Therapeutic approaches for the improvement of peripheral nerve regeneration

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Fabian Szepanowski

aus Kamp-Lintfort

Düsseldorf, Februar 2018

Aus der Klinik für Neurologie
der Heinrich-Heine-Universität Düsseldorf

Gedruckt mit Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Berichterstatter:

1. Prof. Dr. med. Bernd C. Kieseier

2. Prof. Dr. rer. nat. Ulrich Rüther

Tag der mündlichen Prüfung: 20. September 2018

Die vorliegende Arbeit ist eine kumulative Dissertation
gemäß § 6 (4) der Promotionsordnung der
Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf vom 06.12.2013
zur Verleihung des Grades „Doctor rerum naturalium“

“If you need inspiring words, don’t do it.”

- Elon Musk

gewidmet Ellen & Klaus Szepanowski
und Wilma Laqua

Contents

Zusammenfassung

Periphere Nerven ermöglichen die Weiterleitung von efferenten und afferenten Signalen zwischen dem zentralen Nervensystem (ZNS) und den Organsystemen. Mechanische sowie immunvermittelte Schäden können die Funktionalität des peripheren Nervensystems (PNS) dramatisch beeinträchtigen, was mit dem Auftreten schwerwiegender Symptome einschließlich neuropathischer Schmerzen, Parästhesien, Lähmungen sowie vegetativen Störungen einhergehen kann. Im Gegensatz zum ZNS besitzen periphere Nerven zwar eine bemerkenswerte Regenerationsfähigkeit; das Maß an Regeneration nach einer Nervenverletzung oder bei entzündlichen Nervenerkrankungen ist langfristig jedoch häufig unzureichend. Bis heute gibt es keine zugelassene Therapie zur Förderung der Nervenregeneration. Das Ziel dieser Arbeit ist es daher, bereits zugelassene sowie in Testung befindliche immunmodulatorische Medikamente auf ihre neuroregenerativen Eigenschaften zu untersuchen. Verschiedene therapeutische Ansätze werden in begutachteten (‚peer-reviewed') Publikationen vorgestellt.

Es konnte gezeigt werden, dass der Fumarsäureester Dimethylfumarat (DMF) die periphere Nervenregeneration nach einer mechanischen Verletzung in C57BL/6-Mäusen deutlich verbesserte. Die DMF-Behandlung führte zu einer Normalisierung der Griffstärke und einer erhöhten Nervenleitgeschwindigkeit, die mit einer beschleunigten Remyelinisierung korrelierte. Diese Verbesserungen gingen mit der Aktivierung des NF-E2 related factor 2 (Nrf2) Transkriptionsfaktors einher, welcher die Expression des zytoprotektiven und entzündungshemmenden Enzyms Hämoxygenase-1 (HO-1) induziert.

In einem zweiten Ansatz wurde die Wirksamkeit des Immunsuppressivums Fingolimod auf die Nervenregeneration untersucht. Durch die Verwendung von immundefizienten Mausstämmen konnte gezeigt werden, dass Fingolimod, unabhängig vom immunsuppressiven Effekt, die Nervenregeneration fördert. Es kam zu einer Erhöhung des axonalen zyklischen Adenosinmonophosphats, einem förderlichen Faktor für axonales Auswachsen, sowie einer Verbesserung der Myelindicke. Der remyelinisierende Effekt korrelierte mit einer transienten Reduktion der Lysophosphatidsäure (LPA), eines demyelinisierenden Signallipids. Um die demyelinisierende Wirkung von LPA besser zu verstehen, wurden myelinisierte Hinterstrangganglien-Kulturen entweder ausschließlich mit LPA oder in Kombination mit dem LPA_1-Rezeptor-Antagonisten AM095 behandelt. LPA bewirkte eine Demyelinisierung, Schwannzell-Dedifferenzierung und die Induktion von Tumornekrosefaktor alpha (TNF-α), während diese Effekte bei gleichzeitiger Behandlung mit AM095 ausblieben. Des Weiteren wurde AM095 vor der Beibringung einer Nervenverletzung C57BL/6-Mäusen verabreicht, was eine Reduktion der Anzahl dedifferenzierter Schwannzellen sowie eine Verringerung der TNF-α Expression in myeloiden Zellen zur Folge hatte. Die Ergebnisse weisen auf eine Rolle von LPA bei der Entwicklung eines Schwannzell-Phänotyps hin, der charakteristisch für Nervenverletzungen ist und sich typischerweise durch eine Herunterregulierung von myelinisierenden Faktoren sowie der Induktion inflammatorischer Zytokine auszeichnet.

