord, placed on SuperFrost Plus glass slides, and stored at −4°C until immunostaining.

Lesion Volume Calculation

According to the principle of stereoscopy, when an irregular object is cut parallel to enough diminutive, every unit of the object can be looked upon as a stylidium. Section area multiplies dissection interval amounts to unit volume. The volume of the irregular object is the sum of every unit volume, that is to say, the sum of all section area multiplies section internal. Hematoxylin and eosin stain every section. According to the principle, we can calculate the volume dose of every lesion (Krause et al., 2003; Tei et al., 2008).

Immunohistochemistry

The sections were thawed at room temperature until water condensations on the slides had disappeared. A blocking solution (1% bovine serum albumin (w/v), 0.3% Triton X-100, and 0.1% sodium azide in PBS) was gently put on top of each section for 1 h at room temperature. After blocking, the solution was removed and then

sections were incubated overnight at 4°C in the same solution with primary GFAP, MAP-2, ERK1/2 antibodies. The slides were washed three times in PBS (7 min/wash) and then incubated with secondary antibodies. The immune complexes were visualized with Texas Red-conjugated donkey anti-rat IgG (Jackson Immuno-Research Inc.) diluted 1:50 in PBS with 0.3% Triton X-100 and 0.1% sodium azide for 4 h at room temperature. After another round of washing (three times, 7 min/rinse in PBS) the slides were mounted in a mixture of PBS and glycerol (1:1; v/v) containing 0.1 M n-propyl-gallate to minimize photobleaching.

Western Blot Analysis

The rats' spinal cords were removed and segments Th10–11 were used for assay of the protein expression of GFAP, MAP-2, ERK1/2. The spinal cord were quickly isolated and rinsed in sterilized water on ice, and then stored at 80°C until use. Protein determination was performed according to the Lowry method. The obtained protein samples were subjected to 15% sodium dodecylsulfate-polyacrylamide gel electrophoresis, using 7.5–15% polyacrylamide gel, and electrotransferred to polyvinylidene difluoride filter (PVDF) membranes (Millipore Products, Bedford, MA). To reduce non specific binding, the PVDF was blocked for 2 h at room temperature with 5% non fat milk in phosphate-buffered saline (PBS). Then membranes were incubated overnight at 4°C with the primary antibodies for GFAP (anti-rat GFAP mouse monoclonal antibody, 1:500 dilution, Santa Cruz), MAP-2 (anti-rat MAP-2 mouse monoclonal antibody, 1:1000 dilution, Santa Cruz), ERK1/2 (anti-mouse ERK1/2 monoclonal antibody, 1:500 dilution, Santa Cruz), respectively. After incubation with the antibodies, the membranes were washed with PBS-Tween-20 (PBS-T: 10 mmol/L phosphate buffer, pH 7.4, 150 mmol/L NaCl, 0.05% Tween 20) for 30 min and incubated in the relevant horseradish peroxidase-conjugated secondary antibody (1:600 dilution) for 30 min. The membranes were washed again with PBS-T and immunoreactive protein bands were visualized using the enhanced chemiluminescence detection system.

Result

Calculation of Lesion Volume

We quantified cavity volume in Hematoxylin and eosin-stained tissue sections (Fig. 1). Every sample including lesion was cut into 40–50 serial sections. We can see that with the increasing of magnetic stimulation intensity, the lesion volume of Groups A, B, C, and D decreases (Figs. 1 and 2). The quantitative analysis confirmed the marked decrease in the lesion volume in Groups B, C, D compared to Group A ($P < 0.05$).

Figure 1. Cross-section of spinal cord (H&E stain 40X. Scale bar = 200 μm, A: 0 T; B: 1.9 × 40% T; C: 1.9 × 80% T; D: 1.9 × 100% T; E: pre-treated with 10 μM U0126, then exposed to 1 Hz, 1.9 T magnetic stimulation).

Expression of GFAP, ERK1/2, and MAP-2

With the increasing of magnetic stimulation intensity, the number and signal optical density of GFAP and ERK1/2 increase gradually in Groups A, B, C, and D. Significant difference was revealed in the expression of GFAP, ERK1/2 among the four groups (Figs. 3, 4, 5, 6). It was significantly higher in the magnetic stimulation groups than that in the group A ($P < 0.05$). No difference was observed in the percentage of cell positive for MAP-2 in four groups (A, B, C, D). These results suggest that more astrocytes migrate into the lesion areas in Groups B, C, and D.

