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Influence of brief electrical stimulation on peripheral nerve regeneration in heterozygous trkB deficient mice

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1. Introduction

1. Introduction

Trauma, compression, entrapment and ischemia are the most common causes of peripheral nerve injury (Valls-Sole et al. 2010). Despite surgical nerve repair, functional recovery after peripheral nerve injuries is often insufficient (Frostick et al. 1998, Gordon et al. 2003, Lundborg 2003, Lundborg and Rosen 2007, Robinson 2000). First of all, peripheral nerve regeneration requires survival of the neurons after axonal injury, which depends on the following factors: (1) type of neuron, (2) age, (3) degree of injury and (4) distance of the lesion site to the neuronal cell body (for review see Fu and Gordon 1997, Hart et al. 2008). After axonal damage, as a rule more sensory than motor neurons degenerate and adult neurons are more resistant to injury than neonatal neurons. Degree and severity of peripheral nerve injury are linked to neuronal survival and functional recovery (Burnett and Zager 2004). Neurons injured close to the neuronal cell body are more susceptible to axonal damage than neurons injured more distally.

Multiple causes influencing functional recovery after peripheral nerve regeneration have been identified previously: (1) Staggered reinnervation of the distal nerve stump after axonal injury and a limited time period of regeneration support by the environment, including neurotrophic support by Schwann cells (Fenrich and Gordon 2004, Gordon 2009, Gordon et al. 2008, 2009 a). (2) Inappropriate target reinnervation (Brushart 1988, Guntinas-Lichius et al. 2007 a), (3) cortical reorganizations (Lundborg 2003) and (4) polyneuronal innervation of muscle fibres (Guntinas-Lichius et al. 2007 a).

Various approaches have been used to improve neuronal regeneration and functional recovery after axonal injury. For instance, brief electrical stimulation (for review see Gordon et al. 2007, 2008, 2009 a, b), exercise (Asensio-Pinilla et al. 2009, English et al. 2009, 2011, Sabatier et al. 2008, Seo et al. 2009), manual stimulation (Angelov et al. 2007, Bischoff et al. 2009, Guntinas-Lichius et al. 2007 b, Irintchev et al. 2010, Kiryakova et al. 2010, Skouras et al. 2009) as well as application of peptides which functionally mimic glycan moieties (Mehanna et al. 2009, 2010, Simova et al. 2006).

1. Introduction

1.1 Cellular and molecular response after peripheral nerve injury

After injury to a peripheral nerve, characteristic histological and molecular changes are observed in the (1) neuronal cell body, the (2) proximal nerve stump and the (3) distal stump (Fig. 1) (for review see Burnett and Zager 2004, Chen et al. 2007, Frisen 1997, Frostick et al. 1998, Fu and Gordon 1997, Geuna et al. 2009).

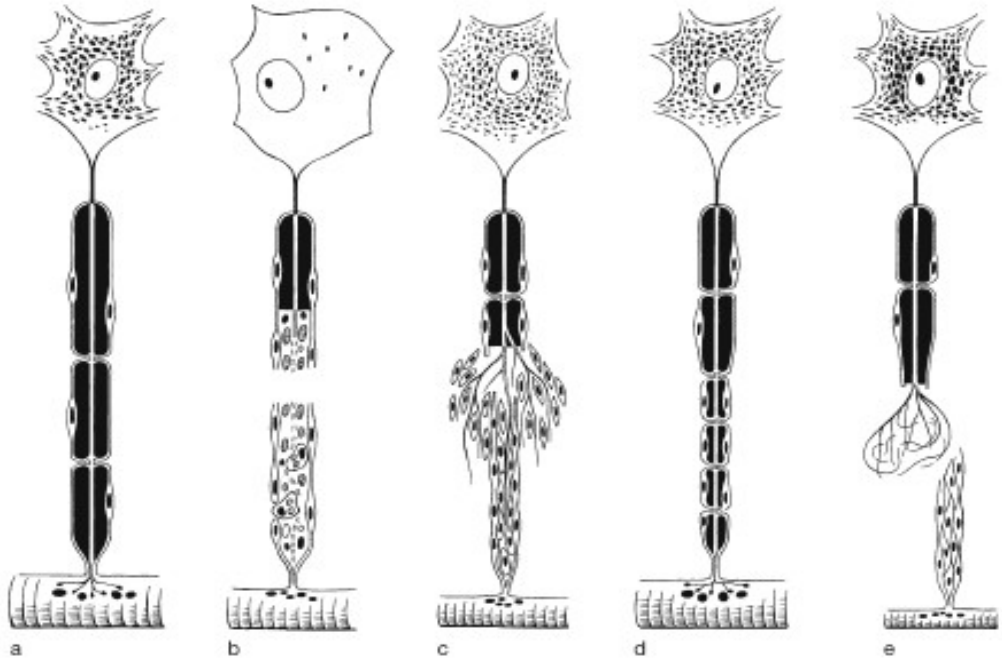

Figure 1. Morphological responses to a peripheral nerve injury (b-e). Intact peripheral nerve (a). Injury to a peripheral nerve causes chromatolysis of the neuronal cell body, a die-back of the proximal nerve stump and Wallerian degeneration of the distal nerve stump (b). Sprouts of axonal branches passing the interstump zone and entering the bands of Büngner (c) to complete end organ reinnervation and reversal of chromatolysis of the neuronal cell body (d). Amputation neuroma after failure of end organ reinnervation (e, adapted from Schiebler 2005).

1. Introduction

1.1.1 Changes in the neuronal cell body

A few hours after peripheral nerve injury, the neuronal cell body undergoes morphological changes known as chromatolysis. These include: (1) swelling of the cell body and nucleus, (2) migration of the nucleus to the periphery of the cell body, and (3) dissolution of the rough endoplasmic reticulum (Fig. 1b, Burnett and Zager 2004, Fu and Gordon 1997, Geuna et al 2009) and reflect the molecular changes after peripheral nerve injury due to enhancement of protein and mRNA synthesis of regeneration-associated genes (RAG). Neurons surviving axonal injury switch from a transmitting to a growth mode (Fu and Gordon 1997). Genes taking part in neurotransmission like choline acetyltransferase (ChAT) are down-regulated, while RAGs, e.g. encoding for the growth-associated protein 43 (GAP-43) and cytoskeletal proteins like actin and tubulin, are upregulated (Fu and Gordon 1997, Geuna et al. 2009).

1.1.2 Changes in the proximal nerve segment

After injury of a peripheral nerve, the proximal nerve stump degenerates back. The extent of this degeneration depends on the severity of the nerve injury. Axonal degeneration is usually limited to the first internodal segment (Fig. 1b, Burnett and Zager 2004, Geuna et al. 2009).

1.1.3 Changes in the distal nerve segment

The responses in the distal nerve stump after axonal damage had been first described by August Waller in 1850 (reprinted in Stoll et al. 2002). These responses, collectively known as Wallerian degeneration, include: (1) Axon degeneration and phagocytosis of axon and myelin debris, (2) Schwann cells switching from a myelinating to non-myelinating mode and (3) aligning of Schwann cells into guiding columns (bands of Büngner) (Fig. 1b, Fu and Gordon 1997). After axonal damage, the

injured axon disintegrates. The loss of axonal contact causes Schwann cell proliferation (Geuna et al. 2009) and a switch to a growth-supportive mode and down-regulation of myelin protein synthesis (Fu and Gordon 1997). Proliferating Schwann cells and macrophages invade the lesion site to phagocytise cellular and myelin debris. This can take up to 3-6 weeks (Geuna et al. 2009). Proliferating Schwann cells also line up as guiding columns (bands of Büngner) to support target reinnervation by up-regulation of regeneration-associated molecules (Fig. 1c). These include, e.g. transcription factors, cell adhesion molecules, neurotrophic factors and their receptors (Fu and Gordon 1997).

1.1.4 Regeneration of peripheral nerves

After axonal die-back in the proximal nerve stump (Fig. 1b, Fu and Gordon 1997) the axonal basal laminae persist and serve as guiding structures for sprouting axons. Axonal sprouts begin to access the endoneurial tubes distal to the injury site 3-42 days after nerve trauma (Fig. 1c, Geuna et al. 2009). Every axon can form up to 50-100 sprouting branches, which mostly degenerate later on. A regeneration unit (Morris et al. 1972) of about five of these sprouting branches, sent by one proximal axon, enters one endoneurial tube of the distal nerve stump (bands of Büngner) after passing the interstump zone (Geuna et al. 2009) and eventually end organ contact is achieved (Fig. 1c). Schwann cells of the distal nerve stump influences target reinnervation by up-regulation of regeneration-associated molecules that create a supportive growth environment in the distal nerve stump (Fu and Gordon 1997). After target reinnervation only one of the regeneration unit axons survives, while the other ones degenerate. Months after successful peripheral nerve regeneration, a reversal of chromatolysis can be observed. The nucleus returns to the centre of the neuronal cell body and the rough endoplasmic reticulum reorganizes to compact Nissl granules (Burnett and Zager 2004), including a switch from regenerative growth mode back to a transmitting mode (Fig. 1d).

1. Introduction

1.2 Regeneration-associated molecules

Regeneration-associated molecules are up-regulated after peripheral nerve injury by the lesioned neurons and by proliferating Schwann cells to promote axonal regeneration and enable end organ reinnervation. These molecules include, e.g. neurotrophic factors and adhesion molecules (Fu and Gordon 1997). Neurotrophic factors are categorized in three groups: (1) the neurotrophin family, (2) the GDNF (glial cell-derived neurotrophic factor) family and (3) neuropoetic cytokines (Boyd and Gordon 2003).

1.2.1 The neurotrophin family

Four members belong to the neurotrophin family in mammals: (1) nerve growth factor (NGF), (2) brain-derived neurotrophic factor (BDNF), (3) neurotrophin-3 (NT-3) and (4) neurotrophin-4/5 (NT-4/5, Barbacid 1994, Boyd and Gordon 2003, Chao 2003, Cui 2006). In fish, neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) have been also described (Götz et al. 1994, Lai et al. 1998). The neurotrophins regulate neuronal cell survival, axonal and dendritic growth and guidance, synaptic structure and connections, neurotransmitter release, long-term potentiation and synaptic plasticity (Boyd and Gordon 2001, Chao 2003). All members of the neurotrophin family are non-covalent homodimers (Yano and Chao 2000). Neurotrophins mediate their effects via multiple high- and a low-affinity receptors. High-affinity receptors are tyrosine receptor kinase A (trkA), tyrosine receptor kinase B (trkB) and tyrosine receptor kinase C (trkC). These belong to the tyrosin receptor kinase (trk) family and bind to their ligands with relatively high specificity (Fig. 2): (1) NGF binds to trkA (Kaplan et al. 1991 a, b, Klein et al. 1991 a), (2) BDNF and NT-4/5 bind to trkB (Klein et al. 1991 b, Klein et al. 1992) and (3) NT-3 binds to trkC (Lamballe et al. 1991). All neurotrophins can bind to the low-affinity p75 neurotrophin receptor ($p75^{NTR}$), which is a member of the tumour necrosis family receptors (Yano and Chao 2000). $P75^{NTR}$ binding by neurotrophins was

shown to induce apoptosis, on one hand, and to modulate trk activation by ligand binding associated with trk, on the other hand (Barker 1998). $P75^{NTR}$ is up-regulated in the distal nerve stump after axonal injury (Heumann et al. 1987) and is involved in remyelination after regeneration (Song et al. 2006). Inhibitory effects after application of high doses of BDNF on peripheral nerve regeneration are mediated by $p75^{NTR}$ (Boyd and Gordon 2001, 2002, 2003, Gordon et al. 2003). Increased motoneuron survival and peripheral nerve regeneration was observed previously in $p75^{NTR}$-/- mice (Ferri et al. 1998, Boyd and Gordon 2001).

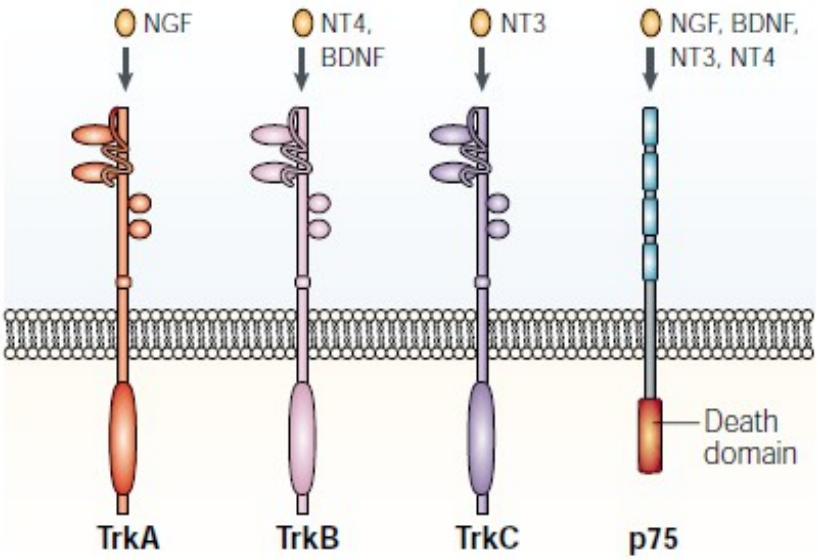

Figure 2. Neurotrophins and their high affinity binding receptors as well as their low affinity receptor $p75^{NTR}$ (adopted from Chao 2003).

1.2.2 Brain derived neurotrophic factor and neurotrophin-4/5

The member of the neurotrophin family BDNF was discovered in 1982 (Barde et al. 1982, Leibrock et al. 1989). Since its identification, numerous observations have shown that BDNF is involved in peripheral nerve regeneration (for review see Boyd and Gordon 2003, Cui 2006, Lykissas et al. 2007, Gordon 2009). The BDNF synthesis is up-regulated in motoneurons after axonal injury (Funakoshi et al. 1993, Kobayashi et al. 1996, Meyer et al. 1992) and this up-regulation promotes motoneuron survival

(Koliatsos et al. 1993, Oppenheim et al. 1992, Sendtner et al. 1992, Yan et al. 1992). Application of antibodies neutralizing BDNF or high doses of BDNF impedes axonal regeneration (Zhang et al. 2000, Boyd and Gordon 2002). The inhibitory effect of high BDNF doses is supposed to be mediated by $p75^{NTR}$ (Boyd and Gordon 2001, 2002, 2003, Gordon et al. 2003). Application of low doses of BDNF has no effect on axonal regeneration after immediate nerve repair (Boyd and Gordon 2002), while BDNF promotes axonal regeneration after delayed suture (Boyd and Gordon 2003). Up-regulation of BDNF synthesis is also observed in denervated Schwann cells (Meyer et al. 1992, Zhang et al. 2000) and brief electrical stimulation of a peripheral nerve after axonal transection and immediate nerve repair promotes up-regulation of BDNF synthesis in injured motoneurons and enhances axonal outgrowth into the distal nerve stump (Al-Majed et al. 2000 a, b).