Insgesamt haben die in diese Arbeit eingepflegten Publikationen zur Identifizierung von therapeutischen Angriffspunkten zur Förderung der Regeneration bei Verletzungen und entzündlichen Erkrankungen des peripheren Nervensystems beigetragen, deren klinische Relevanz es in weiteren Studien zu untersuchen gilt.

Abstract

Peripheral nerves facilitate the propagation of efferent and afferent signals between the central nervous system (CNS) and the organ systems. Mechanical or immune-driven damage can dramatically impair proper functioning of the peripheral nervous system (PNS), which is associated with the emergence of severe symptoms including neuropathic pain, paresthesia, paresis as well as vegetative disorders. In contrast to the CNS, peripheral nerves exert a remarkable ability to regenerate. However, long-term recovery after nerve injury and in patients suffering from neuroinflammatory diseases is often insufficient. No therapy to facilitate peripheral nerve regeneration has been introduced to clinical practice thus far. Therefore, this thesis aims at investigating the neuroregenerative potential of clinically approved as well as emerging classes of immunomodulatory drugs. Different therapeutic approaches are presented in peer-reviewed publications.

It could be demonstrated that the fumaric acid ester dimethyl fumarate (DMF) markedly enhanced peripheral nerve regeneration following mechanical injury in C57BL/6 mice. DMF treatment resulted in a normalization of grip strength and increased nerve conduction velocity paralleled by considerably accelerated remyelination. These improvements were linked to an activation of the NF-E2 related factor 2 (Nrf2) transcription factor driving the expression of the cytoprotective and anti-inflammatory enzyme heme oxygenase-1 (HO-1).

In a second approach, the efficacy of the immunosuppressive drug fingolimod in nerve regeneration was investigated. Using immunodeficient mouse strains, it could be demonstrated that fingolimod promotes nerve regeneration independently of its immunosuppressive potency. Fingolimod treatment increased axonal cyclic adenosine monophosphate, a limiting factor for axonal regrowth, and was associated with improved myelin thickness. The remyelinating effect was correlated with a transient reduction of lysophosphatidic acid (LPA), a demyelinating lipid mediator.

To better understand the demyelinating effect of LPA, myelinated dorsal root ganglia cultures were treated with either LPA alone or in combination with the LPA_1 receptor antagonist AM095. LPA treatment was associated with demyelination, Schwann cell dedifferentiation and the induction of tumor necrosis factor alpha (TNF-α), while concomitant AM095 treatment abolished these effects. Administration of AM095 to C57BL/6 mice prior to nerve injury reduced Schwann cell dedifferentiation and decreased TNF-α expression in myeloid cells. These results suggest a crucial role for LPA in the emergence of a post-injury Schwann cell phenotype typically characterized by the downregulation of myelination-associated genes and the induction of inflammatory cytokine release.

Collectively, these publications have identified therapeutic candidates that may foster regeneration in the injured or inflamed PNS. Understanding the clinical relevance of these findings warrants further investigation.

I. Structure of this thesis

This thesis compiles four publications that explore the therapeutic potential of clinically established and emerging classes of immunomodulatory drugs for the promotion of peripheral nerve regeneration. The publications are presented in the following thematic rather than chronological order:

1. Szepanowski F, Donaldson DM, Hartung HP, Mausberg AK, Kleinschnitz C, Kieseier BC, Stettner M (2017) **Dimethyl fumarate accelerates peripheral nerve regeneration via activation of the anti-inflammatory and cytoprotective Nrf2/HO-1 signaling pathway.** *Acta Neuropathol.* 133(3):489-491 (original research)

2. Szepanowski F, Derksen A, Steiner I, Meyer zu Hörste G, Daldrup T, Hartung HP, Kieseier BC (2016) **Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling.** *J. Neuroinflammation* 13:143 (original research)

3. Szepanowski F, Kieseier BC (2016) **Targeting lysophospholipid signaling as a therapeutic approach towards improved peripheral nerve regeneration.** *Neural. Regen. Res.* 11(11):1754-1755 (perspective article)

4. Szepanowski F, Szepanowski LP, Mausberg AK, Kleinschnitz C, Kieseier BC, Stettner M (2018) **Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling.** *Neurosci. Lett.* 662:136–141 (original research)

As these papers provide background and methodological information by themselves, in the following a brief general introduction into developmental and pathophysiological mechanisms of the peripheral nervous system is given to allow for a broader understanding of the topic. To conclude, the publications will be summarized and set into scientific context.

