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The influence of reduced expression of the TrkB receptor on peripheral nerve regeneration in mice

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"It is difficult to say what is impossible, for the dream of yesterday is the hope of today and the reality of tomorrow"

Robert Goddard (1882 – 1945)

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1 Introduction

1.1 Clinical aspects of peripheral nerve injury

Peripheral nerve lesions, commonly caused by tumors, traumata or surgical interventions are still a challenge for today's high tech medicine (Lundborg, 2003). Injured peripheral neurons in mammals, in contrast to nerve cells in the central nervous system, have the ability to regenerate their axons over long distances with a rate of 1-3 mm per day (Burnett and Zager, 2004). Despite this high potential, functional recovery is often poor. Chronic neuralgia and disabilities due to loss of motor function persist frequently life-long, even after surgical nerve repair (Fu and Gordon, 1997; Lundborg, 2003).

1.2 History of surgical nerve repair

The first efforts in readapting peripheral nerves were made in the 13th century by William de Saiceto and Lanfranchi of Milan (Kretschmer and Richter, 2004). In the following centuries only rare findings were added. In 1872 Silas Weir Mitchell published his experiences with peripheral nerve injuries and neuropathic pain in the civil war. He made the first descriptions of phantom sensations, ascending neuritis, as well as causalgia and discussed various treatment methods (Weaver, 2004). In 1920 the BMRC (British Medical Research Council) published a study in which all results of treatments and outcomes of peripheral nerve injuries of the First World War were gathered. These results showed that transplantations were in the majority of cases without success because of

wound infections and sepsis, which were the main problems at that time (Woodhall and Bebe, 1956).

After the Second World War when antibiotics were commonly used, regeneration success was dependent on the type of peripheral nerve and that, in cases of extensive nerve damage, transplantation of a piece of autologous nerve to bridge the gap had better outcome than superannuated treatments like nerve adaptation under tension (Woodhall and Bebe, 1956).

The next important step was made in the early 1970s when microsurgical methods were developed. By using a surgical microscope, sutures could be made much more precise and gentle by coapting individual nerve fascicles (Kretschmer and Richter, 2004).

Since the 1980s, the molecular biological findings about nerve regeneration increased enormously but they did not result in corresponding development in the clinical field. Clinically, nerve injuries are treated in the same way as 30 years ago (Lundborg, 2000). The interest of scientists has shifted from primarily focusing on surgical repair techniques to basic biological processes regulating and influencing key factors, such as post-traumatic cell death and the influence of the molecular composition of the local microenvironment. Various types of conditioning treatments and extrinsic manipulations have been suggested, such as the application of growth factors, hormonal treatments, electrical stimulation, application of electromagnetic fields and treatment with hyperbaric oxygen. Advanced bioengineering techniques have been applied to develop alternatives to autologous nerve grafts using grafts based on biological or non-biological scaffolds in combination with non-neural cells or neurotrophic factors. Unfortunately, none of these techniques has so far reached the status of an accepted principle for clinical treatment (Lundborg, 2003).

For the future it is important to understand peripheral nerve regeneration mechanisms in much more detail in order to devise novel strategies for optimal treatment of peripheral nerve injuries.

1.3 Peripheral nerve regeneration

Neuronal survival after peripheral nerve injury depends on several factors, such as neuron type, developmental stage and the degree and proximity of the injury (Fu and Gordon, 1997). Generally, sensory neurons are more susceptible than motoneurons (Burnett and Zager, 2004) and neonatal neurons are more susceptible than adult neurons (Brushart, 1988; Le et al., 2001). Further, more severe traumatic injuries induce more cell death than mild injuries (Maggi et al., 2003). Injuries close to the cell body are more damaging than distal injuries (Ygge, 1989): spinal root avulsion injury causes substantial cell death while damage remote to the spinal cord causes little or no neuronal death (Maggi et al., 2003). Furthermore, clinical studies and animal experiments have shown that there are several factors that limit regeneration of injured peripheral nerves and functional recovery. Changes in cortical representations, altered neuronal excitability, regrowth of axons into inappropriate targets, the slow rate of regeneration limited by the rate of slow axonal transport (1-3 mm per day), abnormal spread of excitatory currents in the regenerated nerve and polyneuronal innervation of muscle fibers are potential causes for functional impairment after peripheral nerve injury and repair (Cossu et al., 1999; Fenrich and Gordon, 2004; Guntinas-Lichius et al., 2005; Lundborg, 2003; Navarro et al., 2007).

1.3.1 The cellular and molecular basis for peripheral nerve regeneration

The changes that occur after axotomy of peripheral nerves may be divided into two groups: changes in the distal nerve stump and changes in the cell body and the nerve stump proximal to the lesion side (Burnett and Zager, 2004; Navarro et al., 2007).

1.3.1.1 Changes in the distal nerve stump

Axons that are physically separated from the neuronal cell body after nerve injury undergo Wallerian degeneration (Fig. 1) (Fenrich and Gordon, 2004). The Wallerian degeneration implies a sequence of events in the distal segment of axotomized nerves. One of the first morphological changes is disintegration and degeneration of the axolemma and axoplasma within 24 hours in small and 48 hours in large nerve fibers. Axonal breakdown is mediated by calcium influx via ion-specific channels and involves activation of axonal proteases (Stoll et al., 2002). As a response to axonal degeneration, Schwann cells distal to the lesion side dedifferentiate, down-regulate their myelin protein synthesis within two days. Schwann cells are involved in the removal of myelin debris and, after several division cycles, align into columns inside axonal basal laminae known as bands of Bünger. These bands serve as guiding tubes for regenerating nerve fibers from the proximal nerve stump and are the source of a large number of regeneration associated genes (RAGs), including cell adhesion molecules, cytokines, neurotrophic factors as well as their receptors.

1.3.1.2 Changes in the cell body and proximal nerve stump

While the axons distal to the lesion side undergo Wallerian degeneration, axons of the proximal nerve stump die back to the first node of Ranvier (Fenrich and Gordon, 2004). The cell body of axotomized peripheral motoneurons undergoes characteristic morphological changes that are collectively referred to as "chromatolysis" (Burnett and Zager, 2004). These include the migration of the nucleus from the center to the periphery of the cell body and the breakup and dispersion of the ordered arrays of rough endoplasmatic reticulum, the Nissl substance, within 6 hours (Fig. 1).
These changes are believed to be the precondition for the alterations in mRNA synthesis in the axotomized neurons, concurrent with the

conversion of the neuronal mode of operation from the normal "transmitting" to the "growth" mode that supports axonal regeneration. The production of RAGs, including GAP-43 (growth-associated protein 43), CAP-23 (cytoskeleton-associated protein 23), which both have been shown to be very important mediators of growth cone elongation, is up-regulated (Bomze et al., 2001). Also actin, tubulin and CGRP (calcitonin gene-related protein) are generated more excessively. In contrast, other genes involved in synaptic transmission like ChAT (choline acetyltransferase) are down-regulated.

Regenerating axon sprouts arise from the first node of Ranvier proximal to the injury side. The first nerve fibers emerging from the proximal nerve stump undergo extensive branching. A single axon gives rise to as many as 50 -100 branches, but these new branches often form spirals or ringlets that later disappear. Only an average of 5 sprouts per regenerating axon reaches the distal nerve stump and continues to advance distally as a "regeneration unit" which eventually becomes reconnected with a peripheral target (Fu and Gordon, 1997).

If none of the regenerating and sprouting axons reaches the distal nerve stump due to shortage of guidance cues (Guntinas-Lichius et al., 2002), the regeneration process fails. Amputation neuroma is formed at the distal end of proximal nerve stump and the parent neurons survive in an atrophic state or die.
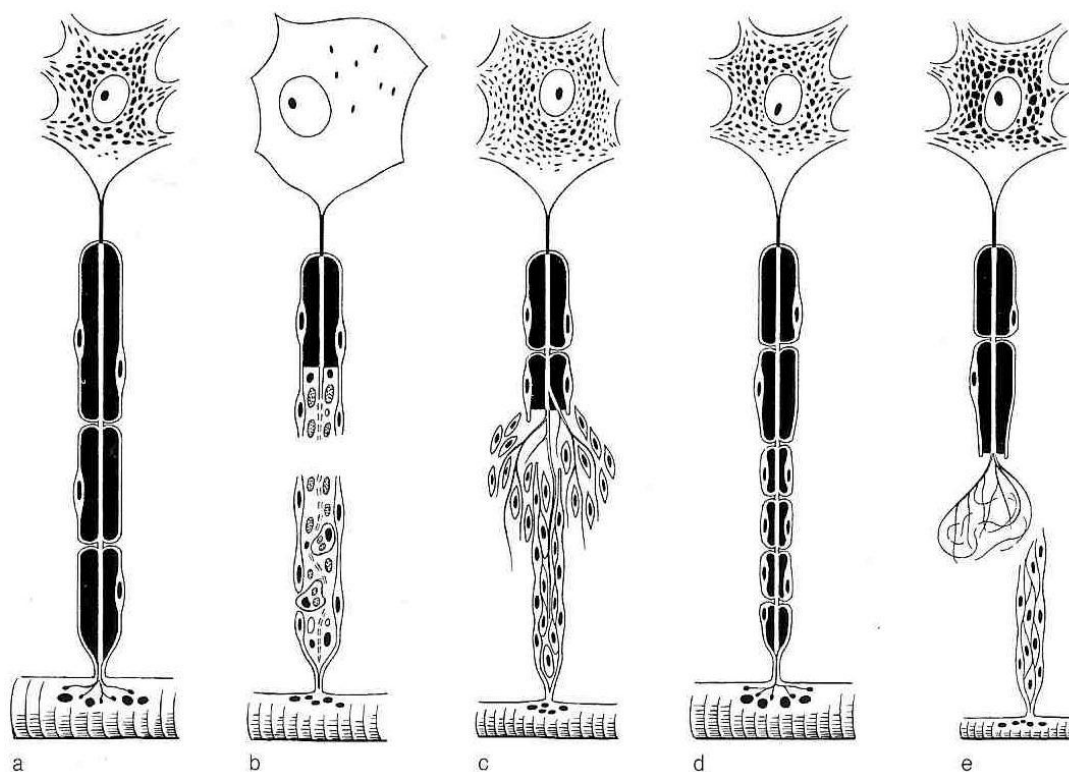
Figure 1: Structural changes in the cell body, the axon, its sheath, as well as in the associated muscle cells (at the bottom of the cartoons) after transection of a peripheral nerve (a – e). Normal anatomical appearance of the neuron (a). Hours after nerve lesion the cell nucleus shifts to the periphery of the perikaryon and the Nissl substance begins to disappear. Distal to the lesion side, the axon degenerates and its remnants are phagocytozed by macrophages (b). The proximal axonal segment rapidly, within hours after injury, forms new collaterals (sprouts). Days after axotomy the denervated muscle fibers begin to atrophy. The Schwann cells throughout the distal nerve stump proliferate and form a compact string which is invaded by the outgrowing axons. Axonal sprouts can also grow to the surrounding area of the lesion side and thus regrow along inappropriate nerve branches (c). Several months after nerve transection the regeneration is eventually successful: cell body, axon and muscle cell have their normal appearance (d). If regeneration fails, the outgrowing axons form an amputation neuroma. The muscle fibers continue to degenerate (e). (From Schiebler and Schmidt, 2003).

