Abstract

The long-term outcome of peripheral nerve injury is often unsatisfactory, especially if the injury resulted in a gap between transected nerve stumps. Brain-derived neurotrophic factor and its receptor, trkB, are strongly implicated in the early phase of axonal regeneration after injury. We examined the role of trkB in long-term functional and morphological outcome of peripheral nerve injury. The sciatic nerve was transected in wild-type and heterozygous trkB-deficient mice. The nerve was either left cut or immediately sewn up or the gap injury model was performed. The gap was provided with autologous or cross (obtained from other genetic group) graft. Sciatic nerve function as well as autotomy was assessed during 16-week follow-up. The long-term functional outcome of nerve cut or immediately rejoined did not differ between wild-type and trkB-deficient mice. Gap injury provided with nerve graft resulted in better functional outcome in trkB-deficient mice than wild-type animals. Sixteen weeks after the surgery, the animals were sacrificed and histological evaluations were performed. The number of nerve fibres regenerating into the distal stump of transected and rejoined nerves did not differ between wild-type and trkB-deficient animals. TrkB deficiency markedly increased the number of Schwann cells as well as mast cells at the injury site and in the distal stump of the regenerating nerve. TrkB deficient nerves also showed higher expression of bcl-2 protein but lower of trkA and NGF than wild-type ones. Our results show for the first time the possible deleterious role of trkB receptor in long-term outcome of peripheral nerve injury.

Key words: peripheral nerve injury, nerve regeneration, nerve fibres, trkB, gap injury, nerve grafting.

Introduction

Despite the general capacity of peripheral nerves to regenerate, complete recovery is rarely achieved [31]. Even if the cut nerve is surgically repaired immediately, functional deficit may persist. Moreover, the outcome is often compromised by neuropathic pain and atrophy of the denervated limb [10]. If the injury results in a gap between proximal and distal stump of the nerve and requires...
nerve bridging, complete recovery is practically impossible [24].

The molecular mechanisms underlying peripheral nerve regeneration are still not clear. Peripheral nerve injury initiates a complex series of reactions, including the up-regulation of trophic factor expression [32]. Neurotrophins are a family of peptides that are involved in neuronal survival, differentiation and regeneration [3]. After peripheral nerve injury, neurotrophins are up-regulated in neurons and non-neuronal components of the distal stump of the nerve [14,16]. Previous studies have shown that specifically brain-derived neurotrophic factor (BDNF) is crucial for nerve regeneration processes and neuropathic pain development [1,5,6,19,39]. BDNF acts by activation of two types of receptors, trkB and p75 [1]. p75 is a member of the tumour necrosis factor receptor family and serves as a low affinity receptor for all neurotrophins. TrkB is a tyrosine kinase containing a receptor that binds BDNF and neurotrophin-4/5 (NT-4/5). TrkB is expressed in axotomized motoneurons and denervated Schwann cells in the distal stump of injured nerve [3]. However, the role of trkB in peripheral nerve repair processes and neuropathic pain phenomenon is not clear.

Genetically modified animals provide a unique model for such evaluations. In the present study, heterozygous trkB-deficient mice were used. We decided to mimic different clinical situations, including nerve transection, immediate surgical repair and gap injury. Presuming the complex role of trkB in consecutive phases of nerve degeneration and regeneration, we chose a long-term observation paradigm and decided to follow up the animals for 16 weeks after nerve injury.

Materials and methods

Mice

Mutant trkB ± (129/SvJ genetic background) mice were generated at the Centre for Molecular Biology, Hamburg University, Germany [11] and generously given by Prof. Melitta Schachner. The animals were housed 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 (450mg/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 [21,22]. In 6 animals of both groups, a 3 mm-long nerve fragment was removed to avoid spontaneous rejoining (TNhz and TNwt groups, 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 (SNhz and SNwt groups, 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 [21]. In 6 animals in each group, the graft was the same nerve piece reversed by 180° (AGhz and AGwt groups, 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 (CGhz and CGwt groups, respectively).

