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AN EXPLORATION OF THE MECHANISMS BEHIND PERIPHERAL NERVE INJURY

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ABSTRACT

Despite surgical innovation, the sensory and motor outcome after peripheral nerve injury is incomplete. In this thesis, the biological pathways potentially responsible for the poor functional recoveries were investigated in both the distal nerve stump/target organ, spinal motoneurons and dorsal root ganglia (DRG). The effect of delayed nerve repair was determined in a rat sciatic nerve transection model. There was a dramatic decline in the number of regenerating motoneurons and myelinated axons found in the distal nerve stumps of animals undergoing nerve repair after a delay of 3 and 6 months. RT-PCR of the distal nerve stumps showed a decline in expression of Schwann cells (SC) markers, with a progressive increase in fibrotic and proteoglycan scar markers, with increased delayed repair time. Furthermore, the yield of SC which could be isolated from the distal nerve segments progressively fell with increased delay in repair time. Consistent with the impaired distal nerve stumps the target medial gastrocnemius (MG) muscles at 3- and 6-months delayed repair were atrophied with significant declines in wet weights (61% and 27% compared with contra-lateral sides). The role of myogenic transcription factors, muscle specific microRNAs and muscle-specific E3 ubiquitin ligases in the muscle atrophy was investigated in both gastrocnemius and soleus muscles following either crush or nerve transection injury. In the crush injury model, the soleus muscle showed significantly increased recovery in wet weight at days 14 and 28 (compared with day 7) which was not the case for the gastrocnemius muscle which continued to atrophy. There was a significantly more pronounced up-regulation of MyoD expression in the denervated soleus muscle compared with the gastrocnemius muscle. Conversely, myogenin was more markedly elevated in the gastrocnemius versus soleus muscles. The muscles also showed significantly contrasting transcriptional regulation of the microRNAs miR-1 and miR-206. MuRF1 and Atrogin-1 showed the highest levels of expression in the denervated gastrocnemius muscle. Morphological and molecular changes in spinal motoneurons were compared after L4-L5 ventral root avulsion (VRA) and distal peripheral nerve axotomy (PNA). Neuronal degeneration was indicated by decreased immunostaining for microtubule-associated protein-2 in dendrites and synaptophysin in pre-synaptic boutons after both VRA and PNA. Immunostaining for ED1-reactive microglia and GFAP-positive astrocytes was significantly elevated in all experimental groups. qRT-PCR analysis and Western blotting of the ventral horn from L4-L5 spinal cord segments revealed a significant up-regulation of apoptotic cell death mediators including caspases-3 and -8 and a range of related death receptors following VRA. In contrast, following PNA, only caspase-8 was moderately up-regulated. The mechanisms of primary sensory neuron degeneration were also investigated in the DRG following peripheral nerve axotomy, where several apoptotic pathways including those involving the endoplasmic reticulum were shown to be up-regulated. In summary, these results show that the critical time point after which the outcome of regeneration becomes too poor appears to be 3-months. Both proximal and distal injury affect spinal motoneurons morphologically, but VRA induces motoneuron degeneration mediated through both intrinsic and extrinsic apoptotic pathways. Primary sensory neuron degeneration involves several different apoptotic pathways, including the endoplasmic reticulum.

Keywords: Peripheral nerve injury, target organ, spinal motoneurons, primary sensory neurons, degeneration

ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by Roman numerals.

I. Jonsson S, **Wiberg R,** McGrath AM, Novikov LN, Wiberg M, Novikova LN, Kingham PJ. Effect of delayed peripheral nerve repair on nerve regeneration, Schwann cell function and target muscle recovery. *PLoS One 2013*;8:e56484.

II. **Wiberg R,** Jonsson S, Novikova LN, Kingham PJ. Investigation of the expression of myogenic transcription factors, microRNAs and muscle-specific E3 ubiquitin ligases in the medial gastrocnemius and soleus muscles following peripheral nerve injury. *PLoS One 2015;*10:e0142699.

III. **Wiberg R**, Kingham PJ, Novikova LN. A Morphological and Molecular Characterization of the Spinal Cord Following Ventral Root Avulsion or Distal Peripheral Nerve Axotomy Injuries in Adult Rats. *Journal of Neurotrauma* July 2016, ahead of print. doi:10.1089/neu.2015.4378.

IV. **Wiberg R**, Novikova LN, Kingham PJ. Evaluation of apoptotic pathways in dorsal root ganglion neurons following peripheral nerve injury. *Manuscript*

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ABBREVIATIONS

α-BTX	Alfabungarotoxin
AChRs	Acetylcholine receptors
AMPK	5´-AMP-activated protein kinase
Apaf-1	Apoptotic protease activating factor 1
ATPase	Adenosine triphosphatase
bHLH	Basic helix-loop-helix
CGRP	Calcitonin gene-related peptide
CNTF	Ciliary neurotrophic factor
CSPG	Chondroitin sulphate proteoglycans
DD	Death domain
DISC	Death-inducing signalling complex
DR	Death receptor
DRG	Dorsal root ganglion
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
Foxo	Forkhead box O transcription factors
FR	Fluorescent tracer Fluoro-Ruby
GFAP	Glial fibrillary acidic protein
HDAC	Histone deacetylase
HSPs	Heat shock proteins
IB4	Isolectin B4
IHH	Immunohistochemistry
L4/L5/L6	Lumbar segments of spinal cord 4/5/6
MAFbx	Muscle atrophy F-box/Atrogin-1
MAP2	Microtubule-associated protein-2
MG	Medial gastrocnemius muscle
MHC	Major histocompatibility complex
miRNA	MicroRNA
MRFs	Myogenic regulatory factors
mRNA	Messenger ribonucleic acid
MuRF1	Muscle RING-finger 1

mTOR	mammalian target of rapamycin
MuSK	Muscle specific kinase
MyHC	Myosin heavy chain
NF-H	High molecular weight neurofilament
NMJ	Neuromuscular junction
NOS	Nitric oxide synthase
PNA	Peripheral nerve axotomy
PNI	Peripheral nerve injuries
RT-PCR	Reverse transcription polymerase chain reaction
SC	Spinal cord
SCs	Schwann cells
SYN	Synaptophysin
TGF-β	Transforming growth factor beta
TRAF2	TNF receptor-associated factor 2
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UPS	Ubiquitin proteasome system
VRA	Ventral root avulsion
WB	Western blot

INTRODUCTION

Clinical background

Peripheral nerve injuries are common, especially in young men [1], with an annual incidence of 13.9 per 100 000 of the population in Sweden [2]. Predominantly the injuries affect the upper limb (80% of total), particularly at the wrist- and hand level (63% of total), resulting in 140 cases of medianus- and ulnaris injuries per year [2]. The majority of the injuries are domestic or occupational accidents with glass or knifes [3], although road traffic accidents, iatrogenic injuries, assault and self-inflicted injuries also are known underlying causes, albeit to a lower extent. Despite advanced microsurgical innovation the motor and especially the sensory outcome is still poor [4], resulting in long lasting disability due to pain, paralyzed muscles and loss of adequate sensory feedback from the target organ [5]. Thus, even with optimal surgical repair, cutaneous innervation will remain reduced and since adequate sensory feedback is a vital component of normal proprioception, this also impacts adversely upon motor function, particularly fine manipulative work [6]. In addition, peripheral nerve injuries are associated with a substantial economic impact, with the total cost for a median nerve injury in Sweden exceeding 51 000 € [3].

There are different techniques used to repair injured peripheral nerves. When the nerve stumps can be aligned without undo tension, the gap is repaired by end-to-end suturing, either through epineural or fascicular repair. However, if the peripheral nerve injury is associated with a significant tissue loss, autologous nerve grafting is performed to bridge the defect. The latter technique is though suboptimal since it requires that a healthy nerve is sacrificed and harvested, resulting in loss of sensation, scarring and sometimes pain from the donor region [7].

Pathophysiology of peripheral nerve injury

Following a peripheral nerve injury, many different events take place in the affected neuron. Axonal injury generates several major signalling cues to the cell body [8], which undergoes morphological changes including swelling of the cell body, nucleolar enlargement, displacement of the nucleus to the periphery and dissolution of Nissl

bodies, the so called chromatolytic reaction [9]. The major signalling cues include interruption of the normal flow of trophic signals from the target organ, exposure of the transected axons to new signals in the extracellular environment which are retrogradely transported to the cell body and injury-induced action potentials through increased membrane permeabilization and rapid calcium influx [8]. The injury-induced signals, which enables the neuron to respond to trauma, mediates a cascade of signalling pathways, culminating in the appearance and nuclear translocation of transcription factors and ultimately activation of regeneration associated genes [8].

Parallel to the events that occur in the cell body, Wallerian degeneration takes place in the distal nerve segment and to the first node of Ranvier in the proximal nerve stump. Soon after injury Schwann cells associated with detached axons release their myelin and proliferate and align within their basal lamina tubes to form bands of Büngner [10]. Schwann cells play an early role in removing myelin debris, which acts as a barrier to regrowing axons in the distal nerve, and they act as the main phagocytic cell during the first 5 days after injury [10]. In addition to proliferating and phagocytosing debris, Schwann cells in the distal nerve stump secrete trophic factors that promote axon growth along with cytokines and chemokines that recruit immune cells into the injured nerve [10]. Within a week of injury, recruited immune cells, especially macrophages, assume a primary role in debris removal and growth factor production [10]. After a lag period, injured axons form a growth cone and begin to regenerate along bands of Büngner, which provide a permissive growth environment and guide extending axons towards potential peripheral targets [10]. During the initial phase of regeneration each axon in the proximal stump sends out multiple sprouts, only one of which survives after target reinnervation [11].

Despite these intrinsic reactions to injury, it has been established that the main neurobiological factors underlying the poor functional outcome after peripheral nerve injury are axotomy-induced retrograde cell death in spinal cord and dorsal root ganglia (DRG), chronic denervation of Schwann cells in the distal nerve stump and degeneration of the target organ [11].

Nerve cell degeneration

Distal peripheral nerve transection in newborn rats almost completely eliminates the corresponding spinal motoneurons within 2 weeks [12, 13], while the same type of injury in adult animals has no effect on motoneuron survival [14, 15]. However, even if the neuronal cell survival is not affected, peripheral nerve lesions induce retrograde changes in the surrounding non-neuronal cells including hypertrophy of astrocytes, stripping of synapses and degeneration of the dendritic tree [16-23]. In contrast to spinal motoneurons, both neonatal and adult primary sensory neurons in the DRG undergo cell death even after distal peripheral nerve injury [15], which is also the case for cranial motoneurons [24]. Moreover, cutaneous afferent neurons are more sensitive to injury-induced degeneration in comparison to muscular afferents [15, 25, 26]. However, more proximal injuries to the spinal nerves, such as spinal nerve transections [27-29] or ventral root avulsions [16, 30] do result in significant neural degeneration in the ventral horn.

