might finally achieve recovery of dynamic laryngeal function (recovery of vocal fold movement). We have not investigated the therapeutic effect of the combination of GDNF and BDNF gene transfer on MNCV and recovery of vocal fold movement. However, this issue is interesting, and we will investigate it in future studies.

To minimize the deleterious effects of early trauma and promote and guide axonal regrowth, the delivery of neurotrophic factors has emerged as a promising strategy to manipulate axonal regrowth in the early phase. This study demonstrated the enhancement of neurofunctional recovery after remote injection of adenovirus vector coding for the GDNF gene into crushed RLNs over the course of a few weeks. The vocal folds are extremely delicate structures, and imperceptible injuries can result in excessive vocal complications. Extended injury results in atrophy of the laryngeal muscles, motoneuron loss in the nucleus ambiguus, and decreases in both motor axon density and nerve–end plate contact. Early recovery from axonal degeneration is important for preservation and recovery of laryngeal function. Again, the present methods achieved good preservation and facilitated recovery of laryngeal function.

Laryngeal paralysis most often occurs clinically as a result of vagal nerve or RLN injury after surgical ablation of a tumor involving the head and neck region. If the nerve is injured during surgery, direct injection of the vector into the nerve might prevent paralysis. Alternatively, when paralysis becomes apparent on extubation, the vector can be injected into the nerve after reintubation and opening of the wound.

The adenovirus vector was used in this study. For clinical applications, controversy remains regarding the potential risks of virus-mediated gene therapy,[28-30] particularly when applied to nonlethal benign diseases such as laryngeal paralysis. To overcome this problem, the safety of the vector must be demonstrated before clinical application. Preliminary experiments of highly safe viral[31] and nonviral gene transfer systems are also currently under way.

REFERENCES

1. Li L, Oppenheim RW, Lei M, Houenou LJ Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J Neurobiol 1994;25:759-66.

2. Shiotani A, O'Malley BW Jr, Coleman ME, Alila HW, Flint PW. Reinnervation of motor endplates and increased muscle fiber size after human insulin-like growth factor I gene transfer into the paralyzed larynx. Hum Gene Ther 1998;9:2039-47.

3. Flint PW, Shiotani A, O'Malley BW Jr. IGF-1 gene transfer into denervated rat laryngeal muscle. Arch Otolaryngol Head Neck Surg 1999;125:274-9.

4. Shiotani A, O'Malley BW Jr, Coleman ME, Flint PW. Human insulinlike growth factor 1 gene transfer into paralyzed rat larynx: single vs multiple injection. Arch Otolaryngol Head Neck Surg 1999;125:555-60.

5. Nakagawa H, Shiotani A, O'Malley BW Jr, Coleman ME, Flint PW Timing of human insulin-like growth factor–1 gene transfer in reinnervating laryngeal muscle. Laryngoscope 2004;114:726-32.

6. Flint PW, Nakagawa H, Shiotani A, Coleman ME, O'Malley BW Jr. Effects of insulin-like growth factor–1 gene transfer on myosin heavy chains in denervated rat laryngeal muscle. Laryngoscope 2004;114:368-71.

7. Henderson CE, Phillips HS, Pollock RA, et al. GDNF: a potent survival factor for motoneurons present in peripheral nerve and muscle. Science 1994;266:1062-4. [Erratum in Science 1995;267:777.]

8. Li L, Wu W, Lin LF, Lei M, Oppenheim RW, Houenou LJ. Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line–derived neurotrophic factor. Proc Natl Acad Sci U S A 1995;92:9771-5.

9. Yan Q, Matheson C, Lopez OT. In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature 1995;373:341-4.

10. Sakamoto T, Watabe K, Ohashi T, et al. Adenoviral vector-mediated GDNF gene transfer prevents death of adult facial motoneurons. Neuroreport 2000;11:1857-60.

11. Nguyen QT, Parsadanian AS, Snider WD, Lichtman JW. Hyperinnervation of neuromuscular junctions caused by GDNF overexpression in muscle. Science 1998;279:1725-9.