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

Foot print test

The functional degree of recovery was monitored every third day for the whole experimental period by evaluating the walking patterns of the mice to obtain a sciatic functional index (SFI), as described elsewhere [8,9,26]. Before the recording, a few conditioning trials acclimatising animals to the track were performed. All animals underwent preoperative walking track analysis. Briefly, the feet of the mouse hindlimbs were wetted with ink and the animals were allowed to walk along a narrow corridor on the walking track covered with scaled paper. Recordings continued until five measurable footprints were collected. All measurements were based on digitized photographs (Mustek Scanner, USA) with a personal computer-assisted system and approximated to the nearest millimetre. The factors used for calculations
were as follows: (i) print length factor PLF = (EPL – NPL)/NPL; (ii) toe spread factor TSF = (ETS – NTS)/NTS; (iii) intermediary toe spread factor ITF = (EIT – NIT)/NIT, where: NPL – normal print length, EPL – experimental print length, NTS – normal toe spread, ETS – experimental toe spread, NIT – normal intermediary toe spread, EIT – experimental intermediary toe spread. The prints were then calculated by the formula of Bain et al. [2]: SFI = −38.8xPLF + 109.5xTSF + 13.3xITF – 8.8. Value of −100 represents total impairment (palsy).

**Autotomy behaviour**

Autotomy is widely accepted as a reliable equivalent of neuropathic pain in humans [4,12,24]. In the present experiment, the mice were assessed for self-mutilation behaviour every third day throughout the follow-up, as described elsewhere [21].

After 16 weeks, all mice were deeply anaesthetized and the animals were perfused transcardially with phosphate-buffered saline (PBS) followed by cold fixative containing 4% formaldehyde in PBS.

**Muscle mass and limb circumference**

To estimate the level of atrophy of denervated muscles, the calf circumference as well as the weight of calf muscles were measured in all animals. The results obtained in the operated limbs were divided by those measured in the intact limbs and expressed as the conservation muscle mass and circumference ratios.

**Histological analysis**

The experimental sites were re-exposed and the repaired nerves were carefully dissected, postfixed, cryoprotected and then embedded in TissueTek (Sakura, Japan). 10 µm thick longitudinal cryostat sections were mounted onto slides (Menzel Glaser, Germany). The sections were treated with: (i) polyclonal antibody against growth associated protein-43 (GAP-43), which labels nerve fibre growth cones, (ii) polyclonal antibody against S-100 protein, which labels Schwann cells. In order to study the role of trkB deficiency on apoptosis regulating proteins, polyclonal (iii) anti-bcl-2 antibody, and (iv) anti-bax antibody were used. In order to find the relationship between nerve growth factor (NGF) and its receptor, trkA and trkB deficiency, monoclonal (v) anti-NGF antibody, and (vi) anti-trkA antibody were used. All primary antibodies were purchased from Chemicon, CA, USA. Secondary goat anti-rabbit IgG antibodies conjugated with Alexa 568 (Molecular Probes, USA) were used. Coverslipped sections were examined under a confocal laser scanning microscope Fluoview (Olympus, Japan). The images were digitally stored and subsequently analyzed.

We evaluated GAP-43 positive nerve endings in the distal stump of operated nerves using semi-quantitative method. Nerve cellularity was assessed as the average number of cells per high-power field.

In order to assess the number of mast cells at the surgery site, the coronal and longitudinal sections were stained with 1% toluidine blue solution, examined under light microscope, photographed and digitally stored.

**Western blot**

Two mice in each group served as a source of protein samples for immunoblotting analyses. Both operated and control (CN) nerves were analyzed.

Tissues were homogenized in RIPA lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) with 50 mM sodium fluoride and 1 mM sodium orthovanadate, supplemented with 1x Complete Protease Inhibitor Tablets (Roche, Indianapolis, IN). In order to avoid differences in sample preparation for Western blotting, all the samples were processed at the same time, in exactly the same conditions.

20 µg of proteins from the lysates were subjected to SDS PAGE in a 10% polyacrylamide gel. Afterwards, the gels were transferred onto PVDF membranes in a semi-dry blotting system (Biorad, Hercules, CA, USA). After blocking with 5% non-fat milk, the blots were subjected to primary and secondary antibodies and enhanced by chemiluminescence detection kit (Amersham, Hercules, CA, USA).

Differences in recovery signs were examined using one-way ANOVA with subsequent multiple comparisons (Tukey test). Statistical significance was set at p<0.05. All data are expressed as mean ± SD.