U0126 Inhibits Up-Regulation of ERK1/2 Which is Stimulated by Magnetic Stimulation

To investigate the role of the ERK/MAPK pathway in injury-induced white matter astrocytes, we assessed the expression of pERK 1/2 after magnetic stimulation. Magnetic stimulation increased the expression of pERK1/2. U0126 is a potent and

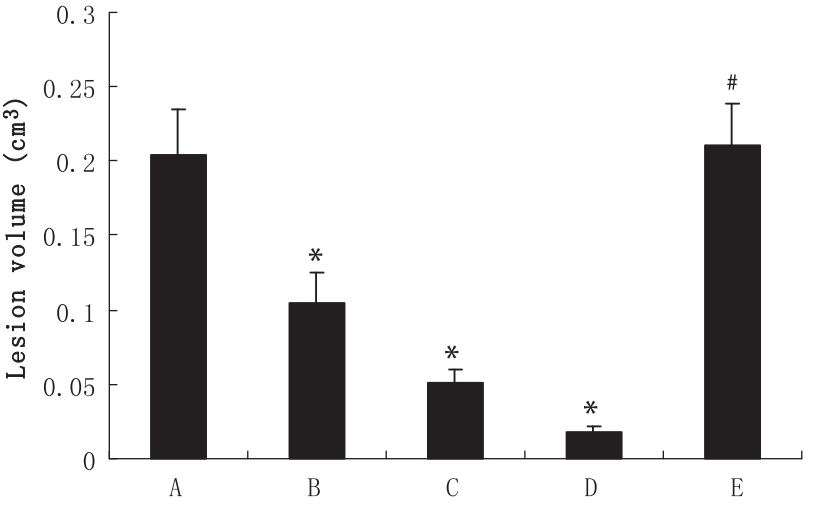

Figure 2. Lesion volume in different groups ($^{*}P < 0.05$, $^{\#}P > 0.05$).

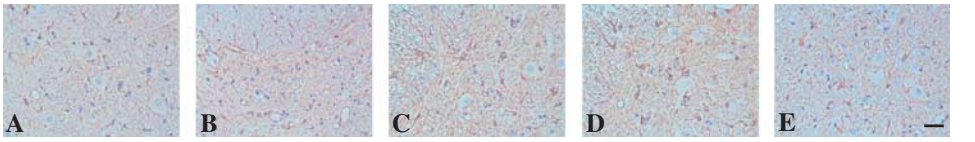

Figure 3. Immunostaining for GFAP in lesion area following magnetic stimulation (Scale bar = 50 μm).

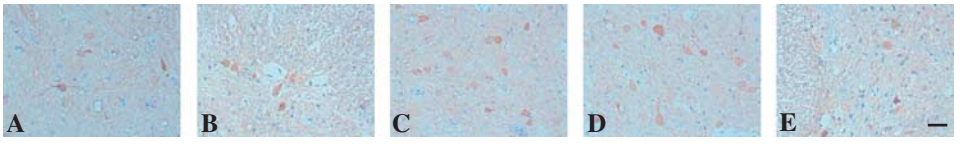

Figure 4. Immunostaining for ERK1/2 in lesion area following magnetic stimulation (scale bar = 50 μm).

selective non competitive MEK 1/2 inhibitor. U0126 inhibited up-regulation of pERK 1/2 which was stimulated by Magnetic stimulation (Fig. 4E). Western blot analysis confirmed the inhibition of ERK1/2 expression in U0126 pre-treated spinal

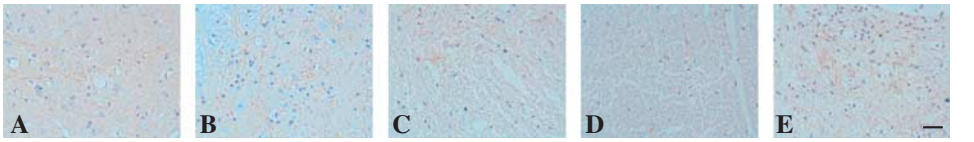

Figure 5. Immunostaining for MAP-2 in lesion area following magnetic stimulation (scale bar = 50 μm).