II. Introduction

II.I. The basic structure of the peripheral nervous system

The vertebrate nervous system is divided into two major compartments: The central nervous system (CNS) comprising the brain and spinal cord, and the peripheral nervous system (PNS) enabling the propagation of efferent and afferent signals between the CNS and the organ systems. The PNS is further subdivided into the autonomic nervous system, which subconsciously impacts the function of internal organs, and the somatic nervous system associated with conscious movements and sensory recognition. The majority of nerves are known to be “mixed” nerves, conducting autonomic, sensory and motor signals. Sensory neurons are contained in structures termed dorsal root ganglia, whereas motor and autonomic neurons are located in the ventral and lateral horn, respectively. Passing from proximal to distal, dorsal and ventral roots combine to form spinal nerves which ultimately give rise to nerve plexus and peripheral nerves (Stewart 2003).

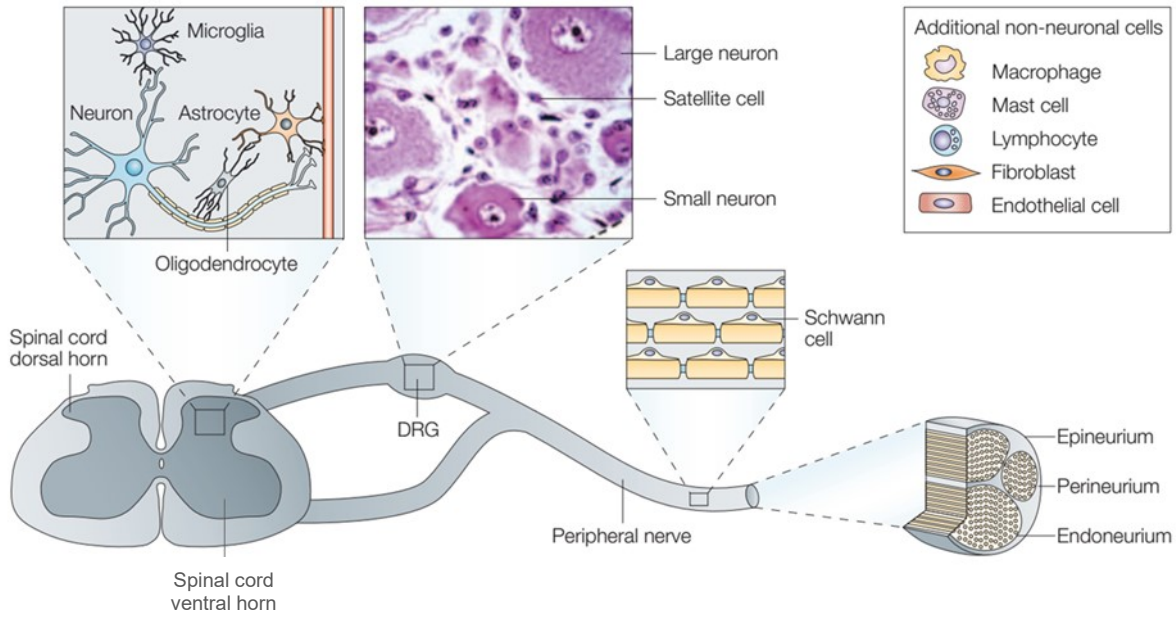


Figure 1: Schematic illustration of the peripheral nervous system. Afferent sensory neurons are contained in dorsal root ganglia (DRGs), efferent motor and autonomic neurons in the ventral and lateral horn. Projecting from proximal to distal, axons of both structures give rise to spinal nerves which finally derive to peripheral nerves. Oligodendrocytes represent the myelinating glial cells of the CNS, and Schwann cells fulfill an equivalent role in the PNS. In addition to myelinating Schwann cells in the peripheral nerve, DRGs home the so-called satellite cells, non-myelinating Schwann cells surrounding sensory neurons to provide a protective milieu. The peripheral nerve is partitioned into endoneurium, perineurium and epineurium. The endoneurium comprises a matrix of connective tissue axons with associated Schwann cells are embedded in. The endoneurium is enclosed by the perineurium to form fascicles. These fascicles and blood vessels supplying the nerve are enclosed by epineurial tissue. (artwork adapted from White et al. 2005).