1.3.1.3 Preferential motor reinnervation

It has been demonstrated that motor axons in mixed nerves have the tendency to selectively reinnervate muscle or muscle nerve branches as opposed to skin or purely sensory nerve branches (Brushart, 1988, 1993). This phenomenon is known as preferential motor reinnervation (PMR). Several studies showed that axons preferentially reinnervate the muscle, rather than the sensory branch when access to both branches is given

equally (Brushart, 1988; Madison et al., 1996). Furthermore, PMR occurs whether or not axons eventually contact muscle or skin, indicating that the nerve pathway itself is competent to generate preferential motor reinnervation (Brushart, 1993; Brushart et al., 1998). It is also known that motor axons initially grow into both nerve branches equally but over time are preferentially retained in the muscle branch, partially by pruning axon collaterals in the inappropriate sensory branch (Brushart, 1993). Muscle afferents, i.e. sensory axons to muscles, also display a preference to reinnervate the muscle branch, although the time course and mechanism of this reinnervation remains unknown (Madison et al., 1996).

Finally, Schwann cells previously associated with motor axons differ from those previously associated with sensory axons in their ability to express a specific molecule, the HNK-1 carbohydrate epitope (human natural killer cell epitope 1), when contacted by regenerating motor axons (Martini et al., 1994).

1.3.2 Regeneration-Associated Molecules

A large number of molecules can potentially regulate axonal regeneration either directly or indirectly via their effects on non-neuronal cells. These molecules can be roughly divided into three groups: neurotrophic factors, cell adhesion molecules and extracellular matrix proteins (Fu and Gordon, 1997).

1.3.2.1 Neurotrophins

The neurotrophins are a family of neurotrophic factors, structurally and functionally related polypeptides, that mediate neuronal survival, neurite outgrowth and differentiation, functional plasticity and development in a wide variety of neuronal populations in the peripheral and central nervous system (Boyd and Gordon, 2001). NGF (nerve growth factor), the prototypical member of the neurotrophin family, was isolated, purified and identified as a diffusible factor which promoted the survival and neurite

outgrowth of sympathetic and sensory neurons both in *vitro* and in *vivo* in classic experiments almost half a century ago (Shooter, 2001). Decades later, a second molecule belonging to this family, the brain-derived neurotrophic factor (BDNF), was purified and cloned (Barde et al., 1982). Recent advantages in cloning and molecular biology allowed the identification of additional neurotrophins and the neurotrophin family now consists of 4 members in mammals: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). Two additional members, NT-6 (neurotrophin 6) and NT-7 (neurotrophin 7) have been identified and cloned in fish, but mammalian homologs have not yet been identified (Götz et al., 1994; Lai et al., 1998). Neurotrophin signaling is mediated via two classes of transmembrane receptors: members of the Trk (tyrosine kinase receptor) receptor family (Huang and Reichardt, 2003) and the p75 neurotrophin receptor, a member of the tumor necrosis factor receptor superfamily (p75NTR; Friedman and Greene, 1999; Lu et al., 2005). Whereas all neurotrophins bind p75 with similar affinity (Rodriguez-Tébar et al., 1990), the three Trk subtypes, TrkA, TrkB and TrkC, show preferential selectivity towards the various neurotrophins (Fig. 2): TrkA is preferentially activated by NGF and NT-3 (Hempstead et al., 1991; Kaplan et al., 1991), TrkB by BDNF and NT-4/5 (Klein et al., 1991; Klein et al., 1992) and TrkC by NT-3 (Lamballe et al., 1991).
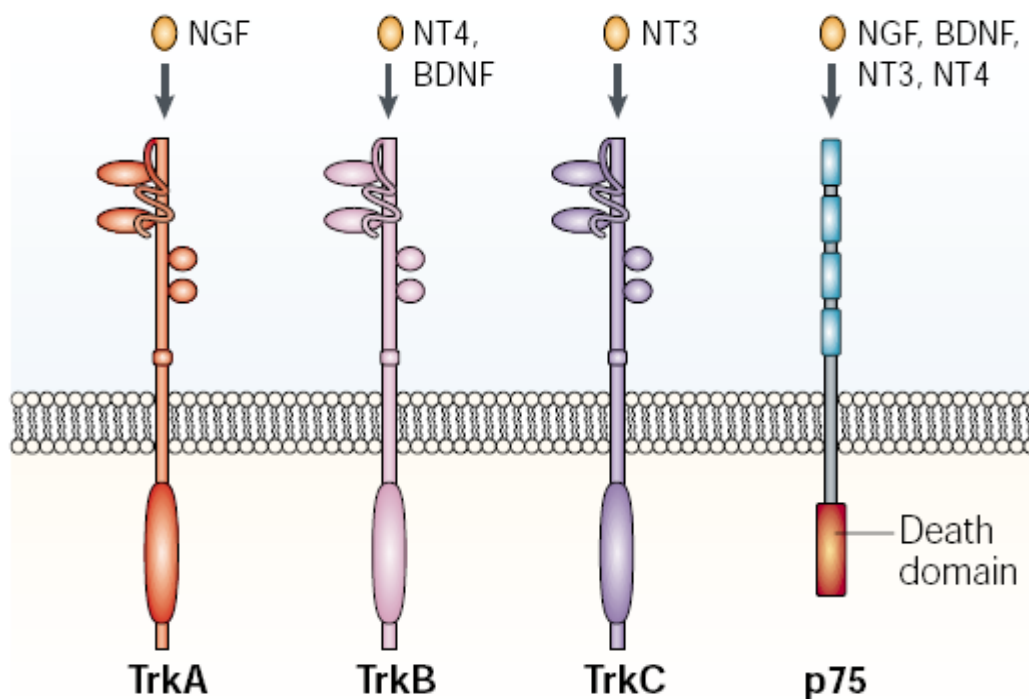
Figure 2: Model of Trk and p75 receptor binding. Every tyrosine kinase receptor is activated by binding of specific members of the neurotrophin family, whereas all neurotrophins bind to the p75 receptor (Chao, 2003).

1.3.2.2 Brain-derived neurotrophic factor and neurotrophin-4/5

First identified in 1982 (Barde et al., 1982), BDNF is a 27-kd homodimer with transcripts found in many organ tissues, including skeletal muscle (Hofer et al., 1990). It has been suggested that BDNF is, for many reasons, a good candidate to promote motor axonal regeneration. The first reason is that BDNF and its receptors, TrkB and p75, are rapidly up-regulated in axotomized motoneurons (Funakoshi et al., 1993) and that denervated Schwann cells also exhibit an up-regulation of BDNF and p75 (Meyer et al., 1992). Second, BDNF plays an important role in the survival of axotomized neonatal motoneurons (Sendtner et al., 1992), as well as adult motoneurons following ventral root avulsion (Novikov et al., 1997). Another reason for the importance of BDNF is that exogenous BDNF maintains the cholinergic phenotype in axotomized adult motoneurons by preventing down-regulation of choline acetyltransferase (ChAT; Friedman et al., 1995). Furthermore, systemic application of an anti-BDNF antibody

reduces the number of regenerated axons and their myelination following a sciatic nerve crush injury (Zhang et al., 2000).

The role of neurotrophin-4/5 in regeneration of peripheral nerves is not as clear as the function of BDNF. But so far, several experiments indicate that NT-4/5 is also required for the growth of regenerating axons in peripheral nerves (English et al., 2005; Simon et al., 2003).

1.3.2.3 Tyrosine kinase receptor B

As described above, the neurotrophins BDNF and NT-4/5 mediate their effects via binding to two different types of receptors: TrkB and p75.

In contrast to TrkB, which is supposed to be important for survival of axotomized nerve fibers, the binding to p75 implies cell death by apoptosis (Lu et al., 2005).

The TrkB receptor, first identified in 1986 (Martin-Zanca et al., 1986), is a gene fusion between the tropomyosin gene and a receptor tyrosine kinase.

Receptor binding leads to dimerization and activation of the intracellular kinase domain of TrkB by phosphorylation of tyrosine residues in its autoregulatory loop. This activation induces further tyrosine phosphorylation, creating adaptor protein docking sites and activating downstream signal transduction pathways, including the Ras/ERK mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol 3'-kinase (PI3K)/Akt pathway and the phospholipase C (PLC)γ pathway, as well as several small G proteins, including Rap-1 and the Cdc-42-Rac-Rho family (Huang and Reichardt, 2003; Lu e al., 2005).

Previous work has shown that continuous low-frequency (20 Hz) electrical stimulation of the proximal nerve stump, performed immediately after nerve transection for a period as short as 1 h, dramatically reduces the period of protracted axonal regrowth and increases precision of reinnervation in the rat (Al Majed et al., 2000a). The accelerated

preferential motor reinnervation after stimulation is associated with an accelerated and enhanced up-regulation of the neurotrophin BDNF and its cognate receptor TrkB in motor neurons (Al Majed et al., 2000b, 2004). These findings suggest that reduced TrkB signalling will have a negative impact on preferential motor reinnervation and functional recovery after femoral nerve injury, a hypothesis tested in this study.

1.4 The femoral nerve regeneration paradigm

A favorable model system to study accuracy of pathway finding and molecular mechanisms determining reinnervation selectivity is the femoral nerve regeneration paradigm (Brushart, 1988; Brushart 1993; Madison et al., 1996).