Sensory neuronal cell death starts shortly after nerve axotomy and experimentally it has been shown that a significant cell loss is already evident in the DRG one week post-injury if repair is not carried out [31]. Loss of sensory neurons increases with time, reaching a value of 35-40% of the total neuronal cell population in the DRG at 2 months post injury [31]. Postmortem studies have suggested that a similar magnitude of loss occurs clinically [32]. In contrast to sensory neurons, motoneurons do not reveal any significant retrograde degeneration up to 13-24 weeks after distal peripheral nerve axotomy [14, 15]. However C7 spinal nerve injury results in delayed motoneuron loss amounting to approximately 20% and 30% after 8 and 16 weeks respectively [28, 29], while ventral root avulsion results in a more rapid degeneration of motoneurons, with 15% to 25% motoneuron loss present already between 8 and 14 days after trauma [16]. Thus, since a prerequisite for axonal regeneration is the survival of the neuron following injury, it is likely that this neuronal cell death is of great importance for the outcome of the axonal regeneration and target organ reinnervation.

Mechanisms of neuronal cell degeneration after peripheral nerve injury

Despite intensive research, data regarding the underlying mechanism behind injury-induced cell death is conflicting, with remaining uncertainties which apoptotic pathways

are involved. One can differ between three different kinds of apoptosis [33], the intrinsic pathway, the extrinsic pathway and apoptosis mediated by endoplasmic reticulum-stress, each pathway culminating in cleavage of the effector caspase-3. The intrinsic pathway is regulated by the balance between pro-apoptotic Bax and anti-apoptotic Bcl-2, which interact at the mitochondrial level determining the membrane permeabilization [33]. When the equilibrium points towards Bax, formation of pores take place in the mitochondrial membrane, allowing the release of cytochrome c [33]. Once released into the cytosol, cytochrome c interacts with apoptotic protease activating factor 1 (Apaf-1) and procaspase-9, forming the apoptosome, resulting in caspase-3 activation and cell death execution [33]. Several kinds of cellular stress are known to initiate the intrinsic pathway including oxidative stress and depletion of neurotrophic factors [33].

The extrinsic pathway is characterised by the presence of death receptors (DRs), which are members of the TNF-receptor superfamily and distinguished by a cytoplasmic region of ~80 residues termed the death domain (DD). When these receptors are triggered by corresponding ligands, a number of molecules are recruited to the DD and subsequently a signaling cascade is activated. So far, eight different DRs have been identified in humans, Fas (CD95) being the most described DR. Upon FasL mediated activation of Fas, FADD recruits procaspase-8 and procaspase-10, forming a death-inducing signalling complex (DISC) [34]. Procaspase-8 is then autoproteolytically cleaved at the DISC, generating an active protease that in turn activates downstream executioner caspase-3 [34]. Type I cells generate high levels of DISC formation and increased amounts of active caspase-8, which results in direct activation of downstream effector caspase-3 [34]. Type II cells on the other hand generate low levels of DISC formation and require an amplification loop through caspase-8-mediated processing of Bid to truncated Bid (tBid), inducing the release of cytocrome c from the mitochondria with subsequent activation of caspase-3 as a result [34].

In addition, apoptosis can be induced via the endoplasmic reticulum. The ER regulates the intracellular calcium (Ca2+) homeostasis as well as serves as the cellular site of newly synthesized secretory and membrane proteins, which must be properly folded and posttranslationally modified before exit from the organelle. Proper protein folding and modification requires molecular chaperone proteins as well as an ER environment

conducive for these reactions. When ER lumenal conditions are altered or chaperone capacity is overwhelmed, so called ER-stress, the cell activates signaling cascades to restore a favorable folding environment, through activation of three different ER membrane receptors, ATF6, IRE1 and PERK [35]. The protective mechanisms include translational attenuation to limit further accumulation of misfolded proteins, transcriptional activation of genes encoding ER-resident chaperones such as BiP/GRP78 and GRP94 and ER-associated degradation (ERAD) which serves to reduce the stress and thereby restore the folding capacity by directing misfolded proteins in the ER back into the cytosol for degradation by the 26S proteasome [35]. If these protective mechanisms are not sufficient, the cytosolic Ca2+ levels are increased through Bax and Bak pore-formation in the ER-membrane [36], resulting in accumulation and activation of m-calpain with subsequent activation of caspase-12 [37]. Activated caspase-12 then cleaves and activates procaspase-9, which in turn activates the downstream caspase cascade including caspase-3 [38].

Distal nerve stump degeneration and Schwann cells atrophy

Prolonged denervation of the distal nerve stump progressively reduces the success of axonal regeneration, which in part is due to distal nerve stump fibrosis and Schwann cell atrophy [11]. Gordon et al demonstrated that prolonged denervation accounts for a 90% reduction in the number of functional motor units following 3 months delayed repair [39], in comparison to a 30% reduction following prolonged axotomy [40]. In addition, the reduced numbers of reinnervated motoneurons after chronic distal nerve and muscle denervation closely matched the reduced numbers of motoneurons that had regenerated their axons into the chronically denervated nerve stumps [41], emphasizing the distal stump as one of the main limiting factors for nerve regeneration after delayed nerve repair. After a peripheral nerve injury, the molecular markers characteristic of myelinating and non-myelinating Schwann cells are altered. Expression of myelin markers decreases dramatically as a consequence of axonal degeneration distal to the injury site, whereas markers of immature and non-myelinating Schwann cells are re-acquired [42], such as nerve growth factor, brain derived neurotrophic factor, neurotrophin-4, glial cell-derived neurotrophic factor and insulin-like growth factor-1 [43]. Chronically denervated Schwann cells, which persist in the denervated nerve stumps, undergo progressive atrophy and loose their capacity to maintain an active pro-

regenerative phenotype, thus failing to support axonal regeneration [44].

Degeneration of the target organ

One contributing factor to the poor outcome following peripheral nerve injury is prolonged denervation of the target organ, leading to apoptosis of both mature myofibres and satellite cells with subsequent replacement of the muscle tissue with fibrotic scar and adipose tissue [45, 46]. Furthermore prolonged denervation results in deterioration of the intramuscular nerve sheaths, which in turn results in failure to attract and provide support for the regenerating axons [39]. In addition, following reinnervation, long term denervated muscle fibres fail to recover entirely from atrophy [39], most likely as a result of reduced satellite cell numbers and impaired satellite cell activity levels [45]. Moreover, muscle regeneration is severely impaired by denervation-induced deposits of extracellular matrix and the spatial separation of satellite cells [46].

The muscle fibre is composed by several myofibrils, which in turn contain repeating sections of sarcomeres. The sarcomeres consist of myosin, which forms the thick filament and actin, which forms the thin filament, together with tropomyosin and troponin. Based on the expression of the myosin heavy chain (MyHC) gene, it is possible to define four different types of muscle fibres including type I, IIa, IIx and IIb [47], which diverge along a continuum of contraction speed and endurance. Type I is slow contracting, with a high capacity for oxidative metabolism and good endurance and type IIb fibres are fast contracting, fatigable and mainly dependent on glycolytic metabolism. Thus, fast and slow fibres contain fast and slow MyHC isoforms that display high or low actin-dependent ATPase activity, respectively [48]. Depending on the biochemical and physiological properties of the muscle it is more, or less, vulnerable to various types of insult, and studies suggest that the muscle phenotype may influence the disease progression [49, 50].

Advances in molecular biology have highlighted the potential role of microRNAs (miRNAs) in influencing clinical outcomes following peripheral nerve injuries [51]. miRNAs are a class of small, 22 nucleotides long non coding single stranded RNAs, that negatively regulate gene expression through post-transcriptional inhibition by complementary base-pair binding of the miRNA seed sequence (2–7 nucleotides) in the

3´untranslated region of target mRNAs [51]. miRNAs down regulate gene expression by two different mechanisms, translational repression and mRNA degradation [51], which is dependent on the degree of complementarity.

The muscle specific miRNAs, miR-1 and miR-206, together with several non-muscle specific microRNAs, are required for muscle proliferation and differentiation through interaction with myogenic factors. MyoD, myogenin, MRF4 and myf5, all myogenic regulatory factors (MRFs), form a family of muscle-specific basic helixloop-helix (bHLH) transcription factors that govern differentiation of muscle cells during development [52]. MyoD and myogenin regulate differential muscle gene expression [53] and in the adult muscle, MyoD is highly expressed in fast muscle fibres [53] and regulates fast muscle development [53]. Conversely, myogenin is highly expressed in slow muscle fibres [42]. Each of the MRFs is capable of activating muscle-specific gene expression, yet distinct functions have not been ascribed to the individual proteins. Furthermore, Forkhead box O transcription factors (Foxo) are the transcriptional activators of the muscle-specific E3 ubiquitin ligases muscle RING-finger 1 (MuRF1) and Atrogin-1/muscle atrophy F-box (MAFbx) [54, 55], which mediates protein degradation through the ubiquitin proteasome system (UPS), and are critical players regarding the functional outcome of the target organ following peripheral nerve injury [56]. Although multiple proteolytic systems are involved in muscle protein breakdown, including mitochondrial proteases, lysosomes and the ubiquitin–proteasome pathway [57], the latter system is indicated to account for up to 80% of the proteolysis during skeletal muscle wasting [58]. Ubiquitin is first activated by E1, after which activated ubiquitin is transferred by E1 to a carrier protein, E2, and finally conjugated to the protein substrate by an E3 catalyzed reaction [57]. The ubiquitin-conjugation reaction is then repeated several times, creating a polyubiquitintail, serving as a degradation signal for 26S proteasome [57]. The polyubiquitination is thus in part performed by the ubiquitin E3 ligases, which tag ubiquitin to specific protein substrates, where Atrogin-1 and MuRF1 are up-regulated in multiple models of skeletal muscle wasting [59]. The expression of Atrogin-1 and MuRF1 is, in addition to Foxo transcription factors [56], regulated by Myogenin [60]. Furthermore, Myogenin and MyoD are transcriptional regulators of miR-206 [61].

Summary

Thus, several factors need to be considered for optimal nerve recovery; the neurons must survive and regenerate axons, the distal nerve stump and its nonneural cells must provide adequate trophic and substrate support for nerve regeneration and regenerating axons must make functional connections with appropriate peripheral targets, which in turn must recover fully from denervation atrophy. Despite surgical innovation, the sensory and motor outcome after peripheral nerve injury is incomplete. In this thesis, the biological pathways potentially responsible for the poor functional recoveries were investigated in the distal nerve stump/target organ, the spinal motoneurons and the DRG.

AIMS OF THE STUDY

The aims of this study were:

- To compare the effect of immediate and delayed peripheral nerve repair on nerve regeneration, Schwann cell properties and target muscle recovery (Paper I).

- To investigate the expression of myogenic transcription factors, muscle specific microRNAs and muscle-specific E3 ubiquitin ligases at several time points following denervation in two different muscles (Paper II).

- To characterize the morphological and molecular changes in spinal motoneurons following ventral root avulsion or distal peripheral nerve transection injuries (Paper III).

- To evaluate different apoptotic pathways in dorsal root ganglion neurons following peripheral nerve injury (Paper IV).

MATERIALS AND METHODS

Experimental animals and ethics statement

The experiments were performed on adult (8–10 weeks old) inbred Fisher F344 rats (Scanbur BK AB, Sweden; Paper I and II) and adult (10–12 weeks old) female Sprague Dawley rats (Paper II, III and IV). The animal husbandry was in accordance to the standards and regulations provided by the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publications No. 86–23, revised 1985) and the European Communities Council Directive (86/609/EEC). All procedures were approved by the Northern Swedish Regional Committee for Ethics in Animal Experiments at Umeå University (protocol number A186-12). Surgery was performed aseptically under general anesthesia using a mixture of Ketamine (Ketalar 50 mg/ml Pfizer, Sweden) and Xylazine (Rompun 20 mg/ml Bayer Health Care, Germany) by intraperitoneal injection. Finadyne (Schering-Plough Animal health 50 mg/ml) and Benzylpenicillin (Boehringer Ingelheim; 60 mg; Paper I) was administered post-operatively. At the end of the survival period the rats were terminally injected with an intraperitoneal overdose of sodium pentobarbital (240 mg/kg, Apoteksbolaget, Sweden). Rats and their well-being were observed throughout the experimental period.