NT-4/5 was identified in 1991 as a new member of the neutrophin family (Hallböök et al. 1991) and it is, like BDNF, up-regulated in the distal nerve stump after axonal injury (Funakoshi et al. 1993). Application of NT-4/5 after peripheral nerve injury improves axonal regeneration (Yin et al. 2001, Simon et al. 2003) and is necessary for axonal outgrowth (English et al. 2005).

1.2.3 Tyrosine kinase B receptor

TrkB belongs to the tyrosine kinase receptor family and has been named after the proto-oncogene tropomyosin-related kinase, that has led to its discovery (Huang and Reichardt 2003). TrkB has a molecular weight of 145 kD (Klein et al. 1989) and mediates BDNF and NT-4/5 effects (Klein et al. 1991 b, Klein et al. 1992). Ligand binding causes receptor dimerization and autophosphorylation of activation loop tyrosine residues (Barbacid 1995, Reichardt 2006). After phosphorylation of additional tyrosine residues, docking sites for adaptor proteins are formed (Huang and Reichardt 2003, Reichardt 2006, Yano and Chao 2000). The adaptor proteins contain phosphotyrosine binding (PTB) or src-homology-2 (SH2) domains (Huang and Reichardt 2003) and modulate receptor signalling. The following intracellular

downstream pathways can be activated via the trkB receptor: (1) the ras/ERK kinase, (2) the PI-3/Akt kinase and (3) the PLC-γ pathway (Kaplan and Miller 2000, Huang and Reichardt 2003, Reichardt 2006).

Two truncated trkB isoforms lacking the intracellular tyrosine kinase domain are known (Middlemas et al. 1991). These truncated isoforms seem to reduce axonal outgrowth by binding BDNF and removing it from the growth promoting environment of the distal nerve stump (Fryer et al. 1997). The truncated as well as the full-length trkB receptors are expressed in numerous structures of the central and the peripheral nervous system (Barbacid 1994). After axotomy, full-length trkB receptor expression in motoneurons is upregulated (Kobayashi et al. 1996, Hammarberg et al. 2000) and brief electrical stimulation was shown to accelerate trkB as well as BDNF expression (Al-Majed et al. 2000 a). No up-regulation of truncated trkB receptors was found in the distal nerve stump after axonal injury (Frisen et al. 1993, Funakoshi et al. 1993, Sebert and Shooter 1993). Reduced expression of trkB in heterozygous trkB mice leads to impairment of motor axonal regeneration (Boyd and Gordon 2001) and worse functional recovery after nerve injury compared with wild-type littermates (Eberhardt et al. 2006, Irintchev at al. 2005). Further investigation have indicated a functional relationship between trkB and the cell adhesion molecule NCAM (neural cell adhesion molecule, Cassens et al. 2010), which is involved in neuronal regeneration (Fey et al. 2010).

1.2.4 Tyrosine kinase B receptor deficient mice

Homozygous trkB deficient mice (trkB-/-) die within the first postnatal week and, thus, cannot be used in regeneration research. These animals show, among other deficits, loss of trigeminal, petrosal and dorsal root ganglion sensory neurons as well as loss of facial and spinal motoneurons (Barbacid 1994, Klein et al. 1993). In contrast, heterozygous trkB mice (trkB+/-) which are vital and do not show apparent structural deficits have been successfully used in several experimental settings (Boyd and Gordon 2001, Eberhardt et al. 2006, Kotulska et al. 2006 a, b, 2007, Xu et al. 2000 a, b).

1. Introduction

1.3 Electrical stimulation and peripheral nerve regeneration

Application of DC currents in vitro and in vivo as well as pulsed electromagnetic fields have been used to influence neurite outgrowth and nerve regeneration in different experimental settings (Hinkle et al. 1981, Politis et al. 1988, Kerns and Lucchinetti 1992, Kerns et al. 1987, 1991, Sisken et al. 1989, Rusovan et al 1992). Improved function recovery after nerve crush injury treated with electrical stimulation was observed by Nix and Hopf (1983) as well as Pockett and Gavin (1985). Further experiments showed that brief electrical stimulation increases axonal sprouting and reinnervation of the distal nerve stump as well as BDNF and trkB expression in motoneurons (Al-Majed et al. 2000 a, b, Brushart et al. 2002). Furthermore, electrical stimulation accelerates and enhances expression of regeneration-associated genes, e.g. Tα1-Tubulin and GAP-43 (Al-Majed et al. 2004). In addition, regeneration of sensory neurons is also improved by electrical stimulation and an up-regulation of BDNF and GAP-43 expression was detected in sensory neurons (Brushart et al. 2005, Geremia et al. 2007). These positive effects of brief electrical stimulation on peripheral nerve regeneration are mediated via the neuronal cell body (Al-Majed et al. 2000 b). Moreover, functional recovery is accelerated as well as the size of motoneuron bodies and the nerve diameter increase after brief electrical stimulation in mice (Ahlborn et al. 2007). The effect of electrical stimulation on myelination is inconsistent. Ahlborn et al. (2007) found a decrease of myelination in motor axons after regeneration. Recent experiments in rats showed, that axonal remyelination after nerve injury in rats is increased after electrical stimulation (Wan et al. 2010 a, b). The brief electrical stimulation paradigm was already tested in a clinical setting. Gordon et al. (2010) observed an accelerated axonal regeneration and target reinnervation in humans after carpal tunnel surgery (Gordon et al. 2010).

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1.4 The femoral nerve model

The femoral nerve model in rodents is a well approved paradigm for evaluation of peripheral nerve regeneration (e.g. Ahlborn et al. 2007, Eberhardt et al. 2006, Guseva et al. 2009, Huang et al. 2009, Irintchev et al. 2005, Irintchev 2011, Malin et al. 2009, Mehanna et al. 2009, Simova et al. 2006). The femoral nerve (Fig. 3) is a mixed peripheral nerve, containing motor and sensory proprioceptive axons. It includes nerve fibres from the L2 to L4 spinal nerves.

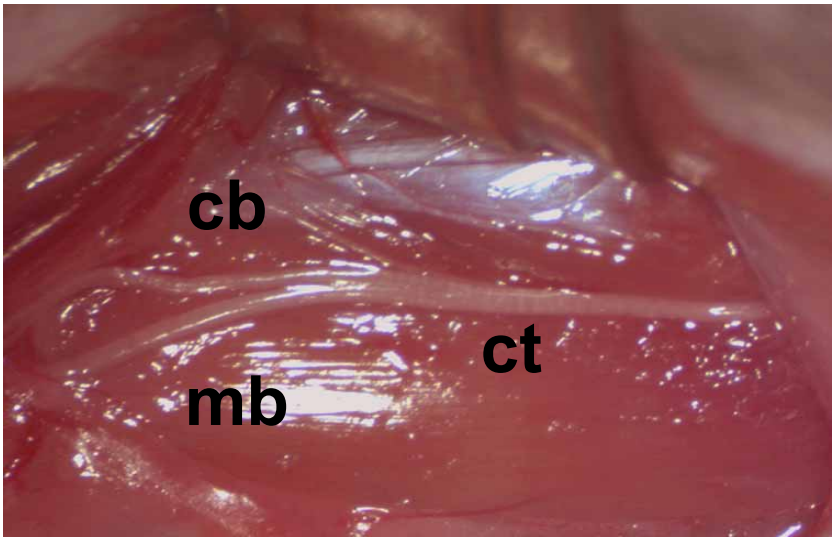

Figure 3. Exposed left femoral nerve. The common nerve trunk (ct) bifurcates into a muscle (mb) and cutaneous branch (cb) after exiting the peritoneal cavity.

After leaving the peritoneal cavity, the femoral nerve bifurcates into two branches. The motor or quadriceps branch (Fig. 3) contains motor and sensory axons neurons that exclusively innervate the ipsilateral quadriceps muscle. Its major function is the extension in the knee joint. The sensory or saphenous branch (Fig. 3) innervates the ipsilateral medial part of the lower limb. Lesions of the femoral nerve proximal to the bifurcation cause (1) gait disability due to ipsilateral quadriceps paresis and (2) sensory deficits. Loss of quadriceps muscle function can be assessed by the single-frame motion analysis (SFMA, Irintchev et al. 2005).

1. Introduction

1.5 Preferential motor reinnervation

Previous experiments have shown that motor axons reinnervate motor pathways of the distal nerve stump in mixed peripheral nerves selectively in rats (Brushart 1988, 1993, 1998). This preferential motor reinnervation (PMR) is accelerated by electrical stimulation (Al-Majed et al. 2000 b) in rats. However, inconsistent results of PMR in mice have been found (Ahlborn et al. 2007, Franz et al. 2005, 2008, Guseva et al. 2009, Mehanna et al. 2009, Simova et al. 2006). PMR is likely associated with the expression of (1) trkB (Eberhardt et al. 2006) and (2) HNK-1 (human natural killer cell glycan, Simova et al. 2006). HNK-1 is expressed exclusively on Schwann cells of motor but not of sensory axons and enhances axonal outgrowth by motoneurons (Martini and Schachner 1986, Martini et al. 1992, 1994). Moreover, (3) PSA-NCAM (α2,8 polysialic acid), which is expressed predominantly on neural cell adhesion molecule (Rutishauser 2008, Franz et al. 2005, 2008) is also associated with PMR.

2. Rationale and aim of the study

Previous work on peripheral nerve regeneration has indicated that brief electrical stimulation is beneficial for motor (Al-Majed et al. 2000 b, Brushart et al. 2002) and sensory axonal regeneration (Brushart et al. 2005, Geremia et al. 2005, 2007). Furthermore, electrical stimulation has been shown to improve functional recovery after axonal injury (Ahlborn et al. 2007) and to be linked to enhanced expression of BDNF and its high-affinity receptor trkB (Al-Majed et al. 2000 a).

The objective of this study was to determine the effect of brief electrical stimulation after femoral nerve injury and immediate nerve repair on (1) functional recovery, (2) neuronal survival and (3) selectively of reinnervation of the distal nerve stump in heterozygous trkB deficient mice compared to their wild-type littermates. Following questions were to be evaluated during this study:

(1) Is the functional recovery,
(2) the neuronal survival and/or,
(3) the selectively of distal nerve stump reinnervation

impaired in heterozygous trkB in mice compared to their wild-type littermates after electrical stimulation of the femoral nerve?

Functional recovery was assessed by the single-frame motion analysis (SFMA, Ahlborn et al. 2007, Eberhardt et al. 2006, Guseva et al. 2009, Huang et al. 2009, Irintchev et al. 2005, Malin et al. 2009, Mehanna et al. 2009, Simova et al. 2006).

Neuronal survival and selectively of reinnervation were investigated using a retrograde labelling procedure in the femoral nerve model (e.g. Ahlborn et al. 2007, Eberhardt et al. 2006, Guseva et al. 2009).

3. Material and Methods

3.1 Animals

Experiments were performed in eight male mice heterozygous for the tyrosine kinase receptor B (trkB+/-) and eight male wild-type (trkB+/+) littermates. All animals were three months of age at the onset of the experiment. The heterozygous trkB and wild-type mice (C57BL/6J background) were bred in the animal facility of the University Hospital of Hamburg and genotyped by standard polymerase chain reaction (PCR) assay as described previously (Xu et al. 2000 a, b). All animals used appeared healthy upon arrival and no structural or behavioural abnormalities were noticed. During experiments mice were kept in Plexiglas-cages (height 14 cm, depth 27 cm, width 20 cm) and had access to standard food and water ad libitum. The animals were kept in an air-conditioned room with a temperature of 22°C and a day-night-rhythm of 12 hours (dark: 19.00 to 07.00). All experiments were conducted in accordance with the German and European laws on protection of experimental animals. The procedures used were approved by the responsible committee of The State of Hamburg.

3.2 Surgical procedure and electrical stimulation

Operation and electrical stimulation of the femoral nerve were performed as described previously (Ahlborn et al. 2007). Animals were anaesthetized by an intraperitoneal injection of 0.4 mg/kg fentanyl (Fentanyl-Janssen, Janssen, Neuss, Germany), 20 mg/kg droperidol (Dehydrobenzperidol, Janssen) and 5 mg/kg diazepam (Valium 10 Roche, Hoffman-La Roche, Grenzach-Wyhlen, Germany). The eyes of the mice were covered with Bepanthen eye ointment (Hoffman-La Roche, Grenzach-Wyhlen, Germany) to prevent drying during operation. Animals were fixed in a supine position on a Styropor plate using adhesive tape. The left lower quadrant of the

3. Material and Methods

abdomen and the left groin were cleaned with ethanol (70%, Walter-CMP, Kiel, Germany), the hair was shaved, and after a skin incision with a length of about 2 cm, the left femoral nerve was exposed (Fig. 3). Teflon coated steel wires were used as stimulation electrodes (A5632, Cooner Wire Company, Chatsworth, CA). The cathode, forming a small loop, was twisted around the proximal nerve stump distal to the nerve's exit from the peritoneal cavity, while the anode was fixed at the muscle close to the femoral nerve. The threshold voltage required to elicit visible contraction of the quadriceps muscle was determined at varying voltage intensities (square pulse of 0.1 ms, duration at 20 Hz) for each mouse individually using an isolated pulse stimulator (Model 2100, A-M Systems, Carlsborg, WA, USA). The femoral nerve was transected with fine scissors 3 mm proximal to the bifurcation of the femoral nerve and 2 mm distal to the electrode. Immediately after transecting, the proximal nerve stump was stimulated for one hour by applying square 0.1 ms pulses at 20 Hz using an amplitude three times above the individually determined threshold level. The pulse amplitude was typically 3-4 V (Fig. 4). During electrical stimulation, the wound was covered with a moistened paper.