A convenient way of understanding the fundamental architecture of a peripheral nerve is its evaluation in a transverse section (figure 1). Here it becomes clear that peripheral nerves essentially home bundles of axons, projections of neurons conducting electrical impulses. These bundles are termed fasciculi. A nerve can be composed of one to multiple fasciculi which are contained within the epineurium, a protective sheath composed mainly of connective tissue. The epineurium fulfills important structural roles by holding fasciculi together and exerting a cushioning effect against mechanical stresses (Sunderland 1990).

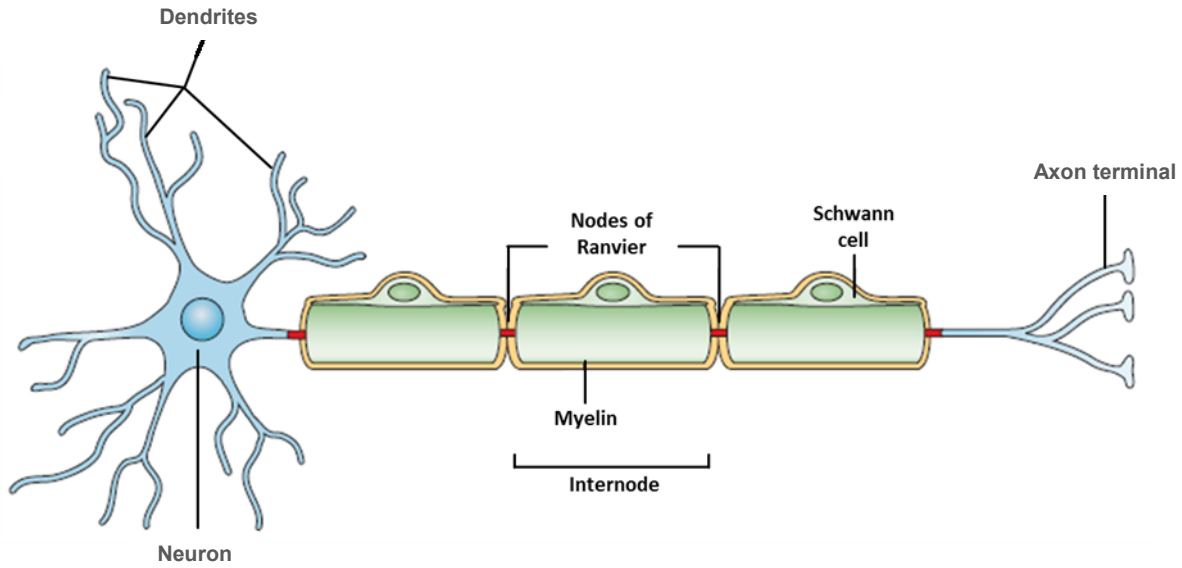


Figure 2: Functional architecture of a peripheral neuron. Neurons are electrically excitable cells that receive, process and transmit information to other neurons or innervated tissues and organs. Neurons basically possess two different types of projections, generally referred to as neurites: multiple branched dendrites function to sense electrochemical stimuli received from other neurons, whereas one long projection, the axon, enables long-distance signal propagation. Axons with a diameter of ≥ 1 µm are typically wrapped by myelin, multilayered lipid-rich sheaths with insulating properties formed by Schwann cells. The myelinated regions are termed internodes; the interjacent uninsulated regions are referred to as nodes of Ranvier. Nodal regions are enriched with voltage-gated but also ATP-dependent ion-channels, facilitating rapid de- and repolarization of the axonal membrane, the action potential. Myelination reduces the current flow along the axonal membrane, allowing fast saltatory conduction from node to node while reducing the metabolic requirements for neuronal activity. (artwork adapted from Poliak and Peles 2003)

Axons are closely associated with Schwann cells, the glial cells of the PNS, which form multilayered insulating myelin sheaths that enable saltatory signal propagation (figure 2). Myelination increases nerve conduction velocity up to 100-fold compared to unmyelinated axons. Moreover, it drastically reduces axonal energy consumption for the restoration of ion gradients by confining the generation of action potentials to only 0.5 percent of the axonal surface area. Schwann cells fulfill a broad range of functions beyond building myelin sheaths. Most notable among these may be their

role in axon development and maintenance by providing metabolic support and the release of neurotrophic factors (Nave 2010[a, b]).