12. Hoke A, Cheng C, Zochodne DW. Expression of glial cell line–derived neurotrophic factor family of growth factors in peripheral nerve injury in rats. Neuroreport 2000;11:1651-4.

13. Friedman WJ, Greene LA. Neurotrophin signaling via Trks and p75. Exp Cell Res 1999;253:131-42.

14. Yano H, Chao MV. Neurotrophin receptor structure and interactions. Pharm Acta Helv 2000;74:253-60.

15. Kishino A, Ishige Y, Tatsuno T, Nakayama C, Noguchi H. BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrowth. Exp Neurol 1997;144:273-86.

16. Oppenheim RW, Houenou LJ, Johnson JE, et al. Developing motor neurons rescued from programmed and axotomy-induced cell death by GDNF. Nature 1995;373:344-6.

17. Chai H, Wu W, So KF, Prevette DM, Oppenheim RW. Long-term effects of a single dose of brain-derived neurotrophic factor on motoneuron survival following spinal root avulsion in the adult rat. Neurosci Lett 1999;274:147-50.

18. Blits B, Carlstedt TP, Ruitenberg MJ, et al. Rescue and sprouting of motoneurons following ventral root avulsion and reimplantation combined with intraspinal adeno-associated viral vector-mediated expression of glial cell line–derived neurotrophic factor or brain-derived neurotrophic factor. Exp Neurol 2004;189:303-16.

19. Saito K, Shiotani A, Watabe K, Moro K, Fukuda H, Ogawa K. Adenoviral GDNF gene transfer prevents motoneuron loss in the nucleus ambiguus. Brain Res 2003;962:61-7.

20. Hangai K, Enomoto K, Kobayashi Y. Postnatal develop-

ment of intrinsic laryngeal muscles and motoneurons of the nucleus ambiguus in rats. Larynx 1995;7:133-8.

21. Moro K, Shiotani A, Watabe K, et al. Adenoviral gene transfer of BDNF and GDNF synergistically prevent motoneuron loss in the nucleus ambiguus. Brain Res 2006;1076:1-8.

22. Araki K, Shiotani A, Watabe K, Saito K, Moro K, Ogawa K. Adenoviral GDNF gene transfer enhances neurofunctional recovery after recurrent laryngeal nerve injury. Gene Ther 2006; 13:296-303.

23. Baumgartner BJ, Shine HD. Neuroprotection of spinal motoneurons following targeted transduction with an adenoviral vector carrying the gene for glial cell line–derived neurotrophic factor. Exp Neurol 1998;153:102-12.

24. Chen ZY, Chai YF, Cao L, Lu CL, He C. Glial cell line–derived neurotrophic factor enhances axonal regeneration following sciatic nerve transection in adult rats. Brain Res 2001; 902:272-6.

25. Varejao AS, Cabrita AM, Meek MF, et al. Functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp. J Neurotrauma 2004; 21:1652-70.

26. Bridge PM, Ball DJ, Mackinnon SE, et al. Nerve crush injuries — a model for axonotmesis. Exp Neurol 1994;127:284-90.

27. Zeng L, Worseg A, Albrecht G, et al. A noninvasive functional evaluation following peripheral nerve repair with electromyography in a rat model. Plast Reconstr Surg 1994;94:146-51.

28. George JA. Gene therapy progress and prospects: adenoviral vectors. Gene Ther 2003;10:1135-41.

29. Check E. Sanctions agreed over teenager's gene-therapy death. Nature 2005;433:674.

30. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Mol Genet Metab 2003;80:148-58.

31. Shiotani A, Fukumura M, Maeda M, et al Skeletal muscle regeneration after insulin-like growth factor I gene transfer by recombinant Sendai virus vector. Gene Ther 2001;8:1043-50.

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Title page

A novel drug therapy for recurrent laryngeal nerve injury using T-588.

