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Effects of Exercise on Axonal Regeneration and Growth-associated Protein (GAP-43) Expression Following Sciatic Nerve Injury in Rats

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Physical activity can improve sensorimotor recovery after peripheral nerve injury. Growth-associated protein 43 (GAP-43) is highly correlated with neuronal development and axonal regeneration and present in large quantities in the axonal growth cone. Using immunofluorescene staining and anterograde and retorgrade techniques, we identified enhanced axonal regrowth in distal stump of the sciatic nerve 3-14 days after crush injury in rats with treadmill training. We also carried out western blot to investigate GAP-43 protein expression in injured sciatic nerve. GAP-43 protein levels were highly induced in the injured sciatic nerve 3, 7 and 14 days compared with sedentary group. Thus, the present data provide a new evidence that treadmill training promoted axonal re-growth after injury and increased GAP-43 protein levels in the regenerating nerve.

Key words – axonal regeneration, retrograde, anterograde, GAP-43

The nervous system is divided into two main subsystems, the central nervous system (CNS), which consists of the brain and spinal cord and the peripheral nervous system (PNS), which includes all nervous tissue outside the central nervous system. These nervous system injuries cause severe sensorimotor impairment and functional disabilities. The ultimate goal of peripheral nerve repair is to restore normal sensory, motor and muscle function. Restoration of function following sciatic nerve injury often requires regrowth of the injured axons over relatively long distances and this situation may differ dramatically from the one encountered in animal experiments where nerve regeneration over a relatively short distance is required[1,2].

PNS has the capacity to regenerate injured axons leading to functional recovery whereas neurons of CNS do not regenerate successfully after injury. An important aspect underlying the opposing outcome of injured PNS and CNS neurons are the different molecular programs initiated in the axotomized neuronal cell bodies[14,15]. One important step for the eventual success or failure of a neuron to regenerate an injured axon is the expression of important regeneration-associated proteins[5,14,15]. Of many proteins expressed during nerve regeneration, GAP-43 protein is highly correlated with neuronal development and regeneration and it has been demonstrated that the protein is present in large quantities in the axonal growth cone[3,4,6,8]. Plantinga et al.[11] reported that the expression of GAP-43 in Schwann cells is up-regulated in degenerating peripheral nerve stumps following sciatic nerve injury. Tetzalff et al. [13] further suggested that GAP-43 protein may be secreted from regenerating axons and may play a role in axon-Schwann cell interactions during axonal development.

To observe the effects of exercise training in recuperation of sensorimotor function following sciatic nerve crush in the rat, various forms of exercise have been studied. Van and coworkers[17] reported that exercise enhances the return of sensorimotor function in the early phase of recovery from peripheral nerve lesion. They further suggested that the beneficial effects of 24 days of exercise after crush injury persist in the late phase of peripheral nerve recovery. While these studies strongly suggest that exercise may enhance axonal regeneration processes after nerve injury followed by improved functional recovery due to the promoted muscle innervation, these has been no report providing direct evidence of increased GAP-43 synthesis in regenerating nerves after animal's exercise. Thus, we have begun to investigate the influence of exercise training on GAP-43 protein expression and histological changes after sciatic nerve injury in adult rats.

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Material and Methods

Experimental animals and surgery

Six-week-old (body weight 200~250 g) male, Sprague-Dawley rats were housed individually in cages in a temperature-controlled room with a 12-h light: 12-h dark cycle and randomly divided into six groups; exercise groups with sciatic nerve injury (3, 7 and 14 days, n=5 in each group), Non-exercise groups with sciatic nerve injury (3, 7 and 14 days, n=5 in each group). Animals were anesthetized with a mixture of ketamin (80 mg/kg) and xylazin (5 mg/kg). Sciatic nerve was exposed and crushed with a pair of forceps held tightly for 30s twice at 1-min intervals[9]. Animals were recovered from anesthesia and sacrificed 3~14 days later. Animals were deeply anesthetized with a mixture of ketamine and xylazine. Sciatic nerves were separately dissected, immediately frozen, and kept at -70℃ until use. For the purpose of immunohistochemistry experiments, the sciatic nerves were prepared by dividing into the proximal stump, 5-mm segments proximal to the injury site, and the distal stump, 5-mm segments distal to the injury site.