Surgical procedures and experimental groups

Sciatic nerve injury and repair

In all experimental models injury was performed on the left side. The sciatic nerve was exposed by bluntly dividing the gluteal muscles of the thigh. Under an operating microscope (Zeiss, Carl Zeiss, Germany) the nerve was either crushed with a fine aortic clamp for 10 sec or transected at a standardized distance from the spinal cord, approximately 5 mm from the most proximal branch of the sciatic nerve. In the group with sciatic nerve transection without repair the nerves were then capped to prevent distal reinnervation. Caps were made out of polyethylene tubes and each nerve stump was introduced and anchored to the cap using the 10–0 Ethilon suture. In the group with immediate nerve repair the nerve stumps were bridged with a 10 mm sciatic reversed autograft. In the group with delayed nerve repair the nerve stumps were ligated approximately 1 mm from the cut end using an 8–0 nonresorbable Ethilon suture and

capped as above to prevent reinnervation. The proximal stump was put under the femoral quadriceps muscle and the distal stump introduced into the popliteal fossa. After 1, 3 or 6 months, the wound was re-opened and the proximal and distal stumps of the sciatic nerve were re-exposed, trimmed by 2–3 mm to remove neuroma/scar tissue and repaired using a 10 mm reversed sciatic nerve graft from a donor Fisher rat. The graft was fixed with four interrupted epineural sutures aligned circumferentially in each anastomosis using micro instruments and a 10–0 nonresorbable Ethilon suture. After surgery the wound was closed in layers.

Ventral root avulsion

Following a lumbar laminectomy and opening of the dura, the left L4 and L5 ventral roots were identified under a dissection microscope and transected close to the dorsal root ganglia. The proximal stump of each cut root was grasped with fine forceps and pulled caudally until it came out in its entire length. The roots were regularly found to rupture at the site of exit from the spinal cord.

Experimental groups

The animals were divided into the following experimental groups: (i) sciatic nerve transection with immediate repair, (ii) sciatic nerve transection with 1 month delayed repair, (iii) sciatic nerve transection with 3 months delayed repair, (iv) sciatic nerve transection with 6 months delayed repair, (v) sciatic nerve transection without repair (vi) sciatic nerve crush injury, (vii) ventral root avulsion and (viii) un-operated controls. All experimental animals with immediate (i) or delayed nerve repair (ii; iii and iv) were allowed to survive for an additional 13 weeks (Papers I & II). In the groups with sciatic nerve transection without repair (v) and sciatic nerve crush injury (vi) the operated animals were allowed to survive for 1, 7, 14 and 28 days (Papers II, III and IV). After ventral root avulsion (viii) animals were sacrificed at 7 and 14 days postoperatively (Paper III).

Labeling with retrograde fluorescent tracer (Paper I)

In order to study the spinal motoneuron regeneration into the distal nerve stump retrograde labelling was performed 12 weeks following repair. The sciatic nerve was

transected in the popliteal fossa 10 mm distal to the repair site and introduced into a small polyethylene tube containing two microlitres of fluorescent tracer Fluoro-Ruby (FR, 10% solution in saline, Invitrogen, Sweden). The tube was fixed to the surrounding muscles using Histoacryl® glue (Braun Surgical GmbH, Germany) and sealed with a mixture of silicone grease and vaseline to prevent leakage. Two hours later the cup was removed, the nerve rinsed in saline and the wound closed in layers. The animals were left to survive for one week before tissue harvest.

Tissue processing (Papers I, II, III & IV)

At the end of the survival period the rats were terminally injected with an intraperitoneal overdose of sodium pentobarbital (240 mg/kg, Apoteksbolaget, Sweden).

For RT-PCR and Western blotting the left ventral half of L4-L5 spinal cords (Papers I & III), DRG L4-L6 (Paper IV), 5 mm long segments of the sciatic nerve from proximal and distal to the nerve graft interface (Paper I) as well as the medial gastrocnemius muscles and the soleus muscles from both the operated and non-operated side (Papers I & II) were harvested and fast frozen in liquid nitrogen.

For muscle morphology analysis (Papers I & II) the muscles were weighed after harvesting, embedded in OCT™ compound and snap frozen in liquid nitrogen. Transverse sections (7-16 μm) of gastrocnemius and soleus muscles from the contra-lateral and operated sides were cut on a cryomicrotome (Leica Instruments, Kista, Sweden), thaw-mounted in pairs onto SuperFrost ® Plus slides, dried overnight at room temperature and stored at -85°C before processing. Samples were either stained with haematoxylin and eosin or fixed with 4% (w/v) paraformaldehyde for 15 min prior to immunostaining.

For morphometric analyses and axon counts (Paper I) the sciatic nerve was removed in its entirety and then three 3 mm pieces of the nerve were cut; 5 mm from the proximal anastomosis, in the middle of the nerve graft and 5 mm from the distal anastomosis. The nerve segments were fixed in 3% glutaraldehyde, post-fixed in 1% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer (pH7.4), dehydrated in acetone and embedded in Vestopal. Semithin transverse sections were cut on a 2128 Ultratome (LKB, Sweden) and counterstained with Toluidine Blue.

For immunohistochemistry the animals were transcardially perfused with Tyrode's

solution followed by 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The left L4-L5 ventral horn, (Paper III), L4-L6 spinal cord segments (Paper I) and DRG L4-L6 (Paper IV) were harvested, post-fixed for 2 – 3 h, cryoprotected in 10% and 20% sucrose for 2 – 3 days and frozen in liquid isopentane. Serial transverse 16 μm thick sections were cut on a cryomicrotome (Leica Instruments, Kista, Sweden), thaw-mounted in pairs onto SuperFrost ® Plus slides, dried overnight at room temperature and stored at -85°C before processing.

For neuron counts (Paper I), immediately after perfusion the spinal cord segments L4-L6 were cut in serial 50 μm thick parasagittal sections on a vibratome (Leica Instruments, Germany), mounted onto gelatin-coated slides and coverslipped with DPX.

Cell culture (Paper I)

Schwann cell (SC) culture

To investigate the number of Schwann cells and their proliferative properties in the proximal and distal nerve stump after nerve repair, a Schwann cell culture was performed. Under a dissecting microsope, the epineurium was removed from the proximal and distal nerve segments. The nerves were further divided into 0.5–1 mm pieces and placed in a petri dish containing Schwann cell growth medium [(Dulbeccos Modified Eagle Medium (DMEM) containing 10% (v/v) foetal calf serum (FCS) and 1% (v/v) pencillin/streptomycin solution (all from Invitrogen) and supplemented with 10 μM forskolin (Sigma) and neuregulin NRG1 (R&D Systems, UK)]. The nerves were incubated for 2 weeks before the addition of 0.0625% (w/v) collagenase type 4 (Worthington Biochemicals, USA) and 0.585 U/mg dispase (Invitrogen) for 24 h. The nerves were triturated, filtered through a 70 μm cell strainer and centrifuged at 800 rpm for 5 min. The pellet was resuspended in 5 ml SC growth media and seeded in a 25 cm^2 flask. The cells were left to incubate at 37°C/5%CO_2 for 7 days and then trypsinised and the cell numbers were counted after separation of contaminating fibroblasts using anti-fibroblast antibody coupled magnetic beads according to the manufacturer's instructions (Miltenyi Biotech, Germany).

Schwann cell proliferation assay

At passage 2, Schwann cells were plated at a density of 7500/well in a 24 well plate and

assessed for proliferation using the AlamarBlue™ assay (Serotec). The optical density of AlamarBlue was obtained by measuring changes of absorbance at 570nm and 600 nm. For the assay, the growth medium in the corresponding wells was replaced by fresh medium (500µl) containing 10% AlamarBlue. After 12 h 100 µl of the AlamarBlue-containing medium was transferred into 96-well culture plates and the plates were subsequently read on a microplate reader (Spectra Max 190, Molecular Device, Albertville, MN, USA) every day at the same time point for 5 consecutive days.

Schwann cell-neuron co-culture

To determine if the Schwann cells isolated from the distal stumps maintained neurotrophic properties, a Schwann cell-neuron co-culture was performed. Schwann cells ($4x10^4$ cells, passage 2) were seeded onto 8 well chamber slides and allowed to settle for 1 day before the addition of $2x10^3$ NG108-15 cells (a neuronal cell line, modeling motor neurons). The two cell types were co-cultured for 48 h. Schwann cell-neuron co-cultures were fixed with 4% (w/v) PFA for 15-20 minutes prior to immunostaining for neurite outgrowth. For RT-PCR the total RNA was isolated from Schwann cell cultures at passage 2**.**

Immunostaining

In vitro cultures: After fixation with 4% (w/v) paraformaldehyde for 20mins at room temperature the Schwann cells co-cultures were washed in PBS and blocked with 5% (v/v) normal serum for 15 minutes and then mouse-anti- βIII-tubulin (Sigma, Poole UK; 1:500) antibody was applied.

Muscle tissue: Transverse sections of gastrocnemius and soleus muscles were blocked with 5% (v/v) normal serum for 15 minutes and stained with primary antibodies: monoclonals raised against fast and slow myosin heavy chain protein (NCL-MHCf and NCL-MHCs, Novocastra, UK; 1:20), rabbit anti-laminin (Sigma; 1:200), rabbit anti-dystrophin (GeneTex; 1:5000), mouse anti-MyoD (BD Pharmingen; 1:200), mouse anti-myogenin (Abcam; 1:200), rabbit anti-pre-synaptic marker SV2A (Abcam, UK, 1:50). In some experiments alpha-bungarotoxin (α-BTX) was also applied.

Nerve tissue: Serial transverse sections from L4-L5 spinal cord segments were blocked with 5% (v/v) normal serum for 15 minutes and treated with the following primary

antibodies: mouse anti-microtubule-associated protein-2 (MAP2; Chemicon; 1:100), rabbit anti-synaptophysin (SYN; Dako; 1:500), rabbit anti-glial fibrillary acidic protein (GFAP; Dako; 1:500) and mouse monoclonal anti-ED1 (ED1; Abcam; 1:100). Sections from L4-L5 DRG were blocked with 5% (v/v) normal serum for 15 minutes and treated with the following primary antibodies: rabbit anti-caspase-3 (Abcam; 1:10), rabbit anti-phosphoPERK (Cell Signaling; 1:200), monoclonal neurofilamentH (Sigma; 1:400). Some sections were also stained with IB4-FITC (Sigma; 1:10).

All primary antibodies were applied for 2 hours at room temperature. After rinsing in PBS, secondary antibodies either goat anti-mouse IgG or goat anti-rabbit IgG Alexa Fluor® 488 and Alexa Fluor® 568 conjugates (Molecular Probes, Invitrogen; 1:300) were applied for 1-2 h at room temperature in the dark. Following washes in PBS, the slides were coverslipped with ProLong anti-fade mounting media containing DAPI (Invitrogen). The staining specificity was tested by omission of primary antibodies.