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Abstract

Objectives/Hypothesis: We have previously shown that gene therapy using Insulin-like growth factor (IGF)-I, glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF), or a combination of these trophic factors, is a treatment option for recurrent laryngeal nerve (RLN) palsy. However, there remain some difficulties preventing this option from becoming a common clinical therapy for RLN injury. Thus, we need to develop novel treatment option that overcomes the problems of gene therapy.

R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-N,N-diethylamino]ethoxy]ethanol hydrochloride (T-588), a synthetic compound, is known to have neuroprotective effects on neural cells. In the present study, the possibility of new drug treatments using T-588 for recurrent laryngeal nerve (RLN) injury was assessed using rat models.

Study Design: Animal study.

Methods: Animals were administered T-588 for 4 weeks. The neuroprotective effects of T-588 administration after vagal nerve avulsion and neurofunctional recovery after recurrent laryngeal nerve crush were studied by motoneuron cell counting, evaluation of choline acetyltransferase immunoreactivity, the electrophysiological examination and the re-mobilization of the vocal fold.

Results: T-588 administration successfully prevented motoneuron loss and ameliorated the choline acetyltransferase immunoreactivity in the ipsilateral nucleus ambiguus after vagal nerve avulsion.

Significant improvement of motor nerve conduction velocity (MNCV) of the RLN and vocal fold movement were observed in the treatment group when compared to controls.

Conclusion: These results indicate that oral administration of T-588 may be a promising therapeutic option in treating peripheral nerve injury.

Keywords

T-588, Avulsion, Crush, Nucleus ambiguus, Laryngeal paralysis, Motoneuron

1. Introduction

Laryngeal paralysis most often occurs as a result of vagal nerve injury or recurrent laryngeal nerve injury after surgical ablation of a tumor involving the head and neck region. Injury to the recurrent laryngeal nerve occurs in 1-2% of all thyroid surgeries. Other surgical procedures utilizing a cervical approach carry a similar incidence of laryngeal paralysis. Patients with unilateral laryngeal paralysis typically present with disabling symptoms related to aspiration, dysphagia and loss of voice (dysphonia). Except for laryngeal reinnervation procedures, surgical options for the management of patients with unilateral laryngeal paralysis (vocal fold injection, thyroplasty and arytenoid adduction) only achieve vocal fold medialization due to static changes in the vocal fold tissue or laryngeal framework, and such deficits can never be neurologically restored.[1] Laryngeal reinnervation procedures have had little impact on the return of dynamic laryngeal function and are still not widely accepted as a treatment option. The failure of reinnervation after recurrent laryngeal nerve injury may be attributed to multiple factors, including decreases in motor fiber density, atrophy of laryngeal muscle, loss of motoneurons in the nucleus ambiguus, and inappropriate or misdirected innervation by antagonistic motoneurons.[2,3] Gene therapy is a potential treatment option for recurrent laryngeal nerve (RLN) palsy. The potential of gene therapy using insulin-like growth factor (IGF)-I [4,5], glial cell line-derived neurotrophic factor (GDNF) [6,7] and brain-derived neurotrophic factor (BDNF), or a combination of these trophic factors [8], has been reported previously. Although we have demonstrated

that gene therapy is very useful for RLN palsy, there are several difficulties preventing it from becoming a common clinical therapy, including ethical problems, technical difficulties and toxicity of viral vectors. Low molecular weight compounds that are safe and convenient to administer are thus desirable alternatives for clinical applications.

Steroids are one of the common agents currently used for the treatment of peripheral nerve palsy, including RLN palsy and idiopathic facial palsy, as a result of their anti-inflammatory and anti-edematous effects. However, these agents produce little benefit in recurrent laryngeal nerve palsy.[9] Donepezil, Galantamine, Memantine and R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-N,N-diethylamino)ethoxy]ethanol hydrochloride (T-588) are recently developed neuroprotective agents against Alzheimer's disease.[10] Among these, we focused on T-588, a novel neuroprotective compound, that delays progression of neuromuscular dysfunction in wobbler mouse motoneuron disease.[11] It has also been demonstrated that oral administration of T-588 improves the survival of injured motoneurons and supports their neuronal function after facial nerve avulsion.[12] In this study, the potential of oral administration of T-588 for RLN injury was assessed using rat models. The neuroprotective effects of T-588 after vagal nerve avulsion and RLN crush were also studied.