The femoral nerve that emanates from the nerve roots L1 to L4 in humans and L2 to L5 in rodents is the largest nerve of the lumbar plexus. In its retroperitoneal passage it includes both, sensory and motor nerve fibers. Along its way through the lacuna musculorum it bifurcates into two major branches, a quadriceps muscle branch containing motor and proprioceptive sensory axons that innervate the quadriceps muscle, and the saphenous nerve, which is a purely sensory nerve branch innervating the skin of the medial part of the lower limb (Fig. 3).
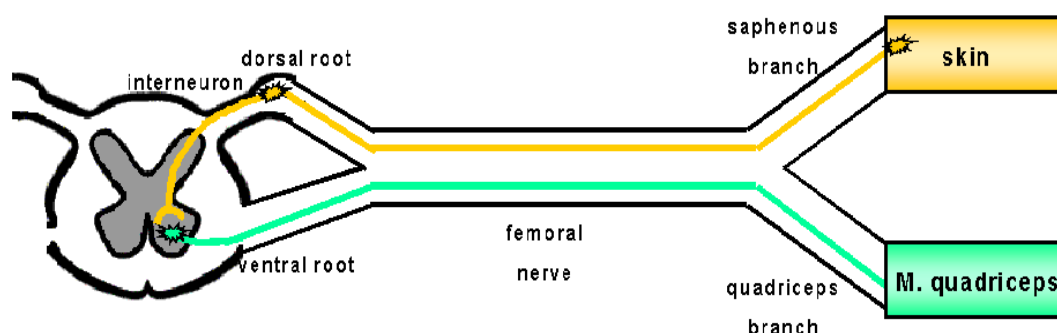
Figure 3: Schematic diagram of the intact femoral nerve with its saphenous and quadriceps nerve branches. Shown in yellow is a sensory nerve fiber in the common femoral nerve trunk and its cutaneous branch and, in green, a motor nerve fiber in the common femoral nerve trunk and the quadriceps nerve branch.

Lesion of the common nerve trunk close to the bifurcation into muscle and cutaneous branches affects a single muscle, the quadriceps muscle. Being the only extensor in the knee joint, the quadriceps muscle has the important function of keeping the knee extended during single-support phases required for the swing of the contralateral leg during walking. Injury of the femoral nerve trunk causes gait disability and loss of skin sensation in the medial aspect of the lower limb.

2 Rationale and aims of the study

Previous studies indicated that the TrkB receptor is involved in peripheral nerve regeneration (Lu et al., 2005), but *in vivo* experiments showing the influence of the TrkB receptor on the morphological and functional nerve regeneration have not been performed.

The aim of this work was to investigate the effects on nerve regeneration of a reduced expression of TrkB receptors in knockout-mice compared to a normal availability of these receptors in wild-type littermates using the femoral nerve paradigm established for rats and mice (Brushart, 1988; Brushart, 1993; Mears et al., 2003; Robinson and Madison, 2003).

In the femoral nerve injury model, retrograde tracing and histological techniques allow qualitative and quantitative morphological assessment of nerve regeneration by analysis of numbers of regenerated axons and degree of their myelination, as well as accuracy of target reinnervation (PMR).

In addition, a recently developed approach, the novel single-frame motion analysis (SFMA), allows precise quantitative evaluation of the functional outcome after femoral nerve transection and regeneration in mice (Irintchev et al., 2005; Simova et al., 2006; Ahlborn et al., 2009).

The experimental design and the available outcome measures were considered adequate to provide answers to the following questions:

Does reduced expression of TrkB receptors in mice lead, as compared with normal TrkB expression to:

1) reduced preferential motor reinnervation,
2) reduced survival of motoneurons,
3) reduced quality of axonal regeneration, and
4) worse functional outcome after transection and surgical repair of the femoral nerve?

The results of these investigations have been published in leading neuroscience journals (Irintchev et al., 2005; Eberhardt et al., 2006).

3 Materials and methods

3.1 Animals

Experiments were performed on six-month old adult mice. Twenty wild-type mice and nineteen heterozygous TrkB-deficient littermates of both genders were divided into three groups for assessment of functional recovery and morphological analyses after femoral nerve transection:

Group A   4 wild-type mice and 4 TrkB deficient littermates were used for morphological analyses without transection of the femoral nerve.

Group B   8 wild-type mice and 7 TrkB deficient mice were subjected to femoral nerve transection and repair and functional recovery was analyzed over a time period of 1 month after injury followed by morphological analyses at 1 month after nerve injury.

Group C   8 wild-type mice and 8 TrkB mutants were treated and analyzed similar to the animals in group B but the time period of observation was prolonged to 3 months.

Randomized code numbers were assigned to the experimental animals to allow blinded analyses throughout the whole experiment.

Before and after surgical treatment all animals were kept on a standard laboratory diet and tap water *ad libitum* with a light-dark cycle of 12 hours light on, 12 hours light off.

All experiments were performed in accordance with the German Law on protection of experimental animals after approval of the procedures by the State of Hamburg animal care committee.

3.2 Transgenic TrkB +/- mice

Homozygous (TrkB -/-) knockout-mice develop abnormally and die within 2 to 3 weeks after birth (Xu et al., 2000b), which makes their use in regeneration experiments impossible. Therefore we used heterozygous TrkB +/- mutants with a reduced expression of tyrosine kinase receptors and wild-type littermates on a C57BL/6J background (Xu et al., 2000a).

3.3 Nerve transection and repair

All procedures were performed under antiseptic conditions.

Mice of all groups were deeply anesthetized by an intraperitoneal injection of fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany, 0.4 mg/kg), droperidol (Dehydrobenzperidol, Janssen, 20 mg/kg) and diazepam (Valium 10 Roche, Hoffman - La Roche, Grenzach-Whylen, Germany, 5 mg/kg).

The left femoral nerve was exposed and sharply transected 3 mm proximal to the bifurcation into saphenous and quadriceps nerve branches under 20x – 40x magnification (surgical microscope OpMi-1, Zeiss, Germany) using fine surgical scissors. The proximal and distal nerve stumps were inserted into a 4-mm-long silastic nerve cuff (0.3 mm inner diameter, Intramedic, Wackersdorf, Germany). By placing a single epineural 11-0 Nylon suture thread (Ethicon GmbH, Norderstedt, Germany), the nerve stumps were fixed microsurgically so that their ends were separated by a gap of 2 mm (Fig. 4). Thereafter the cuffs were filled with phosphate-buffered saline (PBS) and the skin wound was closed by five 6-0 Nylon skin sutures (Ethicon).
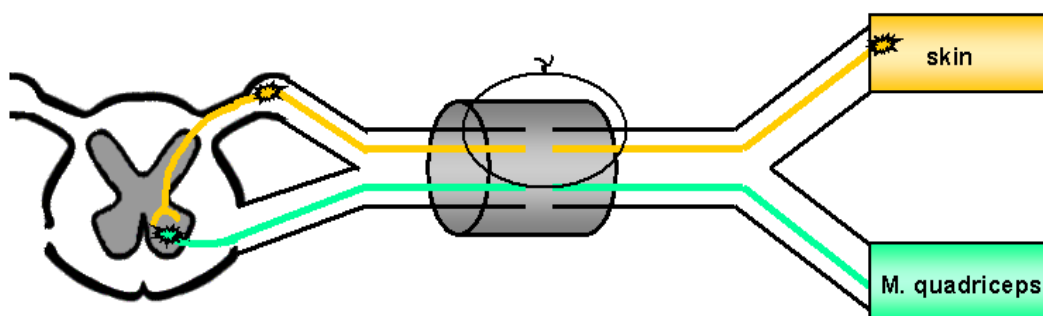
Figure 4: Schematic diagram of the transected and repaired femoral nerve. The proximal and distal nerve stump are inserted in a silastic tube and fixed by a single stitch so that the gap between both ends was 2 mm.

3.4 Analysis of motor function

Functional recovery was assessed by a novel single-frame motion analysis (SFMA) approach recently developed in our laboratory (Irintchev et al., 2005). To evaluate quadriceps muscle function during ground locomotion prior to operation, mice were trained to perform a classical beam walking test. In this test, the animals walk unforced from one end of a horizontal beam (length 1000 mm, width 38 mm; placed 280 mm above the ground) towards their home cage located at the other end of the beam (Fig. 5). The set-up was illuminated by two 60 W lamps placed on both sides of the beam. In order to avoid stress and to ensure normal locomotion pattern. Mice were handled gently and noise disturbances were kept at a minimum.

Figure 5: Side view of the beam walking set-up. A video camera is positioned at one side of a wooden beam and used to record rear views of the mice while they are walking from the right hand side of the beam towards their home cage.
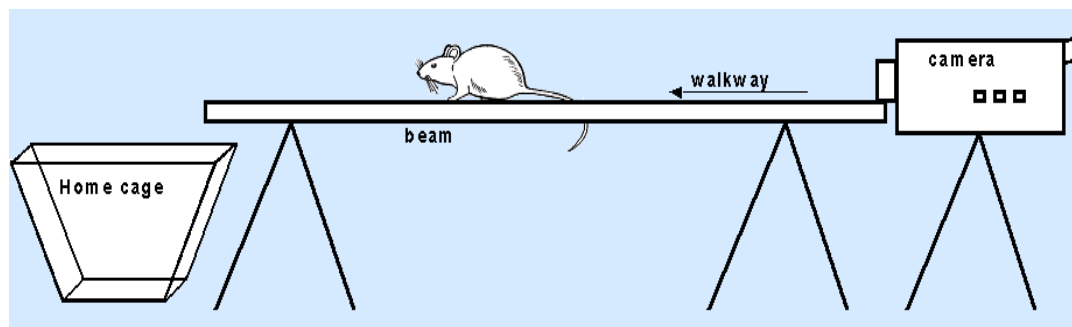
For all mice, a rear view of one walking trial was captured prior to the operation with a Panasonic NV-DS12 camera (Panasonic, Deutschland, Hamburg, Germany) at 25 frames per second and recorded on videotape (video recorder SVL-SE 830, Sony Deutschland, Cologne, Germany). The recordings were repeated 1, 2, 4, 8 and 12 weeks after nerve transection. The video sequences were digitized and examined with VirtualDub 1.5.10 software, a video capture/processing utility (available at http://www.virtualdub.org). Selected frames in which the animals were seen in defined phases of the step cycle (see below) were used for measurements performed with UTHSCSA ImageTool 2.0 software (University of Texas, San Antonio, TX, USA; available at http://ddsdx.uthscsa.adu/dig/).