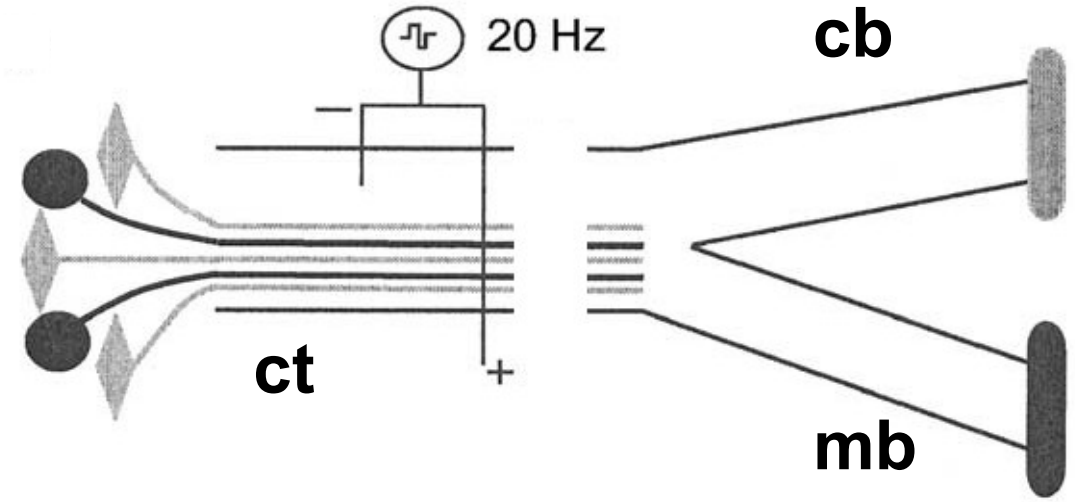

Figure 4. Schematic illustration of the femoral nerve during brief electrical stimulation procedure after femoral nerve transection. Bipolar electrodes are fixed at the common trunk (ct) of the femoral nerve before its bifrucation into the mucle (mb) and cutaneus branch (cb) (Figure 4 adapted from Al-Majed et al. 2000 b).

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After one hour of electrical stimulation, the electrodes were explanted (Fig. 5 A), the proximal and distal nerve stump were readapted with single epidural stitches of 11-0 Nylon suture thread (Ethicon, Norderstedt, Germany) and inserted into a silastic nerve cuff (3 mm length, 0.58 mm inner diameter, Becton Dickinson, Heidelberg, Germany). The proximal and distal nerve stumps were separated by a gap of 2 mm (Fig. 5 B-D). The polyethylene tubing was filled with phosphate-buffered saline (PBS, pH 7.4). After haemostasis, the skin wound was closed with 6-0 sutures (Ethicon, Norderstedt, Germany) and operated animals were kept in a heated room (35 °C) overnight to prevent hypothermia.

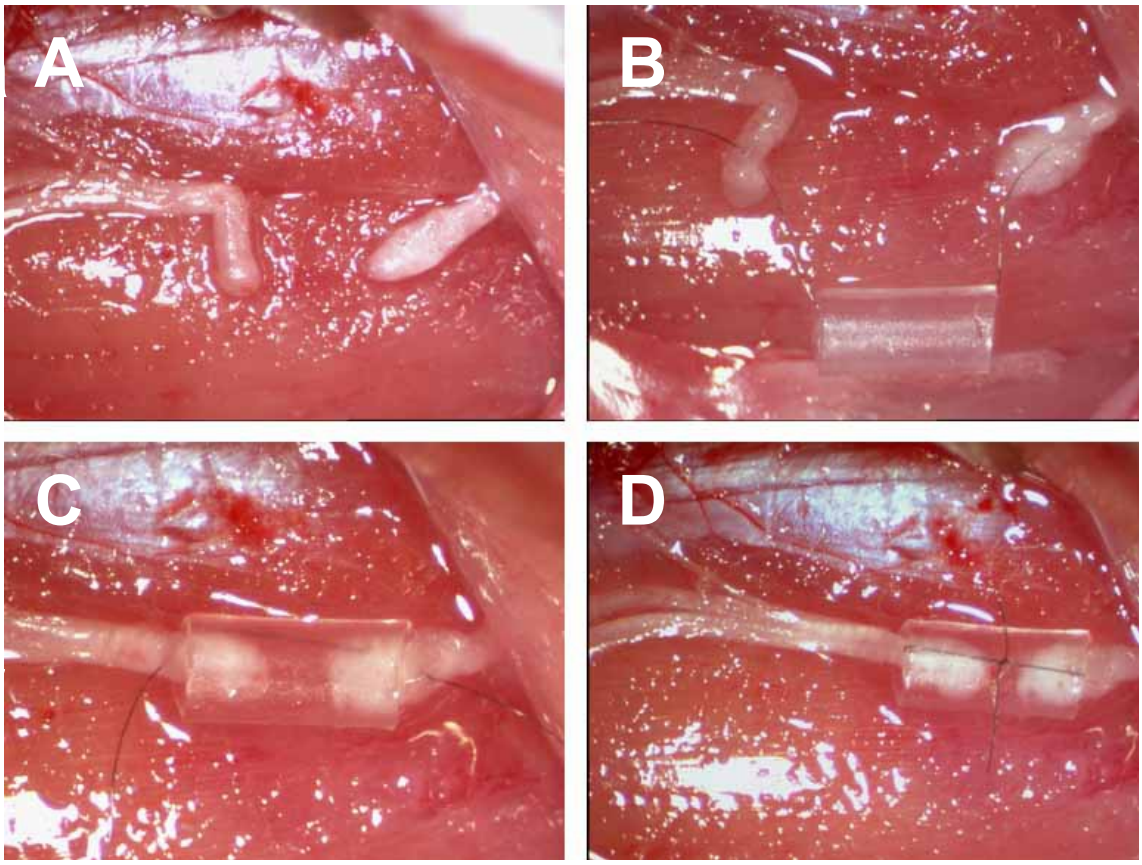

Figure 5. Transected femoral nerve after 1 h of low frequency electrical stimulation at 20 Hz and explantation of the electrodes (A). Distal (left hand side) and proximal (right hand side) nerve stump stitched by suture (B). Distal and proximal nerve stump were inserted into a nerve cuff with a 2 mm gap. The gap between both nerve stumps was filled with PBS (C). Fixation of the suture with a single knot (D).

3. Material and Methods

3.3 Analysis of motor function

Motor functional recovery of the femoral nerve was assessed by the single frame motion analysis (SFMA, Irintchev et al. 2005, Irintchev 2011). The SFMA allows a precise evaluation of the quadriceps muscle function (Ahlborn et al. 2007, Eberhardt et al. 2006, Guseva et al. 2009, Huang et al. 2009, Irintchev et al. 2005, Irintchev 2011, Malin et al. 2009, Mehanna et al. 2009, Simova et al. 2006). Irintchev et al. (2005) showed that deficits in quadriceps muscle function induced by lesions of the femoral nerve and degree of functional recovery in the course of reinnervation can be precisely evaluated in mice.

3.3.1 Video recording and measurements

All animals used in this experiment learned to walk on a wooden beam (1000 mm long, 38 mm wide) leading to their home cage prior to operation (Fig. 6). Rear-view video recordings were taken for each animal prior to surgery and were repeated 1, 2, 4, 8 and 12 weeks after surgical and brief electrical stimulation procedure using a high speed camera (A620fc, Basler, Ahrensburg, Germany) at 100 frames per second and examined using SIMI-Motion 7.0 software (SIMI Reality Motion Systems, Unterschleissheim, Germany) as well as VirtualDub 1.8.1 (written by Avery Lee, free software available at http://www.virtualdub.org). Selected video frames of the step cycle were analysed to measure defined parameters reflecting the quadriceps motor function. The heels-tail angle (HTA) and the foot-base angle (FBA) were measured. Angles were measured using UTHSCSA ImageTool 3.0 program (University of Texas, San Antonio, TX, USA; free software available at http://ddsdx.uthscsa.edu/dig/). Without initial training, voluntary reaching pursuits for estimation of the limb protraction length ratio (PLR) were video recorded. Length measurements were also performed with UTHSCSA ImageTool 3.0.

3. Material and Methods

Figure 6. Experimental setup for video recordings. Shown are high speed camera (arrow), beam walk and an animal home cage. Animals were trained to walk on the wooden beam without exploratory pauses and at a steady speed to their home cage. Rear view of walking was captured with a high speed camera several times: prior to surgery, 1, 2, 4, 8 and 12 weeks after operation of the left femoral nerve and low frequency electrical stimulation at 20 Hz for one hour.

3.3.2 Parameters of functional recovery

When the quadriceps branch of the femoral nerve is injured, only the quadriceps muscle, which extends the leg in the knee joint, is affected. The quadriceps muscle keeps the knee extended during single-support phases required for the swing of the contralateral leg during walking. Three parameters were measured to evaluate the quadriceps muscle function as parameters for the reinnervation of the femoral nerve (Irintchev at al. 2005): (1) the heels-tail angle (HTA), (2) the foot-base angle (FBA) and (3) the limb protraction length ratio (PLR). To evaluate the degree of recovery on an individual basis, the heels-tail angle recovery index, the foot-base angle recovery index, the cumulative stance recovery index and the limb protraction length ratio recovery index were calculated. As an absolute measure of recovery, the stance deficit for the HTA, the FBA and the cumulative stance deficit were calculated.

3.3.2.1 Heels-tail angle

The heels-tail angle (HTA) is formed by the lines connecting the heels with the anus and measured when the leg of the operated and electrically stimulated side is in single support phase and the contralateral extremity has maximum swing attitude with the sole surface parallel to the transverse plane (Fig. 7 A, Irintchev et al. 2005). Quadriceps muscle weakness after femoral nerve injury leads to characteristic changes compared with intact animals: the sole of the injured side is visible in the single support phase, the hip is tiled and the extremity is rotated internally. (Fig. 7 B, Irintchev et al. 2005). Contralateral HTA is not affected by the femoral nerve injury.

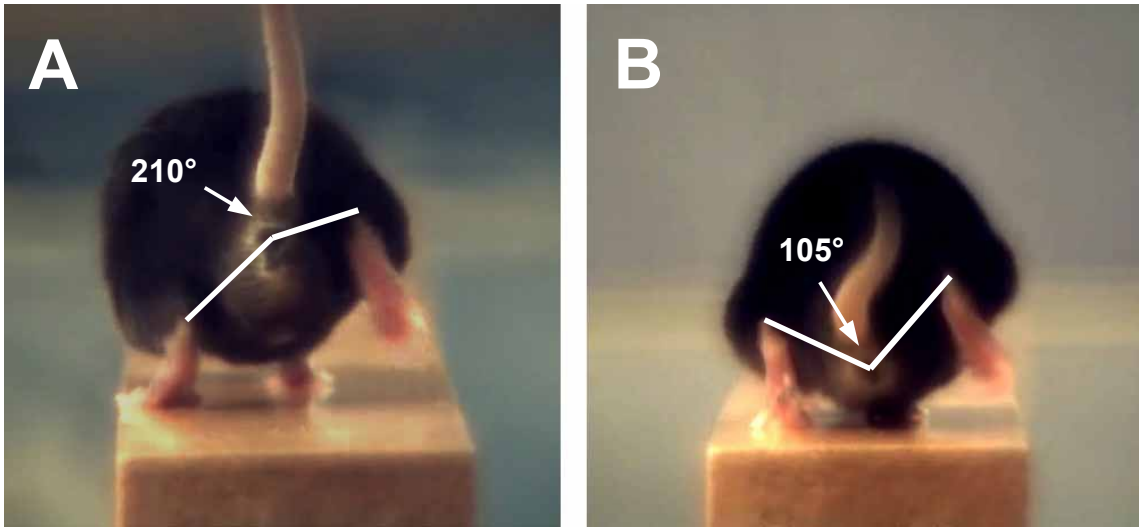

Figure 7. Single video frames from recordings of beam walking (A, B). Lines drawn in the video frames show the heels-tail angle. An animal prior to (A) and 1 week after repair of the left femoral nerve and low frequency electrical stimulation (B).

3.3.2.2 Foot-base angle

The foot-base angle (FBA) is formed by the horizontal line and the line dividing the sole of the operated and electrically stimulated side into two halves symmetrically. The FBA is measured at toe-off position (Fig. 8 A) at the side of repair and electrical stimulation, at which the sole is parallel to the transverse plane (Irintchev et al. 2005).

3. Material and Methods

In injured mice, the ipsilateral FBA is increased due to internal rotation of the foot (Fig. 8 B), which is not observed in intact animals (Fig. 8 A).

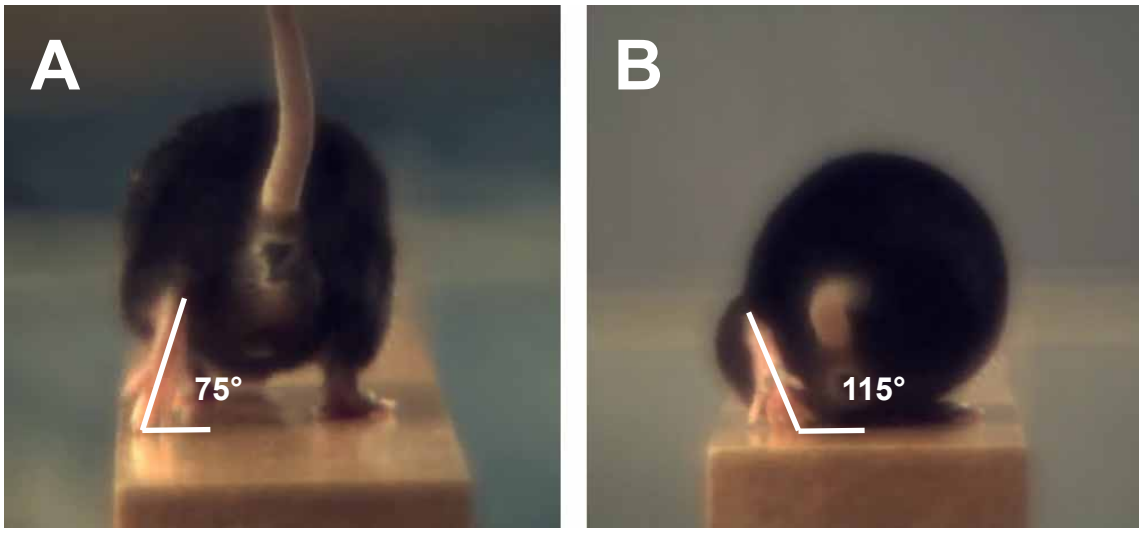

Figure 8. Single video frames from recordings of beam walking (A, B). Lines drawn in the video frames show the foot-base angle. An animal prior to (A) and 1 week after repair of the left femoral nerve and low frequency electrical stimulation (B).