**Results**

All animals survived the surgery and no weight loss or other deterioration symptoms were noted.
The course of sciatic nerve functional regeneration measured by foot-print test and expressed as SFI is presented in Fig. 1. At the end of the study, SFI after nerve transection (TN groups) was worse than in SN, AG, and CG groups (p<0.05), and similar in wild-type and trkB-deficient mice (−103.5±3.8% and −104.2±4.4% in TNwt and TNhz group, respectively). Immediate nerve suturing resulted in better outcome in both wild-type and trkB-deficient mice (−38.9±5.6% and 40.4±13.8% in SNwt and SNhz group, respectively) (p>0.05). Nerve grafting, both autologous and cross, resulted in significantly better functional outcome in trkB-deficient animals than in wild-type mice. Sixteen weeks after surgery, the SFI was −77.7±8.4% in AGwt and −47.7±17.8% in AGhz group (p<0.05). In CGwt and CGhz groups, the results were −63.9±38.1% and −40.1±37.1%, respectively (p>0.05).

### Autotomy behaviour

Autotomy was not observed in any of studied animals throughout 16 weeks’ follow-up.

### Muscle mass and limb circumference

Sixteen weeks after the sciatic nerve transection, the reduction in muscle weight, in comparison to intact limb, was lowest in the SNhz and TNhz groups (21.67±8.4% and 22.03±5.4%, respectively; the difference is not significant) and the differences between these two groups and all the other groups were significant (p<0.05). Nerve transection resulted in higher muscle weight loss in wild-type mice than in trkB-deficient animals (32.27±19.32 and 21.67±8.49%, respectively). Muscle weight reduction after nerve grafting did not differ between mouse strains (Fig. 2). The reduction in the shank circumference was significantly higher in the SNwt group than in all other groups (p<0.05). Limb circumference reduction after nerve transection or grafting did not differ between mouse strains (Fig. 3).

### Histological analysis

Histological examination of the cut and immediately re-joined nerves revealed the presence of numerous, GAP-43-positive axons, penetrating through the junction area into the distal stump of the nerve. There were no significant differences between wild-type and trkB-deficient animals. In AG groups the number of GAP-43 positive fibres in the distal stump of the nerve was lower than in SN groups; however, still no differences between wild-type and trkB-deficient mice was seen (Fig. 4). There were no differences between AG and CG groups.

S-100 staining showed more Schwann cells in the nerves in all Hz groups than in respective Wt groups.

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**Fig. 1.** Sciatic Function Index (SFI) values during 16-week follow-up in wild-type (WT) and trkB-deficient (HZ) mice. Groups: TNhz – nerve was transected and left cut in trkB-deficient mice, TNwt – nerve was transected and left cut in wild-type mice, SNhz – nerve was transected and re-sutured in trkB-deficient mice, SNwt – nerve was transected and re-sutured in wild-type mice, AGhz – autograft was used in trkB-deficient mice, AGwt – autograft was used in wild-type mice, CGhz – wild-type nerve was used as a graft in trkB-deficient mice, CGwt – trkB-deficient nerve was used as a graft in wild-type mice. Asterisk indicates statistically significant difference (p<0.05) between the groups linked by the line.
Anti-Bcl-2 staining revealed numerous positive cells in both distal and proximal nerve stump in all Hz groups. Most of these cells were immunolabelled also for S100, suggesting that they were Schwann cells. Strong anti-Bax immunoreactivity was detected only in TN groups, whereas weakly labelled cells were seen in all other groups.

There were fewer NGF and trkA-positive cells in trkB-deficient mice than in respective wild-type groups (Fig. 4).

In toluidine blue staining the mast cells were also visualized. In all Hz groups the number of mast cells was significantly higher than in the respective Wt groups (Figs. 4, 5).

**Western blot**

The expression of trkB was strong in both TN groups. Wild-type nerves showed stronger trkB expression than trkB-deficient ones, except the AGwt group, in which weak trkB expression was found. Bcl-2 expression was absent in both SN groups and the CNhz group. Bax expression was detected in TNhz, AGhz, SNwt, CGwt groups (Fig. 6).
Fig. 4. Fluorescent microphotograph showing: (A) GAP-43-positive fibres, and S100-positive Schwann cells in distal stump (DS) of operated (group AG) sciatic nerve in trkB-deficient (Hz) mice. Small image shows S100 positive fibres crossing the joint site in wild-type (Wt) animals. (B) BDNF-positive and trkB-positive cells in distal stump (DS) of operated sciatic (AG group) nerve in trkB-deficient (Hz) mice. Small image shows BDNF-positive and trkB-positive cells in AGWt group. (C) NGF-positive and trkA-positive cells at the joint site of operated (SN group) sciatic nerve in trkB-deficient (Hz) mice, and (D) mast cells in TNWt group (toluidine blue staining). Groups: TNhz – nerve was transected and left cut in trkB-deficient mice, TNWt – nerve was transected and left cut in wild-type mice, SNhz – nerve was transected and re-sutured in trkB-deficient mice, SNWt – nerve was transected and re-sutured in wild-type mice, AGhz – autograft was used in trkB-deficient mice, AGWt – autograft was used in wild-type mice, CGhz – wild-type nerve was used as a graft in trkB-deficient mice, CGWt – trkB-deficient nerve was used as a graft in wild-type mice. Magnification 200x, and in small images – 80x.
Discussion