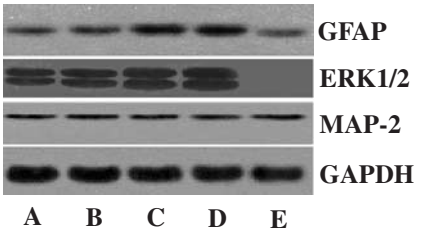

Figure 6. The level of GFAP, ERK1/2, MAP-2 in different groups were analyzed by Western blots.

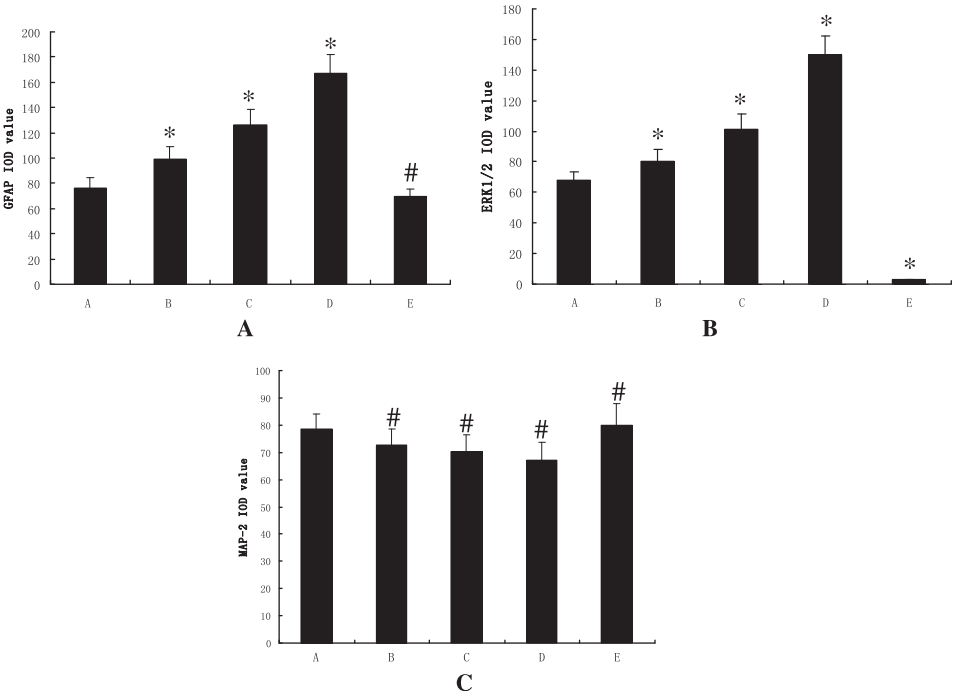

Figure 7. Comparison of the expression GFAP, ERK1/2, MAP-2 in different groups by Western blots.

cord lesion (Fig. 6E). The bands were more intense with the increase of MS, representing the increase in ERK 1/2 expression (Figs. 6A, B, C, D). There were no bands in U0126 pre-treated spinal cord (Figs. 6, 7), indicating that U0126 inhibited the basal levels of ERK 1/2 as well as the MS-induced activation of ERK 1/2 after injury.

U0126 Inhibits Magnetic Stimulated Astrocytes Migration into Spinal Cord Lesion Areas

Then the effect of U0126 on astrocyte migration after magnetic stimulation was investigated. Mice were pre-treated with 10 μM U0126 or 0.1% DMSO and examined 14 days post-injury. The data show that 10 μM U0126 partially inhibited the migration of astrocyte into the lesion area (Figs. 1E, 2, 3E, 4E). Cells were stained with Coomassie blue prior to Discovery-1 analysis. Quantification and comparison of lesion area measurements by Discovery-1 showed that U0126 significantly inhibited the migration of astrocyte after magnetic stimulation, suggesting the involvement of the ERK/MAPK mitogenic pathway in the migration of astrocyte after magnetic stimulation.

Discussion

In this research, we chose ethidium bromide (EB) to make focal spinal cord injury model. After injury oligodendrocytes, myelin and astrocytes are deleted quickly in injuried region (Blakemore et al., 1982; Faber-Elman et al., 1996; Fushimi et al., 2002), so that the incremental cells in the injured region is that migrating into the area newly.