2. Materials and Methods

2.1. Animals and surgical procedures

Forty-two Sprague-Dawley male rats (12-weeks-old, 340-360 g) were used in this study. Animals were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) during all surgical procedures. The vagal nerve avulsion model was utilized to assess the neuroprotective effects of T-588 in the nucleus ambiguous after severe vagal/RLN surgery. Under a dissecting microscope, the left vagal nerve was exposed at the jugular foramen. Using microhemostat forceps, the proximal vagal nerve was avulsed and removed from the distal vagal nerve by gentle traction and skin was suture closed[7]

The nerve crush model was utilized to assess the potential of T-588 to promote neurofunctional recovery of RLN after detrition injury. Following a midline vertical cervical incision, the left RLN was exposed and dissected circumferentially just inferior to the left lobe of the thyroid gland. At 10 mm proximal from the inferior of the thyroid gland, the nerve was crushed with a forceps for exactly 60 seconds. To confirm left vocal fold paralysis, direct laryngoscopy was performed. Subsequently, strap muscles and overlying fascia were replaced, and the skin was suture closed. Animals were cared for and used in accordance with protocols approved by the Animal Care and Use Committee of Keio University School of Medicine (Tokyo, Japan).

2.2. T-588 administration

T-588 was supplied by Toyama Chemical Co. Ltd. (Tokyo, Japan). After avulsion or crush of the left vagal nerve, rats were freely administered water containing 0.05% T-588 for 4 weeks. The total daily amount of T-588 consumed by the rats thorough freely available 0.05% solution was set to be equivalent to the daily dose for humans.[12, 13]

2.3. Histological analysis

For motoneuron cell counting, rats were anesthetized with a lethal doze of ketamine and were transcardially perfused with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer at 4 weeks after vagal nerve avulsion. Brain stem tissue was harvested and immersion fixed in the same fixative for 2 h. Subsequently, samples were embedded in paraffin, and serial transverse sections were cut at 7 μm. Every fifth section (28-μm interval) was collected, deparaffinized and stained with Toluidine Blue, and ambiguus motoneurons having nuclei containing distinct nucleoli on both sides of the nucleus ambiguus were counted in 20 sections. We did not apply any correction factors for data analysis, as the ambiguus neurons have a maximum diameter of 21.8±4.96 μm [14], and these neurons were counted only once in every fifth section, at a 28-μm interval. Data are expressed as means ± S.D. from eleven animals and statistical significance was assessed between the treatment group and control group by Mann-Whitney U test.

For immunostaining of Choline acetyltransferase (ChAT), animals were perfused at 4 weeks after

treatment. Subsequently, samples were embedded in paraffin, serial transverse sections were cut at 7 μm, and sections were pretreated with 0.3% H_2O_2 in PBS, rinsed in 0.1% Triton X-100 in PBS (T-PBS) and preincubated in 3% normal goat serum in T-PBS. Next, sections were incubated overnight at 4°C with mouse monoclonal antibody against ChAT (Chemicon, mouse anti-ChAT, Temecula, CA, USA) at a dilution of 1:100, followed by incubation with HRP-labeled anti-mouse polymer (Dako Cytomation, Carpinteria, CA, USA). Treated sections were visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution and counterstained with Hematoxylin.

2.4. Neurofunctional analysis

Motor nerve conduction velocity (MNCV) was calculated to assess the neurological functional recovery of RLN after crush injury. Animals were anesthetized and the left recurrent laryngeal nerve was exposed inferior to the left lobe of the thyroid gland, as described above. The strap muscles were sectioned to expose the larynx, and laryngeal fissures were made. The left thyroarytenoid (TA) muscle was pierced through the fissure with a needle concentric electrode for recording. To stimulate the left RLN, two bipolar hook electrodes were placed to hook the dissected left RLN. One was placed inferior to the left lobe of the thyroid as a distal stimulator and the other was placed 16 mm proximal to the distal electrode as a proximal stimulator. The nerve was maximally stimulated and

compound muscle action potential in TA muscle was recorded using a Power Lab computer-assisted electromyography machine (AD Instruments, Colorado Springs, CO, USA). Maximal stimulation was achieved by increasing the current output until no further changes in amplitude of the compound action potential occurred. A 0.01-millisecond current impulse was delivered. Maximum MNCV was calculated based on derived latency and distance between the two stimulating points (16 mm).