Morphological analysis

Counts of retrogradely labelled spinal motoneurons (Paper I)

Nuclear profiles of labeled motoneurons were counted in all sections at x250 magnification under a Leitz Aristoplan fluorescence microscope using filter block N2.1. The total number of nuclear profiles was not corrected for split nuclei, since the nuclear diameters were small in comparison with the section thickness used. It has previously been demonstrated that the accuracy of this technique in estimation of retrograde cell death in spinal cord is similar to that obtained with the physical dissector method [29] and counts of neurons reconstructed from serial sections [14]. Preparations were photographed with a Nikon DXM1200 digital camera. The captured images were resized, grouped into a single canvas and labeled using Adobe Photoshop CS4 software. The contrast and brightness were adjusted to provide optimal clarity.

Counts and area assessment of myelinated axons (Paper I)

Axonal count and area assessment were performed on myelinated axons in the proximal and distal nerve stumps, as well as in the middle of the nerve graft. Myelinated axons were counted at x1000 final magnification using the fractionator probe in Stereo

Investigator™ 6 software (Micro-BrightField, Inc., USA). The axonal area was calculated by the software after manually marking the outer border of single axons. The area was then described as a mean figure after measuring and summarizing the area of 30 axons at four random sites per cross section and dividing the total area with the number of axons counted.

Neurite outgrowth assay in Schwann cell-neuron co-culture (Paper I)

The slides were photographed with a Nikon DXM1200 digital camera attached to a Leitz microscope and an average of 150 NG108-15 cell bodies for each condition were analyzed (n=4 replicates) for neurite outgrowth using Image-Pro Plus software (MediaCybernetics, UK). Neurites were recorded using the trace function. The mean number of neurites per neuron and mean neurite length together with mean length of the longest neurite were calculated.

Morphometric analysis of muscles (Papers I & II)

Images were captured with Nikon Elements Imaging Software. Morphometric analysis of muscle sections was performed on coded slides without knowledge of their source. Five random fields were chosen using the 16X objective (Paper I) or 20X objective (Paper II). Images for the immunolocalisation of myosin heavy chain type and laminin were captured using the appropriate emission filters, and combined to provide dual-labelled images. Each image contained at least 10 individual muscle fibers for analysis. Image-Pro Plus software was calibrated to calculate the mean area in μm for each muscle. The injured side was expressed relative to the contra-lateral control side and the relative mean % ± SEM calculated for each group.

Spinal cord analysis (Paper III)

For quantitative analysis of spinal cord the images were captured from the left ventral horn of L4-L5 spinal cord segments at 40X magnification using a Nikon DXM1200 digital camera. The resulting images had a size of 1280 X 1024 pixels, which corresponded to 3.8 pixels per 1 μm tissue length. The area occupied by immunostained profiles was calculated using Image-Pro Plus software (Media Cybernetics, Inc., USA). In total 20 pictures were analyzed for each staining with 5-10 sections analyzed per rat

and one picture taken per spinal cord section. Sections that were folded or damaged in any other way were excluded from the analyses. The frame was positioned along the outer border of the ventral horn, and only motoneurons localized in the dorso-lateral region of the ventral horn on the border with the lateral funiculus, which supply the distal hind limb, were included for analysis. The neuronal area and diameter was counted by manually marking the outer border of the cells immunopositive for MAP- 2, excluding the exit of the dendrites. All cells with clear margins were analysed per picture (in total 20 pictures including 39-71 cells per group).

Gene expression analyses

In order to assess the molecular composition, total RNA was isolated from distal nerve segments (Paper I), muscle (Papers I & II), spinal cord (Paper III) and DRGs (Paper IV) using a RNeasy^{TM} kit or miRNeasy mini kit (Qiagen, Sweden). The purified RNA was quantified by determining the absorbance at 260 nm using a Nanodrop 2000/2000c spectrophotometer (ThermoScientific, Sweden).

Muscle (Paper II) and spinal cord samples (Paper III) from rats in each experimental group were pooled and 1-5 ng total RNA was converted to cDNA using a First-Strand cDNA Synthesis Kit (Bio-Rad iScript cDNA synthesis kit). The reaction mix was incubated in a thermal cycler under the following conditions; 5 min at 25°C, 30 min at 42°C and finally 5 min at 85°C. qRT-PCR was subsequently performed using SsoFast^{TM} EvaGreen Supermix (BioRad) according to the manufacturer recommendations (enzyme activation at 95 °C for 30 s followed by up to 40 cycles of denaturation (95 °C for 5 s) and annealing/extension (5s at optimal temperatures shown below). All previously published and validated primers were purchased from Sigma, UK (Table 1).

For semi-quantitative RT-PCR, muscle (Paper I) and spinal cord samples (Paper III) from rats in each experimental group were pooled and 1-5 ng total RNA was incorporated into the One-Step RT-PCR kit (Qiagen) per reaction mix. A thermocycler (Biometra, Germany) was used with the following parameters: a reverse transcription step (50°C, 30 min), a nucleic acid denaturation/reverse transcriptase inactivation step (95°C, 15 min) followed by 35 cycles of denaturation (95°C, 30 sec), annealing (30 sec) and primer extension (72°C, 1 min) followed by final extension incubation (72°C, 5 min). PCR amplicons were electrophoresed (50 V, 75 min) through a 1.5% (w/v)

agarose gel and the size of the PCR products estimated using Hyperladder IV (Bioline, UK). Samples were visualised under UV illumination following GelRed™ nucleic acid stain (Bio Nuclear, Sweden) incorporation into the agarose. All primers were purchased from Sigma, UK (Table 1).

For microRNA analysis, muscle (Paper II) and spinal cord samples (Paper III) from rats in each experimental group were pooled and 1-10 ng total RNA was converted to cDNA using a TaqMan MicroRNA Reverse Transcription Kit (Taqman® Small RNA Assays). The reaction mix was incubated in a thermal cycler under the following conditions; 30 min at 16°C, 30 min at 42°C and finally 5 min at 85°C. qRT-PCR was subsequently performed using Taqman® Universal PCR Master Mix according to the manufacturer recommendations (enzyme activation at 95 °C for 10 min followed by up to 40 cycles of denaturation (95°C for 15 s) and annealing/extension (60°C for 60 s). The Taqman® MicroRNA assay, rno-miR-21, hsa-miR-206 and rno-miR-1 were purchased from Invitrogen, Sweden.

Table 1. Primer sequences for RT-PCR and annealing temperatures (°C).

Factor	Forward Primer (5′→3′)	Reverse Primer (5′→3′)	°C
S100B	GTTGCCCTCATTGATGTCTTC	AGACGAAGGCCATAAACTCCT	57.9
erbB2	AACCTTTCCTTGCTGCTTGA	GTTCCCTCCAGACCTCTTCC	59.9
erbB3	AGAGGCTTGTCTGGATTCT	AGGAGTAAGCAGGCTGTGT	55.9
erbB4	AACCAGCACCATACCAGAGG	TTCATCCAGTTCTGCTCGTG	62.1
TGF-β	CTAATGGTGGACCGCAACAAC	CGGTTCATGTCATGGATGGTG	67.8
Tenascin C	GCCTCAACAACTGCTACAATCGTG	TCAGCCCCTGTGAACCCATC	66.1
Collagen I	GTGAACCTGGCAAACAAGGT	CTGGAGACCAGAGAAGCCAC	64.1
Phosphacan	GAATTCTGGTCCACCAGCAG	GGTTTATACTGCCCTCTTTAGG	59.0
Versican	ACACAGGGAGAAACCCAGGA	TGTCTTGTTTTCTCTGACCT	61.0
α-nAChR	TGTGTCTCATCGGGACGC	GGGCAGAGGGAGGCTTAGTTC	64.0
β-nAChR	CCGTGTCACTGCTGAATCTGT	CTCAAAGGACACCACGACAT	60.9
γ-nAChR	TGTCATCAACATCATCGTCCC	CGAGGAAAAGGAAGACGGT	61.8
δ-nAChR	TGTGGAGAGAAGACCTCG	AGCCTCTTGGAGATAAGCAAC	57.0
ε-nAChR	AACTGTCTGACTGGGTGCGT	GAAGATGAGCGTAGAACCGAC	61.8
MuSK	TGAAGCTGGAAGTGGAGGTTTT	GCAGCGTAGGGTTACAAAGGAA	63.3
18S	TCAACTTTCGATGGTAGTCGC	CCTCCAATGGATCCTCGTTAA	62.1
Actin	ACTATCGGCAATGAGCGGTTC	AGAGCCACCAATCCACACAGA	64.1
MyoD	TGTAACAACCATACCCCACTC	AGATTTTGTTGCACTACACAG	60.6
Myogenin	CACATCTGTTCGACTCTCTTC	ACCTTGGTCAGATGACAGCTT	58
Atrogin	GAACAGCAAAACCAAAACTCAGTA	GCTCCTTAGTACTCCCTTTGTGAA	60
MuRF	TGTCTGGAGGTCGTTTCCG	ATGCCGGTCCATGATCACTT	64
SDHA	AGACGTTTGACAGGGGAATG	TCATCAATCCGCACCTTGTA	60.9
HSPCB	GATTGACATCATCCCCAACC	CTGCTCATCATCGTTGTGCT	61.9
Caspase-8	CTGGGAAGGATCGACGATTA	CATGTCCTGCAGTTTTGATGG-	61
Caspase-3	GGACCTGTGGACCTGAAAAA	AGTTCGGCTTTCCAGTCAG	60.5
Diablo	CTCGGAGCGTAACCTTTCTG	TCCTCATCAGTGCTTCGTTG	64
PPIA	CACCGTGTTCTTCGACATCAC	CCAGTGCTCAGAGCACGAAAG	64
Fas	ATGGCTGTCCTGCCTCTGGT	ACGCTCCTCTTCAACTCCAAA	67
TNFR1	ACCAAGTGCCACAAAGGAAC-	CTGGAAATGCGTCTCACTCA	64
TRAIL-R	TGATGAAGAGTGCCAGAAATAGC	CCAGGTCCATCAAATGCTCA	65
FasL	TTTTTCTTGTCCATCCTCTG	CAGAGGGTGTGCTGGGGTTG	65
TNFα	TCAGCCTCTTCTCATTCCTGC	TTGGTGGTTTGCTACGACGTG	67
TRAIL	TGATGAAGAGTGCCAGAAATAGC	CCAGGTCCATCAAATGCTCA	65
Caspase-12	GGCAGACATACTGGTACTATTTGG	GCTCAACACACATTCCTCATCTGT	61
Caspase-7	TACAAGATCCCGGTGGAAGCT	CTGGGTTCCTCCACGAATAAT	62
Calpain	TGCTCTGCCGAAGTGTCACC	GCACTGGATGGCCTGGAGTT	66

Western blotting

The protein expression was analysed using western blot (Papers III and IV). Individual lysates of ventral horn dissected from L4-L5 spinal cord (Paper III) and DRG L4-L6 (Paper IV) were prepared by homogenisation in 100-300µl lysis buffer. Samples were left on ice for 15 min and were then centrifuged at 13000 rpm for 10 min at 4°C to produce a supernatant of soluble proteins which were then stored at -80°C. The supernatants or whole tissue lysates were analysed for protein content using a commercially available protein assay kit (Bio-Rad). 10-30 µg protein were prepared per sample, combined with Laemmli buffer and denatured at 100°C for 5 minutes. Proteins were resolved on 10% sodium dodecyl sulphate-polyacrylamide gels. Electrophoretic transfer to nitrocellulose was achieved at 30V for 1.5 h. Then, membranes were blocked for 1 hour in either 5% (w/v) non-fat dry milk or 5% (w/v) BSA in TBS-Tween (10mM Tris PH 7.5, 100 mM NaCl, 0.1% (v/v) Tween), and then incubated overnight at 4°C with either monoclonal anticaspase-8 antibody (Santa Cruz Biotechnology; 1:200), polyclonal active caspase-8 antibody (Novus; 1:1000), polyclonal phospho-IRE1α antibody (Novus; 1:1000) or polyclonal phosphoPERK antibody (Cell Signaling; 1:1000). Following 6 x 5 minute washes in TBS-Tween, membranes were incubated for 1 hour at room temperature with HRP-conjugated secondary antibody (Cell Signaling Technology; 1:1000). Membranes were washed as previously and treated with ECL chemiluminescent substrate (GE Healthcare) for 1 minute and developed using an Odyssey® Fc imaging system (LI-COR). Confirmation of equal protein loading was confirmed by staining the membrane with Ponceau S.