3.3.2.3 Limb protraction length ratio

The limb protraction length ratio (PLR) is measured as the ratio of the relative length of the intact to the lesioned limb. PLR is measured when a mouse is held by its tail and is allowed to grasp a pencil with its paws. Doing this, the mouse extends both hind limbs simultaneously. The distance is measured between the most distal mid-point of the extremity and the anus (Irintchev et al. 2005). After nerve injury on one side, the ipsilateral side can not be extended maximally (Fig. 9 B), while the contralateral hind limb can be extended to its maximum. (Fig 9 A).

3. Material and Methods

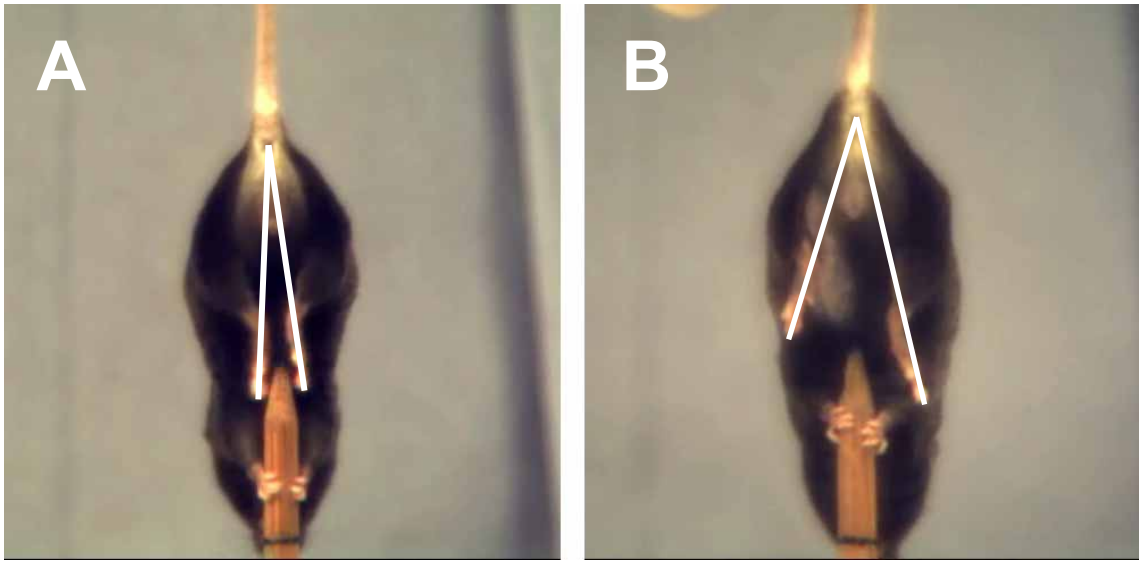

Figure 9. Single video frames from video recordings of limb protractions (A, B). Lines drawn in the video frames show limb length used for calculation of the limb protraction length ratio (PLR). An animal prior to (A) and 1 week after repair of the left femoral nerve and low frequency electrical stimulation (B).

3.3.2.4 Recovery indices

As relative measure of functional recovery, different recovery indices were calculated: the (1) HTA, (2) FBA, (3) PLR and (4) stance recovery index. These recovery indices allow evaluation of recovery on an individual animal basis and are more advantageous than absolute values in order to compare the degree of recovery, because of excluding interindividual differences like gender, body weight and age that could impede comparison of absolute values (Irintchev et al. 2005).

3.3.2.4.1 Heels-tail angle, Foot-base angle and Protraction length ratio recovery index

The heels-tail angle, foot-base angle and limb protraction length ratio recovery indices are calculated in percent, as:

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

X_{pre} is the value prior to operation and low frequency electrical stimulation at 20 Hz for one hour, X_{den} during the state of denervation (7 days after injury), and X_{reinn} at any time-point of reinnervation. In this experiment the recovery indices were calculated for four different time-points: 2, 4, 8 and 12 weeks.

3.3.2.4.2 Stance recovery index

The stance recovery index is calculated as the mean of the heels-tail angle and the foot-base angle recovery index after 2, 4, 8 and 12 weeks of regeneration.

3.3.2.5 Stance deficit

As an absolute measure of recovery, the (1) heels-tail angle, (2) foot-base angle and (3) cumulative stance deficit were calculated (Simova et al. 2006).

3.3.2.5.1 Heels-tail angle stance deficit

The heels-tail angle stance deficit is calculated as the difference between the pre- and postoperative HTA values at any time-point of reinnervation after operation and electrical stimulation (HTA_{reinn}):

$$\text{HTA stance deficit} = HTA_0 - HTA_{reinn}$$

3.3.2.5.2 Foot-base angle stance deficit

The foot-base angle stance deficit is calculated as the difference between the postoperative FBA_{reinn} values at any time-point of reinnervation after surgery and electrical stimulation and the preoperative FBA_0 values:

$$\text{FBA stance deficit} = FBA_{reinn} - FBA_0$$

3.3.2.5.3 Cumulative stance deficit

The cumulative stance deficit is calculated as the sum of the FBA and HTA stance deficit (Simova et al. 2006):

$$\text{Cumulative stance deficit} = (FBA_{reinn} - FBA_0) + (HTA_0 - HTA_{reinn})$$

3.4 Retrograde labelling of motoneurons

The retrograde labelling procedure was performed to (1) differentiate between correctly and incorrectly projecting motoneurons, (2) evaluate the degree of preferential motor reinnervation and (3) assess neuronal survival 3 months after nerve injury and brief electrical stimulation.

Retrograde labelling is a standard technique to quantitatively evaluate reinnervation in peripheral nerve research (Hayashi, 2007). The retrograde labelling has been described in detail previously (e.g. Ahlborn et al. 2007, Al-Majed et al. 2000 a, b, 2004, Eberhardt et al. 2006, Guseva et al. 2009, Malin et al. 2009, Mehanna et al. 2009, Simova et al. 2006).

3. Material and Methods

Twelve weeks after axonal transection and brief electrical stimulation all animals used for functional analysis were anaesthetized deeply by application of 0.4 mg/kg fentanyl (Fentanyl-Janssen, Janssen, Neus, Germany), 20 mg/kg droperidol (Dehydrobenzperidol, Janssen) and 5 mg/kg diazepam (Valium 10 Roche, Hoffman-La Roche, Grenzach-Wyhlen, Germany) into the peritoneal cavity. The eyes of the mice were covered with Bepanthen eye ointment (Hoffman-La Roche, Grenzach-Wyhlen, Germany) to prevent drying during operation. Animals were fixed in a supine position on a Styropor plate using adhesive tape. The left lower quadrant of the abdomen and the left groin were cleaned with ethanol (70%, Walter-CMP, Kiel, Germany) and the hair was shaved. After a skin incision with a length of about two cm, the left femoral nerve with its muscle and cutaneous branch was exposed. Pieces of Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) were placed underneath the two nerve branches distal to the bifurcation. The quadriceps and saphenous branch were cut with a fine pair of scissors 5 mm distal to the bifurcation of the femoral nerve (Fig. 10 A). Fluoroscence retrograde tracers were applied in powder for the transected nerve ends. Fluoro-Gold (Fluorochrome, Denver, Colorado, USA) was applied to the muscle branch, while Fast-Blue (EMS-Chemie GmbH, Großumstadt, Germany) was applied to the cutaneous branch (Fig. 10 B). Thirty minutes after application, the quadriceps and saphenous stumps were rinsed with PBS and gently blotted with filter paper. After haemostasis, the skin wound was closed with 6-0 sutures (Ethicon). After the retrograde back labelling and surgical procedure, the animals were kept in a heated room (35 °C) overnight to prevent hypothermia.

3. Material and Methods

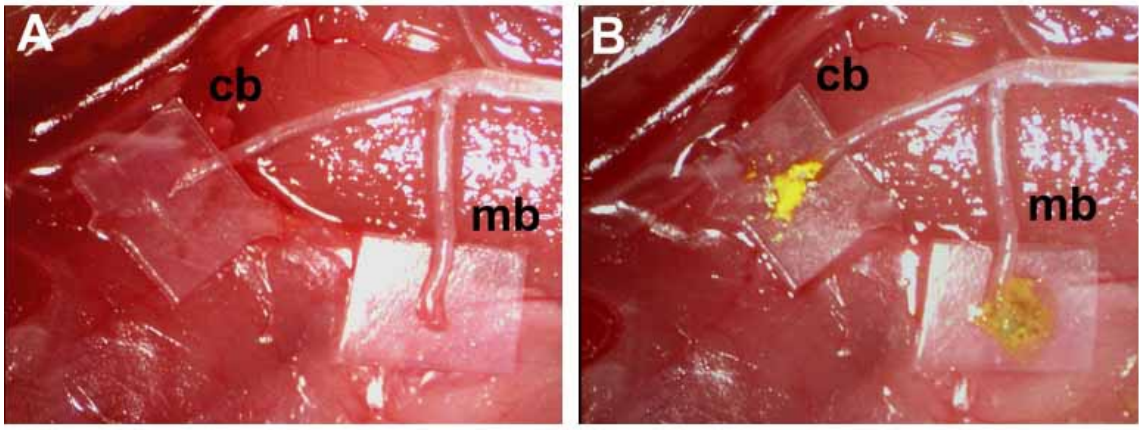

Figure 10. Exposed left femoral nerve with its muscle (mb) and cutaneous (cb) branch during the retrograde labelling procedure (A, B) 12 weeks after nerve repair and 1h low frequency electrical stimulation. Pieces of Parafim underneath the transected branches (A). Fluoroscence retrograde tracers were applied to both branches of the femoral nerve. Fluoro-Gold was applied to the muscle branch and Fast-Blue was applied to the cutaneous branch (B).

3.5 Transcardial perfusion and tissue preparation

One week after the retrograde labelling, mice were anaesthetized by an intraperitoneal application of 5 µl/g of a 16% solution of sodium pentobarbital (Narcoren®, Merial, Hallbermoos, Germany). The deeply anaesthetized animals were transcardially perfused using a peristaltic pump (Ismatec SA, Glattbrugg, Switzerland) with PBS for 1 minute followed by a fixative (4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3) for 15 minutes at room temperature. The lumbar spinal cord was dissected, removed and post-fixed for 18-24 hours at 4°C in the same fixative (4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3). The spinal cords were cut transversely on a vibratom (VT1000S, Leica Instruments, Nußloch, Germany). Serial section of 50-µm thickness were mounted on glass slides (SuperFrost®Plus, Roth, Karlsruhe, Germany). The sections were stored in boxes at -20°C until morphological analysis.

3. Material and Methods

3.6 Morphological analysis of the spinal cord sections

The lumbar spinal cord sections were studied using a fluorescence microscope (Axiophot 2, Zeiss, Oberkochen, Germany) with an ultraviolet filter (excitation: 365 nm) appropriate for both retrograde labelling tracers. Fluoro-Gold and Fast Blue were used to back-label motor neurons. Motoneurons that were labelled with Fluoro-Gold had a bright yellow fluorescence of the cytoplasm and the dendritic processes (Schmued et al. 1986, Hayashi et al. 2007). Neurons retrogradely labelled by Fast Blue showed a bright blue fluorescence of the stained cytoplasm and the dendritic processes (Bentivoglio et al. 1980, Hayashi et al. 2007). Sections were studied by counting single (Fast Blue or Fluoro-Gold only) and double-labelled (Fast Blue and Fluoro-Gold) neurons. To be sure that no cells were double counted a stereological method was used as described previously (Ahlborn et al. 2007, Eberhardt et al. 2006, Mehanna et al. 2009, for detailed description see Simova et al. 2006).

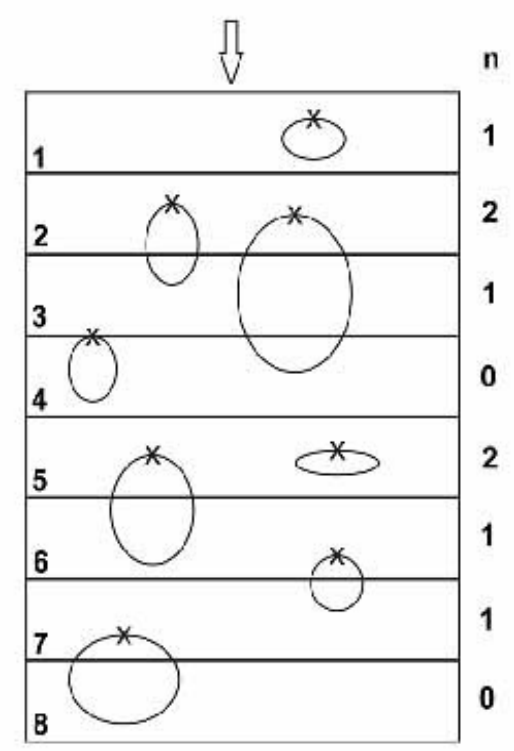

Figure 11. Stereological method to avoid double counting of retrogradely labelled neurons. Shown are eight sections (number of slices are shown on the left hand side) cut from a continuous piece of tissue (containing eight cells to be counted). Starting from the top sections were examined by focusing through the depth of each section (arrow). Labelled cells were counted when their profiles were not visible on top of the sections. Total number of counted cells (n) are shown on the right hand side (Figure 11 from Simova et al. 2006).

3. Material and Methods

All sections were examined by focusing through the section thickness starting from the top using a 40x magnification. All visible motoneurons were counted except those profiles visible on the top of the section (Fig. 11).

3.7 Photographic documentation

Images of the surgical procedure were taken using a Stemi 2000 microscope (Zeiss, Oberkochen, Germany) equipped with a digital camera Axiocam (Zeiss, Oberkochen, Germany). Processing of images was made by using Adobe® Photoshop® 7.0 (Adobe Systems Inc., San Jose, California, USA).