Various kinds of CNS injury can induce astrocyte reaction, that is to say, reactive gumnosis (Malhotra et al., 1990). Astrocyte reactive gumnosis has dualism: induction in naïve period and inhibitory action in maturation period. When nerve fiber regenerates, astrocytes are in naïve period whose synthesis is active, construct reticulate framework, and support nerve fiber regeneration (Hinkle et al., 1997). When nerve fiber hypoplasia, astrocyte gumnosis transforms from naive period to maturation period. Astrocytes transfer to structural glial cell which forms machinery barrier and stops nerve regeneration. In adult CNS, the formation of glial scars is because of astrocyte migration into lesion area but not proliferation (Joanne et al., 2007; Kurtis et al., 2007). GFAP, the marker of astrocytes, is a kind of microfilament whose molecular mass is 55 ku and is used for labeling post-injury gum response. In our study with increasing of magnetic stimulation intensity, the focal spinal cord injury lesion volume wound down. On the edge of cavity, GFAP took on positive expression, which suggested that magnetic stimulation raise astrocytes migration and promote the formation of the glial scar.

Different from GFAP, magnetic stimulation caused no remarkable expression changes of MAP-2. Microtubule-associated protein (MAP-2) is one of structural protein of microtubular. Research showed that MAP-2 participated in neuron development,structure stability,apophysis formation, and synaptic plasticity regulation, which was important to the generation,elongation, and stability of neuron axon and dendrite (Ma et al., 1999). MAP-2 highly express in CNS neurons chiefly. In addition, MAP-2 also express a small quantity in dorsal root ganglias, activated glial cells, and oligodendrocytes. The effects of MAPs in CNS include two aspects: one is that they interact with microtubules and promote the assembly and

stability of microtubules; the other is that they are relevant to axonal transportation. Research found that the activity descending of MAP-2 could cause microtubule to degenerate and accumulate, impact cytoskeleton integrity, and disturbance the axonal transportation of mitochondrion which leads neurons to death in the end.

Consistent with the changes of GFAP, magnetic stimulation increased the expression of ERK1/2. Furthermore, there was a positive correlation between the expression of ERK1/2 and GFAP. Extracellular signal-regulated kinase (ERK) is one of important members of mitogen activated protein kinase signal transduction passageway group, mainly mediating proliferative reaction including cellular cleavage, growth, migration, and differentiation (Talmor et al., 2000; Song et al., 2006; Su et al., 2009). The positive correlation between ERK1/2 and GFAP suggested that astrocyte migration was relevant to mitogen activated protein kinase signal pathway. U0126, potent MEK 1/2 inhibitor, blocked ERK expression. However, the migration of white matter astrocytes was inhibited completely, suggesting that other pathways are also involved in this process.

With the increasing of magnetic stimulation intensity, the lesion volume of focal spinal cord decreases. On the edge of lesion, GFAP and ERK1/2 had identical change tendency and took on positive expression, but MAP-2 did not express. It suggests that magnetic stimulation promote astrocytes migration but not neurons. What's more, with the enhancement stimulation intensity, the ability of astrocytes to migrate into lesion areas was strengthened, which may be relevant to ERK1/2 pathway.

The research of Fang et al. (2006) showed that remyelination induced by EB and mediated by oligodendroglia relies on the existence of astrocyte. Astrocytes' rapid colonization may help to promote nerve regeneration. According to this, the increasing of astrocyte migration ability by magnetic stimulation may conduce to repair quickly gum limiting membrane after central nerve injury and abate the formation of cavity after spinal cord injury as far as possible. However, it is pending to investigate that what degree astrocyte migrate to help to remyelination or lead to glial scar formation and stop nerve regeneration.

In conclusion, we find that magnetic stimulation play an important role in astrocyte migration in CNS pathophysiology. It help us to change the reaction after CNS injury, improving the function restoration of injured central nerve. However, the effect of magnetic stimulation depends on various factors, such as stimulation intensity, frequency, volume dose of stimulation, etc. In this research, we only investigate the effect of different intensity stimulation on astrocyte migration. Dose-effect relationship of frequency and stimulation dose needs further investigation.

Acknowledgments

Dr Fang Zhengyu was supported by the National Natural Science Foundation of China (NSFC, No. 30801220).

