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The role of trkB receptor in the formation of post-traumatic neuroma

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Abstract

The outcome of peripheral nerve injury is often impaired by post-traumatic neuroma developing at the injury site. Neuroma is usually accompanied by neuropathic pain, which is usually resistant to most analgesics and presents a serious clinical problem. The mechanisms underlying post-traumatic neuroma remain unclear, but they are likely associated with regeneration processes. Brain-derived neurotrophic factor (BDNF) and its receptor, trkB, are strongly implicated in axonal regeneration after injury. The aim of this work was to examine the role of trkB in post-traumatic neuroma formation. 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 an autologous or cross (obtained from another genetic group) graft. Sixteen weeks after surgery, the animals were sacrificed and histologic evaluations were performed. We found very limited or no neuroma formation in wild-type animals, regardless of the surgical procedure. In the majority of trkB-deficient mice, the post-traumatic neuroma was found at the end of the proximal stump of the transected nerve. In the gap injury model, in trkB-deficient animals receiving wild-type graft, there was no neuroma at the join site between the graft and distal stump of the nerve. In contrast, if the graft was autologous, neuroma formed at both joints. We also noticed many more mast cells accumulated at the surgery site in trkB-deficient than in wild-type animals. These results indicate the important role of BDNF receptor in post-traumatic neuroma formation.

Key words: *peripheral nerve injury, nerve regeneration, nerve fibres, BDNF, mutant mice, nerve grafting.*

Introduction

Despite the general capacity of peripheral nerves to regenerate, complete recovery of function after nerve injury is rarely achieved. Moreover, the outcome of peripheral nerve injury is often impaired not only by persistent functional loss but also by neuroma developing at the end of the proximal stump of the damaged nerve [15, 22]. Post-traumatic neuroma is often associated with neuropathic pain, chronic, highly unpleasant and resistant to most therapeutic strategies that may further impair peripheral nerve repair [5]. So far, the best and practically only method of post-

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traumatic neuroma treatment is to remove it surgically [11,15]. It requires, however, an extra surgical procedure and rarely provides satisfactory outcome, as a piece of nerve has to be cut off and, usually, replaced by graft. This may induce further neuroma and the need for multiple interventions [15].

Therefore, techniques of neuroma prevention are currently under investigation [11,14,15,20]. The ideal technique of nerve repair should provide rapid and complete regeneration without any side effects. Alas, despite much progress in neuroscience and microsurgery, methods introduced so far usually are neither effective nor painless.

Neuroma is a bulb-shaped thickening created by improperly, irregulary regenerating nerve fibres [15]. The molecular and cellular background of neuroma development after nerve injury is not clear, but several mechanisms are widely accepted to be involved. Peripheral nerve injury initiates a complex series of reactions. First, soon after the injury, the axons in the distal stump of the nerve begin to degenerate. When the myelin and neural debris is cleared, Schwann cells proliferate and form so-called Buengner bunds, the paths for regenerating axons [22]. It is likely that neuroma formation is related to improper nerve fibre regrowth resulting at least partly from impaired injury site clearing. During Wallerian degeneration, neurotrophic factors produced in the distal nerve stump diffuse and attract nerve fibres from many different directions. Also, the forming connective tissue scar additionally disperses them, contributing to neuroma development.

Previous studies have shown that brain-derived neurotrophic factor (BDNF) and its receptor trkB are crucial for nerve regeneration processes [2,3,10,21,28]. Moreover, we have shown that BDNF plays an important role in post-traumatic neuroma development (unpublished data). The aim of the present work was to establish the role of trkB receptor in neuroma formation 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.

Materials and methods

Mice

Mutant trkB +/- (129/SvJ genetic background) mice were generated at the Centre for Molecular Biology, Hamburg University, Germany [6] 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). In 6 animals of both groups, a 3 mm-long nerve fragment was removed to avoid spontaneous rejoining and muscles and skin were closed in layers (4/0, Ethicon, USA), then the animals were placed back in separate cages (groups TN_{hz} and TN_{wt}, respectively).

