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TrkB deficiency increases survival and regeneration of spinal motoneurons after axotomy in mice

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Abstract

Persisting motor function deficit after peripheral nerve injury often results from axotomized motoneuron death. Brain-derived neurotrophic factor (BDNF) and its receptor, trkB, are known to promote peripheral nerve regeneration. However, the requirement of BDNF and trkB for adult motoneuron survival after peripheral nerve injury is not established. We studied the number of surviving and regenerating motoneurons after sciatic nerve transection in wild-type and heterozygous trkB-deficient mice. The nerve was either left cut or immediately sewed up or the gap injury model was performed. The gap was provided with an autologous or cross (obtained from other genetic group) graft. Sixteen weeks after surgery, the animals were sacrificed and histological evaluations were performed. In order to study the number of regenerating motoneurons, immunofluorescent tracer was applied to the distal stump of the operated nerve. We found that in wild type mice, the decrease in motoneurons after nerve transection was markedly higher than in trkB-deficient animals, regardless of the operation procedure. Nerve transection resulted in the highest decrease in motoneuron number in wild type mice. This decrease was lower if the nerve was re-joined using a cross-graft obtained from a trkB-deficient animal. Interestingly, in trkB-deficient animals, the decrease in motoneuron count did not depend on type of operation and was similar after nerve transection, re-joining or grafting. The number of regenerating motoneurons after nerve transection and re-joining in wild type animals was lower than in trkB-deficient mice. The number of regenerating motoneurons after nerve grafting did not differ between groups. These results provide further evidence for the role of trkB receptor in spinal motoneuron survival and regeneration.

Key words: *motoneuron, survival, regeneration, peripheral nerve injury, trkB receptor, peripheral nerve injury*

Introduction

In contrast to central neurons, peripheral nerves of adult mammals do not lose the potential to regenerate. However, it is clinically recognized that peripheral nerve injuries are rarely followed by complete recovery [10,21]. Deficits in motor axon function are especially debilitating, as they result not

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only in paralysis but also in denervated muscle atrophy. Therefore, there is considerable academic as well as practical value in understanding the mechanisms underlying motor neuron survival and regeneration after nerve injury.

Neurotrophins are a family of neurotrophic factors that play an important role in the development, differentiation, survival and plasticity of both central and peripheral neurons. Recently, many reports have emphasized the potential use of neurotrophins to promote axonal regeneration after peripheral nerve injury [9,16,21].

Evidence is growing that brain-derived neurotrophic factor (BDNF) and its receptor trkB are crucial for nerve regeneration processes [4,5,21]. BDNF is known to promote the survival of axotomized motoneurons in many experimental paradigms [18,19]. Axotomized motoneurons up-regulate trkB and BDNF mRNA, starting from the 3rd day after the injury. This increase lasts for approximately three weeks [11,12]. Activation of the trkB receptor by trkB leads to activation of an intracellular phosphorylation cascade resulting in specific transcriptional and translational events [2]. It was reported that survival of axotomized neonatal motoneurons depends on the expression of trkB [1]. Recently, we have shown that in trkB-deficient mice the regrowing nerve fibres are arranged more haphazardously, resulting in more frequent neuroma formation at the injury site [13]. However, the role of trkB in adult motoneuron survival after axotomy is not established.

The aim of the present work was to study the role of trkB receptor in spinal motoneuron survival and regeneration after peripheral nerve injury in mice. Genetically modified animals provide a unique model for such evaluations. In the present study, heterozygous trkB-deficient mice were used.