Two parameters were measured: the foot-base angle (FBA, Figs. 6a, b) and the heels-tail angle (HTA, Figs. 6c, d). The foot-base angle, measured at toe-off position, is defined by a line dividing the sole surface into two halves and the horizontal line, and is measured with respect to the medial aspect. The heels-tail angle is measured when one leg is in the single support phase and the contralateral extremity has maximum swing altitude, and is defined by the lines connecting the heels with the anus. The angle is measured with respect to the dorsal aspect. Compared to intact animals (Figs. 6a, c), the two angles change significantly after lesion of the femoral nerve (Figs. 6b, d). Both parameters are directly related to the ability of the quadriceps muscle to keep the knee joint extended during

contralateral swing phases (Irintchev et al., 2005). As a relative measure of functional recovery at 4 and 12 weeks after lesion, we calculated the stance recovery index, which is a mean of the recovery index (RI) for the HTA and the FBA. The index is calculated, in percent, as:

$$RI = [(X_{reinn} - X_{den}) / (X_{pre} - X_{den})] \times 100,$$

where X_{pre}, X_{den} and X_{reinn} are values prior to operation, during the state of denervation (7 days after injury), and at 4 or 12 weeks of reinnervation, respectively.

A third parameter, the limb protraction length ratio (PLR), was evaluated from video recordings of voluntary pursuit movements of the mice (Figs. 6e, f). The mouse, when held by its tail and allowed to grasp a pencil with its fore paws, tries to catch the object with its hind paws and extends simultaneously both hind limbs. In intact animals, the relative length of the two extremities, as estimated by lines connecting the most distal mid-point of the extremity with the anus, is approximately equal and the PLR (ratio of the right and left limb length) is close to 1 (Fig. 6e). After denervation, the limb cannot extend maximally (left side of the animal shown in Fig. 6f), and the PLR increases significantly above 1.
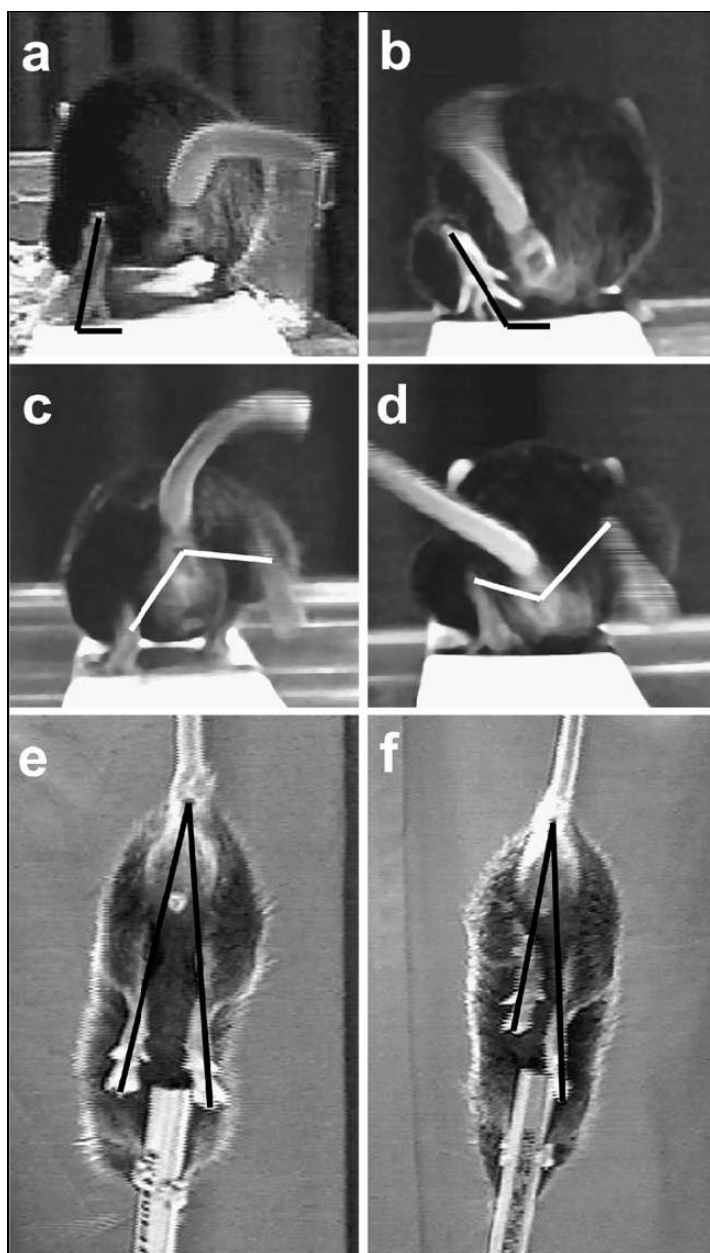
Figure 6: Single video frames from recordings of beam walking (a –d) and voluntary limb protractions (e, f). Shown are intact animals (a, c, e) and animals with transection of the left femoral nerve at 7 days after lesion (b, d, f). The lines drawn in the video frames show the foot-base angle (FBA, a, b), the heels-tail angle (HTA, c, d) and the limb lengths used for calculation of the limb protraction length ratio (PLR, e, f). HTA is measured always with respect to the dorsal aspect, FBA with respect to the medial aspect. For further explanations, see the main text.

3.5 Retrograde labeling of motoneurons

At the end of the 3-month observation time-period, animals were anesthetized as described above. After re-exposing the left femoral nerve, two separate pieces of Parafilm (Pechiney, Plastic Packaging, Chicago, IL, USA) were laid underneath the muscle and sensory nerve trunks distal to the bifurcation. Both nerve branches were dissected free and transected 5

mm distal to the bifurcation using 20x – 40x magnification. Fluorescence retrograde tracers were applied for 30 min to the cut proximal nerve ends in powder form: Fluoro-Ruby (FR) (tetramethylrhodamine dextran, D-1817, Molecular Probes, Eugene, OR, USA)to the muscle branch and Fast-Blue (FB) (EMS-Chemie GmbH, Großumstadt, Germany) to the cutaneous branch (Fig. 7). Subsequently, the application sites were rinsed thoroughly with PBS and gently blotted with filter paper. The wound was closed by five 6-0 Nylon skin sutures (Ethicon).
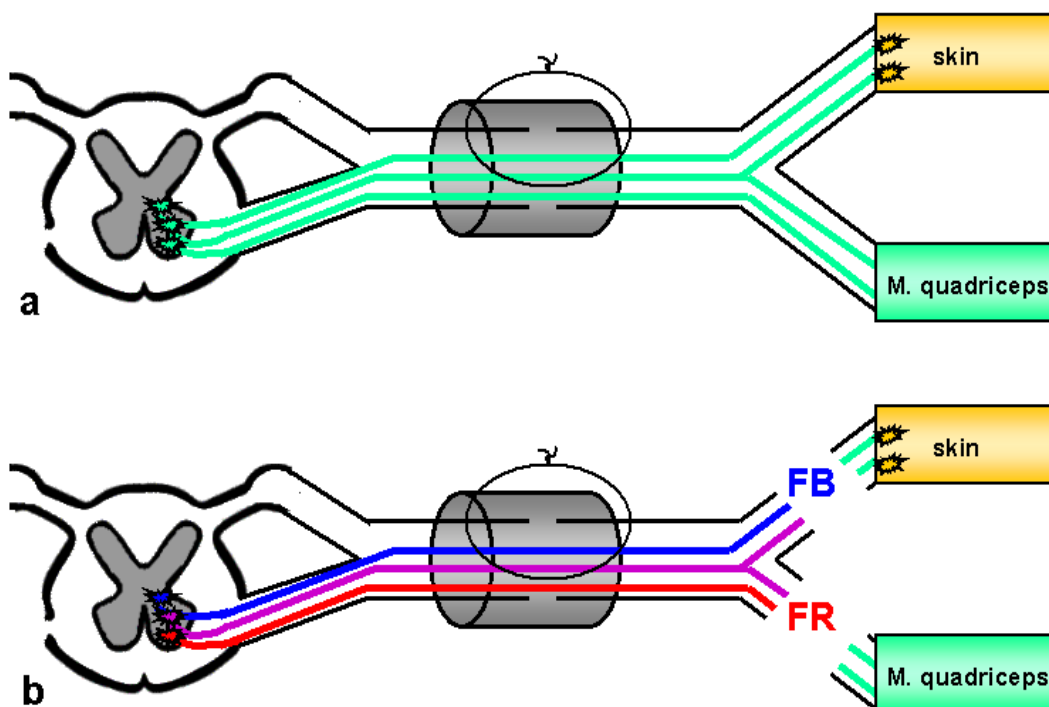
Figure 7: Schematic diagram of the retrograde labeling. The upper panel shows the transected and surgically repaired femoral nerve with motor axons which have regrown into the muscle branch only, the sensory branch only or into both branches (a). For retrograde labeling, the muscle and cutaneous nerve branches were cut 5 mm distal to the bifurcation, as shown in the lower panel (b). Fast-Blue was applied to the proximal end of the cut sensory nerve branch and Fluoro-Ruby was applied to the muscle nerve branch. The use of two different retrograde tracers allows subsequent identification in spinal cord sections of motoneurons projecting correctly (to the quadriceps branch only, red) as well as incorrectly projecting motoneurons with axons regrown into the saphenous nerve branch only (blue) or both into the saphenous and the quadriceps nerve branches (purple) (b).

Spinal cords were dissected from the mice after a survival period of 7 days. Previous experiments (Franz et al., 2005; Simova et al., 2006) have

shown that this time period is sufficient for a maximal retrograde transport and accumulation of the tracers in the motoneuronal cell bodies.

3.6 Morphological analyses

3.6.1 Preparation of tissue

Mice were deeply anesthetized by an intraperitoneal injection of sodium pentobarbital (Narcoren, Merial GmbH, Hallbergmoos, Germany, 0.05 ml per 100 g body weight) and perfused transcardially through the left heart ventricle with PBS for 1 minute, followed by 4% formaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 20 minutes. Two hours later the lumbar spinal cord between L1 and L5, which contains the entire pool of femoral motoneurons, was dissected. The common femoral nerve trunk with its both branches was also discected up to the level of transaction for dye application.

3.6.2 Analysis of retrograde labeled motoneurons

3.6.2.1 Preparation of vibratome sections of spinal cord

After post-fixation overnight at 4°C in the fixative used for perfusion, the spinal cord was embedded in 6% Agar (Fluka Chemie GmbH, Buchs, Switzerland) and cut transversely (serial section of 50 µm thickness) on a Leica VT 1000 M Vibratome (Leica, Nussloch, Germany). The sections were collected in PBS. The sections were serially mounted on glass slides and coverslipped with Fluoromount-G (Southern Biotechnology Associates, Biozol, Eching, Germany). The slides were stored in the dark at 4°C.