In 6 animals of the Hz as well as Wt group, the transected nerve was immediately sewn up (groups SN_{hz} and SN_{wt}) 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. In 6 animals in each group, the graft was the same nerve piece reversed by 180° (groups AG_{hz} and AG_{wt}). In order to establish the role of trkB in the graft in neuroma formation, 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).

Autotomy behaviour

Autotomy is widely accepted as a reliable equivalent of neuropathic pain in humans [4, 12, 23]. In the present experiment, the mice were assessed for self-mutilation behaviour every third day throughout the follow-up.

Histologic analysis

After 16 weeks, the animals were sacrificed and perfused through the heart with saline followed by

fixative mixture (saline and 4% paraformaldehyde). The experimental sites were re-exposed and the repaired nerves were carefully dissected, postfixed, cryoprotected and then embedded in TissueTek (Sakura, Japan). 10μm-thick longitudinal cryostat sections were mounted onto slides (Menzel Glaser, Germany).

In order to find neuroma development at the surgery site, the coronal and longitudinal sections were stained with 1% toluidine blue solution staining, examined under light microscope, photographed and digitally stored. On toluidine blue microphotographs, the number of mast cells seen at the joint site was counted. The regenerating fibres were also visualized by immunofluorescent labelling with anti-GAP-43 antibody, as described elsewhere [12]. Briefly, the sections were treated with rabbit polyclonal antibody against growth associated protein-43 (GAP-43), which labels nerve fibres and? growth cones and then with secondary goat anti-rabbit IgG antibody conjugated with Alexa 568 (Molecular Probes, USA). Coverslipped sections were examined under a confocal laser scanning microscope Fluoview (Olympus, Japan). The images were digitally stored and subsequently analyzed.

Table I. Incidence of neuroma formation in individual groups

Group			Neuroma incidence n (%)
WT	TN_{wt}		2 (33.4)
	SN_{wt}		0 (0)
	AG_{wt}	proximal joint	1 (16.7)
		distal joint	0 (0)
	CG_{wt}	proximal joint	1 (16.7)
		distal joint	0 (0)
Hz	TN_{hz}		6 (100)
	SN_{hz}		5 (83.3)
	AG_{hz}	proximal joint	5 (83.3)
		distal join	5 (83.3)
	CG_{hz}	proximal joint	4 (66.7)
		distal joint	0 (0)

Results

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

Whole dissected operated nerves in all Hz groups were markedly thicker than in Wt groups, while uninjured nerves did not differ.

Post-traumatic neuroma was recognized macroscopically as swelling of the nerve ending or the operation site, together with adhesions to surrounding tissues. Microscopically, it was recognized as bulb-shaped thickening, with chaotically arranged nerve fibres, growing in all directions. The incidence of post-traumatic neuroma formation found at the injury site by immunohistochemical and histological analysis is presented in Table I. In wild-type animals significantly fewer neuromas were found than in trkB-deficient mice, regardless of the surgical procedure. Only 2 (33.4%) mice in the TN_{wt} group developed a neuroma at the end of the proximal nerve stump. In all animals in the TN_{hz} group haphazardously organized outgrowing fibres were observed at the end of the proximal stump of the transected nerve (Fig. 1). In the majority (5 of 6, 83.3%) of mice from the SN_{hz} groups and no mouse in SN_{wt}, neuromas were present at the joint site (Fig. 2). In autologically grafted nerves of Hz animals (AG_{hz} group), neuromas were found at both joint sites in the majority (5 of 6, 83.3%) of animals. In 4 (66.7%) cross-grafted Hz mice (Cg_{hz} group) neuromas were present at the joint between the proximal stump and the graft. In both AG_{wt} and CG_{wt} groups neuromas were present at the proximal joint in 16.7% of animals. No neuromas were found at the joint between the graft and the distal stump of the grafted nerve.