At the time of laryngeal fissure creation, recovery of vocal fold movement was also assessed. Recovery was only considered present when equal vocal fold movement on the denervated side was observed when compared to the vocal fold on the contralateral non-denervated side. Limited recovery was considered to be the absence of recovery.

2.5. Statistical analysis

Data are expressed as means ± S.D. Statistical comparison of motoneuron loss and MNCV were performed by Mann-Whitney U test. Recovery of vocal fold movement was statistically compared by χ^2-test for independence. The level of significance was set at $p<0.05$.

3. Results

3.1. Neuroprotective effects of T-588 administration

The left vagal nerves of adult rats were avulsed and removed at the level of the jugular foramen.

Animals were freely administrated water containing 0.05% T-588 solution after surgery. Four weeks after surgery, the number of surviving motoneurons in the nucleus ambiguus was counted using Nissl staining in order to evaluate the neuroprotective effects of T-588 (T-588, n=6; control, n=5). There was marked atrophy and loss of motoneurons in the nucleus ambiguus of the lesion side (Fig.1). The number of motoneurons decreased and reached 57.9±4.8% when compared with the contralateral side in the control group. Oral administration of T-588 successfully prevented the motoneuron loss, i.e., the number of residual motoneurons in the ipsilateral nucleus ambiguus (69.0±3.5%) was significantly higher in the treatment group when compared to controls (P=0.0062). (Fig. 2). ChAT immunoreactivity is known to rapidly decrease in the motoneurons after nerve injury.[15-19] Although marked decreases in immunoreactivity in the nucleus ambiguous was observed in the control group, improved ChAT immunoreactivity was observed in the treatment group at 4 weeks after vagal nerve avulsion (Fig. 3).

3.2. Neurofunctional recovery after T-588 administration

Effects of T-588 administration on neurofunctional recovery were examined at 4 weeks after RLN crush. Shorter latency, together with shorter time lag of latency was observed in the treatment group when compared to controls (Fig. 4). Mean (±S.D.) MNCV in the treatment group (32.07 ± 16 m/s) was significantly higher than in the control group (20.47 ± 5.02 m/s, P=0.015) (Fig. 5).

3.3. Recovery of vocal fold movement

The number of rats displaying obvious recovery of ipsilateral vocal fold movement was 9/12 in the treatment group and 2/9 in the control group. Statistically better recovery was observed in the treatment group when compared to controls (P=0.016) (Table. 1).

4. Discussion

T-588, a synthetic derivative of acetylcholine[20], has been developed as a candidate neuroprotective agent against neurodegenerative diseases. Clinical trials using T-588 to treat dementia associated with Alzheimer's disease are currently underway.[10] T-588 is efficiency transported into the central nervous system (CNS)[11] , and it has been reported to delay the progression of Alzheimer's disease in wobbler mouse[11] and to exert neuroprotective effects against ischemia/reperfusion-induced brain damage in vivo[21]. Oral administration of T-588 improves the survival of injured motoneurons and supports their neuronal function after facial nerve avulsion.[11] In vitro, this compound enhances neurite outgrowth and ChAT activity in primary explant cultures of the ventral spinal cord[22] and activates the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) pathway in cultured rat newborn astrocytes, inhibiting astrocyte apoptosis induced by Ca^{2+}stress.[23] These data suggest that T-588 exerts neuroprotective effects in damaged motoneurons.