3.6.2.2 Fluorescence microscopy

Each section was examined under an Axiophot 2 microscope (Zeiss, Oberkochen, Germany) using 40 x magnification and filter sets appropriate for Fluoro-Ruby and Fast-Blue. Changing the fluorescent filter sets allows identification of motoneuron cell bodies containing FR only, FB only or both tracers (Fig. 8). About 45 – 50 serial cross-sections contained retrogradely labeled cells. All sections were examined by focusing through the section thickness starting from the top surface and all single and double-labeled cells counted except those profiles visible on top of sections. The application of this simple stereological principle prevents double counting of labeled cells and allows an unbiased evaluation of cell numbers which does not rely on assumptions or requires corrections (Simova et al., 2006).
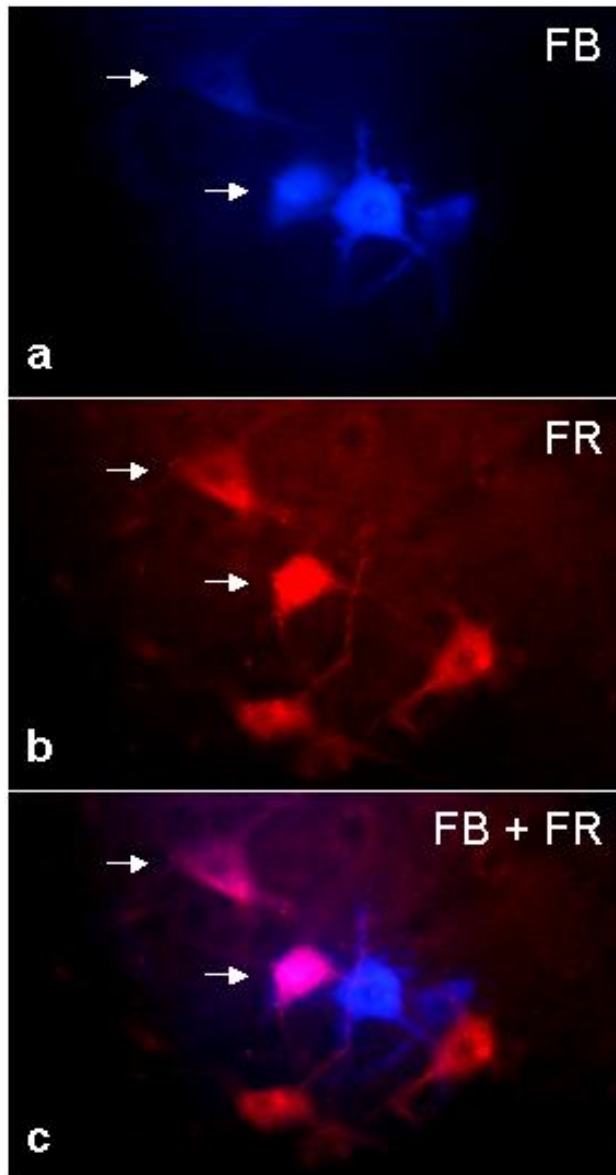
Figure 8: One cross-section of the lumbar spinal cord containing cellbodies of retrogradely labeled motoneurons (a-c) observed with filter sets for Fast-Blue (a), Fluro-Ruby (b) and overlay of both (c). Arrows indicate cell bodies to be labeled with both, FR and FB which appear purple in (c). Motoneurons labeled with FR (red) or FB (blue) only have axons projecting into the quadriceps (correct) and the saphenous (incorrect) branch of the femoral nerve.

3.6.3 Analysis of axons in regenerated nerve branches

3.6.3.1 Preparation of semithin sections of nerve branches

The dissected femoral nerve trunk with both nerve branches was post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.3, for one hour at room temperature and embedded in Epon according to a routine protocol. Semithin (1µm) transverse sections were cut from the motor and sensory nerve branches on a Reichert Ultracut UCT (Leica) at a

distance of approximately 3 mm distal to the bifurcation and stained with 1% Toluidine blue/ 1% borax in distilled water.

3.6.3.2 Light microscopy

One cross-section of both nerve trunks per animal (Fig. 9) was examined on an Axioskop microscope (Zeiss) equipped with a motorized stage and Neurolucida software-controlled computer system (MicroBrightField Europe, Magdeburg, Germany). Using a 100x objective, the total number of myelinated axons in the muscle and cutaneous nerve branches was counted.

For measuring the orthogonal diameter of axons and the degree of myelination, a grid with line spacing of 60 μm was projected into the microscope visual field using the Neurolucida software. To obtain a randomized sample, only myelinated axons crossed by or attaching the vertical grid lines were measured. For each myelinated axon, the orthogonal axon plus myelin sheath) were measured using the Neurolucida software. The mean orthogonal diameter is the averaged length of the longest axis of the structure and the axis passing through the middle of the longest one at right angle (Irintchev et al., 1990).
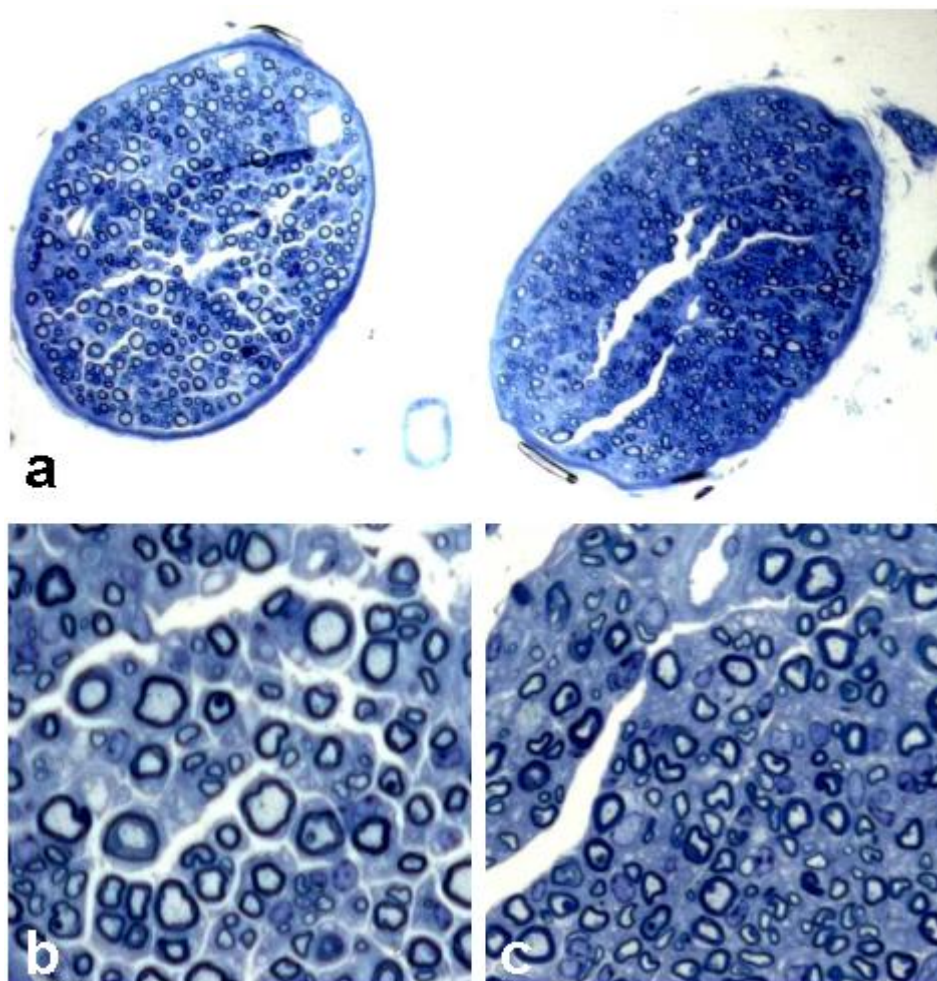
Figure 9: Semithin cross-sections of regenerated femoral nerve branches of a wild-type mouse obtained 3 mm distal to the bifurcation at 3 months after nerve injury. Shown are low- (20x objective) and high-power (100x oil objective) magnifications of the muscle (quadriceps) and the skin (saphenous) branches (a, b and c respectively). Note that large-diameter myelinated fibers are more frequent in the quadriceps than in the saphenous branch.

3.7 Photographic documentation and statistical analyses

Photographic documentation was performed on an Axiophot 2 microscope equipped with a digital camera AxioCam HRC and AxioVision software (Zeiss). The digital images were additionally processed using Adobe Photoshop 8.0 software (Adobe Systems Inc., San Jose, CA, USA). Throughout the text and in the figures all numerical data are presented as mean values with standard errors of mean (SEM). Parametric tests (*t* test,

analysis of variance, ANOVA, or multifactor analysis of variance, MANOVA for repeated measurements, with subsequent Tukey *post hoc* tests) were used to compare group mean values as appropriate. Frequency distributions were compared with the non-parametric Kolmogorov-Smirnov test. Analyses were performed using the SYSTAT 9 software package (SPSS, Chicago, IL, USA). The accepted level of significance of differences between groups was 5%. Sigma Plot 8 software (SPSS) was used for graphic presentation of data.

4 Results

4.1 Preferential motor reinnervation

If TrkB signaling is causally related to preferential motor reinnervation, one should expect that reduced levels of TrkB expression will compromise selectivity of reinnervation. To test this hypothesis, we performed double retrograde labeling of motoneurons that had reinnervated either the saphenous or the quadriceps branch only or both branches 3 months after nerve surgery. In TrkB-deficient mice (TrkB +/-, n=8, Fig. 10), equal numbers of motoneurons had innervated either the motor or sensory branch, and a minority had innervated both branches. While numbers of motoneurons labeled via the sensory branch alone or via both branches were similar in wild-type mice (TrkB +/+, n=8, Fig. 10) and TrkB +/- littermates, neurons innervating the motor branch were significantly more numerous in TrkB +/+ versus TrkB +/- mice ($P < 0.05$, t test). These findings show a preferential motor reinnervation of the quadriceps nerve branch in TrkB +/+ mice (index of preferential motor reinnervation, PMR, bar pair to the right hand side in Fig. 10), but not in TrkB +/- mice (Fig. 10, $P < 0.05$ compared to TrkB +/+ littermates, t test).