In toluidine blue staining the mast cells were also visualized. In all Hz groups the number of mast cell was significantly higher than in the respective Wt groups (Figs 1, 3).

Discussion

Comparing the post-traumatic neuroma formation in wild-type and trkB-deficient mice, we found that trkB deficiency created favourable conditions for haphazardous regrowth of axotomized nerve fibres. However, this did not correlate with the autotomy

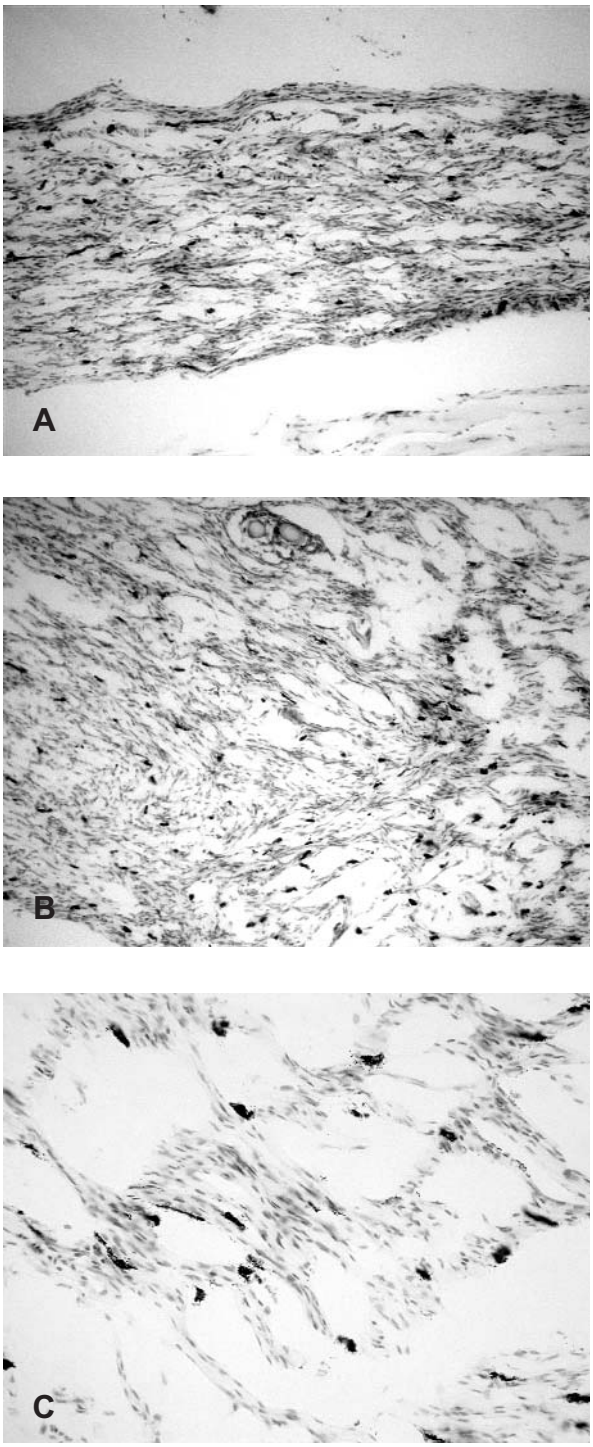


Fig. 1. Microphotograph showing toluidine blue stained nerve fibres: (A) – regularly, parallel arranged in SN_{wt} group, and (B) – haphazardly organized in SN_{hz} group, (C) – mast cells at injury site in SN_{wt} group. Magnification 200x – (A) and (B), 400x – (C)