In this study we investigated the impact of trkB receptor on long-term functional and morphological outcome of peripheral nerve injury. We used various experimental paradigms in order to mimic nerve transection without rejoining the stumps, immediate surgical repair of the cut nerve and gap injury. We also observed the animals for autotomy behaviour and atrophy of denervated limb.

In our study, the long-term functional outcome of nerve cut or immediately rejoined did not differ between wild-type and trkB-deficient mice. Gap injury provided with nerve graft resulted in better functional outcome in trkB-deficient mice than wild-type animals. Our results may seem contradictory to those obtained recently by Eberhardt et al. [11] and Irintchev et al. [17]. They showed that heterozygous trkB-deficient mice presented worse recovery of quadriceps muscle than wild-type littermates after peripheral nerve injury. However, our experimental paradigm differed from theirs in several aspects. First, our study lasted much longer: 16 weeks versus up to 4 weeks in aforementioned experiments. In 8-week-long observations the influence of trkB deficiency on motor neuron regeneration was complex: initial increased motoneuron regeneration, followed by early plateau and eventually poorer outcome [5]. Therefore, further time-dependent changes in peripheral nerve regeneration progress in trkB-deficient mice cannot be excluded. 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 [13]. mRNA for BDNF is up-regulated in the nerve after injury, but its level begins to increase 1 week after nerve damage and decreases after 4 weeks [14]. In the early phase of regeneration, only a limited amount of BDNF is available for outgrowing neurites. Therefore, the decreased number of inhibitory truncated trkB receptors may increase the availability of BDNF to regenerating fibres and thus enhance neurite outgrowth. Sixteen weeks after nerve injury, the impact of BDNF and its receptors on axon regeneration may be reversed.

Second, in the studies of both Eberhardt et al. [11] and Irintchev et al. [17], the cut nerve stumps were separated from the vicinity by a silicone tube. The injury site was at least partly protected from cells and factors that normally migrate there during Wallerian degeneration. Some of them may act differently in trkB-deficient and wild-type animals; thus the influence of the environment may contribute to the discrepancies between our results and those of Eberhardt et al. [11] and Irintchev et al. [17]. Moreover, in the Eberhardt et al. [11] experiment nerve regeneration was stimulated electrically, whereas we did not use any additional stimulants.

Atrophy of a denervated limb may markedly impair the functional outcome of peripheral nerve injury. In our study, after nerve transection and immediate repair we found more extensive decrease in muscle weight and limb circumference in wild-
-type mice than in trkB-deficient animals. Gap injury resulted in similar muscle atrophy in both strains of mice. The process of muscle atrophy depends mostly on successful reinnervation; however, direct effect of trkB deficiency should also be kept in mind. There are no data existing on the muscle pathology related to trkB deficiency. Mousavi et al. [29] showed that BDNF rescued muscle fibres after neonatal nerve injury, but they did not study the role of trkB receptor. The beneficial effect of trkB-deficiency on muscle atrophy after nerve injury requires further elucidation.

Throughout the whole follow-up we did not observe any signs of autotomy in operated animals. Autotomy behaviour in animals has been discussed as a reliable equivalent of neuropathic pain in humans [7,18], although it can also be observed in humans, especially neonates and elderly people [4]. Our results are a bit surprising, as in many other experiments self-mutilation behaviour was observed during regeneration processes [21,22]. Moreover, our recent study [21] showed increased neuroma formation in trkB-deficient mice in comparison to wild-type animals after peripheral nerve transection. Neuroma developing at the injury site is strongly implicated in the neuropathic pain phenomenon [24]. However, it is well established that a tendency for autotomy behaviour varies between mice strains [30]. It cannot be excluded that 129SvJ mice are highly resistant to autotomy. Another possible explanation of this finding is the involvement of membrane-bound trkB receptors in the spinal cord in the neuropathic pain. These receptors’ expression is increased after peripheral nerve injury leading to protein kinase C (PKC) cascade activation [25]. It is documented that spinal cord PKC activation plays a critical role in pain-related behaviour in mice [34-36]. Therefore, it cannot be excluded that in trkB deficient animals, autotomy behaviour may be attenuated because of a limited number of membrane bound trkB receptors in the spinal cord.