3.8 Statistical analysis

Data are presented as mean values with standard errors of mean (S.E.M.). One way analysis of variance (ANOVA) for repeated measurements with Tukey post hoc test and two-sided t-test for independent groups were used to compare group mean values. The accepted level of significance was set at 5%. Mean was used as representative value, when two or more measurements per animal were made. For all comparisons, the degree of freedom was determined by the number of animals. Statistical analysis was performed with SigmaStat 3.5 (Systat Software Inc., San Jose, California, USA). Linear regression analysis was performed using SigmaPlot 10 (Systat Software Inc., San Jose, California, USA).

4. Results

4.1 Functional recovery

To evaluate functional recovery in heterozygous trkB animals and their wild-type littermates after femoral nerve transection and brief low frequency electrical stimulation, the quadriceps motor function was assessed by using the single-frame motion analysis (SFMA, Irintchev et al. 2005). The heels-tail angle (HTA), the foot-base angle (FBA) and the limb protraction length ratio (PLR) were calculated. Measurements were performed at several time points: prior to and 1, 2, 4, 8 and 12 weeks after nerve repair and electrical stimulation. To evaluate the degree of recovery on an individual basis, for each animal the recovery index for the HTA, the FBA, the PLR and the stance recovery index were calculated. As an absolute measure of recovery, the stance deficit for the HTA, the FBA and the cumulative stance deficit were calculated.

4.1.1 Heels-tail angle

After an initial decrease of mean angle values immediately after axonal injury and electrical stimulation in both groups, a significantly delayed increase of the HTA mean values in trkB+/- mice compared to the control group was detected at 28 ($p<0.001$, ANOVA) and 56 ($p<0.001$, ANOVA) days (Fig. 12). No statistically significant differences were found after 7 ($p=0.082$, ANOVA), 14 ($p=0.110$, ANOVA) and 84 ($p=0.437$, ANOVA) days of recovery between heterozygous trkB mice and their wild-type littermates.

4. Results

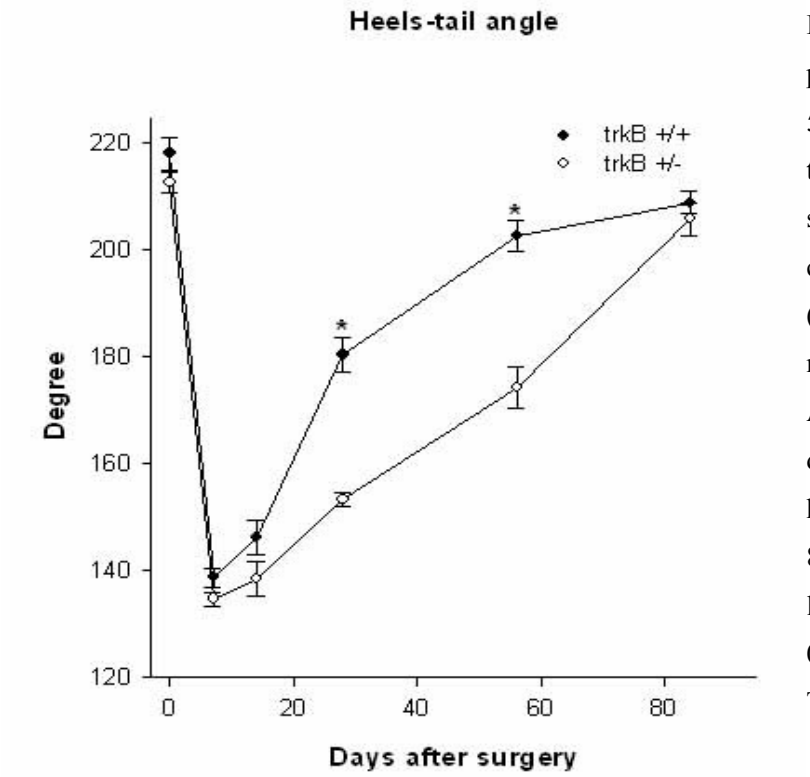

Figure 12. Heels-tail angle prior to operation, 7, 14, 28, 56 and 84 days after nerve transection and electrical stimulation in trkB+/+ (black circles) and trkB+/- animals (open circles). Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n = 8) and their wild-type littermates (n = 8) ($p < 0.001$, one way ANOVA with Tukey post hoc test).

4.1.2 Foot-base angle

Following an initial increase of FBA mean values after axonal injury followed by brief electrical stimulation in both groups, a significantly delayed decrease of FBA mean values in trkB+/- mice compared to their wild-type littermates was found at 14 ($p<0.001$, ANOVA) and 28 ($p<0.001$ ANOVA) days. No statistically significant differences comparing both groups were found after 7 ($p=0.07$, ANOVA), 56 ($p=0.088$, ANOVA) and 84 ($p=0.117$, ANOVA) days of regeneration after femoral nerve injury and brief electrical stimulation (Fig. 13).

4. Results

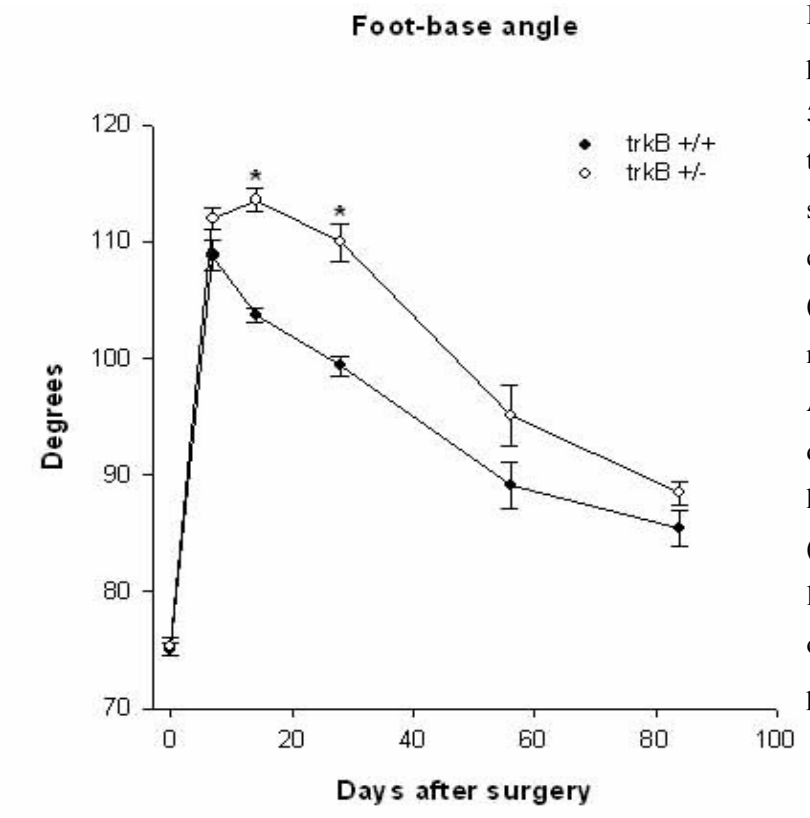

Figure 13. Foot-base angle prior to operation, 7, 14, 28, 56 and 84 days after nerve transection and electrical stimulation in trkB+/+ (black circles) and trkB+/- animals (open circles). Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.001$, one way ANOVA with Tukey post hoc test).

4.1.3 Limb protraction length ratio

Comparison of the heterozygous trkB and the control group showed, after an initial increase of PLR mean values after femoral injury followed by brief electrical stimulation, a significantly delayed decrease of PLR mean values in trkB+/- animals after 14 ($p=0.048$, ANOVA) and 28 ($p<0.001$, ANOVA) days of regeneration. No statistically significant differences between both groups were found 7 ($p=0.670$, ANOVA), 56 ($p=0.839$, ANOVA) and 84 ($p=0.138$, ANOVA) days after femoral lesion followed by brief electrical stimulation (Fig. 14).

4. Results

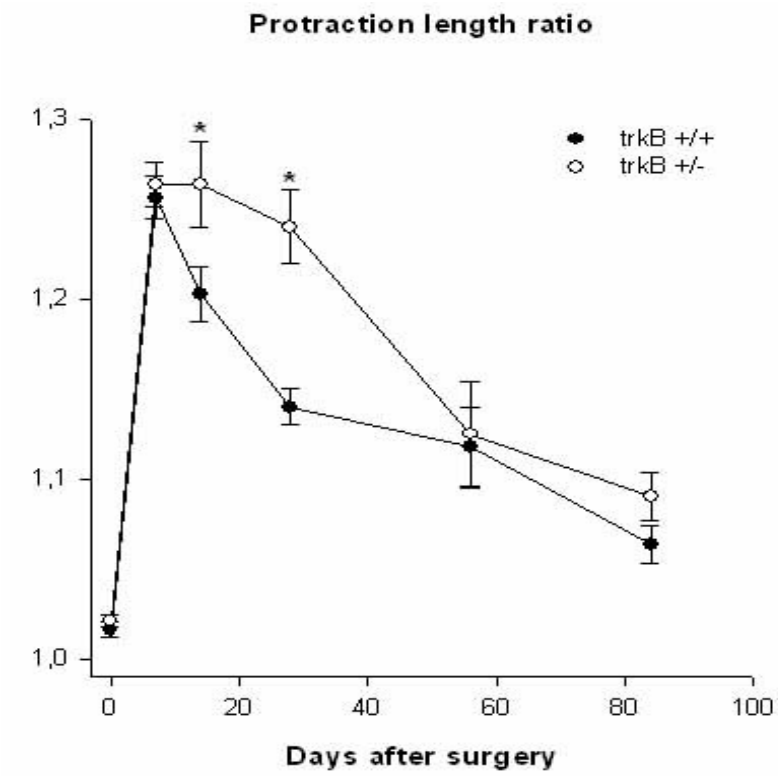

Figure 14. Limb protraction length ratio prior to operation, 7, 14, 28, 56 and 84 days after nerve transection and electrical stimulation in trkB+/+ (black circles) and trkB+/- animals (open circles). Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.05$, one way ANOVA with Tukey post hoc test).

4.1.4 Recovery indices

After 2, 4, 8 and 12 weeks of regeneration (1) the HTA, (2) the FBA, (3) the stance recovery index as well as (4) the PLR recovery index were calculated to evaluate the degree of regeneration on an individual basis.

4.1.4.1 Heels-tail angle recovery index

A significantly better recovery for the HTA index was found in trkB+/+ littermates compared with trkB+/- mice at 4 ($p<0,001$, t-test) and 8 weeks ($p<0,001$, t-test) but not at 2 and 12 weeks of regeneration (Fig. 15).

4. Results

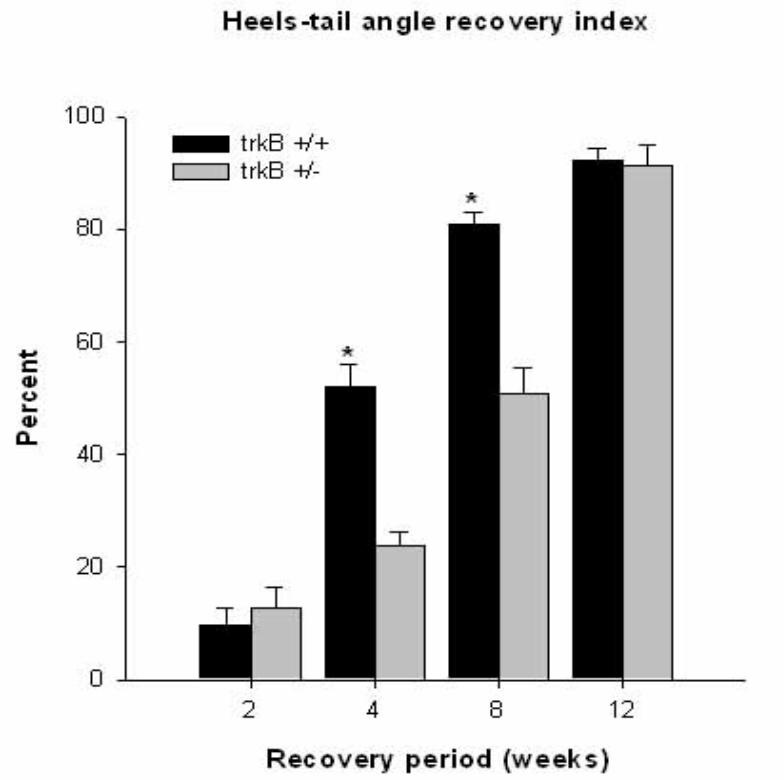

Figure 15. Heels-tail angle recovery index after 2, 4, 8 and 12 weeks of recovery after operation and electrical stimulation in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.001$, two-sided t-test for independent groups).

4.1.4.2 Foot-base angle recovery index

A statistically significant difference between the two groups, with better recovery in trkB+/+ than trkB+/- mice, was found only after 4 weeks of recovery ($p=0.003$, t-test, Fig. 16).

4. Results

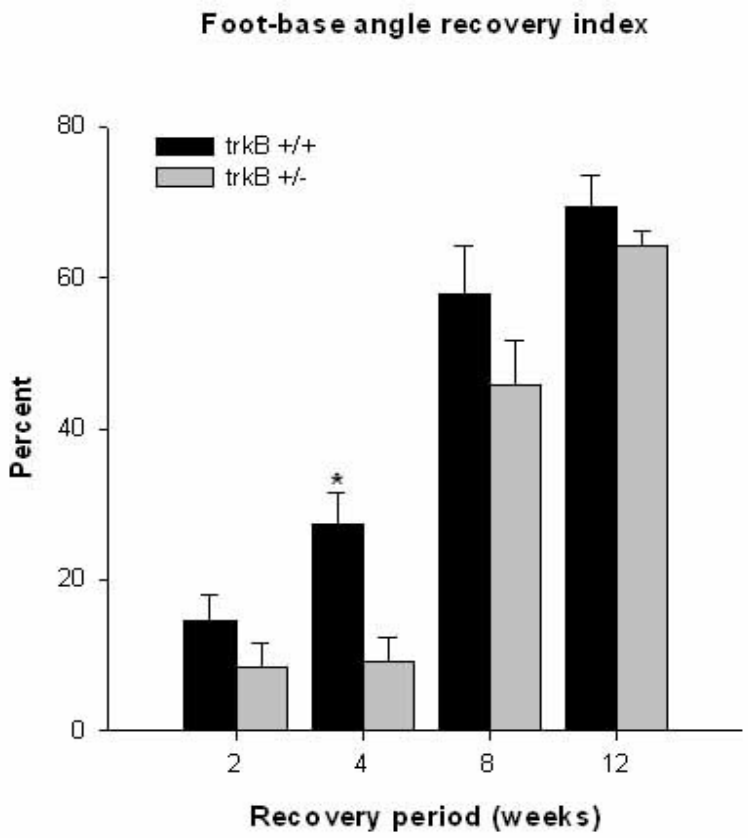

Figure 16. Foot-base angle recovery index after 2, 4, 8 and 12 weeks of recovery after operation and electrical stimulation in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p = 0.003$, two-sided t-test for independent groups).