To estimate the number of motoneurons normally innervating the quadriceps muscle in TrkB +/+ and TrkB +/- mice, we transected the femoral nerve in 4 animals of each genotype, applied tracer and perfused the animals 1 week later. No difference in the size of the quadriceps motoneuron pool was found between TrkB +/- mice (184 ± 13 motoneurons) and TrkB +/+ littermates (187 ± 11, not significant, t test). Compared with these control values, the total number of retrogradely labeled motoneurons after a regeneration period of 3 months (Fig. 10) was reduced by 30% and 20% in TrkB +/- and TrkB +/+ mice, respectively ($P <$

0.05 for both genotypes, *t* test). Finally, we should note that the total number of motoneurons that had regenerated their axons into the motor nerve branch, i.e. neurons labeled through the motor branch plus double-labeled neurons, was significantly lower in the TrkB +/- mice (71 ± 6) versus TrkB +/+ mice (93 ± 9, *P* < 0.05, *t* test). These numbers represent only 39% and 50%, respectively, of the motoneurons which normally innervate the quadriceps muscle (around 185 motoneurons found in control animals of both genotypes, see above), a finding important with regard to the degree of functional recovery achieved after nerve injury (see 4.4).
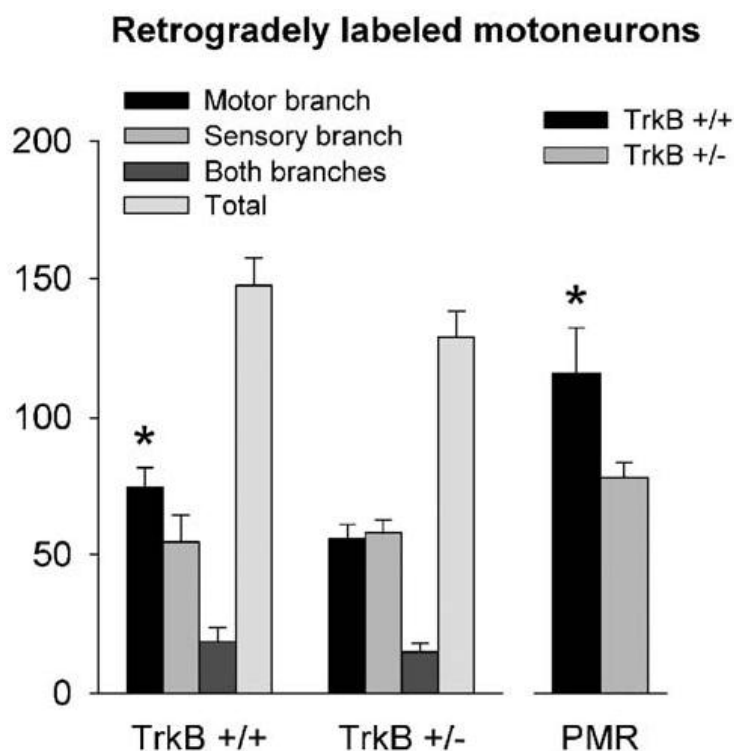
Figure 10: Morphological evaluation of muscle reinnervation after nerve transection in TrkB +/+ and TrkB +/- mice. The two groups of bars on the left hand side show numbers of motoneurons innervating the motor or sensory branches only, or both branches, as well as the total number of regenerated motoneurons 3 months after nerve lesion. The number of motoneurons with axons regenerated into the motor branch only (black bars) is significantly higher in TrkB +/+ (n = 74 ± 7) compared to TrkB +/- (n = 56 ± 5, asterisk, *P* < 0.05, *t* test) mice while no significant differences were found for the other categories of regenerated neurons (*t* test). The degree of preferential motor reinnervation (bar pair on the right hand side, values multiplied by 100 to fit the scaling), calculated as ratio of the number of motoneurons regenerated in the motor branch only to those regenerated into the sensory branch, is also significantly higher in TrkB +/+ animals (black bar) compared to TrkB +/- (gray bar, *P* < 0.05, *t* test) mice.

4.2 Numbers of myelinated axons in regenerated nerves

One month after nerve transection, numbers of myelinated axons in both, motor and sensory nerve branches, were lower (33% and 24%, respectively) in TrkB +/- (n = 7) versus TrkB +/+ (n = 8) littermates (Fig. 11, $P < 0.05$, t test). While the values for TrkB +/+ mice did not change between 1 and 3 months of reinnervation (differences of about 5% for both nerve branches, Fig.11), numbers of myelinated axons in TrkB +/- (n = 6) nerves increased to reach near levels of wild-type animals (n = 6) at 3 months after nerve lesion (Fig. 11). Therefore, partial deficiency in TrkB expression causes a significant delay in axonal regrowth and myelination.
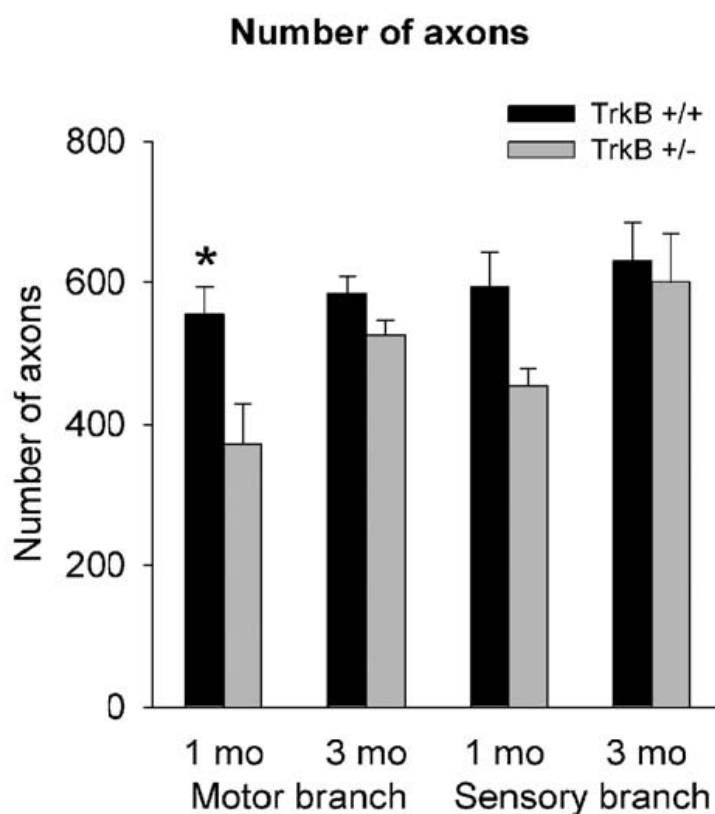
Figure 11: Morphological evaluation of nerve regeneration after nerve transection in TrkB +/+ and TrkB +/- mice. The diagram shows numbers of myelinated axonal profiles in semithin sections from the motor and the sensory nerve branches 1 and 3 months after nerve transection. The asterisk indicates significant difference between the mean group values ($P < 0.05$, t test). The number of regenerated axons in the motor branch of TrkB +/- mice (n = 373 ± 56) is significantly lower than in TrkB +/+ animals (n = 555 ± 37) at 1 month after nerve transection, but not at 3 months after lesion (TrkB +/- n = 534 ± 22 versus TrkB +/+ n = 584 ± 24).

4.3 Quality of nerve regeneration

The estimation of the degree of myelination and axonal diameters of regenerated motor nerve fibers determines the quality of the regrown axons. Shown are only results after three months of regeneration since measurement after one month was too imprecise due to the very small axonal diameters and thin myelin sheaths.

As compared to intact nerves of wild-type mice, thickness of myelin sheaths in the motor branches of transected and repaired femoral nerves of both genotypes were significantly reduced ($P < 0.05$, one-way ANOVA with Tukey's post hoc tests, Fig. 11a), a well known consequence of peripheral nerve injury. But there was no difference in the thickness of the myelin sheaths between TrkB +/+ (n=6) and TrkB +/- littermates (n=6, $P > 0.05$, one-way ANOVA with Turkey's post hoc tests).

The same was true for the axonal diameters in the motor branch of regenerated nerves. Diameters of regenerated axons were significantly reduced compared to intact nerves ($P < 0.05$, one-way ANOVA with Tukey's post hoc test, Fig. 11b), but there is no difference between knockout-mice (n=6) and wild-type littermates (n=6).

The frequency distribution revealed a unimodal distribution for transected nerves of TrkB +/+ and TrkB +/- mice, with peak within the range of 1 to 2 µm and no difference between the genotypes ($P > 0.05$, Kolmogorov-Smirnov test, Fig. 11c). In contrast, the distribution for intact nerves was multimodal with peaks at 2, 4-5 and 6-7 µm (significantly different from both groups of regenerated nerves, $P <= 0.05$, Kolmogorov-Smirnov test, Fig. 11c).

These results show no difference in the morphology of regenerated nerve fibers in the motor nerve branch between the genotypes three months after nerve transection and repair. Compared with intact nerves, however, axonal myelination and axonal diameters were strongly reduced in both genotypes.