Statistical analysis

In order to determine the statistical difference between the groups one-way analysis of variance (ANOVA) complemented by Newman-Keuls test (Prism Graph-Pad software) (Paper I) and Bonferroni test (Prism-Graph-Pad software) (Papers II & III) were used. Two-way ANOVA was applied to further compare soleus and gastrocnemius muscles in each of the analyses (Paper II). Statistical significance was set as *$p<0.05$, **$p<0.01$, ***$p<0.001$.

RESULTS

Analysis of regenerating motoneurons following immediate and delayed nerve repair (Paper I)

Sciatic nerve transection was performed on rats followed by either immediate repair or delayed repair (at 1, 3, or 6 months) using 10 mm nerve grafts and then 13 weeks later motoneuron regeneration was analysed. Motoneurons which regenerated their axons across the nerve graft were identified and counted after labeling with a fluorescent dye, Fluoro-Ruby (Fig. 1A–D). Following immediate nerve repair or delayed repair for 1 month, 1027±31 and 1041±26 motoneurons had regenerated into the distal nerve stump (Fig. 1E). In contrast, delayed 3 months and 6 months nerve grafting drastically reduced the number of regenerating spinal motoneurons to 374±34 and 253±19, respectively (Fig. 1E). The proximal stump of nerves from animals undergoing immediate repair showed large diameter axons and sections from the 1 month delayed repair animals appeared similar (Fig. 2). In contrast, the 3 and 6 months delayed repair sections showed more numerous axons which were smaller in size (Fig. 2). The distal stumps showed a progressive reduction in the number of axons from immediate repair through to the 6 months delay repair animals (Fig. 2). Sections from the mid-point of the graft appeared similar throughout all time points (Fig. 2). These observations were quantified using Stereo Investigator software (Fig. 3). The axonal number in the middle of the graft showed no significant difference between any of the groups. In the proximal segment no difference in the number of axons was observed between the immediate repair and 1-month groups. At 3 and 6 months however the number of axons showed a significant 2-3-fold increase (Fig. 3A). In the distal segment the number of axons showed a minimal decline after 1 month delayed repair compared with immediate repair. However by 3 months delay the number of distal axons was significantly reduced by approximately 80–90% compared with both the immediate repair and 1-month groups (Fig. 3A). The axonal numbers were further statistically reduced between the 3- and 6-months groups. The axonal area in the distal stump showed no statistical difference between any of the groups (Fig. 3B). In the middle of the graft a small decline in axon area was observed in the 3 and 6 months delayed repair groups when compared with the immediate and 1 month delay repair groups. Furthermore, the proximal stump showed a progressive and

statistically significant decrease in axonal area at 3- and 6-months delayed repair (Fig. 3B).

Analysis of the distal nerve stump following immediate and delayed nerve repair (Paper I)

The molecular composition of the distal nerve stumps after immediate or delayed nerve repair was investigated using RT-PCR (Fig. 4). Compared with the immediate and 1 month delay repair groups there was a noticeable reduction in S100B (Schwann cell marker) mRNA in the 3 and 6 months delay repair groups. The levels of neuregulin/glial growth factor receptors, erbB2-4, were also determined. There was no apparent change in erbB2 expression under the different experimental conditions. In contrast, there was a modest reduction in erbB3 and significant reduction of erbB4 mRNA levels in the distal stumps of animals undergoing delayed repair after 3 and 6 months (Fig. 4). A range of fibrotic and scar associated molecules were also assessed. Transforming growth factor beta (TGF-β) was barely detectable in the immediate repair groups but was progressively up-regulated with increase in the time of delayed repair. Similarly, tenascin C expression was only observed in the 3 and 6 months delayed repair animals (Fig. 4). Collagen I mRNA was expressed in all distal stumps as was the extracellular matrix proteoglycan versican. Another chondroitin sulphate proteoglycan (CSPG), phosphacan, was only significantly elevated in the 6 months delayed repair group (Fig. 4).

Analysis of the Schwann cells in the proximal and distal nerve stump following immediate and delayed nerve repair (Paper I)

Schwann cells were isolated from the proximal and distal nerve stumps and expanded in vitro (Fig. 5). In all experimental groups a consistent number of Schwann cells could be isolated from the proximal stumps (Fig. 5A). In contrast, with a longer delay to nerve repair there was a progressive reduction in the number of Schwann cells which could be isolated from the distal stumps (Fig. 5A). This reduction in Schwann cell numbers was paralleled by an increase in the number of contaminating fibroblasts. To determine if these changes were the result of decreased responsiveness to glial growth factors, a proliferation assay was performed (Fig. 5B). When cultured in the presence of forskolin and neuregulin, there were no significant differences in the growth rate of Schwann

cells isolated from the distal stumps of animals undergoing immediate or delayed nerve repair. Furthermore, the cells from these animals grew similar to those isolated from control uninjured rats. Consistent with these observations the expression levels of erbB receptors were similar in Schwann cells isolated from all groups (Fig. 5C). The Schwann cells neurotrophic activity was then determined using a previously published model in which NG108-15 cells (a model motor neuron cell line) are cocultured on a monolayer of Schwann cells [62]. Schwann cells isolated from uninjured control nerve significantly enhanced neurite outgrowth of the NG108-15 cells (Fig. 6). The Schwann cells obtained from the animals undergoing either immediate nerve repair or delayed nerve repair also significantly enhanced the neurite outgrowth and there were no statistically significant differences between the groups (Fig. 6).

Muscle analysis (Paper I and Paper II)

The ipsilateral and contralateral medial gastrocnemius muscles from animals undergoing sciatic nerve transection followed by either immediate repair or delayed repair (at 1, 3, or 6 months) using 10 mm nerve graft were harvested 13 weeks postoperatively and sectioned and stained with antibodies directed against fast and slow type myosin heavy chain protein (Fig. 7, paper I). Contra-lateral muscles showed a well organized structure, predominantly populated by fast type muscle fibers. The muscle from the operated side of animals undergoing immediate repair also showed a normal muscle morphology but there was evidence of slow muscle fiber type grouping (Fig. 7, paper I). In contrast, muscle samples taken from animals undergoing nerve repair after a 6 months delay showed irregular morphology and an apparent reduction in muscle fiber size (Fig. 7, paper I). Quantitative analysis of the muscle fiber size was performed (Fig. 8, paper I) and showed a significant reduction in fast type fiber area with 1 month delayed repair and a progressively smaller fiber size in the 3 and 6 months delayed repair animals (Fig. 8A, paper I). The size of the slow type fibers was not significantly reduced until the 6 month delay repair time point. Similar data were obtained when the fiber diameters were measured (Fig. 8B, paper I). Animals with immediate nerve repair or after 1 month delay, showed an approximate 20% reduction in the wet weight of the operated side muscles compared with the contralateral side (Fig. 8C, paper I). Consistent with the reductions in muscle fiber size, there was a significant reduction in the wet weight of muscles harvested from the 3 and 6 months delayed repair groups

(Fig. 8C, paper I). The muscles were also examined immunohistochemically (Fig. 9A, paper I) using the presynaptic marker, SV2A and ∝-bungarotoxin (∝-BTX) to label the post-synaptic acetylcholine receptors (AChRs). SV2A immunostaining was coincident with the ∝-BTX binding sites in control muscles. Similar neuromuscular junction (NMJ) structures were detected in animals undergoing delayed repair at 1, 3 and 6 months; however at the later time points the NMJs were noticeably sparser. Furthermore, qualitatively the NMJs appeared smaller with increased time-delay to nerve repair. Changes in motor end plate size and quantity of NMJs are known to be reflected in altered expression levels of genes normally associated with formation of AChRs. To semi-quantitatively measure differences in the NMJs the transcript levels of the nicotinic AChR subunits (∝, β, γ, δ and ε) were determined in the muscles following immediate and delayed nerve repair. There were differential changes in expression of the various subunits compared with control muscle (Fig. 9B, paper I). Of significant note was the detection of the embryonic specific γ subunit in muscle from the animals undergoing 3 and 6 month delayed nerve repair. The receptor tyrosine kinase, MuSK, an intrinsic protein of the NMJ, is regulated by the innervation state of the muscle. There was a progressively increased expression of the MuSK gene with increase in the delay repair time (Fig. 9B, paper I).

Since the soleus and gastrocnemius muscles appeared to respond differently to injury, the muscles were studied in more detail following both nerve transection and nerve crush injury without repair with 1, 7, 14 and 28 days survival. The muscles were stained with haematoxylin and eosin (Fig. 1, paper II) and as early as 7 days after injury there were noticeable morphological changes; the muscle fibre showed a more rounded shape compared with the characteristic mosaic pattern still evident at day 1. The muscles harvested 28 days after nerve transection injury showed the greatest signs of morphological atrophy; small muscle fibres were surrounded by large numbers of other cells, presumably inflammatory cells and fibroblasts. In the animals treated with nerve crush injury, the morphological changes were less marked at 28 days. Interestingly, the soleus muscles 28 days after crush injury looked almost identical to the muscles 1 day after injury, suggesting a significant recovery with time (Fig. 1, paper II). Animals undergoing sciatic nerve transection showed a progressive decline in the medial

gastrocnemius wet weight over the period of 1– 28 days post-injury (Fig. 1, paper II). By day 28, there was a 70.57% ± 1,39 reduction in the weight of the operated side compared with the contra-lateral side (Fig 1, paper II). Animals undergoing crush injury showed much less loss of muscle weight (45.19% ± 2.6 at day 28). There was a similar progressive weight loss in the soleus muscle of animals with sciatic nerve transection (Fig. 1, paper II). In contrast, in the crush injury treated animals, after an initial reduction in weight by 36.43% ± 2.67, the weight of the soleus muscles recovered and showed just 19.13% ± 2.90 loss at day 28 (Fig. 1, paper II).