II.II. Developmental mechanisms of axon growth and myelination

A plethora of signaling molecules, receptors and transcription factors orchestrates the complex processes of axon growth, guidance and myelination. Understanding the fundamentals of these in a developmental context appears crucial to identify therapeutic targets that may have relevance for the improvement of nerve regeneration.

The most basic question may be how neurons are polarized to build one axon and how axon outgrowth is subsequently maintained. With regard to neuron-intrinsic processes, the answer appears as simple as fascinating: subcellular accumulations of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) determine whether an axon or dendrites will be formed. In that process, cAMP and cGMP reciprocally inhibit each other via an activation of specific phosphodiesterases catalyzing the degradation of the respective nucleotides. The development of only one axon per neuron but multiple dendrites has been attributed to the remarkable phenomenon that cAMP accumulation in one neurite causes long-range inhibition of cAMP formation in the remaining neurites, which show a corresponding increase in cGMP. This long-range inhibitory mechanism is exclusively observed for cAMP, but not cGMP, providing a conclusive scenario of how neurite identity is specified (Shelly et al. 2012).

How is the outgrowth of axons and guidance to innervation targets accomplished? Numerous guidance molecules have been identified, including netrins, semaphorins and ephrins, most of which have been studied in the nematode *Caenorhabditis elegans* or the fruit fly *Drosophila melanogaster*. Semaphorins and ephrins represent chemorepulsive cues, whereas netrins mediate chemoattraction or –repulsion, depending on the type of netrin receptor expressed on the axonal growth cone (Dickson 2003). Upon binding to the netrin receptor DCC (Deleted in Colorectal Cancer), axons elongate and turn towards the netrin gradient. In contrast, the netrin receptor UNC-5 mediates repulsion. Interestingly, co-expression of UNC-5 and DCC in the same growth cone switches DCC-mediated attraction to UNC-5/DCC receptor complex mediated repulsion (Hong et al. 1999). The attractant and elongating effect of netrin on axons can be inhibited by addition of competitive cAMP analogues or

inhibition of the common cAMP downstream target protein kinase A (Ming et al. 1997), suggesting that an accumulation of cAMP is not only required to determine an axonal fate for neurites, but subsequently facilitate its elongation. The developmental outgrowth of axons is further promoted by neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF; despite its name also expressed in the PNS) and neurotrophin-3 (NT-3), all of which signal through tropomyosin receptor kinases (Tucker et al. 2003).

Certainly, one of the best-understood regulators of myelination is Neuregulin/ErbB signaling, which has been prominently featured in the development of the peripheral nervous system over the past decade (figure 3).

Schwann cells are known to derive from neural crest cells arising from the dorsal neural tube. Neural crest cells migrate to various sites and differentiate into a great number of diverse cell types. The ErbB3 receptor subtype is highly expressed in neural crest cells and appears to be involved in their migratory processes. While ErbB3 expression is lost in most neural crest derivatives over time, it is maintained in Schwann cells and seems critical for Schwann cell migration along developing axons as well as adjusting Schwann cell proliferation (Birchmeier 2009). These processes are dependent on the axonal presentation of the ErbB ligand neuregulin-1 (Nrg-1). Nrg-1 can bind to ErbB3 and ErbB4 receptors, of which only ErbB4 has tyrosine kinase activity. The ErbB2 receptor has such a functional kinase domain as well, but cannot bind Nrg-1. Given that ErbB4 expression is negligible in Schwann cells, Nrg-1 signaling is widely considered to be mediated by a heterodimeric ErbB2/ErbB3 receptor complex (Nave and Salzer 2006).