References

Blakemore, W. F. (1982). Ethidium bromide induced demyelination in the spinal cord of the cat. *Neuropathol. Appl. Neurobiol.* 8:365–375.

Chan, P., Feng, L., Lee, Y. *et al.* (1999). Effects of pulsed magnetic stimulation on GFAP levels in cultured astrocytes. *J. Neurosci. Res.* 55:238–244.

Crowe, M. J., Sun, Z. P., Battocletti, J. H. *et al*., (2003). Exposure to pulsed magnetic fields enhances motor recovery in cats after spinal cord injury. *Spine (Phila Pa 1976)* 15:2660–2666.

Faber-Elman, A., Solomon, A., Abraham, J. A. *et al*. (1996). Involvement of wound-associated factors in rat brain astrocyte migratory response to axonal injury: in vitro simulation. *J. Clin. Invest*. 97:162–171.

Fang, Z., Duthoit, N., Wicher, G., *et al*. (2006). Intracellular calcium-binding protein S100A4 influences injury-induced migration of white matter astrocytes. *Acta Neuropathol*. 111:213–219.

Fushimi. S., Shirabe, T. (2002). The reaction of glial progenitor cells in remyelination following ethidium bromide-induced demyelination in the mouse spinal cord. *Neuropathology* 22:233–242.

Hinkle, D., Baldwin, S., Schef, S. *et al*. (1997). GFAP and S100 gene expression in the codex and hippocampusin response to mild comical contusion, *J. Ncurotrauma* 14:729–738.

Joanne, H., Hannah, M., Gibbon, S. *et al*. (2007). Extracellular signal-regulated kinase involvement in human astrocyte migration. *Brain Res*. 1164:1–1213.

Koch, G., Brusa, L., Carrillo, F. *et al*. (2009). Cerebellar magnetic stimulation decreases levodopa-induced dyskinesias in Parkinson disease. *Neurology* 73:113–119.

Krause, P., Straube, A. (2003). Repetitive magnetic and functional electrical stimulation reduce spastic tone increase in patients with spinal cord injury. *Supp., Clin. Neurophysiol*. 56:220–225.

Kurtis, I., Songwan, J., Kazunori, U. *et al*. (2007). Greatly impaired migration of implanted aquaporin-4- deficient astroglial cells in mouse brain toward a site of injury. *FASEB J*. 21:108–116.

Ma, D., Chow, S., Obrocka, M. *et al*. (1999). Induction of microtubule associated protein 1B expression in Schwann cells during nerve regeneration. *Brain Res*. 823:141–153.

Malhotra, S., Shnitka, T., Elabrink, J. (1990). Reactive astrocytes: a review. *Cytobios* 61:133–160.

Nardone, R., Bergmann, J., Tezzon, F. *et al*. (2008). Cholinergic dysfunction in subcortical ischaemic vascular dementia: a transcranial magnetic stimulation study. *J. Neural Transm*. 115:737–743.

Pekny, M., Nilsson, M. (2005). Astrocyte activation and reactive gliosis. *Glia* 50:427–434.

Silver, J., Miller, J. H. (2004). Regeneration beyond the glial scar. *Nat. Rev. Neurosci*. 5:146–156.

Song, H., Moon, A. (2006). Glial cell-derived neurotrophic factor (GDNF) promotes low-grade Hs683 glioma cell migration through JNK, ERK-1/2 and p38 MAPK signaling pathways. *Neurosci. Res*. 56:29–38.

Su, C. M., Lu, D. Y., Hsu, C. J. *et al*. (2009). Glial cell-derived neurotrophic factor increases migration of human chondrosarcoma cells via ERK and NF-kappaB pathways. *J. Cell. Physiol*. 220:499–507.

Talmor, D., Applebaum, A., Rudich, A. *et al*. (2000). Activation of mitogen-activated protein kinases in human heart during cardiopulmonary bypass. *Circ. Res*. 86:1004–1007.

Tei, R., Kaido, T., Nakase, H., Sakaki, T. (2008). Protective effect of C1 esterase inhibitor on acute traumatic spinal cord injury in the rat. *Neurol. Res*. 30:761–767.

Tsai, P. Y., Wang, C. P., Chiu, F. Y. *et al*. (2009). Efficacy of functional magnetic stimulation in neurogenic bowel dysfunction after spinal cord injury. *J. Rehab. Med*. 41:41–47.