These results suggested that the soleus, a predominantly slow fibre type muscle, was less susceptible to injury (showing a recovery from day 7), so further experiments investigated whether there were any specific differences between fast type and slow type fibres in the mixed fibre type gastrocnemius muscle. Contra-lateral muscles from animals undergoing nerve transection or crush injury followed by 7 or 28 days without repair showed a well-organized structure, predominantly populated by fast type muscle fibres (Fig. 2, paper II). The muscles from the operated side of animals undergoing nerve transection showed a much more affected morphology and an apparent reduction in muscle fibre size compared to the operated side muscles from animals undergoing crush injury, especially in respect to fast type fibres (Fig. 2, paper II). Furthermore, following nerve transection indication of muscle fibre grouping was observed with time. Quantitative analysis showed that the animals undergoing sciatic nerve transection exhibited a 16.20% ± 8.26 and 83.25% ± 1.48 reduced fast type fibre area in the gastrocnemius muscle 7 and 28 days after injury respectively (Fig. 2, paper II). There was also a reduction in size in the slow type fibres, but to a lower extent (66.25% ± 4.55 at 28 days). In the crush injury model no significant difference in fibre size was shown between 7 and 28 days and neither a difference between fast and slow type fibres (Fig. 2, paper II).

To further investigate differences between the two muscle types, the expression of the myogenic transcription factors MyoD and myogenin as well as the microRNAs miR-1 and miR-206 were investigated. The expression of the myogenic transcription factors MyoD and myogenin showed a dynamic pattern over time, however with contrasting expression patterns in the different muscle phenotypes. Further studies focussed on two time-points, 7 days (the point after which recovery occured in crush injured soleus

muscles) and 28 days (when maximal atrophy was observed in the transection model). 7 days following crush injury, the expression levels of MyoD were increased 54.23 ±1.03 and 4.55 ± 0.19 fold in the soleus and the gastrocnemius muscle respectively (Fig. 3, paper II). 7 days following nerve transection, the expression levels of MyoD were increased 14.74 ± 0.82 and 2.86 ± 0.14 fold in the soleus and the gastrocnemius muscle respectively (Fig. 3, paper II). At 28 days after injury, the MyoD expression in the soleus muscle returned to near control levels in the crush injury model but was 30.23 ± 0.76 fold higher in the transection injury model (Fig. 3, paper II). Immunostaining showed that the increased gene expression levels correlated with protein changes - large numbers of MyoD positive nuclei were detected in injured muscles but these were absent in the control muscles (Fig. 3, paper II). In contrast to MyoD, the reverse gene expression pattern was observed regarding myogenin which was increased 7.51 ± 0.12 and 22.79 ± 0.90 fold in the soleus and the gastrocnemius muscle following crush injury respectively at 7 days, with decreased expression with time (Fig. 4, paper II). Following nerve transection, there was also a significantly larger increase in myogenin expression in the denervated gastrocnemius versus soleus muscles (Fig. 4, paper II). Interestingly, myogenin expression was 12.50 ± 0.75 fold higher in the control soleus muscles compared with the control gastrocnemius muscles (S1 Fig., paper II). As with MyoD, an elevated number of myogenin positive nuclei correlated with the injury-induced gene expression changes (Fig. 4, paper II). Taken together these quantitative analyses thus showed a pronounced upregulation of myogenin in the gastrocnemius muscle 7 days following injury, regardless of injury type, whilst a pronounced up-regulation of MyoD was observed in the soleus muscle 7 days following injury.

In the delayed nerve repair model, MyoD expression (Fig. 5, paper II) was up-regulated 3.93 ± 0.28 fold in the soleus muscle after 3 months delayed repair, whilst MyoD was up-regulated only after 6 months in the gastrocnemius muscle, but then to a more extensive degree (30.89 ± 1.19). A similar pattern was observed with myogenin expression which was up-regulated 2.74 ± 0.10 fold in the soleus muscle after 3 months delayed repair, in contrast to the gastrocnemius muscle, which showed only a significant 7.40 ± 0.42 fold increase after 6 months of delayed nerve repair (Fig. 5, Paper II). These quantitative analyses thus showed in the long-term that myogenin and MyoD were significantly up-regulated after 3 months delayed repair in the soleus muscle, in contrast

to the gastrocnemius muscle, in which case the myogenic transcription factors was significantly up-regulated only after 6 month delayed nerve repair.

As with the myogenic transcription factors, miR-1 and miR-206 exhibited opposite expression patterns in the different muscle phenotypes. Following crush injury and nerve transection, miR-1 was increased 1.99 ± 0.03 and 2.43 ± 0.08 fold respectively in the gastrocnemius muscle in comparison to the control at 7 days, whilst the expression was decreased in the soleus muscle (Fig. 6, paper II). miR-206 was markedly elevated (10.06 ± 0.06 fold) in the gastrocnemius muscle at 28 days following crush injury (Fig. 6, paper II). Compared to the gastrocnemius muscle, the level of miR-206 in the soleus muscle was 8.20 ± 0.23 fold higher prior to injury (S1 Fig., paper II). In contrast, miR-1 expression levels were similar in the control soleus and gastrocnemius muscles (data not shown). In summary, the soleus and the gastrocnemius muscle showed contrasting transcriptional regulation of miR-1 which was down-regulated in the soleus muscle 1 week post injury, regardless of injury type, whilst the gene expression was significantly up-regulated in the gastrocnemius muscle.

As with the myogenic transcription factors and the microRNAs, the expression pattern of the muscle-specific E3 ubiquitin ligases MuRF1 and Atrogin-1 differed in the two muscle phenotypes. Following crush injury and nerve transection, MuFR1 was increased 5.02 ± 0.09 and 6.98 ± 0.18 fold respectively in the gastrocnemius muscle in comparison to the control at 7 days, whilst only moderate increases were observed in the soleus muscle (Fig. 7, paper II). Similar data was obtained when Atrogin-1 was analysed following cut injury (Fig. 7, paper II), levels were increased 6.87 ± 0.29 fold in the gastrocnemius muscle in comparison to the control at 7 days, in contrast to in the soleus muscle, where the increase was less prominent, 3.56 ± 0.18. Levels of MuRF1 and atrogin-1 were 2.67 ± 0.13 ($p < 0.001$) and 4.03 ± 0.22 (not significantly different) fold higher in the control soleus muscles compared with the control gastrocnemius muscles (S1 Fig, paper II).

Spinal cord analysis (Paper III)

The aim of paper III was to gain more insight about the mechanism behind injury-induced motoneuron degeneration. Morphological and molecular changes in spinal motoneurons were compared after L4-L5 ventral root avulsion (VRA) and distal peripheral nerve axotomy (PNA) at 7 and 14 days postoperatively. Morphological changes were assessed by using quantitative immunohistochemistry and molecular changes were assesesed by qRT-PCR and western blot.

Quantitative analysis of ventral horn neuropil of spinal cord indicated that the synaptic frequency as well as the frequency of dendritic branches were significantly reduced following both VRA and PNA. The synaptic frequency was reduced by 52% and 57%, 1 and 2 weeks following VRA in comparison to the respective controls (Fig. 1 and Fig. 2A). The amount of synapses was also significantly changed after PNA 7 and 14 days, however to a lower extent; 24% at both time points (Fig. 2A). The same pattern was observed in regard to MAP2 staining. After VRA 7 and 14 days the expression was decreased by 42% and 60% respectively (Fig. 1 and Fig. 2B). Following PNA 7 and 14 days the MAP2 expression was decreased by 28% and 32% respectively (Fig. 1 and Fig. 2B). A significant soma atrophy in spinal motoneurons was detected at all time points, independently of injury type (Fig. 1 and Fig. 2C, D).

The level of immunoreactivity for ED1-positive microglial cells was up-regulated 19.80 ± 2.30 and 19.61 ± 1.56 fold following VRA 7 and 14 days in comparison to un-operated control (Fig. 3 and Fig. 4A). After PNA ED1 reactivity was increased by 11.45 ± 1.93 and 10.65 ± 0.93 fold at 7 and 14 days respectively in comparison to un-operated control (Fig. 3 and Fig. 4A). VRA elevated immunoreactivity of GFAP-positive astrocytes in the ventral horn of spinal cord by 2.02 ± 0.07 and 2.06 ± 0.10 fold at 7 and 14 days respectively in comparison to un-operated control (Fig. 3 and Fig. 4B). After PNA GFAP reactivity was increased by 1.85 ± 0.05 and 1.82 ± 0.07 fold at 7 and 14 days respectively in comparison to un-operated control (Fig. 3 and Fig. 4B)

The molecular changes in the spinal cord evoked by the two types of injuries were investigated. Following VRA 7 and 14 days the expression levels of caspase-3 was increased 2.43 ± 0.20 and 1.91 ± 0.28 fold respectively (Fig. 5A), in contrast to after PNA, for which no

significant up-regulation was observed. The gene expression of caspase-8 was significantly up-regulated even after PNA, however to a significantly lower extent in comparison to after VRA. Following VRA 7 and 14 days the expression level of caspase-8 was increased 3.65 ± 0.51 and 3.34 ± 0.46 fold respectively (Fig. 5B) and after PNA 7 and 14 days the expression level of caspase-8 was increased 2.03 ± 0.18 and 2.38 ± 0.37 fold respectively (Fig. 5B). Western blotting showed that the increased caspase-8 gene expression correlated with activation of the enzyme. Both the caspase-8 precursor and the active fragment p18 were increased 14 days following VRA (Fig. 5C).

In order to assess whether any death receptors and their corresponding ligands were expressed in the injured spinal cord, RT-PCR was performed. The expression level of the death receptors TRAIL-R, TNF-R and Fas was up-regulated following VRA 7 and 14 days, in contrast to after PNA (Fig. 6). However, the expression of the corresponding ligands was not increased following either VRA or PNA.

With a view to further elucidating the molecular pathways activated after the different types of injury the expression levels of Diablo, a mitochondrial protein that promotes cytochrome-c dependent activation, and microRNA-21 (miR-21), a microRNA that prevents neuronal death by targeting FasL, were investigated. The gene expression of Diablo was significantly up-regulated first 14 days after VRA, 1.80 ± 1.10 fold (Fig. 7A) and the gene expression of miR-21 was more pronounced following VRA in comparison to after axotomy. Following VRA 7 and 14 days the gene expression was increased 2.56 ± 0.69 and 4.3 ± 0.23 respectively (Fig. 7A). A significant increase of miR-21 was also observed after PNA 7 days, however, the expression returned to normal level 14 days post-operatively (Fig. 7B).

DRG analysis (Paper IV)

In order to get a better understanding of the apoptotic mechansisms underlying sensory neuronal cell death in the DRGs, molecular changes were compared 7,14 and 28 days following peripheral nerve injury. Analyses by qRT-PCR showed that at 7, 14 and 28 d following nerve transection the expression of caspase-3 was increased 2,66 ± 0,19, 2,62 ± 0,10 and 2,21 ± 0,28 fold respectively in comparison to control (Fig. 1A). Immunohistochemistry showed that the increased caspase-3 expression correlated with activation of the enzyme (Fig. 1B). Caspase-8, which is a member of the extrinsic

apoptotic pathway, was increased 1,56 ± 0,16, 2,72 ± 0,19 and 1,35 ± 0,10 fold in comparison to control 7, 14 and 28 days respectively following nerve transection (Fig. 2A). Western blotting showed that the caspase-8 protein was also up-regulated by injury but we were unable to detect significant quantities of the active caspase-8 fragment (Fig. 2B). At 7, 14 and 28 d following nerve transection the gene expression of caspase-12 was increased 2,91 ± 0,22, 2,20 ± 0,42 and 3,16 ± 0,69 fold respectively in comparison to control (Fig. 3A). Western blotting showed that the increased caspase-12 gene expression correlated with activation of the enzyme. Both the caspase-12 precursor and the active fragment were increased 14 days following nerve transection (Fig. 3B). The increased expression of caspase-12 was parallelled with an increased expression of calpain, a known activator of caspase-12 [29]. At 7, 14 and 28 d following nerve transection the expression of calpain was increased 2,10 ± 0,13, 1,55 ± 0,08 and 1,41 ± 0,05 fold respectively in comparison to control (Fig. 3C). In addition to calpain, the increased expression of caspase-12 was parallelled with an increased expression of caspase-7, which is another known activator of caspase-12 [53]. At 7, 14 and 28 d following nerve transection the expression of caspase-7 was increased 1,83 ± 0,12, 1,93 ± 0,20 and 1,62 ± 0,29 fold respectively in comparison to control (Fig. 3D). The ER-stress response is mediated by three different ER membrane receptors, ATF6, IRE1 and PERK [27], and this study showed that the phosphorylation of IRE1 and PERK was increased 14 days following nerve transection (Fig. 4A). Analysis of the DRGs by immunohistochemistry indicated that phosphoPERK expression was mainly localised to IB4 positive neurons in control tissue (Fig. 4B). After nerve injury, the staining for phosphoPERK was more widely distributed through the different neuronal populations (Fig. 4B).