Exercise paradigm

Exercise was performed by having rat run on a treadmill (Qmc, Quinton Inc., USA) at a speed of 18 meters/min on a horizontal platform for 30 min a day during 3 to 14 days. The animals were normally adapted to treamill working for 10 min/day on the second day before the start of this experiment.

Western blot analysis

Nerve was washed with ice-cold PBS, and sonicated under 50~200 μl of triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 25 mM β-glycerophosphate, pH 7.14, 2 mM sodium pyrophosphate, 2 mM EDTA, 1 mM Na3VO4, 1% Triton X-100, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μM benzamidine, 0.5 mM DTT, 1 mM PMSF). Ten micrograms of proteins were used for Western analysis using anti-GAP-43 antibody (H-100, Santa Cruz Biotech.). Electrophoresis of membrane proteins was preformed using a 12% SDS-polyacrylamide gel (SDS-PAGE) and then the proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Pall Corporation, U.S.A). Transferred to polyvinylidene difluoride (PVDF) membrane (Pall Corporation, U.S.A). Blocking of unspecific bindings was done using 3% BSA, 0.1% Tween 20 in TBS buffer for 1h at room temperature; then, the membrane was incubated overnight at 4℃. The membrane was washed and then incubated in a solution containing a 1:1000 dilution of polyclonal antibodies specific for the C-terminal of rat GAP-43 for one and half hour at room temperature. The membrane was washed again and then incubated in a solution containing a 1:1000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz, U.S.A) for one and half hour at room temperature. After washing, blotting proteins were visualized using Western blotting detection system, and then exposed to a Kodak Scientific Imaging Film (Esteman Kodak Co., U.S.A).

Histology and immunofluorescence staining

Nerve segments were embedded and frozen at -20℃. Sections (20-μm thickness) were cut on a cryostat and mounted on positively charged slides. For immunofluorescence staining, sections were fixed with 4% paraformaldehyde, 4% sucrose in PBS at room temperature for 40 min, permeablilized with 0.5% nonidet P-40 in PBS, and blocked with 2.5% horse serum and 2.5% bovine serum albumin for 4h at room temperature. Sections were incubated with anti-neurofilament-200 antibody (NF-200, clone no. N52, Sigma), then incubated with fluorescein-goat anti-mouse secondary antibody (Molecular probes) in 2.5% horse serum and 2.5% bovin serum albumin for 1h at room temperature and cover-slipped with gelatin mount medium. In the present experiment, we always included the control sections treated with secondary antibody alone, which usually did not have any visible images. In cases when the nonspecific signals were high, all the data from that experiment were not further analyzed. Sections were viewed with a Zeiss fluorescent microscope and the images were captured by using Axioskop camera. The software Adobe was used to acquire images from the digital camera, and the software Adobe Photoshop (version 5.5) was used to process images. To all the section from the individual experiments, the brightness and contrast of the green and red color images were adjusted essentially to the equal extent when necessary, and the merged images were produced by using layer blending mode options of the Photoshop program.

Retrograde tracing of motor neurons in the spinal cord

The sciatic nerve of anesthetized rats with ketamine and

xylazine was exposed and DiI (5 μl of 3% in Dimethylsulfoxide) was applied to 1 cm distal to the injury site with microsyringe. The incision was sutured, and the animals were returned to their cages after they had recovered from the narcosis. DiI labeled motor neurons were visualized and counted under fluorescence microscope by an observer unaware of the experimental treatment.

Antergrade tracing of motor axons in sciatic nerve

The spinal cord of anesthetized rats was exposed and DiI (10 μl of 3% in Dimethylsulfoxide) was injected between thoracic 12 and lumbar 1 level with microinjector. sciatic nerves were prepared after 3 days and sections (20-μm thickness) were cut on a cryostat. DiI labeled motor axons were visualized under fluorescence microscope.