4.1.4.3 Stance recovery index

Statistically significant differences between the two groups were found after 4 ($p<0.001$, t-test) and 8 weeks ($p=0.002$, t-test) of regeneration due to slower recovery in the heterozygous trkB mice than in the control group (trkB+/+, Fig. 17).

4. Results

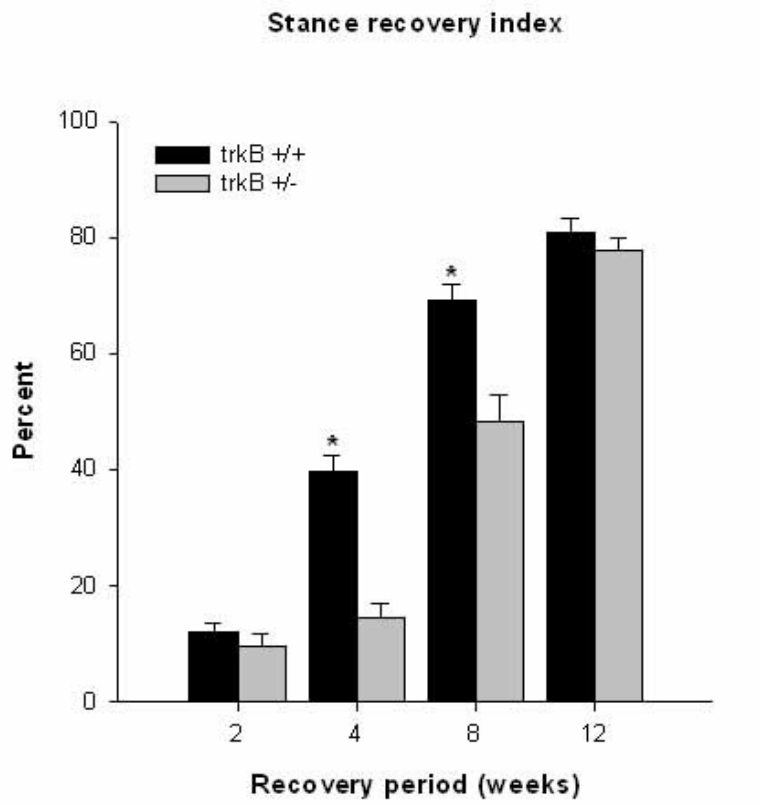

Figure 17. Stance recovery index, an overall estimate of quadriceps muscle function during stance after 2, 4, 8 and 12 weeks of recovery after operation and electrical stimulation in trkB+/+ (black bars) and trkB+/- (grey bars) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.05$, two-sided t-test for independent groups).

4.1.4.4 Limb protraction length ratio recovery index

Comparison of trkB+/- and trkB+/+ animals showed a significantly better recovery in trkB+/+ than trkB+/- mice after 4 weeks of regeneration ($p<0.001$, t-test) but not at 2, 8 and 12 weeks of regeneration (Fig. 18).

4. Results

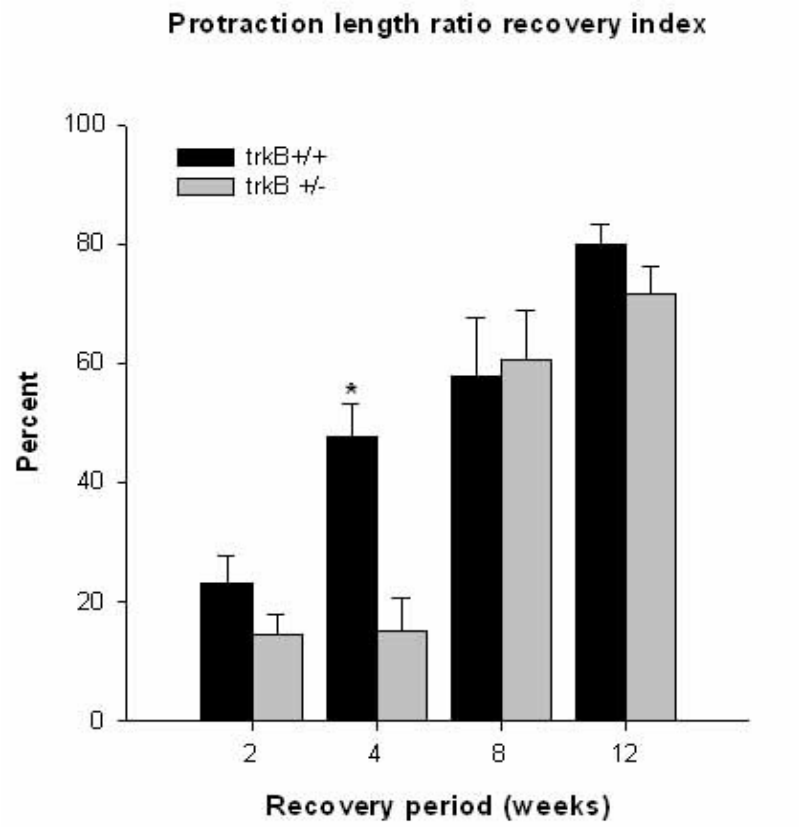

Figure 18. Limb protraction length ratio recovery index after 2, 4, 8 and 12 weeks of recovery after operation and electrical stimulation in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.001$, two-sided t-test for independent groups).

4.1.5 Stance deficit

(1) The HTA, (2) the FBA and (3) the cumulative stance deficit were calculated as absolute measure of recovery 1, 2, 4, 8 and 12 weeks after operation of the left femoral nerve followed by low frequency electrical stimulation at 20 Hz for one hour.

4.1.5.1 Heels-tail angle stance deficit

Statistically significant differences comparing the heterozygous trkB animals with their wild-type littermates were found after 4 ($p=0.001$, t-test) and 8 weeks ($p<0.001$, t-test) of regeneration due to a delayed decrease of the HTA stance deficit in trkB+/- animals (Fig. 19).

4. Results

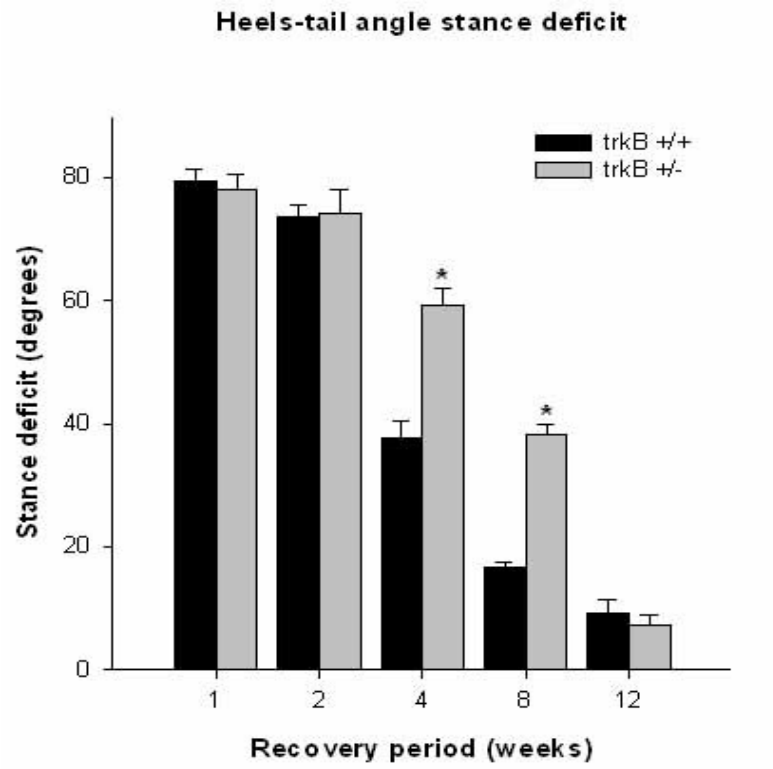

Figure 19. Heels-tail angle stance deficit after 1, 2, 4, 8 and 12 weeks of recovery after operation of the left femoral nerve and low frequency electrical stimulation at 20 Hz for one hour in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.05$, two-sided t-test for independent groups).

4.1.5.2 Foot-base angle stance deficit

Comparison of trkB+/- and trkB+/+ mice showed statistically significant differences after 2 ($p<0.001$, t-test) and 4 weeks ($p<0.001$, t-test) for the FBA stance deficit due to a delayed decrease of FBA stance deficit in heterozygous trkB animals in comparison to their wild-type littermates (Fig. 20).

4. Results

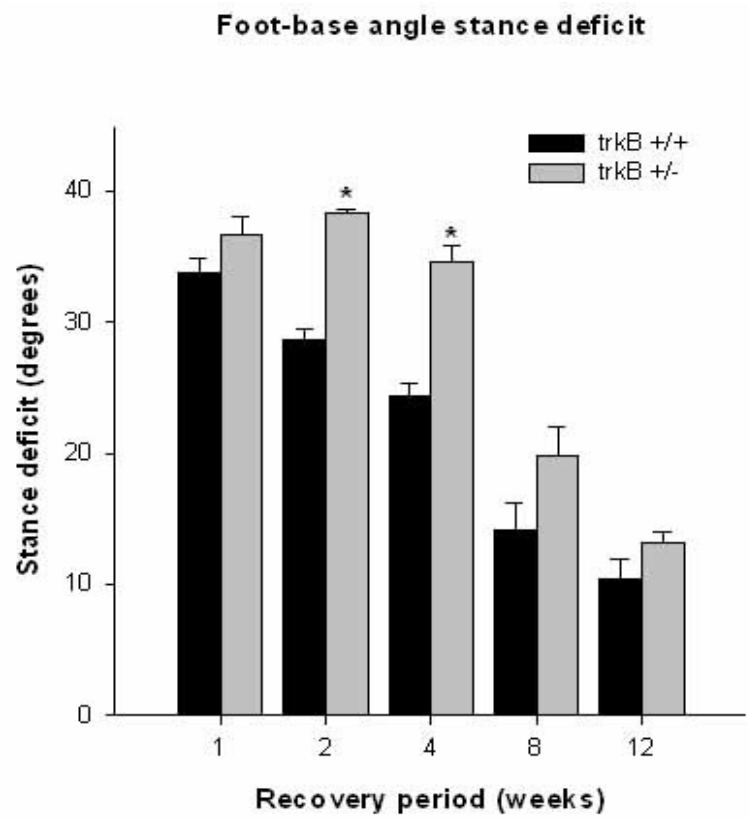

Figure 20. Foot-base angle stance deficit after 1, 2, 4, 8 and 12 weeks of recovery after operation of the left femoral nerve and low frequency electrical stimulation at 20 Hz for one hour in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice (n=8) and their wild-type littermates (n=8) ($p < 0.05$, two-sided t-test for independent groups).

4.1.5.3 Cumulative stance deficit

Comparison of the two groups showed a significantly larger deficit after 2 ($p=0.023$, t-test), 4 ($p<0.001$, t-test) and 8 weeks ($p<0.001$, t-test) of regeneration between trkB+/- animals and their wild-type littermates (Fig. 21).

4. Results

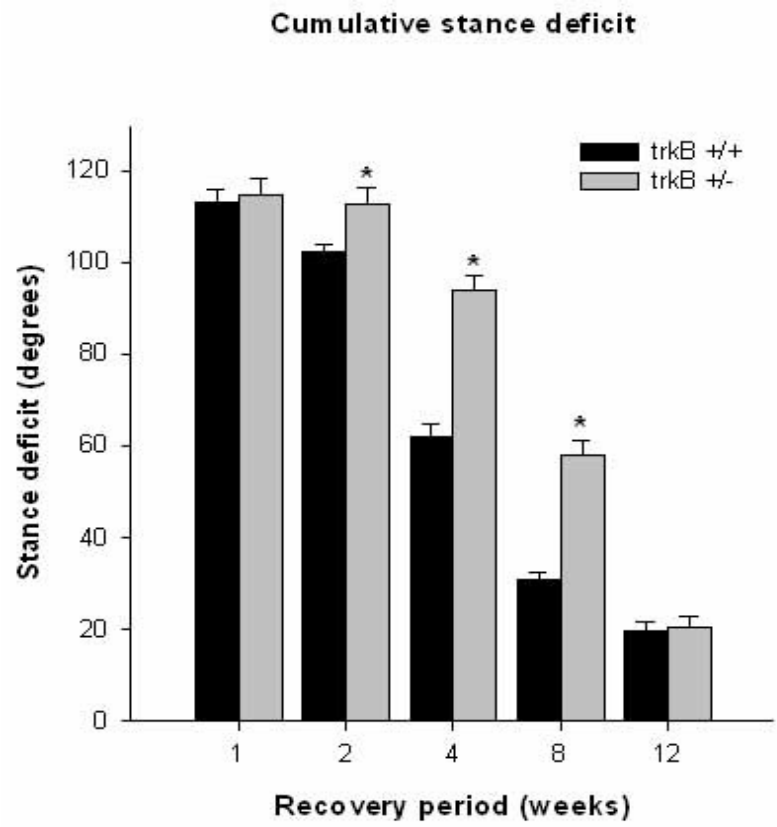

Figure 21. Cumulative stance deficit after 1, 2, 4, 8 and 12 weeks of recovery after operation of the left femoral nerve and low frequency electrical stimulation at 20 Hz for one hour in trkB+/+ (black bars, n=8) and trkB+/- (grey bars, n=8) animals. Shown are mean values ± S.E.M. Asterisks indicate significant differences between heterozygous trkB mice and their wild-type littermates ($p < 0.05$, two-sided t-test for independent groups).