a. Thickness of myelin sheaths in the motor branch

b. Averageas of mean axonal diameter in the motor branch

c. Frequency distribution of axonal diameters in the motor branch
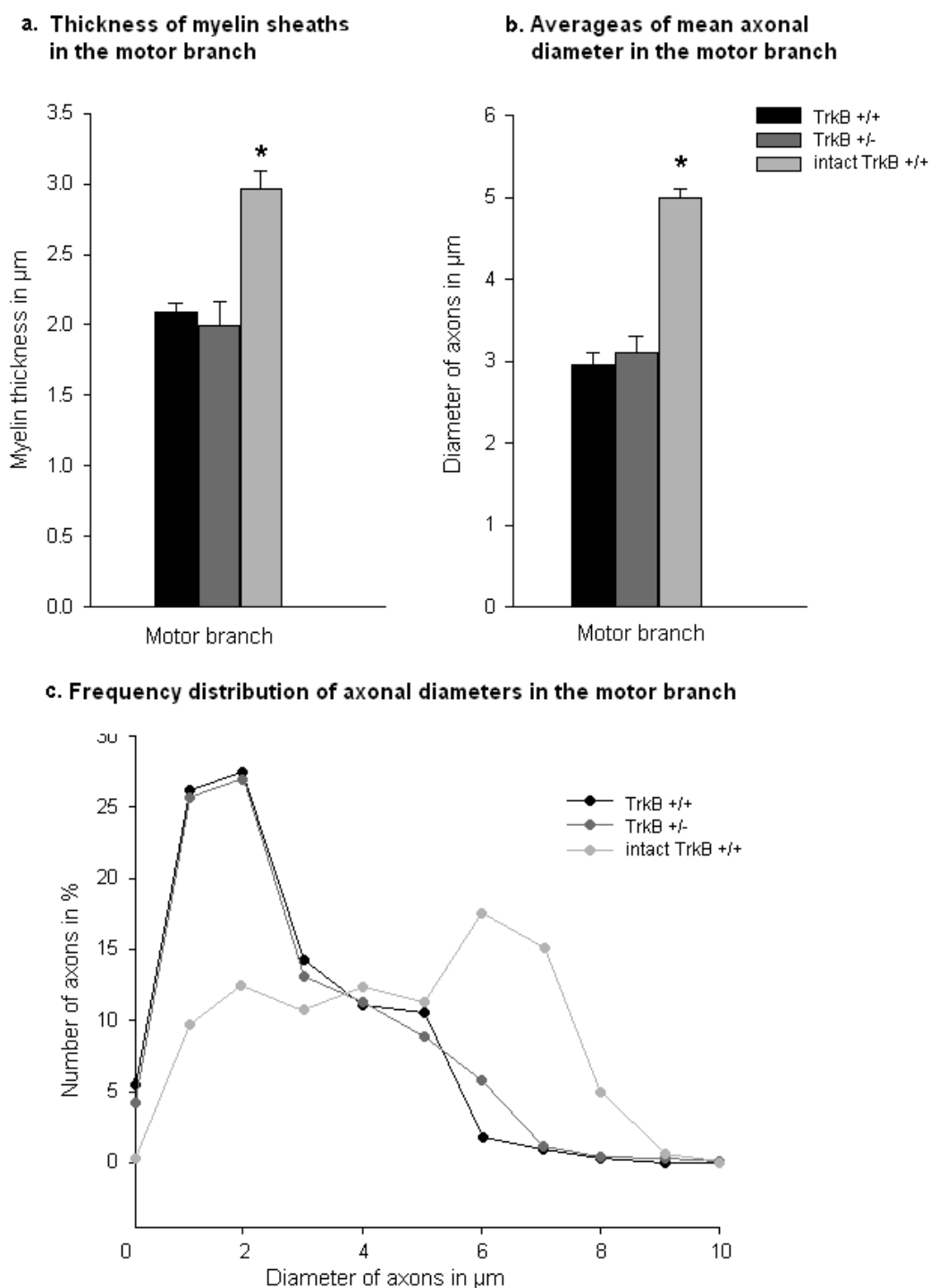
Figure 11: Morphological evaluation of quality of nerve regeneration in TrkB +/+ and TrkB +/- mice three months after nerve transection. Shown are mean values (+SEM) of myelin sheath thickness (a), axonal diameters (b) in the regenerated motor branch of the femoral nerve three months after nerve repair. The asterisks indicate significant difference ($P < 0.05$, one-way ANOVA with Tukey's post hoc tests, Fig.11 a, b) between intact nerves (3.02 ± 0.16 µm myelin thickness, 5.03 ± 0.47 µm axonal diameter), on the one side, and injured nerves of TrkB +/+ (2.09 ± 0.14 µm myelin thickness, 2.97 ± 0.33

μm axonal diameter) and TrkB +/- (1.99 ± 0.53 μm myelin thickness, 3.12 ± 0.16 μm axonal diameter) mice, on the other side.
Panel c shows frequency distributions of axonal diameters in regenerated nerves of both genotypes and intact nerves of wild-type mice.

4.4 Recovery of motor function

The finding of delayed and less precise reinnervation in the TrkB +/- mice raised the question as to whether also motor function recovery is delayed and less complete in TrkB +/- compared to TrkB +/+ mice. Using a novel single-frame motion analysis approach, we have recently provided evidence that TrkB deficiency causes impairment of functional recovery at early time-periods after nerve-injury (Irintchev at al., 2005). To confirm this result and extend the observations to long-term recovery periods, we evaluated the extensor function of the quadriceps muscle in mice of both genotypes (16 wild-type mice and 15 TrkB deficient littermates) prior to nerve transection, and at 1, 2, 4, 8 and 12 weeks after surgery (Figs. 12 a-c). Three parameters were used for evaluation, the foot-base angle (FBA, Figs. 6a, b, 12a), the heels-tail angle (HTA, Figs. 6b, c, 12b) and the limb protraction length ratio (PLR, Figs. 6e, f, 12c). The first two parameters, evaluated from video recordings of beam walking trials, reflect the ability of the quadriceps muscle to keep the knee extended during single support phases of the gate cycle and, for the FBA, to counteract in addition the rotational moments in the hip caused by the swing of the contralateral extremity. These abilities were severely compromised after femoral nerve injury as seen from the changes in the angle values at 7 days after nerve transection compared to the preoperative values (day 0) of the same animals (Figs. 12a, b). Multifactor analysis of variance (MANOVA) for repeated measurements showed that both genotypes (TrkB +/+ versus TrkB +/-) and recovery period have a strong impact on the two parameters, with a significant interaction between the two factors. This shows that functional recovery is affected by reduced TrkB expression. As indicated by multiple comparisons between mean group values using the

stringent post hoc test of Tukey, the most pronounced difference was observed at 4 weeks after lesion, when motor function recovery in TrkB +/- animals was by far less than in TrkB +/+ littermates (Figs. 12a, b, asterisks). The large difference between the two groups at 4 weeks is even more apparent for the stance recovery index (Fig. 12d, see Material and methods for calculation of the index). Functional improvement at 3 months after injury in both groups of animals (Fig. 12d) is remarkable in view of the fact that by that time the quadriceps muscle is reinnervated by only 50% and 39% of the intact motoneuron pool in TrkB +/+ and TrkB +/- mice, respectively (see results of retrograde tracing experiments above). The high degree of recovery despite low numbers of motoneurons is likely to be associated with enlargement of reinnervated motor units that compensates for their reduced numbers (Rafuse and Gordon, 1996). There was a tendency for less complete recovery of FBA in TrkB +/- versus TrkB +/+ littermates.

In addition to the FBA and HTA, which evaluate quadriceps function during ground locomotion, we studied a third functional parameter, the limb protraction length ratio (PLR). This parameter estimates the ability to stretch the knee joint during target-reaching movements performed without body weight support. Denervation-induced functional impairment, which is similar in both genotypes, was found also for these parameters (Fig. 12c, compare values at 0 and 7 days). As indicated by MANOVA analysis, PLR was significantly affected by recovery period but not by genotype. Thus, recovery of the ability to use the limb without bearing body weight was not compromised in TrkB +/- versus TrkB +/+ littermates.
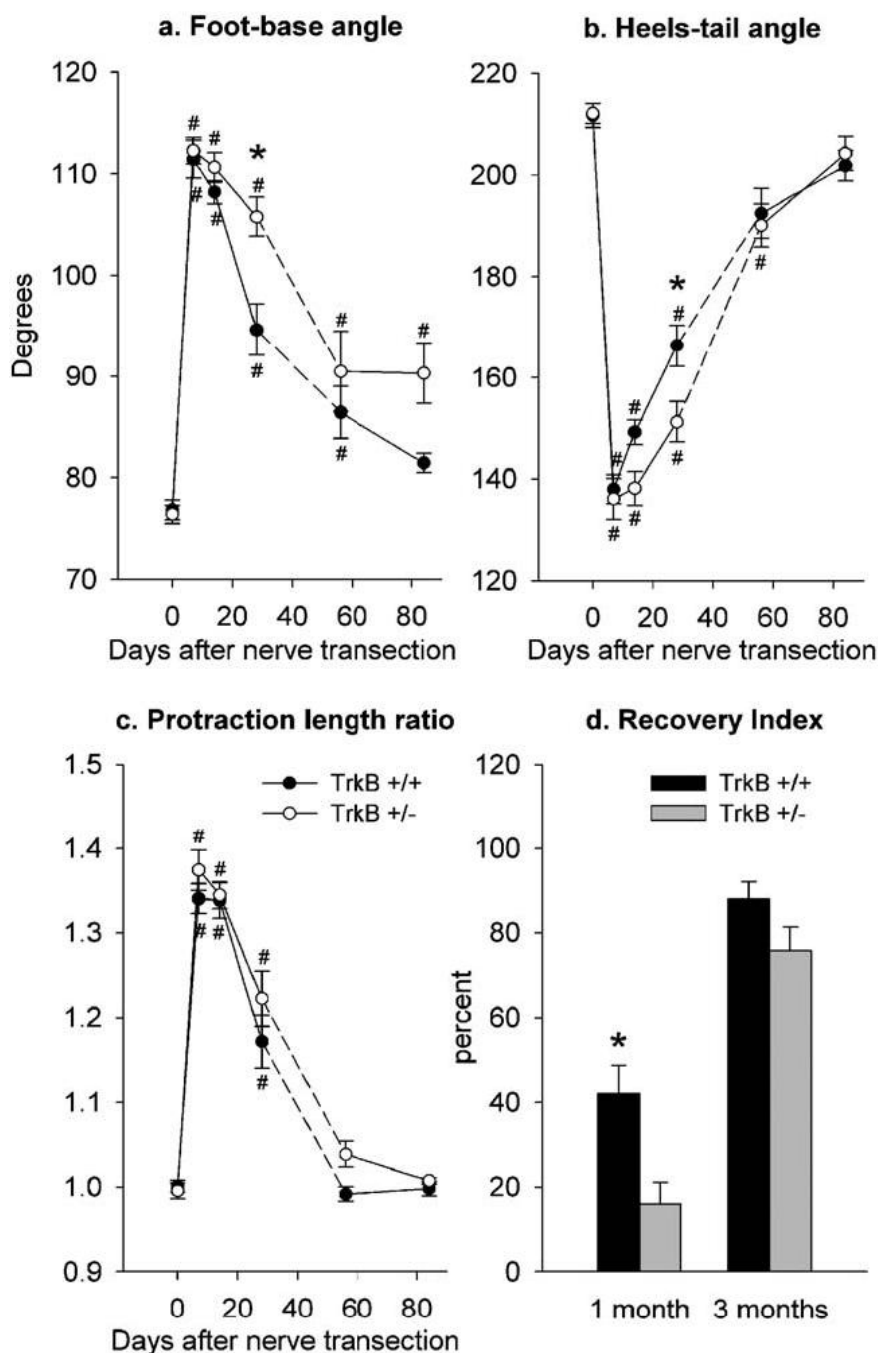
Figure 12: Functional evaluation of quadriceps muscle reinnervation. Functional performance of TrkB +/+ (black circles and bars) and Trkb +/- mice (open circles and gray bars) was evaluated by measuring foot-base angle (FBA, a), heels-tail angle (HTA, b) and limb protraction length ratio (PLR, c) before lesion (day 0) and during 3 months after nerve transection. Ten TrkB +/+ and nine TrkB +/- animals were studied over a period of 1 month, additional 6 mice per genotype were followed up to 3 months after nerve transection. Statistically significant differences by post hoc Tukey's test analysis performed after multifactor analysis of variance (MANOVA) for repeated measurements are also indicated for group mean values different from: (#) the within-group preoperative value or (*) from both the within-group preoperative value and the corresponding postoperative value of the other group. (d) Stance recovery index, an overall estimate of

quadriceps muscle function during stance, at 1 and 3 months after nerve transection in the TrkB +/+ (black bars) and TrkB +/- (gray bars) mice shown in panels a-c. The asterisk shown in panel d indicates a statistically significant difference between the two groups ($P < 0.05$, t test).