Statistical analysis

All data are reported as means±SE. Student's t-test was used to test for difference of the mean numbers of DiI-labeled motor neuron in individual animals and GAP-43 expression after sciatic nerve injury for each time point. The significance level was set at $P<0.05$.

Results

Axonal regeneration is promoted after animal's exercise

To examine if the sciatic nerve injury was correctly performed and the nerves were regenerated after surgery, cross- or longitudinal sections were prepared after surgery and immunostained with anti NF-200 antibody. As shown in Fig. 1, the nerve fibers (i. e., axons) were clearly visualized. It was observed that the number of regenerating axons was much increased in exercise group compared to non-exercise group (Fig. 1A). Axonal regrowth rate was faster in exercise group than in non-exercise group (Fig. 1B). Thus, these data suggest that exercise contributed to axonal regeneration.

Number of DiI-labeled motor neurons in spinal cord is elevated by animals exercise

To quantitate the extent of axonal regeneration, nerves were labeled with retrograde dye DiI. DiI was injected at 1cm distal to the lesioned site and the spinal cord sections between thoracic 12 level and lumbar 1 level were prepared and observed under fluorescence microscope. We found that the number of DiI labeled motor neurons was increased as exercise periods were extended. Importantly, exercise group revealed significant increase in cell numbers compared to corresponding non-exercise group in seven day group (Fig. 2).

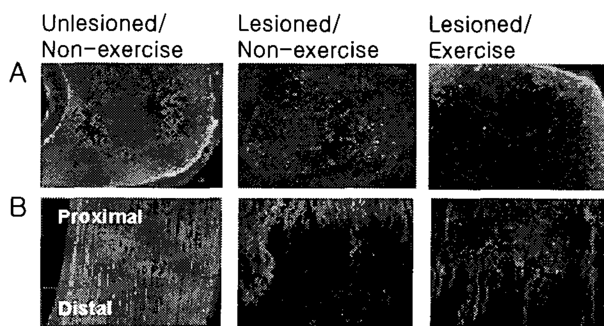
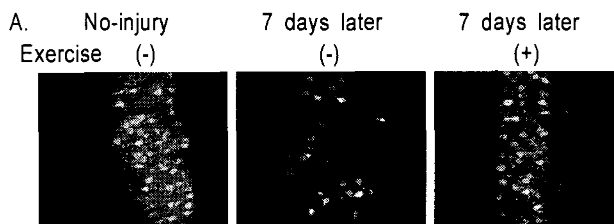


Fig. 1. Immunofluorescence staining of regenerating axon with NF-200. (A) Sciatic nerve was prepared 3 days after injury and 20-μm sections crossing at 3-mm distal to the injury site were used for immunostaining with NF-200. (B) Longitudinal sections were prepared from regenerating nerves 3 days after crush at 3-mm distal to the injury site.



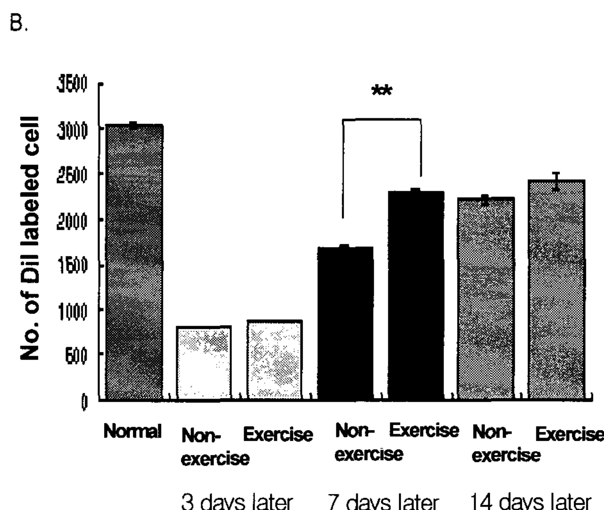


Fig. 2. Number of DiI labeled neurons on individual sections and summated. Error bars denote standard error (** $p<.01$, student's t-test)