In this study, the vagal nerve avulsion model was utilized to assess the neuroprotective effects of T-588. Marked motoneuron loss was observed in the ipsilateral nucleus ambiguus after surgery, as reported previously.[7, 8] Similar findings have been reported in the facial nucleus after facial nerve avulsion and in the ventral horn after spinal root avulsion.[18, 19] Treatment with T-588 significantly prevented the loss of vagal motoneurons when compared to controls. The presence of ChAT is associated with the viability of motoneurons and ChAT immunoreactivity is known to decrease rapidly at 1 week after facial nerve or spinal nerve avulsion. Thereafter, ChAT immunoreactivity gradually decreases for 7weeks.[15-19] It has been reported that oral administration of T-588 after facial nerve avulsion improved ChAT immunoreactivity in adult rats.[12] In the present study, the decrease in ChAT immunoreactivity was attenuated by the T-588 treatment in the nucleus ambiguus. These findings are indicative of the neuroprotective effects of T-588 on vagal nerve motoneurons after severe vagal nerve injury.

Detrition injury to vagal/recurrent laryngeal nerves can be caused by surgery utilizing a cervical or mediastinal approach. Nerve crush consistently induces Sunderland second-degree injury (axonotmesis), yielding Wallerian degeneration of the nerve distal to the injury site.[24, 25] Bridge *et al.* demonstrated that the functional and histological responses to crush are identical in the various methods to deliver crush injury to the rat sciatic nerve.[24, 25] MNCV for the injured nerve is a commonly used physiological measure to evaluate functional recovery of peripheral nerves after

injury.[24-26] The present study assessed neurofunctional recovery at 4 weeks after crush injury to RLN. Treatment with T-588 resulted in significant improvement in MNCV and vocal fold movement when compared to control animals. It has been reported that it takes 8 weeks for physiological and histological recovery of peripheral nerves after crush injury.[24-26] Reducing the RLN functional recovery period with T-588 administration may ameliorate speech/swallowing problems more quickly and prevent atrophy of the internal laryngeal muscles.

5. Conclusion

We demonstrated that oral administration of T-588 prevents motor neuron loss in the nucleus ambiguus and supports neurofunctional recovery after vagal/recurrent laryngeal nerve injury. Oral administration of T-588 is thus a promising therapeutic approach for various peripheral injuries.

6.References

1. Isshiki N. Progress in laryngeal framework surgery. *Acta Otolaryngol* 2000; 120:120-7.

2. Crumley R. L. Mechanisms of synkinesis. *Laryngoscope* 1979; 89: 1847-54.

3. Flint P. W., Downs D. H. and Coltrera, M. D. Laryngeal synkinesis following reinnervation in the rat. Neuroanatomic and physiologic study using retrograde fluorescent tracers and electromyography. Ann Otol Rhinol Laryngol 1991;100: 797-806.

4. Shiotani A., O'Malley B. W., Jr., Coleman M. E., et al. Reinnervation of motor endplates and increased muscle fiber size after human insulin-like growth factor I gene transfer into the paralyzed larynx. Hum Gene Ther , 1998; 9: 2039-47.

5. Shiotani A., O'Malley B. W., Jr., Coleman M. E., et al. Human insulinlike growth factor 1 gene transfer into paralyzed rat larynx: single vs multiple injection. Arch Otolaryngol Head Neck Surg 1999; 125: 555-60.

6. Araki K., Shiotani A., Watabe K., et al. Adenoviral GDNF gene transfer enhances neurofunctional recovery after recurrent laryngeal nerve injury. Gene Ther 2006; 13: 296-303

7. Saito K., Shiotani A., Watabe K., et al. Adenoviral GDNF gene transfer prevents motoneuron loss in the nucleus ambiguus. Brain Res 2003; 962: 61-7

8. Moro K., Shiotani A., Watabe K., et al Adenoviral gene transfer of BDNF and GDNF synergistically prevent motoneuron loss in the nucleus ambiguus. Brain Res 2006; 1076: 1-8.

9. Wang L. F., Lee K. W., Kuo W. R., et al. The efficacy of intraoperative corticosteroids in recurrent laryngeal nerve palsy after thyroid surgery. World J Surg 2006; 30: 299-303.