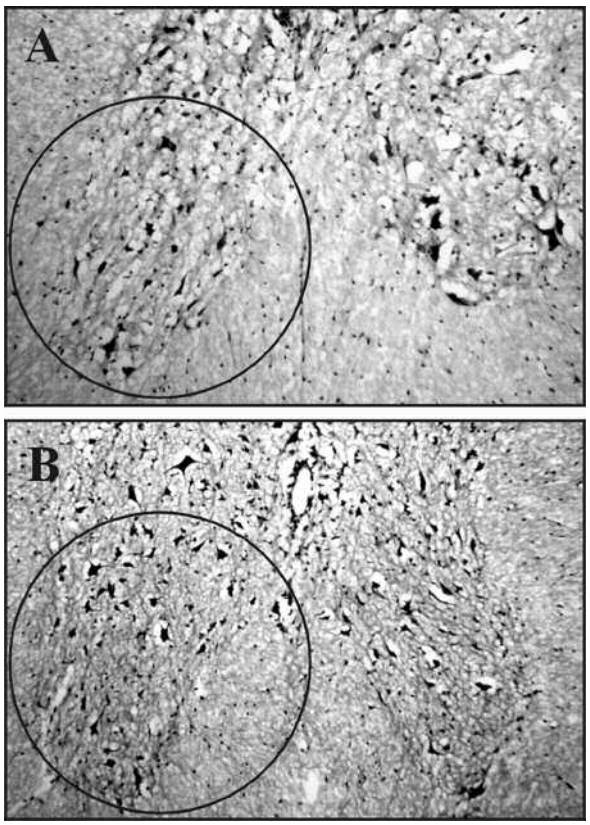

Fig. 1. Microscopic view of spinal cord after nerve transection in wild-type (TN_{wt} group) (A) and trkB-deficient mouse (TN_{hz} group) (B). Operated side is circled. Magnification 200x

Materials and methods

Mice

Mutant trkB ±(129/SvJ genetic background) mice were generated at the Centre for Molecular Biology, Hamburg University, Germany [5], and generously given by Prof. Melitta Schachner. The animals were bred at the Department of Physiology, Medical University of Silesia.

Twenty-four adult heterozygous (Hz group) mutant mice were used for this study. Control mice (Wt group, n=24) were the wild-type littermates.

All experiments were carried out in accordance with the European Council Directive regarding care and use of laboratory animals and they were approved by the local Ethics Committee. The surgical procedures were performed under intraperitoneal Avertine (Sigma) anaesthesia (450 mg/kg b.w.).

Peripheral Nerve Surgery

Under anaesthesia, in all animals the right sciatic nerve was exposed and cut at mid-thigh level with microscissors (Chifa, Poland), as described elsewhere [15]. In 6 animals of both groups, a 3 mm-long nerve fragment was removed to avoid spontaneous rejoining (groups TN_{hz} and TN_{wt}, respectively) and muscles and skin were closed in layers (4/0, Ethicon, USA) and the animals were placed back in separate cages.

In 6 animals of the Hz as well as Wt group, the transected nerve was immediately sewn up (groups SN_{hz} and SN_{wt}, respectively) with a single suture (10/0, Ethicon, USA).

In order to mimic the gap injury of the nerve, in 12 animals of both genotypes a 5 mm-long piece of the nerve was removed and replaced by graft, as described elsewhere [14]. In 6 animals in each group, the graft was the same nerve piece reversed by 180° (groups AG_{hz} and AG_{wt}, respectively). In order to establish the role of trkB in the graft in motoneuron survival, cross-grafting was performed, i.e. 6 Hz animals received Wt grafts and 6 Wt mice received Hz grafts (groups CG_{hz} and CG_{wt}, respectively).

All surgical procedures were performed under operative microscope (Nikon, Japan).

Histological analysis

After 16 weeks, the animals were sacrificed and perfused through the heart with saline followed by fixative mixture (saline and 4% paraformaldehyde). In order to find the number of surviving motoneurons, L4–L6 segments of spinal cords of all experimental animals were carefully dissected, postfixed, cryoprotected and then embedded in Tissue-Tek (Sakura, Japan). 20 mm thick transverse sections were mounted on gelatin-coated slides (Menzel Glaser, Germany) and treated with aqueous 1% toluidine blue solution for 30 s, examined under light microscope, photographed and digitally stored. Neuronal counting was done according to Mohammed and Santer technique [17]. To avoid double counting every second section was taken into consideration. Only cells in which nuclei were clearly visible were counted in 5 of 10 consecutive sections. To determine the ratio of surviving motoneurons, the number of motoneurons from the injury side was divided by the number of motoneurons on the intact side and expressed as a percentage.

In order to study the number of regenerating motoneurons, two days before sacrifice, 2 animals in each group were reanaesthetized, and a microcrystal of DiI (1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine chlorate, Molecular Probes, USA) was placed on the distal stump of the operated nerve. The wound was resutured and the rats were placed back in the cages.

Forty-eight hours after dye administration, mice were sacrificed, perfused transcardially with phosphate-buffered saline (PBS) (0.2 L), followed by cold fixative containing 4% formaldehyde in PBS (0.4 L). The L4–L6 segments of spinal cords were immediately dissected, postfixed, cryoprotected and embedded in Tissue-Tek (Sakura, Japan). Serial 20 µm thick cross sections were made by means of cryostat (Cryotome 620, Anglia Scientific, England) and mounted on gelatin-coated slides. Then, air-dried sections were dehydrated and coverslipped in di-n-butyl-phthalate xylene (DPX) (Gurr, England). Sections were subsequently examined under fluorescence microscope (Labophot-2 Nikon, Japan) at the wavelength 550 nm, and photographed.