DiI-tracing motor axons in sciatic nerve is elongated by animal's exercise

To examine a regenerative motor axons in sciatic nerve, nerves were labeled with anterograde dye DiI. DiI was injected between thoracic 12 and lumbar 1 level. After 3 days, sciatic nerves were prepared and observed under fluorescence microscope. As shown in Fig. 3, DiI labeled motor axons in sciatic nerve showed clear elongation in the exercise group compared to non-exercise group when measured 6 days after surgery (Fig. 3). Also Dii labeled motor axons were increased as exercise periods were extended (data not shown).

Exercise up-regulates GAP-43 expression following sciatic nerve injury

To examine whether GAP-43 protein is involved in axonal regeneration during exercise, GAP-43 protein levels in regenerating nerves were analysed by Western blotting. Animals were subject to running exercise on a treadmill for different time periods. As shown in Fig. 4, bilateral comparison of GAP-43 protein showed clear increases in the ipsilateral side compared to the contralateral side when measured 3, 7 and 14 days after surgery and also up-regulation in the exercise group compared to the non-exercise group.

Discussion

Numerous studies have documented the involvement of axonal growth-associated protein GAP-43 during axonal regeneration. Induction of GAP-43 gene expression was demonstrated in regenerating peripheral nerve stumps following nerve injury[11] and in dorsal root ganglia and sciatic nerve during regenerative sprouting[16]. In exercise physiology studies, exercise training was shown to promote motor nerve conduction velocity after sciatic nerve injury in the rat and accompany functional recovery[17]. While these studies imply the positive association of the improved scores of axonal regeneration with increased GAP-43 synthesis, there has been no report providing direct evidence on this issue. Here, we examined levels of GAP-43 in sciatic nerves after injury and investigated the effects of exercise on changes of GAP-43 and pattern of axon regeneration.

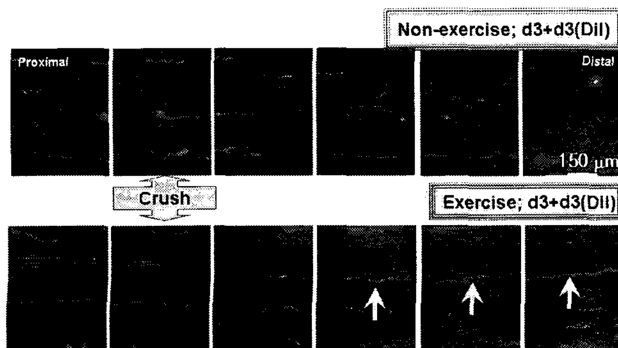


Fig. 3. DiI labeled motor axons in sciatic nerve is elongated by ainmal's exercise. Arrows are a regenerative motor axons.

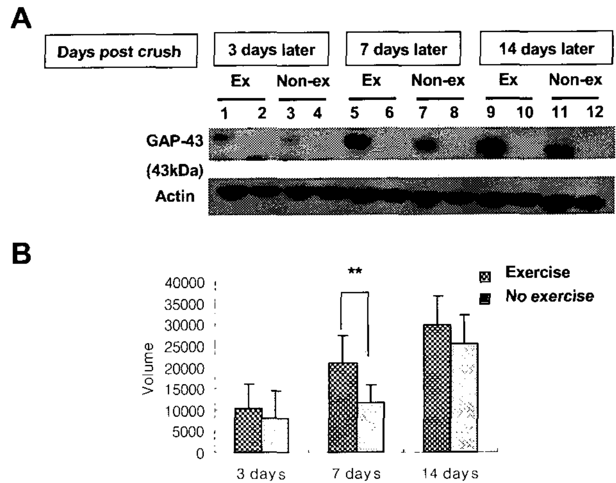


Fig. 4. GAP-43 protein levels are increased in sciatic nerve after crush. Odd numbers are the ipsilateral side and even numbers from the contralateral side. Ex, Exercise group; Non-ex, Non-exercise group. Actin western was used as a loading control. $^{**}p \leq .01$, Student's t-test.