10. Hirouchi M. Current status and perspectives on the development of therapeutic agents for Alzheimer's disease. Nippon Yakurigaku Zasshi 2004; 123: 421-7.

11. Ikeda K., Iwasaki Y., Kinoshita M., et al. T-588, a novel neuroprotective agent, delays progression of neuromuscular dysfunction in wobbler mouse motoneuron disease. Brain Res 2000; 858: 84-91.

12. Ikeda K., Sakamoto T., Marubuchi S., et al. Oral administration of a neuroprotective compound T-588 prevents motoneuron degeneration after facial nerve avulsion in adult rats. Amyotroph Lateral Scler Other Motor Neuron Disord 2003; 4:74-80.

13. Shimada K., Mitamura K. and Kitama N. Quantitative determination of 25-hydroxyvitamin D3 3-sulphate in human plasma using high performance liquid chromatography. Biomed Chromatogr 1995; 9: 229-32.

14. Hangai K., Kobayashi Y. and Nonaka S. Developmental changes in histochemical properties of intrinsic laryngeal muscles in rats.Auris Nasus Larynx 1999; 26: 467-78.

15. Yan Q., Matheson C. and Lopez O. T. In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature 1995; 373: 341-4.

16. Kishino A., Ishige Y., Tatsuno T., et al. BDNF prevents and reverses adult rat motor neuron degeneration and induces axonal outgrowth. Exp Neurol 1997; 144: 273-86

17. He J. W., Hirata K., Kuraoka A., et al. An improved method for avulsion of lumbar nerve roots as an experimental model of nitric oxide-mediated neuronal degeneration. Brain Res Brain Res Protoc 2000; 5: 223-30.

18. Sakamoto T., Watabe K., Ohashi T., et al. Adenoviral vector-mediated GDNF gene transfer prevents death of adult facial motoneurons. Neuroreport 2000; 11: 1857-60.

19. Watabe K., Ohashi T., Sakamoto T., et al. Rescue of lesioned adult rat spinal motoneurons by

adenoviral gene transfer of glial cell line-derived neurotrophic factor. J Neurosci Res 2000; 60: 511-9.

20. Ono S., Kitamura K., Maekawa M., et al. Protective effect of R(-)-1-(benzo[b]thiophen-5-yl)-2-[2-(N,N-diethylamino)ethoxy]ethanol hydrochloride (T-588), a novel cerebral activator, against experimental cerebral anoxia. Jpn J Pharmacol 1993; 62: 81-6.

21. Ono S., Yamafuji T., Chaki H., et al. A new cognition-enhancing agent, (R)-(-)-1-(benzo[b]thiophen-5-yl)- 2-[2-(N,N-diethylamino)ethoxy]ethanol hydrochloride. Effects on memory impairment in rats generated by cerebral embolization and basal forebrain lesions. Biol Pharm Bull 1995; 18: 1779-83.

22. Iwasaki Y., Ikeda K., Ichikawa Y., et al. T-588 enhances neurite outgrowth and choline acetyltransferase in cultured rat spinal ventral horn neurons. Neurochem Res 2002; 27: 225-8.

23. Takuma K., Fujita T., Kimura Y., et al. T-588 inhibits astrocyte apoptosis via mitogen-activated protein kinase signal pathway. Eur J Pharmacol 2000; 399: 1-8.

24. Bridge P. M., Ball D. J., Mackinnon S. E., et al. Nerve crush injuries--a model for axonotmesis. Exp Neurol 1994;127: 284-90.

25. Varejao A. S., Cabrita A. M., Meek M. F., et al. Functional and morphological assessment of a standardized rat sciatic nerve crush injury with a non-serrated clamp. J Neurotrauma 2004; 21: 1652-70.

26. Zeng L., Worseg A., Albrecht G. et al. A noninvasive functional evaluation following peripheral nerve repair with electromyography in a rat model. Plast Reconstr Surg 1994; 94: 146-51.