DISCUSSION

It is well established that the main neurobiological factors underlying the poor functional outcome after peripheral nerve injury are axotomy-induced retrograde cell death in spinal cord and DRG, chronic denervation of Schwann cells in the distal nerve stump and degeneration of the target organ [11]. Despite advanced microsurgical innovation the motor and sensory outcome is still poor, creating a need for additive pharmological approaches [31]. By further investigating the underlying mechanisms behind the above mentioned impeding neurobiological factors, we aimed to gain more knowledge about the complex network underlying peripheral nerve injury degeneration.

Axotomy-induced retrograde degeneration

The cell type and the distance between the cell body and the lesion site are important parameters influencing the degree of retrograde cell death. Distal injury to the sciatic nerve in adult rodents does not induce any detectable loss of spinal motoneurons [14, 15], while the same type of injury in primary sensory neurons in DRGs results in sigificant cell degeneration [15]. However, more proximal injuries to the spinal nerves [16, 27-30] are associated to prominent cell death in the ventral horn. Furthermore, the time of delay is of big importance for the regenerative potential. Results showed that following nerve transection with immediate (0) or 1, 3 or 6 months delayed repair with a nerve graft, the number of regenerating spinal motoneurons projecting into the distal stump were drastically reduced following 3 and 6 month delayed repair (Paper I).

Peripheral nerve injuries are followed by an increased expression of neurotrophic factors in Schwann cells, as well as an increased retrograde axonal transport of neurotrophins from the lesioned neurons [63]. Immature motoneurons are more dependent of trophic support from their target muscles, since ciliary neurotrophic factor (CNTF) and other neurotrophic factors are present at insufficient levels at early postnatal life [64]. The difference in neuronal cell death following nerve transection and ventral root avulsion may reflect the deprivation of trophic support from Schwann cells associated with the peripheral nerve following avulsion [64], which is further emphasized by the reduced motoneuron degeneration observed following implantation

of a peripheral nerve graft at the site of avulsion [64], as well as after exogenous delivery of neurotrophic factors [65]. Despite intensive research, data regarding the underlying mechanism behind the injury-induced motoneuron death is conflicting, with remaining uncertainties whether motoneuron death is mediated through apoptosis or necrosis [13, 66, 67].

Spinal cord changes

Peripheral nerve lesions induce retrograde changes not only in the affected motoneurons but also in surrounding non-neuronal cells. Astrocytes hypertrophy and within a few days most neurons are extensively enwrapped by the astrocytic processes. In parallel with the glial reaction, stripping of synapses from the perikaryon and dendrites of affected cells occurs and both activated microglia and astrocytes have been suggested to play a role in this process. The precise signaling pathways for the synaptic stripping are however mainly unknown, but activated microglia have been attributed an important role for the synaptic detachment [17]. Blinzinger and Kreutzberg [17] hypothesized that reactive microglial cells are involved in the removal of synaptic terminals from axotomized motoneurons since they can be found in close contact with motoneuron perikarya. However, this hypothesis has been contradicted by later studies. Svensson and Aldskogius [18] demonstrated that following infusion of an antimitotic agent in the cerebrospinal fluid at the brainstem level, which completely blocked the microglial response, the presynaptic terminals were displaced to the same extent as in animals in which saline was infused. Other studies implicate that reactive astrocytes are involved in the removal of synapses, based on the observation that astrocytic processes rather than microglial cells appear to be in the most intimate structural relationship to the neuronal membrane following peripheral nerve injury [19]. The functional implication of synapse removal from axotomised neurons is shifting from a transmitting electronically activated phenotype to a more regenerative phenotype, promoting axonal regeneration. In agreement with earlier studies work in this thesis demonstrated that the amount of synapses [16-21], as well as the expression of GFAP [19, 21, 22], a protein expressed in normal and reactive astroglia [22], were significantly changed following both peripheral nerve transection and ventral root avulsion (Paper III). Furthermore there was an increased expression of ED1, a protein expressed in activated microglia, following both VRA and PNA, although to a higher extent following VRA, which is

linked to the presence of degenerating motoneurons. The results concerning up-regulation of activated microglia vary between different research groups. In contrast to the results in Paper III, some studies have demonstrated that ED1 immunoreactivity is totally absent following axotomy [24, 68], which has been suggested to be due to the absent need of phagocytic cells following such mild trauma, while others have demonstrated a significant upregulation [69]. Results in Paper III showed that the frequency of dendritic branches were significantly reduced following both VRA and PNA, which is in line with previous studies [23]. Thus, even following less pronounced trauma, shrinking of the dendritic tree takes place, creating a need for reactive microglia for scavenging debris.

The molecular mechanisms behind injury-induced motoneuron degeneration are not fully understood, although it appears that trophic factor deprivation [12, 65] and nitric oxide– mediated oxidative stress [30] is of critical value. Spinal motoneurons express nitric oxide synthase (NOS) after VRA, in contrast to after ventral root transection [30], where the motoneuron loss can be reduced by inhibition of NOS [70]. Li et al [13] demonstrated that TUNEL labeling was detected in neonatal mice following axotomy, whereas TUNEL labeling was not observed following either neonatal or adult avulsion. Nevertheless, the morphological characteristics of motoneuron death following neonatal axotomy and avulsion were similar, and both resembled most closely apoptosis. By contrast, adult avulsion resulted in a necrotic degeneration. Although caspase inhibitors may rescue motoneurons after a VRA in neonatal rats, this pharmacological treatment approach has been unsuccessful to protect motoneurons subjected to the same injury in adult rats [66]. Inconsistent with the above mentioned data, other studies have demonstrated that injection of a Bcl-2-expressing vector 1 week prior to root avulsion increases the survival of lesioned motor neurons by 50% [71]. Furthermore, several studies have shown an enriched caspase-3 expression in motoneurons following VRA [67, 72]. In Paper III it was demonstrated that the gene expression of caspase-3, caspase-8 and related death receptors TRAIL-R, TNFR and Fas were significantly up-regulated following VRA 7 and 14 days. In contrast, following PNA the gene expression regarding caspase-3 and the death receptors did not differ from the control, and only a modest increased expression of caspase-8 was observed. Moreover the altered gene expression correlated with protein changes. This indicates that injury-

induced motoneuron degeneration is mediated through apoptosis, and that both the intrinsic and the extrinsic pathway is involved. One hypothesis is therefore that motoneuron degeneration in the adult spinal cord following ventral root avulsion is mediated by apoptosis involved in association with oxidative stress, possible due to trophic factor deprivation, as well as by activation of death receptors.

DRG changes

Just as there are uncertainties whether injury-induced motoneuron death is mediated through apoptosis or necrosis, current data regarding the underlying mechanism behind injury-induced sensory neuronal cell death is conflicting, although the emphasis on previous research has been on the intrinsic apoptotic pathway [73, 74], with the role of other apoptotic pathways being largely overlooked.

ER-stress is known to be induced in response to spinal cord injury [75]. In Paper IV it was demonstrated that caspase-12 mRNA was significantly up-regulated in sensory neurons following nerve transection, as well as calpain and caspase-7 (Paper IV). Furthermore phosphorylation of IRE1 and PERK was increased, indicating that the ER might be involved in the injury-induced apoptosis following peripheral nerve injury (Paper IV). Bax and Bak associate not only with mitochondria membranes but also with the ER [36], and mouse fibroblasts lacking both Bax and Bak ($Bax^{-/-}/Bak^{-/-}$) are resistant to apoptosis induced by thapsigargin, tunicamycin, and brefeldin A [76]. During ER stress, Bax and Bak undergo conformational changes and oligomerization in the ER membrane [36], releasing Ca2+ from the ER to the cytoplasm [36]. The increase in Ca2+ concentration in the cytosol activates m-calpain, which cleaves and activates procaspase-12 [37]. Activated caspase-12 then cleaves and activates procaspase-9, which in turn activates the downstream caspase cascade including caspase-3 [38]. Calpain-deficient mouse fibroblasts have reduced ER stress-induced caspase-12 activation and are resistant to ER stress-associated apoptosis [77]. Furthermore, caspase-7 translocates from the cytosol to the cytoplasmic side of the ER membrane in response to ER-stress where it cleaves the prodomain to generate active caspase-12, resulting in increased cell death [78]. Moreover, co-transfection of cells with caspase-12 and the catalytic mutant of caspase-7 not only blocked the cleavage of caspase-12, it also attenuated the cell death [78].

In addition to the role of calpains and caspase-7 in the activation of caspase-12, TNF receptor-associated factor 2 (TRAF2) has been shown to form a stable complex with procaspase-12 in unstressed cells [79]. Upon ER-stress, activated IRE1 recruits TRAF2, allowing dissociation of TRAF2 from caspase-12 and subsequent caspase-12 activation [79]. It should be noted that although caspase-12 is activated during ER stress, the involvement of caspase-12 in ER stress-induced apoptosis is still controversial. Some studies show that fibroblasts from caspase-$12^{-/-}$ mice are resistant to apoptosis in response to ER stress [80], while others claim that caspase-12 does not affect the survial rate, but rather the degree of inflammation [81]. Furthermore, Sanges et al [82] have proposed that ER stress-induced apoptosis is mediated by calpain, but not by caspases, based on the observation that calpain inhibitors, but not a pan-caspase inhibitor, block tunicamycin and thapsigargin-induced apoptosis.

Adult DRG comprise a heterogenous pool of sensory neurons that can be divided into subpopulations according to several parameters including neuronal size, neurochemistry, trophic requirements and sensory modality [83]. It is possible to distinguish between small unmyelinated afferents and large myelinated afferents; the former type comprise two populations of cells differentiated by their potential to synthesise neuropeptides; peptidergic afferents, which are predominantly of nociceptive function and identifed by their immunoreactivity for calcitonin gene-related peptide (CGRP), and non-peptidergic afferents, which are exclusively nociceptive and identified by their binding of isolectin B4 (IB4). Myelinated afferents innervate peripheral mechanoreceptors and proprioceptors and are identified by their binding of the high molecular weight neurofilament (NF-H). Specific sensory neuronal subpopulations show contrasting responses to peripheral nerve injury, as shown by the axotomy-induced death of many cutaneous sensory neurons whilst muscular sensory afferents survive an identical insult [15, 25, 26]. Furthermore the proportion of IB4-positive neurons decreases in axotomised DRGs [84]. Reid et al al [73] demonstrated that medial gastrocnemius neurons markedly downregulate caspase-3 mRNA in response to axotomy, whilst sural neurons upregulate their gene expression at 1 week post-injury, which was hypothesized to be attributable to the differing IB4-positive composition of the subpopulations described. In Paper IV it was demonstrated that a significant increase of phosphorylated PERK occurred following axotomy, which was mainly

restricted to IB4-positive neurons, raising the possibility that the increased suspectibily seen in these cells is due to activation of ER-stress.