There are three major findings from the present study. First, we found that animal's exercise promoted axonal regeneration. Immunofluorescence staining of sciatic nerves around the injury site clearly showed more regenerating nerve fibers in the exercise group. Retrograde labeling data which revealed increased numbers of motor neurons in the exercises group compared to non-exercise group further confirmed above interpretation. Second, levels of GAP-43 protein were clearly elevated in regenerating sciatic nerves from exercise group compared to non-exercise group at every time point examined. It was particularly noted that the nerves prepared 3 and 7 days after injury showed larger difference in GAP-43 protein levels between exercise and non-exercise group, and became similar at 14 days after surgery (see Fig. 2). Nothing that the number of Di-I labeled motor neurons is significantly higher in the exercise group than non-exercise group at 7 days after surgery and then similar between two groups 14 days after surgery, it

is speculated that animal's exercise may contribute to improve axonal regeneration mostly within 1 week though its effect on functional recovery might be extended for at least several week period. Further studies on changes in axonal regeneration pattern and GAP-43 protein levels for longer period should be helpful to clarify this issue. Third, an evidence on the improvement of functional recovery in an exercise group after sciatic nerve injury was obtained by measuring foot print (data not shown), implicating that indeed, our histological data on an improved axonal regeneration by exercise are well correlated with GAP-43 up-regulation and behavioral recovery.

Protein GAP-43 is expressed in non-neuronal cells in the nervous system such as Schwann cells as well as in the neuronal cells[11]. Since we determined GAP-43 protein in nerve segments around injury, possible induction of GAP-43 protein in the Schwann cells near the injury site cannot be excluded. Our studies on in situ hybridization of spinal cord sections spanning motor nerves of sciatic nerves have shown clear induction of GAP-43 mRNA expression in the motor neuron of sciatic nerves (data not shown). We are currently examining GAP-43 protein and mRNA in spinal cord sections after animal's exercises.