4.1.6 Summary

To evaluate quadriceps muscle reinnervation in heterozygous trkB mice (trkB+/-) compared to control mice (trkB+/+) after femoral nerve transection and brief electrical stimulation numerous functional parameters were evaluated at several points in time. A delayed functional recovery was found in heterozygous trkB mice compared to their wild-type littermates in all parameters. After 12 weeks of regeneration all functional recovery parameters were similar in trkB+/- and trkB+/+ animals.

4. Results

4.2 Morphological evaluation of reinnervation

Three month after nerve transection followed by low frequency electrical stimulation at 20 Hz, morphological analysis of the spinal cord was performed in mice used for evaluation of the functional recovery by SFMA.

No statistically significant differences between trkB+/- and trkB+/+ animals were found for number of motoneurons labelled via the motor, sensory or through both branches (Fig. 22). The total number of retrogradely labelled motoneurons was also similar in the two groups (Fig. 22) and no preferential motor reinnervation (PMR), estimated by the ratio of motoneurons regenerated into the muscle branch only to those regenerated into the sensory branch – was observed (Figs. 22 and 23).

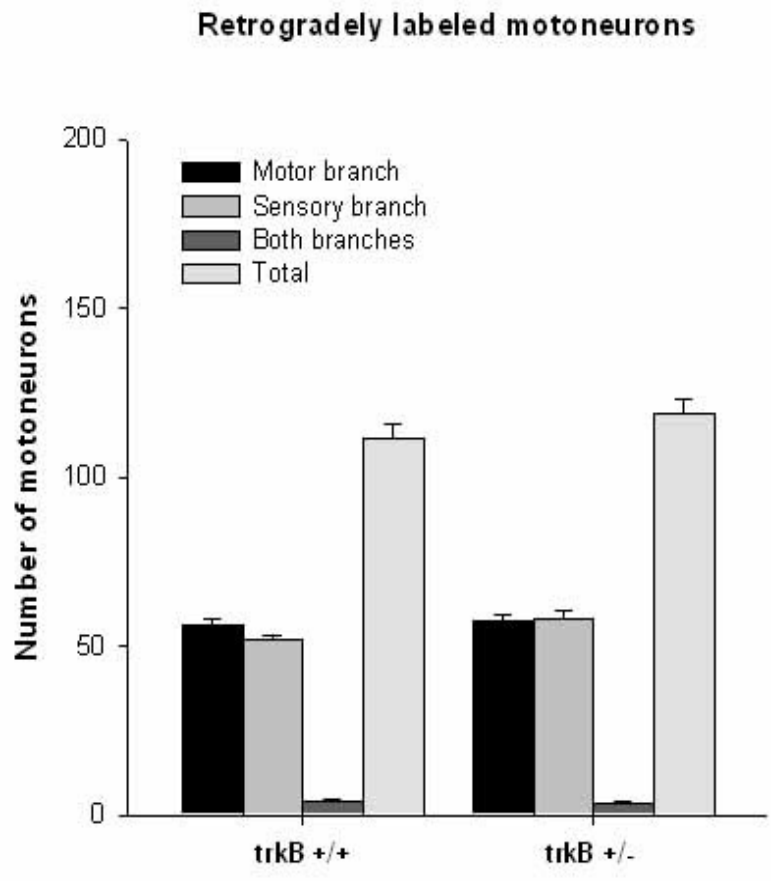

Figure 22. Morphological evaluation of motoneuron regeneration 3 month after operation and electrical stimulation in trkB+/+ (n=8) and trkB+/- (n=8) animals. Shown are cells labelled via the motor branch (black bar) or sensory branch (first grey bar) only, through both branches (second grey bar), as well as the number of regenerated neurons (third grey bar). Shown are mean values ± S.E.M. No differences between the groups were found ($p > 0.05$, two-sided t-test for independent groups).

4. Results

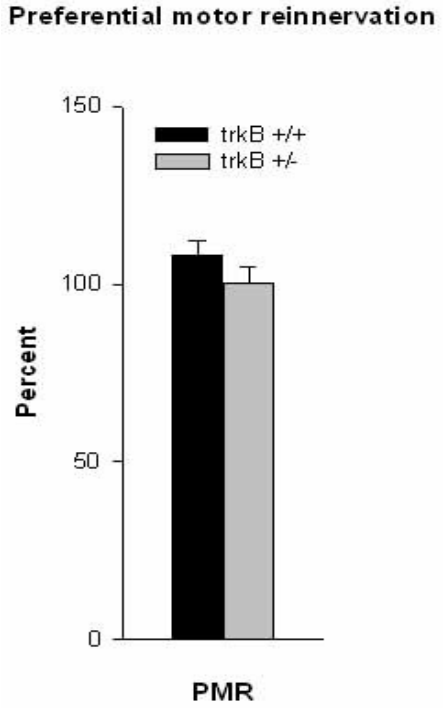

Figure 23. Degree of preferential motor reinnervation (PMR) 3 months after nerve transection of the left femoral nerve and low frequency electrical stimulation at 20 Hz for one hour in trkB+/+ (n=8) and trkB+/- (n=8) animals. PMR is calculated in percent as ratio of neurons regenerated into the muscle branch only to those regenerated into the sensory branch. No statistically significant difference between the groups was found ($p > 0.05$, two-sided t-test for independent groups).

4.3 Co-variation of recovery indices and structural parameters

Regression analysis was performed to detect if functional parameters correlate with morphological parameters. As functional parameters the stance recovery index and the limb protraction length ratio recovery index were correlated with different structural parameters: (1) the total number of labelled motoneurons, (2) the number of motoneurons projecting into the muscle branch and (3) the index of preferential reinnervation 3 months after surgery and electrical stimulation.

No co-variation between the estimates of functional recovery and morphological parameters was found when data of individual mice from both groups were analysed 3 month after surgical procedure followed by low frequency electrical stimulation (Tab. 1 and Fig. 24).

4. Results

Tab. 1. Degree of co-variation of recovery indices and structural parameters. Shown are results of regression analyses including correlation coefficients (r), coefficients of determination (r^2) and statistically significant probability values (p) for regression calculated by ANOVA.

Stance recovery index versus	3 Months after surgery
Total number of motoneurons	$r = -0.181$, $r^2 = 0.03$, $p > 0.05$
Number of motoneurons projecting into the motor branch	$r = -0.01$, $r^2 = 0.0001$, $p > 0.05$
Index of preferential motor reinnervation	$r = 0.134$, $r^2 = 0.02$, $p > 0.05$
Limb protraction length ratio recovery index versus	**3 Months after surgery**
Total number of motoneurons	$r = 0.165$, $r^2 = 0.03$, $p > 0.05$
Number of motoneurons projecting into the motor branch	$r = 0.254$, $r^2 = 0.06$, $p > 0.05$
Index of preferential motor reinnervation	$r = 0.157$, $r^2 = 0.02$, $p > 0.05$

4. Results

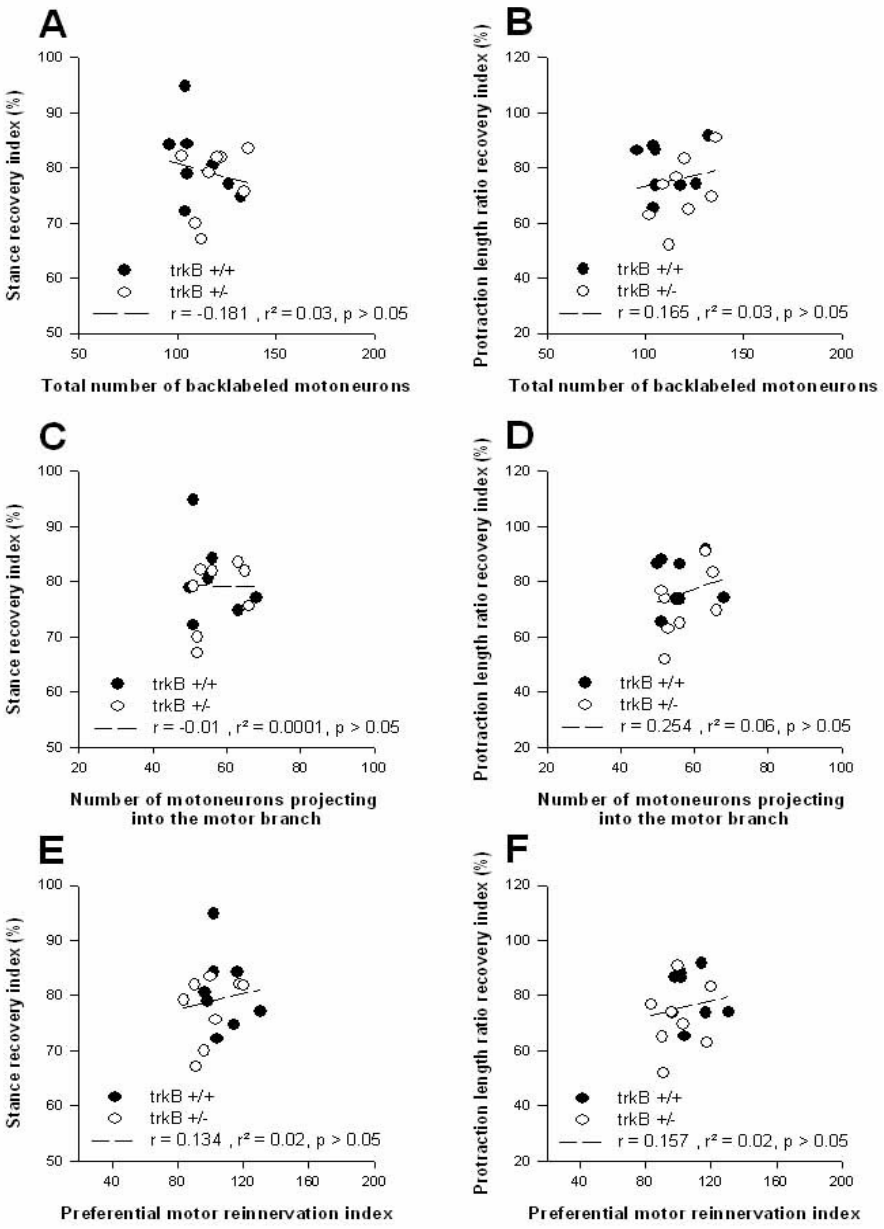

Figure 24. Correlation between functional and structural parameters. Stance recovery index (A, B, C) and recovery index of limb protraction length ratio (D, E, F) studied 3 months after operation and electrical stimulation are plotted against the total number of labelled motoneurons (A, D), the number of motoneurons projecting into the motor branch (B, E) and the index of preferential motor reinnervation (C, F). Shown are regression lines, coefficients of regression (r) and determination (r2) and probability values of regression analysis by ANOVA. No statistically significant difference among the groups was found ($p > 0.05$, one way analysis of variance, ANOVA).

5. Discussion

This study provides first quantitative data on functional recovery and motoneuron regeneration of heterozygous tyrosine kinase B mice compared to their wild-type littermates after transection of the left femoral nerve followed by a low frequency electrical stimulation at 20 Hz for one hour and microsurgical nerve repair. A brief summary of results is given in Table 2. These results show that a reduction in trkB expression does not cause failure in recovery in motor function and end organ reinnervation, but leads to a delay at early time points in functional motor recovery suggesting that trkB signalling is essential for achievement of the previously reported acceleration of peripheral nerve regeneration after brief electrical stimulation.

Table 2. Summary of differences found between trkB+/- and trkB+/+ animals in functional recovery 1-12 weeks after nerve injury and in morphological parameters after 12 weeks of recovery. Statistically significant lower (↓) or higher (↑) values in mutant animals as compared to control animals. = no statistically significant difference among both groups. Two-sided t-test for independent groups or one way ANOVA with post hoc Tukey test. / not studied.

Functional recovery	1 week	2 weeks	4 weeks	8 weeks	12 weeks
Heels-tail angle	=	=	↓	↓	=
Foot-base angle	=	↓	↓	=	=
Protraction length ratio	=	↓	↓	=	=
HTA recovery index	/	=	↓	↓	=
FBA recovery index	/	=	↓	=	=
PLR recovery index	/	=	↓	=	=
Stance recovery index	/	=	↓	↓	=
HTA stance deficit	=	=	↑	↑	=
FBA stance deficit	=	↑	↑	=	=
Cumulative stance deficit	=	↑	↑	↑	=

5. Discussion

Morphological analysis	1 week	2 weeks	4 weeks	8 weeks	12 weeks
Correctly projecting motoneurons (labelled through the motor branch)	/	/	/	/	=
Incorrectly projecting motoneurons (labelled through the sensory branch)	/	/	/	/	=
Double-labelled motoneurons	/	/	/	/	=
Total number of motoneurons	/	/	/	/	=
Preferential motor reinnervation	/	/	/	/	=

5.1 Functional recovery

To evaluate functional recovery of the quadriceps muscle after femoral nerve injury followed by brief electrical stimulation and nerve repair at different time points, the single frame motion analysis (SFMA, Irintchev et al. 2005) was used. It was shown to be a reliable and sensitive method to estimate functional recovery and deficits after femoral nerve injuries in mice (Ahlborn et al. 2007, Eberhardt et al. 2006, Guseva et al. 2009, Huang et al. 2009, Irintchev et al. 2005, Irintchev 2011, Malin et al. 2009, Mehanna et al. 2009, Simova et al. 2006). The results showed that functional recovery is delayed at early time periods in heterozygous trkB-deficient mice compared to their wild-type littermates after femoral nerve transection and one hour of low frequency electrical stimulation at 20 Hz (Table 2).

5. Discussion

Electrical stimulation enhances trkB and BDNF mRNA after nerve injury (Al-Majed et al. 2000 a) and accelerates functional recovery (Ahlborn et al. 2007). Partial trkB deficiency alone causes a delay in functional recovery at early time points after femoral nerve injury. From these findings it could have been expected that functional recovery in electrically stimulated trkB+/- might lead to a persistent deficit in functional recovery compared to their stimulated wild-type littermates. Two factors that have influenced functional recovery in previous experiments and this study have to be considered: (1) electrical stimulation and (2) genotype.