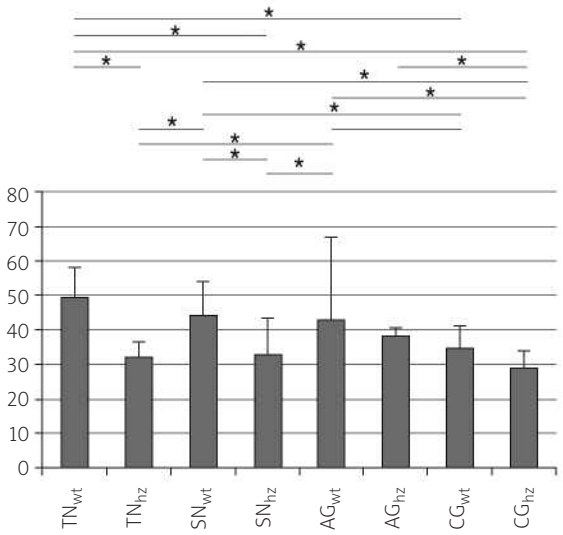

Fig. 2. Decrease in motoneuron number after peripheral nerve transection in wild-type (Wt) and trkB-deficient (Hz) mice. Groups: TN – nerve was transected and left cut, SN – nerve was transected and re-sutured, AG – autograft used, CG – cross-graft used (for details, see text). The number of motoneurons from the injury side was divided by the number of motoneurons on the intact side and expressed as a percentage

Statistical analysis was performed using Student's *t*-test. Significance was set at $p<0.05$.

Results

All animals survived the surgery and no weight loss or other deterioration symptoms were noted. Autotomy was not observed in any of the studied animals throughout 16 weeks of follow-up.

Whole dissected spinal cords did not differ in size in Wt and Hz mice.

We found that in Wt mice, the decrease of motoneurons after nerve transection was markedly higher than in Hz animals, regardless of the operation

procedure (Fig. 1). Nerve transection resulted in the highest decrease in motoneuron number in Wt mice. This decrease was lower if the nerve was re-joined using a cross-graft obtained from a trkB-deficient animal. Interestingly, in Hz animals, the decrease in motoneuron count did not depend on type of operation and was similar after nerve transection, re-joining or grafting. The percentage of surviving motoneurons in individual groups is presented in Fig. 2.

The number of regenerating motoneurons after nerve transection and immediate surgical repair was significantly higher in Hz animals than in wild type mice. No differences in regenerating motoneurons rate between Hz and Wt mice were observed after nerve grafting. In groups in which the nerve was transected and left cut, no motoneuron was found to outgrow its fibre into the distal stump of the nerve (Fig. 3).

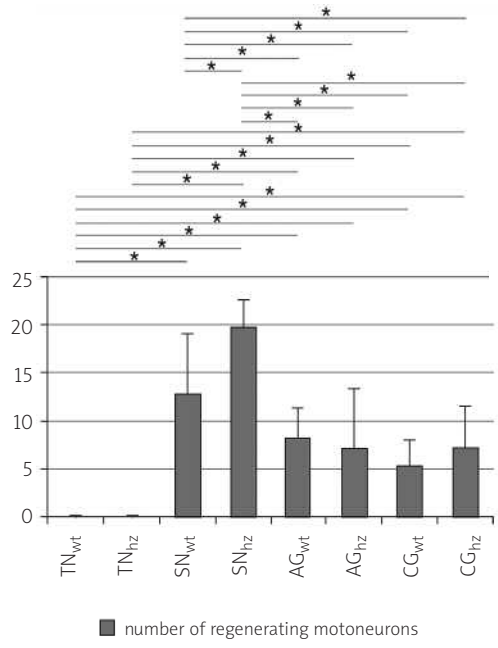

Fig. 3. The number of regenerating motoneurons that regrew their axons to the distal stump of the injured nerve and transported DiI to the cell body in wild-type (Wt) and trkB-deficient (Hz) mice. Groups: TN – nerve was transected and left cut, SN – nerve was transected and re-sutured, AG – autograft used, CG – cross-graft used (for details, see text). Asterisk indicates statistical significance between groups ($p<0.05$)

Discussion

Our study shows for the first time the possible deleterious role of trkB receptor in motoneuron survival and regeneration. We showed that a long time after peripheral nerve injury, the loss of motoneurons is markedly higher in wild-type mice than in trkB-deficient animals.