Our results also indicate that trkB deficiency increases the number of mast cells invading the injury site in the chronic phase of regeneration. The role of mast cells in peripheral nerve regeneration is complex. They are attracted by several factors involved in the nerve regeneration processes, including BDNF, VEGF and transforming growth factor-beta-1 (TGF-beta-1) [38]. In trkB-deficient animals the availability of BDNF, at least in the early phase of regeneration, is higher than in wild-type mice. One can presume that it may stimulate mast cells accumulation. On the other hand, mast cells release histamine, leukotriens,
tumour necrosis factor-alpha (TNF-alpha), vascular endothelial growth factor (VEGF), various proteases and other substances [23,39]. Some of these factors, such as VEGF, are beneficial for neurite outgrowth. Mast cell accumulation may contribute to neuroma formation at the injury site, as intensive mast cell accumulation in neuromas and neurofibromas was formerly reported [38]. Our previous report [20] showed that trkB-deficient animals are more prone to develop neuroma after peripheral nerve injury than wild-type mice.

In our study, histological evaluations revealed that the number of nerve fibres regenerating into the distal stump of transected and rejoined nerve did not differ between wild-type and trkB-deficient animals. Nor were any differences between the two mice strains observed after gap injury and nerve grafting. Our results indicate that trkB deficiency did not influence the neurotrophic activity of the distal stump of the injured nerve. These findings may seem confusing; however, the poor correlation between morphological and functional features of regeneration is well documented [8]. Moreover, in our study the expression of trkB in the distal stump of the transected nerve was similar in wild-type and trkB-heterozygous mice, as shown in Western blot analysis.

We found that trkB deficiency markedly increased the number of Schwann cells in the distal stump of the regenerating nerve 16 weeks after the injury. This may result from changed behaviour of Schwann cells per se, or may reflect disturbed signalling from neurons, mast cells and denervated target. The impact of trkB deficiency on Schwann cells has not been studied yet. Given the important role of BDNF in myelination and remyelination in the peripheral nervous system [39], it is likely that trkB is strongly involved in these processes. Many Schwann cells found in trkB-deficient animals were bcl-2 positive. Moreover, bcl-2 positive cells were more numerous in trkB-deficient than in wild type nerves, regardless of the surgical procedure. Bcl-2 protein is one of the strongest cell death inhibitors and is able to protect different mammal cells from apoptosis induced by various factors [33]. In contrast, Bax protein, belonging to the same superfamily of cell death regulators, promotes apoptosis [15,33]. An increase in Bax expression was reported in cells dying due to various apoptotic stimuli. According to recent studies, Bax expression seems to be pivotal in motoneuron apoptosis induced by various stimuli [15]. In this experiment, we demonstrated strong Bax expression in transected and not rejoined nerves and this may reflect apoptosis of neurons transected and deprived from their targets.

The role of the other trkB ligand, NT-4/5, in peripheral nerve regeneration is not well established. mRNA level for NT-4/5 is elevated in the distal stump of the transected nerve, beginning from the 4th day after injury [14]. Application of NT-4/5 to the transected nerve immediately after injury resulted in enhanced motoneuron regeneration [21]. This effect was especially marked on motoneurons innervating type 1 and 2a muscle fibres. The impact of truncated trkB receptors found on Schwann cells in the distal stump of the regenerating nerve on NT-4/5 availability is not known, but one can presume that it may be similar to the one observed for BDNF.

The level of BDNF in the nerve after lesion is up-regulated by NGF [27,28]. Therefore, we studied the role of trkB-deficiency on NGF and its receptor, trkA expression during nerve regeneration. We found that in trkB-deficient mice the expression of both NGF and trkA was decreased in comparison to wild-type animals, regardless of the surgical procedure. The possible reciprocal relationship between these two neurotrophins and their receptors remains to be clarified.

In conclusion, our study shows for the first time the possible deleterious role of trkB receptor in long-term outcome of peripheral nerve injury. Decreased expression of trkB seems to be beneficial specifically in gap injury that requires nerve grafting. TrkB deficiency results in increased accumulation of mast cells at the injury site; however, the contribution of this effect on long-term outcome of peripheral nerve injury requires further studies.

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