(1) In rats, Al-Majed et al. (2000 b) were able to demonstrate that electrical stimulation accelerates reinnervation of the distal nerve stump after femoral nerve injury and further research confirmed that electrical stimulation enhances axon regeneration (for review see e.g. Gordon et al. 2007, 2008, 2009 a, b). Brief electrical stimulation was shown to accelerate functional recovery after 4 and 6 weeks of regeneration in C57BL/6J mice compared to sham-stimulated animals (Ahlborn et al. 2007). The results presented in this study do confirm the findings of Ahlborn et al. (2007) about functional recovery in trkB+/+ mice after brief electrical stimulation. No statistically significant differences were found comparing the stance recovery indices, which allow comparison of different experimental datasets at different points in time (Irintchev at al. 2005), calculated in the study of Ahlborn et al. (2007) and the data presented in this study (after 2 weeks: 12% versus 7%, $p>0.05$, t-test; after 4 weeks 40% versus 33%, $p>0.05$, t-test, after 8 weeks: 69% versus 68%, $p>0.05$, t-test; after 12 weeks: 81% versus 73% , $p>0.05$, t-test). This comparison suggests that brief electrical stimulation in this study was effective in accelerating functional recovery in wild-type mice. However, the functional outcome at 12 weeks after injury in stimulated trkB+/+ was deficient, compared with preoperative values (Figs. 15-21), similar to the outcome reported for wild-type mice with or with-out electrical stimulation in previous studies (Simova el al. 2006, Ahlborn et al. 2007). Although brief electrical stimulation after carpal tunnel release surgery in patients with median nerve compression accelerates axonal regeneration and target reinnervation (Gordon et al. 2010), long-term improvement in a clinical setting has still not been demonstrated.

5. Discussion

(2) A number of studies have pointed out the role of trkB and its ligand BDNF in peripheral nerve regeneration (for review see Gordon 2009, Gordon et al. 2003). An early delay in functional recovery in trkB+/- mice, as compared with wild-type littermates, has been shown after nerve transection and immediate microsurgical repair without electrical stimulation (Eberhardt et al. 2006, Irintchev et al. 2005). These results indicate that functional recovery is delayed by partial trkB deficiency. Comparison of calculated stance recovery indices in trkB+/- animals (Eberhardt et al. 2006) and heterozygous trkB mice that received brief electrical stimulation of the femoral nerve in the present study do not show an effect of brief electrical stimulation on functional recovery (4 weeks 15% versus 14%, p=0.946 and 12 weeks 78% versus 78%, p=0.950). This could be interpreted as a confirmation of the important role of trkB and its signaltransduction pathway in functional recovery and peripheral nerve regeneration and its fundamental relation to electrical stimulation.

In conclusion, the present results indicate that partial trkB deficiency decelerates functional recovery possibly as a result of reduced, as compared with wild-type mice, up-regulation of the trkB signal transduction pathway.

5.2 Axonal regeneration and preferential motor reinnervation

In the present study, no influence of partial trkB deficiency in electrically stimulated animals on motoneuron regeneration at 12 weeks after injury compared to their wild-type littermates was found. Equal numbers of neurons have regenerated via the motor, sensory and both branches and no effect of partial trkB deficiency in electrically stimulated mice on precision of reinnervation was observed. These results on anatomical features of regeneration are in agreement with the finding of similar degree of functional recovery at 12 weeks after injury in the two genotypes.

5. Discussion

Previous studies showed a total number of <120 motoneurons that reinnervated the distal nerve stump after femoral injury in wild-type mice without electrical stimulation (Simova et al. 2006). Here we counted a total number of about 110 neurons reinnervating the distal nerve stump after 12 weeks of regeneration and brief electrical stimulation in both genotypes. The present results on total number of regenerated neurons and numbers of neurons the regenerated via the motor, sensory or both branches in electrically stimulated trkB+/+ and trkB+/- mice are similar to results of Ahlborn et al. (2007) on electrically stimulated C57BL/6 mice ($p>0.05$, t-test). Thus, our data supports previous findings indicating that electrical stimulation in mice does not improve motoneuron survival (Ahlborn et al. 2007).

Preferential motor reinnervation (PMR) was found in previous femoral nerve injury studies (Brushart 1988, 1993) and inappropriate reinnervation of end organs was considered as a reason for poor functional recovery after nerve injury. Brief electrical stimulation was observed to enhance the staggered process of motoneuron regeneration in association with preferential motor reinnervation and enhancement of trkB and BDNF mRNA (Al-Majed 2000 a, b) in a rat femoral nerve injury model. Studies in mice to elucidate the phenomenon of PMR showed controversial results. On the one hand, studies performed in mice did not detect PMR in a femoral nerve model (Mehanna et al. 2009, Robinson 2003, 2005, Simova et al. 2006), while other studies in mice found a preferential motor reinnervation (Eberhardt et al. 2006, Franz et al. 2005). Missing PMR in trkB+/- mice was interpreted as an affect of genotype. Electrical stimulation in rats is known to accelerate preferential motor reinnervation (Al-Majed et al. 2000 b). In mice, controversial results were observed. Franz et al. (2008) reported about PMR in mice after brief electrical stimulation while other experiments did not show PMR in electrically stimulated and sham-stimulated wild-type mice (Ahlborn et al. 2007). Correlation between functional recovery and preferential motor reinnervation in mice is still controversial and strain-related differences might account for the controversies (Mehanna et al. 2009).

5. Discussion

5.3 Co-variation of functional and structural parameters

In the present study, no co-variation between functional and structural parameters was detected comparing trkB+/- and trkB+/+ animals 12 weeks after femoral nerve injury. This stands in line with previous findings of Simova et al. (2006) showing that functional and anatomical measures of regeneration do not correlate at 3 months of regeneration after nerve injury. The only exception is a correlation between the stance recovery index and mean soma area of motoneurons reported in previous experiments with (Ahlborn et al. 2007) and without (Simova et al. 2006) brief electrical stimulation in mice. No estimates of motoneuron size were performed in this study but, considering the similar outcome in the two genotypes, it is unlikely to expect differences in motoneuron soma size in the two experimental groups.

5.4 Potential mechanisms of electrical stimulation and their relationship to trkB

Reinnervation of the peripheral nerve stump after nerve transection and immediate nerve repair is a staggered process, which can be accelerated by brief electrical stimulation of the injured nerve. Electrical stimulation supports the early sprouting of motor (Al-Majed et al. 2000 b) and sensory neurons (Brushart et al. 2005, Geremia et al. 2007) and enhances the staggered reinnervation of the distal nerve stump, which was accompanied by an increase and acceleration in BDNF and trkB mRNA expression (Al-Majed et al. 2000 a, b). In addition, brief electrical stimulation was shown to accelerate functional recovery (Ahlborn et al. 2007). The increase of BDNF and trkB mRNA expression is considered as the mechanism underlying the enhanced reinnervation of the distal nerve stump. But application of low doses of BDNF and GDNF did not lead to accelerated reinnervation of the distal nerve stump (Boyd and Gordon 2002, 2003) while application of high doses of BDNF or GDNF resulted in

inhibition of axonal regeneration highly likely mediated via the p75 receptor (Boyd and Gordon 2001, 2002, 2003, Gordon et al. 2005, 2003), so that target connections might be formed and neurite outgrowth is decelerated (Boyd and Gordon 2003, Gordon et al. 2003, 2005). Electrical stimulation of peripheral nerves is followed by an increase of cAMP which is induced by an activity-dependent calcium influx via membrane depolarization. Via protein kinase A gene transcription and translation growth-associated protein levels like actin, tubulin, GAP-43 (Al-Majed et al. 2004) and trkB and BDNF (Al-Majed et al. 2000 a) are upregulated (for review see Aglah et al. 2008, Gordon 2009, Gordon et al. 2010). Aglah et al. (2008) showed that rolipram mimics the effect of brief electrical stimulation immediately after nerve transection which proves a cAMP-depended effect of brief electrical stimulation.

Reduced trkB signaling by partial trkB deficiency could influence expression of other molecules having impact on peripheral nerve regeneration and functional recovery. Possible candidates are (1) the neural cell adhesion molecule L1 and (2) the human natural killer cell glycan (HNK-1). The neural cell adhesion molecule L1 was shown to promote neurite outgrowth and neuronal survival and to be regulated by neurotrophin signaling (Maness and Schachner 2007) in neurons and glial cells. L1 was shown to reduce Schwann cell proliferation after nerve injury, which causes a delay in functional recovery (Guseva et al. 2009). Previous studies showed that HNK-1 is expressed on motor myelin sheath but not sensory myelin sheath in mice and that it enhances motoneuron outgrowth (Martini and Schachner et al. 1986, Martini et al. 1992, 1994). HNK-1 is up-regulated after electrical stimulation and is associated with PMR in wild-type mice that did not undergo electrical stimulation. This effect was not observed in trkB+/- and BDNF+/- mice (Eberhardt et al. 2006). From these findings it has been concluded that HNK-1 expression is dependent on trkB signalling (Eberhardt et al. 2006). Functional recovery and PMR is enhanced after HNK-1 application to an injured femoral nerve (Simova et al. 2006).

Application of functional mimetic of the polysialic acid (PSA), a glycan associated with the neural cell adhesion molecule (NCAM), was shown to promote

functional recovery, remyelination of regenerating axons and Schwann cell proliferation was enhanced in vitro and in vivo, while no effect on precision of reinnervation or motoneuron survival was observed in mice (Mehanna et al. 2009). Electrical stimulation increases axon diameters but reduces the degree of myelination of regenerated axons compared to sham-stimulated animals (Ahlborn et al. 2007). Further experiments on myelination after peripheral nerve injury in partial trkB deficient mice, with and without electrical stimulation could elucidate the correlation of L1, trkB and brief electrical stimulation.

5.5 Perspective

Our results are in line with previous findings indicating that trkB is an important factor in peripheral nerve regeneration associated with effects of brief electrical stimulation. Electrical stimulation accelerates functional recovery but does not improve the final outcome (Ahlborn et al. 2007). On the other hand, application of HNK-1 (Eberhardt et al. 2006, Simova et al. 2006) and PSA mimetics (Mehanna et al. 2009) to injured peripheral nerves improve the motor outcome of nerve injury without accelerating effects during early time periods of reinnervation. Further studies combining mimetic treatments with electrical stimulation seem promising for improving the functional outcome of peripheral nerve injury.

6. Summary

Functional recovery after peripheral axonal injury is often insufficient and several treatments to improve neuronal regeneration have been investigated. Brief electrical stimulation, for instance, has previously been shown to accelerate axonal sprouting and distal nerve stump reinnervation, up-regulate regeneration-associated genes and accelerate functional recovery after peripheral nerve injury. These effects are believed to be mediated by BDNF and its high-affinity receptor trkB, which are up-regulated after axonal injury.

Here, the effects of short-term low-frequency brief electrical stimulation (1 h, 20 Hz) after femoral nerve injury and immediate nerve repair on (1) functional recovery, (2) neuronal survival and (3) precision of reinnervation were investigated in heterozygous trkB deficient mice (trkB+/-) compared to their wild-type littermates (trkB+/+) in the femoral nerve paradigm. Recovery of the quadriceps muscle function was assessed by single-frame motion analysis over a post-operative period of 3 months, while axonal reinnervation of the distal nerve stump was evaluated morphologically using retrograde labelling after 12 weeks of recovery.

The results showed that functional recovery was worse in trkB+/- mice compared to their wild-type littermates at 2-8 weeks after injury but the final outcome at 12 weeks was similar in the two genotypes. Morphological analysis revealed, in accordance with the functional data, a similar degree of motoneuronal regeneration and no preferential motor reinnervation in the two genotypes.

The findings of worse functional recovery during the first 2 month after injury in heterozygous trkB versus wild-type mice is apparently related to reduced trkB expression and provides further evidence that enhances trkB signalling underlies the effect of brief electrical stimulation on nerve regeneration. The amount of trkB expressed in the heterozygous mouse is, however, obviously sufficient to compensate, at late time points after injury, for initial deficits in regeneration.

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8. Abbreviations

8. Abbreviations

BDNF	Brain-derived neurotrophic factor
cb	Cutaneous branch
ChAT	Choline acetyltransferase
cm	Centimetres
ct	Common nerve trunk
e.g.	Example given
FBA	Foot-base angle
FBA_{reinn}	postoperative FBA value at any time-point of reinnervation
FBA_0	FBA value prior to operation and electrical stimulation
Fig.	Figure
g	Gram
GAP-43	Growth-associated protein 43
GDNF	Glial cell line-derived neurotrophic factor
h	Hour
HNK-1	Human natural killer cell glycan 1
HTA	Heels-tail angle
HTA_{reinn}	postoperative HTA value at any time-point of reinnervation
HTA_0	HTA value prior to operation and electrical stimulation
Hz	Hertz
kD	Kilodalton
kg	Kilogram
M	Molar
mb	Muscle branch

8. Abbreviations

mg	Milligram
µl	Microlitre
mm	Millimetre
µm	Micrometre
mRNA	Messenger ribonucleic acid
ms	Millisecond
NCAM	Neural cell adhesion molecule
NGF	Nerve growth factor
nm	Nanometre
NT-3	Neurotrophin-3
NT-4/5	Neurotrophin-4/5
NT-6	Neurotrophin-6
NT-7	Neurotrophin-7
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PLR	Limb protraction length ratio
PMR	Preferential motor reinnervation
PSA	α2,8 Polysialic acid
PTB	Phosphotyrosine binding
$p75^{NTR}$	P75 neurotrophin receptor
RAG	Regeneration associated genes
RI	Recovery index
SEM	Standard errors of mean
SFMA	Single-frame motion analysis
SH2	Src-homology-2
trk	Tyrosine receptor kinase
trkA	Tyrosine receptor kinase A
trkB	Tyrosine receptor kinase B
trkC	Tyrosine receptor kinase C
V	Volt

8. Abbreviations

X_{reinn}	Value at any time-point of reinnervation
X_{den}	Value during the state of denervation (7 days after injury)
X_{pre}	Value prior to operation and electrical stimulation
ZMNH	Zentrum für Molekulare Neurobiologie Hamburg
°C	Degree Celsius
%	Percent

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9. Acknowledgements

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10. Curriculum Vitae

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Entfällt aus datenschutzrechtlichen Gründen.

11. Eidesstattliche Versicherung

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