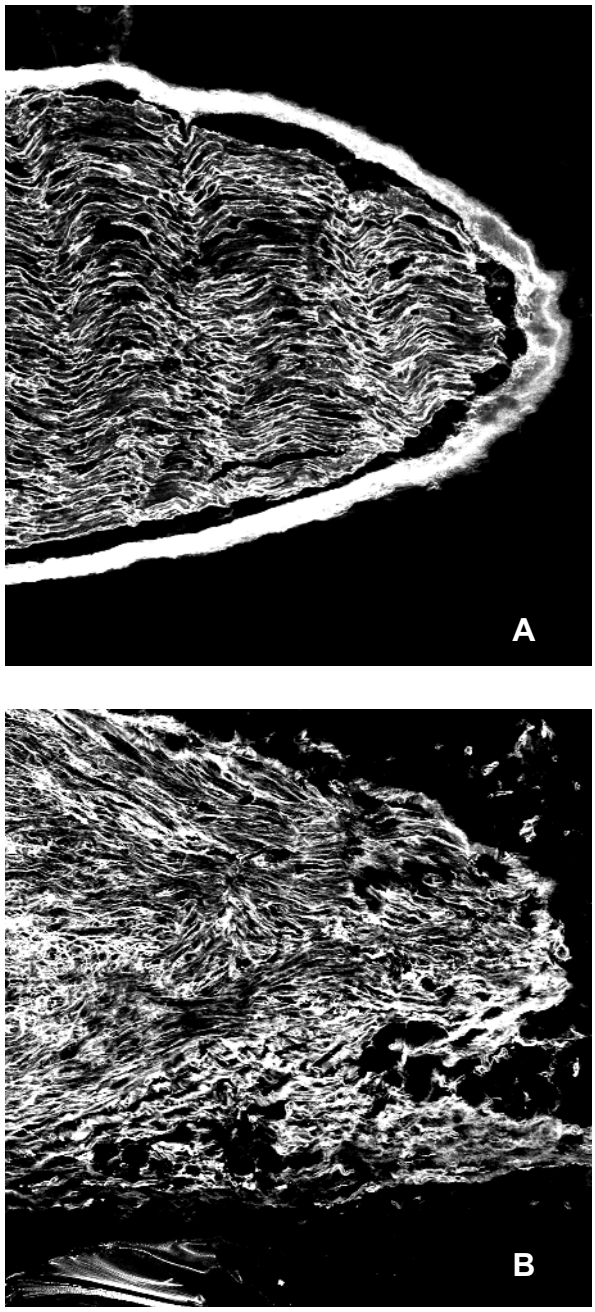
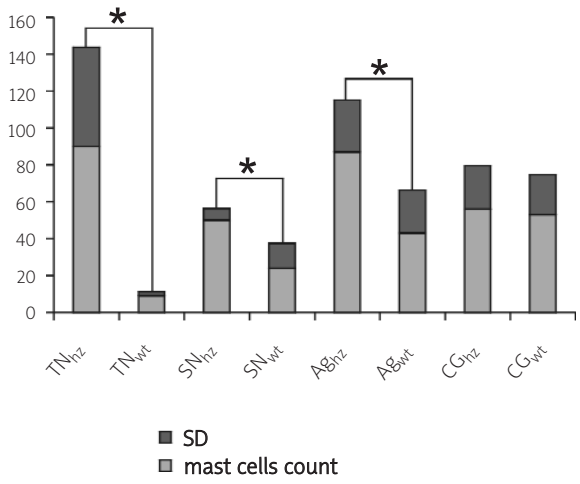


Fig. 2. Fluorescent microphotograph showing GAP-43-positive nerve fibre cells in distal stump of operated sciatic nerve in TN_{wt} (A) and TN_{hz} (B) mice. Note regularly arranged fibres as well as well-defined connective tissue capsule around proximal stump of transected nerve in TN_{wt} group. In TN_{hz} group fibres are dispersed in all directions and there is no visible capsule. Neuroma was attached to vicinity. Magnification 400x



Hz – heterozygous animals, wt – wild-type animals, TN – transected nerve, SN – sutured nerve, AG – nerve provided with autologous graft, CG – nerve provided with cross-graft (see text for details) proximal joint – joint between proximal nerve stump and graft, distal joint – joint between graft and distal stump of nerve