Progressive decline in the capacity of motoneurons to regenerate their axons after axotomy, together with motoneuron death, contributes to poor recovery of motor function after peripheral nerve injury. Immediate surgical repair, although providing the best environment for axons to regenerate, is not always possible. Regeneration is particularly compromised, the injury resulting in a gap between the proximal and distal stump of the nerve [14,21].

BDNF, like other nervous system growth factors, is involved in the growth, maintenance and differentiation of neurons [4,21,22,23]. It is especially important for motoneuron survival and regeneration. Exogenous BDNF enhances the regeneration of chronically axotomized motoneurons. Interestingly, it does not improve motoneuron regeneration if immediate surgical repair was performed [4]. There is also other evidence indicating the complex role of BDNF in motor axonal regeneration. BDNF activates two types of receptors, trkB and p75 [1]. Both receptors, and BDNF itself, are rapidly up-regulated in axotomized motoneurons [11,12]. The role of these two receptors in peripheral nerve regeneration, however, seems to be different. p75 is a member of the tumour necrosis factor receptor family and serves as a low affinity receptor for all neurotrophins. BDNF, when applied in low doses, was shown to promote motoneuron regeneration and survival [4]. High doses of BDNF inhibited motor axonal regeneration and this effect was mediated by activation of the p75 receptor [4]. It was also shown that expression of p75 was not crucial for motoneuron survival or prompt regeneration following facial nerve injury [7]. TrkB is a tyrosine kinase containing a receptor that binds BDNF and neurotrophin-4/5 (NT-4/5) [2]. It was shown that the expression of trkB receptors is pivotal for immature motoneuron survival after axotomy. TrkB-deficient mice show reduced regeneration of motor fibres after femoral nerve transection followed by surgical repair and electrical stimulation [5]. The influence of trkB deficiency on motor neuron regeneration, however,

seems to be complex: initial increased motoneuron regeneration, followed by early plateau and eventually poorer outcome after 8 weeks, is observed [3]. It was suggested that this early increase in regeneration results from the limited number of non--neuronal truncated trkB receptors. These receptors expressed in the distal stump of injured nerve are believed to inhibit neurite outgrowth by removing trkB ligands from the environment of the regenerating axon [8]. BDNF is up-regulated in the nerve after injury, but its level begins to increase 1 week after nerve damage [9]. In the early phase of regeneration, only a limited amount of BDNF is available for outgrowing neurites. Therefore, in the early phase of regeneration, the decreased number of inhibitory truncated trkB receptors may increase the availability of BDNF and enhance neurite outgrowth. The discrepancies between the aforementioned [3,5] and our results may originate from different duration of the study. We observed the mice for much longer, i.e. 16 weeks, and found more regeneration motoneurons in trkB-deficient animals. Given the documented multiphasic activity of trkB on motoneuron regeneration in the first 8 weeks after injury, further time-dependent changes cannot be excluded. Interestingly, in our study the beneficial effect of trkB deficiency was observed only when immediate surgical repair was performed. In the gap injury model we found no correlation between motoneuron regeneration and trkB expression.

The most surprising finding of our experiment refers to motoneuron survival after axotomy. Sixteen weeks after peripheral nerve injury, we found that in wild-type animals nearly half of motoneurons died. This loss was not attenuated by immediate surgical repair, but appeared to be markedly lower, when a trkB-deficient graft was inserted in the gap in the injured nerve. It is possible that the trkB deficient graft increases the availability of BDNF, thus creating a promising environment for the survival of axotomized motoneurons. These findings, however, require further studies.

TrkB also serves as a receptor for another ligand, NT-4/5. Recently, some reports indicated its important role in peripheral nerve regeneration [6]. mRNA level for NT-4/5 is elevated in the distal stump of the transected nerve, beginning on the 4th day after injury [9]. Application of NT-4/5 to the transected nerve immediately after injury resulted in enhanced motoneuron regeneration [20]. It cannot be excluded that NT-4/5 promoted motoneuron survival in our experimental paradigm.

In conclusion, our study is the first to show the possibly deleterious effect of trkB receptor expression on motoneuron survival and regeneration after peripheral nerve injury.

Acknowledgements

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