5 Discussion

The results of this study show that even partially reduced expression of TrkB leads to compromised preferential motor reinnervation, delayed axonal regrowth into the distal nerve stump and delayed functional recovery after femoral nerve injury. In conjuction with previous data, these results show that TrkB signalling is a major factor regulating the functional outcome after nerve injury.

5.1 The importance of TrkB receptor for preferential motor reinnervation

Previous work has suggested that preferential motor reinnervation after injury of the femoral nerve in rodents is associated with the HNK-1 carbohydrate. This carbohydrate is carried by or binds to different neural recognition molecules among them the myelin-associated glycoprotein, several laminin isoforms, amphoterin, NCAM, L1, P0, and highly acidid glycolipids (Kleene and Schachner, 2004). Interactions of the HNK-1 epitope with chondroitin sulphate proteoglycans enhance neuronal cell adhesion and neurite outgrowth (Miura et al., 1999). The HNK-1 epitope is specifically expressed in motor, but not sensory, axon pathways and it has been proposed that this expression contributes to the preferential motor reinnervation of the quadriceps as opposed to the saphenous branch of the femoral nerve in rodents (Martini et al., 1992, 1994). Recent work has provided direct evidence for the importance of the HNK-1 carbohydrate in motor reinnervation. Application of a HNK-1 peptide mimic to the transected femoral nerve of wild-type mice enhances preferential motor reinnervation, HNK-1 carbohydrate expression in the motor nerve branch,

survival of quadriceps motoneurons and functional recovery compared to control groups (Simova et al., 2006). The expression of the HNK-1 epitope is strongly enhanced by short-term electrical stimulation applied to the nerve immediately after transection (Eberhardt et al., 2006), a procedure that also leads to improved reinnervation (Al Majed et al., 2000a). The HNK-1 up-regulation induced by electrical stimulation is dependent on BDNF and its receptor TrkB (Eberhardt et al., 2006). All these findings and the present observations that even partially reduced expression of TrkB leads to compromised preferential motor reinnervation indicate that TrkB signalling is a major factor regulating precise targeting of motor axons after nerve injury and that the HNK-1 carbohydrate plays an essential role in the TrkB signalling pathway.

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5.2 The influence of TrkB receptor on speed of axonal regrowth

Our morphological analysis showed that axonal regrowth in TrkB deficient mice is delayed. Currently, there is no straightforward explanation of the mechanisms via which the neurotrophin receptor influences axonal elongation. One possibility that should be pointed out is that reduced neurotrophin signaling has a negative impact on the expression of regeneration-promoting molecules. Neurotrophins have been shown to influence expression of adhesion molecules, such as the potent neurite outgrowth promoting molecule L1 in neurons. Regulation of L1 synthesis can be affected by both neurotrophins and electrical stimulation (Seilheimer and Schachner, 1987; Scherer et al., 1992; Itoh et al., 1995, 1997) and L1 expression is conductive to axon growth when expressed and up-regulated not only in neurons, but also in glial cells of the peripheral and central nervous system (Mohajeri et al., 1996; Yazaki et al., 1996). These findings indicate that neurotrophins and electrical activity in

nerve regeneration provide the overall conductive environment for axonal growth. In the case of constitutive partial TrkB deficiency, as present in the TrkB +/- mice, reduced neurotrophin signalling in both neurons and Schwann cells may be sufficient to alter the permissiveness of the environment for axonal regrowth and thus reduce speed of axonal regeneration.

5.3 Influence of TrkB receptor on functional recovery

Assessment of motor functions, for example gait, provide the most clinically relevant outcome measures in neurological animal models and could greatly aid proper understanding of factors promoting or impeding recovery. Our recently described method for analysis of ground locomotion and voluntary movements without body weight support provides for the first time objective and precise estimates of functional recovery after femoral nerve injury in mice (Irintchev et al., 2005; Eberhardt et al., 2006). Applied to the TrkB +/- mouse, this analysis revealed delayed recovery of gait, but not compromised final outcome, as compared with TrkB +/+ mice. These findings are in good agreement with the observation of reduced numbers of regenerated axons at one but not at three months after injury in TrkB +/- mice compared with wild-type mice. Therefore, it can be concluded that delayed regrowth of axons in injured TrkB +/- mice causes a delay in functional recovery. While in this case the morphological data are in good agreement with and help explain the functional results, another anatomical estimate of regeneration used in this study, the preferential motor reinnervation, was less coherent with the functional estimates. At three months after injury, the degree of preferential motor reinnervation in TrkB +/- mice was strongly reduced compared with TrkB +/+ littermates but gait parameters were similar in both genotypes. This finding could be interpreted as an indication that precision of motor reinnervation, i.e. number of motoneurons reinnervating the correct target,

is not that crucial for functional recovery. In agreement with this notion, Simova et al. (2006) have reported that there is no correlation between degree of preferential motor reinnervation and gait parameters estimated in individual mice. Functional improvement at 3 months after injury in both genotypes analyzed in this study is remarkable in view of the fact that by that time the quadriceps muscle is reinnervated by only 50% and 39% of the intact motoneuron pool in TrkB +/+ and TrkB +/- mice, respectively. The high degree of recovery despite low numbers of motoneurons, and irrespective of preferential motor reinnervation, is likely to be associated with enlargement of reinnervated motor units that compensates for their reduced numbers (Rafuse and Gordon, 1996). Most important in the context of this investigation is, however, the finding that reduced TrkB expression in mice has significant, though temporary, functional consequences in the course of nerve regeneration.

5.4 Clinical aspects and outlook

The identification of the TrkB signalling as a major factor influencing functional nerve regeneration in this and previous studies has given impetus to test strategies to promote recovery after nerve injury. In particular, two approaches likely involving this signalling pathway have been tested. A single intra-operative application of a HNK-1 glycomimetic during nerve repair improves the functional outcome in the femoral nerve injury paradigm in mice (Simova et al., 2006). In the same paradigm, one hour electrical stimulation of the proximal nerve stump immediately after injury leads to acceleration of functional recovery (Ahlborn et al., 2007). As both procedures do not completely restore normal function, it appears warranted to test the potential of a combined treatment with electrical stimulation and HNK-1 glycomimetic application to further improve the final outcome of nerve repair.

6 Summary

Peripheral neurons have the ability to regenerate their axons after axotomy but functional recovery is often poor. Knowledge of cellular and molecular factors limiting or promoting restoration of function and design of efficient therapeutic approaches remain serious challenges for neuroscience and medicine.

This study was designed to analyze the influence of the TrkB receptor on peripheral nerve regeneration *in vivo*. Axonal regeneration and functional recovery were analyzed at different time points after transection and surgical repair of the femoral nerve in heterozygous TrkB +/- mice, animals with a reduced amount of available TrkB receptors, and in wild-type (TrkB +/+) littermates.

One month after nerve injury significantly lower numbers of myelinated axons were found in the motor branch of the femoral nerve of TrkB +/- compared with TrkB +/+ mice. This difference disappeared at three months after nerve injury, a time-point at which also axonal diameters and myelin thickness were similar in the two genotypes. Thus, although partial TrkB deficiency does not compromise axonal regeneration, leads to a delay of axonal regrowth and/or remyelination.

Retrograde labelling of regenerated motoneurons three months after injury revealed that in TrkB +/- mice more, as compared with TrkB +/+ mice, motor axons have regrown into the wrong target pathway, the saphenous nerve branch, and less have correctly reinnervated the quadriceps nerve branch of the femoral nerve. This finding shows that, in addition to delay in axonal regrowth, reduced expression of TrkB leads to reduced precision of muscle reinnervation.

Finally, functional analysis revealed gait deficits in TrkB +/- versus TrkB +/+ mice at one but not at three months after nerve injury. Thus, similar to

axonal regrowth, a reduced expression of TrkB leads to delayed functional recovery.

In summary, this work shows that TrkB signaling is a major factor regulating the axonal regrowth and precision of target reinnervation after nerve repair. Strategies leading to stimulation of TrkB signaling may be a promising way to improve the functional outcome after traumatic nerve injuries in clinical settings.

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8 Abbreviations

BDNF	Brain derived neurotrophic factor
BMRC	British Medical Research Council
CAP	Cytoskeleton-associated protein 23
CGRP	Calcitonin gene-related protein
ChAT	Choline acetyltransferase
FB	Fast-Blue
FBA	Foot-base angle
FR	Fluoro-Ruby
g	gram
GAP-43	Growth associated protein 43
HNK-1	Humane Natural Killer cell epitope 1
HTA	Heels-tail angle
kg	kilogram
MAPK	Mitogen-activated protein kinase
mg	Milligram
min	Minutes
mm	Millimeter
µm	Micrometer
mRNA	Messenger ribonucleic acid
NGF	Nerve growth factor
NT	Neurotrophin
PBS	Phosphat buffered saline
PCR	Polymerase chain reaction
PLC	Phospholipase C
PLR	Limb protraction length ratio
PMR	Preferential motor reinnervation
RAGs	Regeneration associated genes
RI	Recovery Index

SEM	Standard errors of mean
SFMA	Single-frame motion analysis
TrkA	Tyrosine kinase receptor A
TrkB	Tyrosine kinase receptor B
TrkC	Tyrosine kinase receptor C
VA	Veterans administration
W	Watt
ZMNH	Zentrum für molekulare Neurobiologie Hamburg

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Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.

Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

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