Fig. 3. Number of mast cells seen at injury site. In all groups, mast cells were counted in toluidine blue microphotographs. In TN groups, mast cells were counted at the end of the proximal stump of the transected nerve; in SN group, at the joint site. In Ag and CG groups, mast cells were counted at the joint between proximal nerve stump and graft. Asterisk indicates statistically significant difference; $p<0.05$

behaviour, which was not observed in either of the animals. The latter finding may be related to the strain of animals. It is well established that a tendency for autotomy behaviour varies between mice strains [19]. It was not examined in 129/SvJ mice, but given our results one may presume that this strain is particularly resistant to self-mutilation.

BDNF is a member of the neurotrophin family, binding 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 an injured nerve (10). However, the role of trkB in peripheral nerve repair processes is not clear. It was shown that the expression of trkB receptors is pivotal for motoneuron survival after axotomy. In a recent report, heterozygous trkB-deficient mice after peripheral nerve injury presented decreased precision of neurite regrowth [6]. Interestingly, the influence of trkB deficiency on motor neuron regeneration is complex: initial increased motoneuron regeneration, followed by early plateau and eventually poorer outcome, is observed [2]. 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 upregulated in the nerve after injury, but its level begins to increase one week after nerve damage [9]. In the early phase of regeneration, only a limited amount of BDNF is available for outgrowing neurites. In the peripheral nervous system, BDNF plays a pivotal role in axotomized neuron survival and regeneration [28]. 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. In our previous experiment, exogenous BDNF applied to the proximal stump of injured nerve caused an increase in neuroma development in rats (unpublished data). Together, these observations may at least partly explain the higher incidence of neuroma formation in trkB deficient mice in comparison to wild-type animals observed in the present study.

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 on the 4th day after injury [9]. Application of NT-4/5 to the transected nerve immediately after injury resulted in enhanced motoneuron regeneration [21]. This effect was especially marked in 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. Therefore, not only BDNF, but also NT-4/5 increased availability may be responsible for neuroma formation in trkB-deficient mice.

Our results indicate also that trkB-deficiency increases the number of mast cells invading the

injury site in the chronic phase of regeneration. Accumulation of intensive mast cells in neuromas and neurofibromas was formerly reported [18, 27]. The role of mast cells in peripheral nerve regeneration is complex. They are attracted by several factors involved in nerve regeneration processes, including BDNF, VEGF and Transforming Growth Factor-beta-1 (TGF-beta-1) [27]. In trkB-deficient animals the availability of BDNF, at least in the early phase of regeneration, is higher in comparison to wild-type mice. One can presume that it may stimulate mast cell 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 [18]. Some of these factors, like VEGF, are beneficial for neurite outgrowth. Others, like histamine, contribute to neuropathic pain phenomenon. Moreover, in neuroma there is an alternative way of mast cell degranulation, by direct mechanical irritation. In this context, most interesting is the absence of autotomy behaviour in association with neuroma formation in our experiment. One possible explanation of this finding is the involvement of membrane-bound trkB receptors in the spinal cord in neuropathic pain. Expression of these receptors is increased after peripheral nerve injury leading to protein kinase C (PKC) cascade activation [26]. It is documented that spinal cord PKC activation plays a critical role in pain-related behaviour in mice [16, 24, 25]. Therefore, it cannot be excluded that in trkB deficient animals, autotomy behaviour may be attenuated because of the limited number of membrane-bound trkB receptors in the spinal cord.

It should also be kept in mind that mast cell degranulation stimulates collagen depositions by fibroblasts and therefore contributes to neuroma formation [13]. Fibrosis is usually associated with mast cell degranulation. Together with dispersion of regenerating fibres by forming connective tissue scar this creates a real vicious circle.

This study provides the first direct evidence that trkB receptor plays an important role in neuroma formation after total nerve transaction and further validates BDNF/trkB signalling as a potential target for therapeutic intervention in such cases.

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