The different apoptotic pathways interact, where, in parallel with the caspase-12 activation, ER stress triggers caspase-8 activation, resulting in cytochrome c/caspase-9 activation via Bid processing [85]. An increased expression of caspase-8 mRNA was detected following sciatic nerve transection (Paper IV), indicating that also the extrinsic pathway might be involved in the injury-induced apoptosis following peripheral nerve injury. In CNS studies, Huang et al [86] demonstrated that Z-IETD-FMK, a specific caspase-8 inhibitor, almost completely blocked the apoptosis induced by phenylalanine in cortical neurons, as well as the increased expression of caspase-3 and caspase-8. Furthermore, treatment with neutralizing antibodies against Fas signaling significantly decreased both the number of apoptotic cells and the expression levels of cleaved caspase-8 and cleaved caspase-3. Morever Martin-Villalba et al [87] showed that mice injected with a mixture of neutralizing anti-TNF and anti-FasL antibodies 30 min after induction of stroke exhibit a reduced infarct volume as well as an almost 70% reduced mortality.

In additon to the results of Paper IV suggesting the involvement of caspases 3,8 and 12 in injury-induced sensory neuron cell death, Vignesvara et al [88] observed significantly elevated levels of cleaved caspase-2, compared to cleaved caspase-3, following sciatic nerve transection. Furthermore, siRNA mediated down regulation of caspase-2 protected 50% of DRG neurons from apoptosis after serum withdrawal, while downregulation of caspase-3 had no effect on DRG neurons survival.

In summary, since adult DRG comprise a heterogenous pool of sensory neurons, it is likely to assume that several apoptotic pathways are involved in the injury-induced cell death. It can be speculated that the intrinsic apoptotic pathway, the extrinsic apoptotic pathway and the ER-stress responses are involved.

Chronic denervation of Schwann cells in the distal nerve stump

The timing of surgery is one factor influencing the extent of recovery [28, 89], where delayed surgical repair results in distal nerve stump fibrosis and Schwann cell atrophy, with progressively reduced axonal regeneration [11]. It is however not clearly defined how long a delay may be tolerated before repair becomes futile. There are clinical cases where there is doubt whether the nerve injury is operable or not and whether it is worth to operate if there has been a time delay after injury. An example where this is an important consideration is the case of obstetrical brachial plexus injuries. Even with modern imaging techniques it is difficult to diagnose and to differentiate between preganglionic and postganglionic plexus injury and microsurgical reconstruction of the brachial plexus is often performed with 3–6 months delay [90] leading to poor recovery of sensation and reduced motor function in the affected limb [91]. The results of paper I demonstrated that following nerve transection with immediate (0) or 1, 3 or 6 months delayed repair with a nerve graft, the number of myelinated axons projecting into the distal stump were drastically reduced following 3 and 6 month delayed repair. The failure for axons to continue from the graft into the chronically denervated distal stump is therefore most likely to be one the main impediments to reinnervation of peripheral targets. In addition, the reduced numbers of reinnervated motoneurons seen after chronic distal nerve and muscle denervation closely match the reduced numbers of motoneurons that regenerate their axons into chronically denervated nerve stumps [41], suggesting either decreased neurotrophic support from Schwann cells or an increase in the expression of molecules inhibitory to regeneration. There was a decline of the Schwann cell marker S100 with time, as well as a reduced number of Scwann cells isolated from the distal nerve stump (Paper I). However, cultured SC from all groups proliferated at similar rates and irrespective of the delay in time to repair all Schwann cell cultures could support neurite outgrowth of the NG108-15 motor neuron like cell line (Paper I). In addition, the reduced number of SCs in the distal nerve stump was parallelled by a progressive increase in fibrosis and proteoglycan scar markers in the distal nerve with increased delayed repair time (Paper I). In summary it can be concluded that one of the main limiting factors for nerve regeneration after delayed repair is the distal stump, where the critical time point after which the outcome of regeneration becomes too poor appears to be 3-months.

Degeneration of the target organ

Another contributing factor to the poor outcome following peripheral nerve injury is prolonged denervation of the target organ, leading to apoptosis of both mature myofibres and satellite cells with subsequent replacement of the muscle tissue with fibrotic scar and adipose tissue [45, 46]. Several studies suggest that muscle phenotype may influence disease progression and a number of clinical studies have reported increased vulnerability of fast fatiguable fibres [49, 50]. However, the precise molecular mechanisms and signaling pathways that control the expression of the key regulators of muscle protein turnover have not been fully defined. In Paper I it was demonstrated that following sciatic nerve transection with delayed repair, the size of fast type fibres was significantly reduced after one month delayed repair, whilst the slow type fibres were not significantly reduced in size until 6 month delayed repair. Thus either the slow type fibers have a greater capacity to maintain their size upon denervation or they recover faster/are preferentially reinnervated than the fast type fibers. Since the soleus and gastrocnemius muscles appeared to respond differently to injury, the muscles were studied in more detail following both nerve transection and nerve crush injury without repair with 1, 7, 14 and 28 days survival (Paper II).

MyoD and myogenin are myogenic transcription factors preferentially expressed in adult fast and slow muscles, respectively [53]. Up-regulation of myogenin in denervated skeletal muscle promotes the expression of acetylcholine receptors [50, 92] and Park et al [49] have demonstrated that myogenin gene transfer into muscle supports spinal cord motors neuron survival and endplate innervation, while myoD gene transfer decrease survival and enhances motor neuron degeneration and muscle denervation. In contrast with the above mentioned study [49], Moresi et al [60] have demonstrated that myogenin binds and activates the promoter regions of the MuRF1 and Atrogin-1 genes, and adult mice lacking myogenin are resistant to neurogenic atrophy. Histone acetylation has been implicated to affect the denervation-dependent changes in skeletal muscle gene expression [50, 92, 93]; HDAC4 and HDAC5 repress the expression of Dach2, which in turn is a myogenin transcriptional repressor [50, 93]. Following nerve injury, several microRNAs are up regulated in an injury dependent pattern, including the muscle specific miRNAs miR-206 and miR-1. miR-206 represses the expression of HDAC4 and thus, in accordance to Moresi et al [60], prevents muscle atrophy. In

addition, Williams et al [61] demonstrated that miR-206 delays ALS progression and promotes regeneration of neuromuscular synapses in mice, possibly through a HDAC4 dependent mechanism. The mice in which HDAC4 was selectively deleted were reinnervated more rapidly than those of controls following nerve crush or cut. However, Soares et al [94] showed that overexpression of miRNA-206 was sufficient to induce a 10% decrease of fibre size in innervated muscles when compared with controls, which was further confirmed by another experiment showing that inhibition of miRNA-206 was sufficient to induce 10% hypertrophy of innervated muscle.

The expression of Atrogin-1 and MuRF1 is, in addition to myogenin [60], regulated by the forkhead box subfamily O (FOXO) transcription factors [56]. Senf et al [56] demonstrated that specific inhibition of FOXO in rat soleus muscle during disuse prevented 40% of muscle fibre atrophy, demonstrating the importance of FOXO signaling in muscle atrophy. Akt inhibit the action of FOXO transcription factors through phosphorylation, leading to FOXO transcription factor retention in the cytoplasm and absent target gene activation [95]. HSPs bind to and prevent dephosphorylation of AKT and thus prohibit FOXO3a nuclear localization [56, 96], where miR-1 appears to mediate an increased protein degradation through HSPs targeting [96].

In Paper II it was demonstrated that the medial gastrocnemius muscle, in comparison to the contralateral side, underwent a much more pronounced atrophy 28 days after injury, compared with the soleus muscle. In addition, in the crush injury model, the soleus muscle showed significantly increased muscle weights 14 and 28 days after injury, which was not the case for the gastrocnemius muscle who continued to atrophy (Paper II). Furthermore the results demonstrated that the gastrocnemius exhibited a pronounced up-regulation of myogenin following injury, whilst a significant up-regulation of MyoD was observed in the soleus muscle at the same time point. In addition the soleus and the gastrocnemius muscles exhibited a contrasting transcriptional regulation of miR-1 which was markedly down regulated in the soleus muscle 1 week post injury, whilst the gene expression was substantially up regulated in the gastrocnemius muscle (Paper II). miR-206 was also markedly elevated in the gastrocnemius 28 days after crush injury (Paper II). Moreover it was demonstrated that MuRF1 and Atrogin-1 were up-regulated

to a significantly higher extent in the gastrocnemius muscle compared with the soleus muscle (Paper II).

Earlier research regarding the role of myogenic transcription factors and microRNAs are conflicting. One could hypothesize that the more pronounced atrophy seen in the gastrocnemius muscle following denervation is due to a higher expression level of miR-1 through a muscle-specific E3 ubiquitin ligases dependent mechanism. miR-1 can mediate down-regulation of HSP, followed by dephosphorylation and nuclear translocation of FOXO and up-regulation of FOXO downstream target genes including MuRF1 and Atrogin-1 with ultimately an increased protein degradation [96]. Another possible explanation for the increased vulnerability of fast fatiguable fibers is that myogenin is expressed in a higher extent in the gastrocnemius muscle compared to the soleus muscle following injury, since myogenin mediates denervation-induced atrophy by binding the promoter regions of the MuRF1 and Atrogin-1 genes [60]. However, as mentioned above, up-regulation of myogenin in denervated muscle promotes the expression of acetylcholine receptors [50, 61] and thus enables a bi-directional signaling between motor neurons and skeletal muscle neuromuscular junctions. Several studies implicate miR-206 in promoting regeneration of neuromuscular synapses and preventing muscle atrophy through a HDAC4 dependent mechanism, raising the possibility that miR-206 might represents an inadequate protecting mechanism, and that the soleus muscle is more resilient to injury because the levels of miR-206 are higher prior to injury [61].

CONCLUSIONS

On the basis of the experimental data presented in this thesis, the following conclusions can be made:

- The distal nerve segment is of critical importance regarding the limitation of nerve regeneration and reinnervation after delayed repair. The ‘‘cut-off’’ point in the experimental rat model seems to be at 3 months, after this point the functional outcome of delayed nerve repair is likely to be limited.

- Following traumatic nerve injuries, the soleus and the gastrocnemius muscles exhibit a contrasting transcriptional regulation of microRNAs, myogenic transcription factors and muscle-specific E3 ubiquitin ligases, which might influence the degree of atrophy.

- Although distal nerve transection has no effect on the survival of the corresponding spinal motoneurons, cell atrophy, synaptic shedding and degeneration of dendrites is induced. Furthermore the results suggest that injury-induced motoneuron degeneration is mediated through apoptosis, involving both the intrinsic and the extrinsic pathways.

- Injury-induced sensory neuronal cell death seems to involve several different apoptotic pathways including the intrinsic and the extrinsic apoptotic pathway, as well as ER-stress mediated apoptosis.

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