References

1. Aldskogius, H., Arvidson, J., Grant, G. 1992. Axotomy-induced changes in primary sensory neurons. *Sensory Neurons: Diversity, Development, Plasticity. Oxford Univ. Press, Oxford.* 363-383.
2. Aldskogius, H., Svensson, M.1993. Neuronal and glial cell responses to axon injury. *Adv. Struct. Biol.* **2**, 191-223
3. De Graan PN, van Hooff CO, Tilly BC, Oestreicher AB, Schotman P, Gispen WH. 1985. Phosphoprotein B-50 in nerve growth cones from fetal rat brain. *Neurosci Lett.* Nov 11; **61(3)**, 235-41.
4. De la Monte SM, Federoff HJ, Ng SC, Grabczyk E, Fishman MC. 1989. GAP-43 gene expression during development: persistence in a distinctive set of neurons in the mature central nervous system. *Brain Res Dev Brain Res.* Apr 1; **46(2)**, 161-8.
5. Herdegen T, Skene P, Bahr M. 1997. The c-Jun transcription factor-bipotential mediator of neuronal death, survival and regeneration. *Trends Neurosci.* May; **20(5)**, 227-31. Review.
6. Katz F, Ellis L, Pfenninger KH. 1985. Nerve growth cones isolated from fetal rat brain. III. Calcium-dependent protein phosphorylation. *J Neurosci.* Jun; **5(6)**, 1402-11.
7. Medinaceli, L., W. J. Freed, and R. J. Wyatt. 1982. An index of the functional condition of rat sciatic nerve based on measurement made from walking tracks. *Exp. Neurol.* **77**, 634-643.
8. Meiri KF, Pfenninger KH, Willard MB. 1986. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polypeptide of a subcellular fraction enriched in growth cones. *Proc Natl Acad Sci U S A.* May; **83(10)**, 3537-41. Erratum in: Proc Natl Acad Sci U S A 1986 Dec; **83(23)**, 9274.
9. Namgung U, Routtenberg A. 2000. Transcriptional and post-transcriptional regulation of a brain growth protein: regional differentiation and regeneration induction of GAP-43. *Eur J Neurosci.* Sep; **12(9)**, 3124-36.
10. Namgung U., Choi B.H., Park S.H., Lee J.U., Seo H.S., Suh B.C., Kim K.T. 2004. Activation of cyclin-dependent kinase 5 is involved in axonal regeneration, Mol. Cell. Neurosci, **25**, 422-432.
11. vPlantinga LC, Verhaagen J, Edwards PM, Hol EM, Bar PR, Gispen WH. 1993. The expression of B-50/GAP-43 in Schwann cells is upregulated in degenerating peripheral nerve stumps following nerve injury. *Brain Res.* Jan 29; **602(1)**, 69-76.
12. Redistribution of B-50/growth-associated protein 43 during differentiation and maturation of rat hippocampal neurons in vitro. *Neuroscience.* Dec; **51(3)**, 601-19.
13. Tetzlaff W, Zwiers H, Lederis K, Cassar L, Bisby MA. 1989. Axonal transport and localization of B-50/GAP-43-like immunoreactivity in regenerating sciatic and facial nerves of the rat. *J Neurosci.* Apr; **9(4)**, 1303-13.
14. Tetzlaff W, Alexander SW, Miller FD, Bisby MA. 1991. Response of facial and rubrospinal neurons to axotomy: changes in mRNA expression for cytoskeletal proteins and GAP-43. *J Neurosci.* Aug. **11(8)**, 2528-44.
15. Tetzlaff W, Kobayashi NR, Giehl KM, Tsui BJ, Cassar SL, Bedard AM. 1994. Response of rubrospinal and corticospinal neurons to injury and neurotrophins. *Prog Brain Res.* **103**, 271-86. Review.
16. Van der Zee, C.E., Nielander, H.B., Vos, J.P., Lopes da Silva, S., Verhaagen, J., Oestreicher, A.B., Schrama, L.H., Schotman, P., Gispen, W.H., 1989. Expression of growth-associated protein GAP-43 in dorsal root ganglia and sciatic nerve during regenerative sprouting. *J Neurosci.* Oct; **9(10)**, 3505-12.
17. Van Meeteren, N.L., Brakkee, J.H., Hamers, F.P., Helder, P.J., Gispen., 1997. Exercise training improves functional recovery and motor nerve conduction velocity after sciatic nerve crush lesion in the rat. *Arch. Phys. Med. Rehabil.* Jan; **78**, 70-77.

초록 : 좌골신경 손상 후 운동이 쥐의 축색 재생과 성장관련 단백질(GAP-43) 발현에 미치는 영향

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Growth-associated protein (GAP-43)은 손상된 신경 재생 시 axonal growth를 확인할 수 있는 가장 널리 알려진 단백질이다. 본 연구의 목적은 좌골신경 손상을 준 쥐에 저강도 운동 수행 후 좌골신경에서 발현되는 GAP-43 단백질의 양을 정량적으로 분석하고, 손상된 부분으로부터 신경섬유의 재생 정도를 조직학적으로 분석함으로써 운동이 말초신경 손상을 가한 쥐에 미치는 영향을 분석하는 것이다. GAP-43의 정량적인 분석은 western blotting을 통해 확인하였고, 좌골신경의 조직학적인 분석은 immunofluorescence staining과 anterograde로 DiI 주입, 척수 내의 motor neuron은 retrograde로 DiI를 주입 한 후 형광현미경에서 관찰하였다. 본 연구에서는 좌골신경이 손상된 쥐에게 운동을 수행시킴으로써 GAP-43 단백질이 비운동 그룹보다 더 많은 양이 발현되고, 손상된 부위에서 재생하는 신경섬유의 길이와 양이 더 많은 것을 확인할 수 있었다. 또한 운동을 수행한 그룹에서 DiI에 염색된 motor neuron의 수가 더 많이 증가하는 것을 관찰하였다. 따라서 이러한 결과들은 운동이 좌골신경이 손상된 쥐의 재생을 촉진시킬 것으로 생각된다.