Neuregulin/ErbB signaling between axons and Schwann cells provides an elegant explanation of how Schwann cells regulate myelin thickness with respect to axon diameter and why very small caliber axons (< 1 µm in diameter) are typically non-myelinated. The regulation of myelination requires the expression of membrane-associated Nrg-1 type III on axons and expression of ErbB2/3 receptors on Schwann cells. Whereas ErbB2 and ErbB3 receptors are expressed at saturating levels, the amount of Nrg-1 type III presented by axons correlates with myelin thickness (Nave and Salzer 2006). Studies employing mice that are heterozygous for Nrg-1 display a significant reduction in myelin thickness, whereas overexpression in Nrg-1 transgenic mice results in hypermyelination; in these mice, small caliber non-myelinated axons may become myelinated as well (Michailov et al. 2004; Taveggia et al. 2005). As such, axon diameters of approximately 1 µm apparently set a threshold level for

myelination to occur in wildtype mice. Above this threshold, the correlation of Nrg-1 expression and axon diameter (and therefore its surface area) determines myelination and myelin thickness, respectively. Schwann cells associated with very small caliber sensory axons will typically differentiate into Remak cells, a specialized non-myelinating type of Schwann cell. Remak cells engulf multiple sensory axons to provide trophic support and structural integrity in Remak bundles (Nave and Salzer 2006).

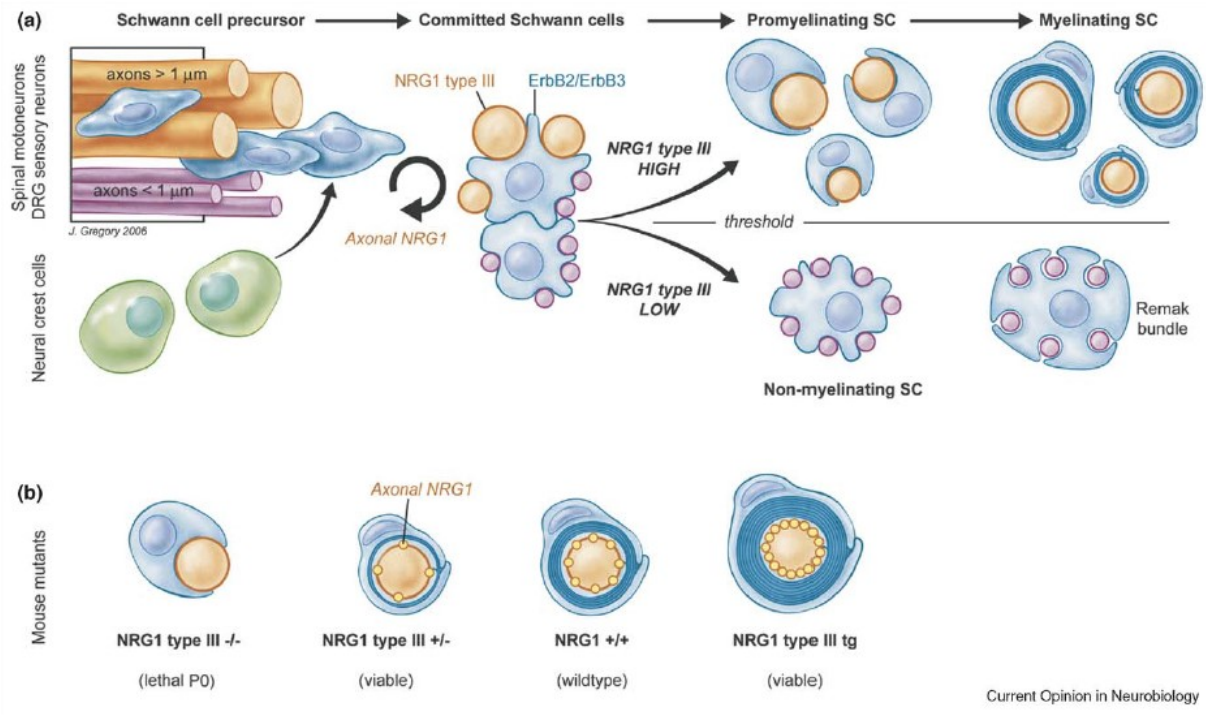


Figure 3: Axonal Neuregulin-1 type III (Nrg-1) levels determine myelin sheath thickness. (a) Presentation of axonal Nrg-1 to ErbB2/3 expressing Schwann cells is essential throughout the development of the peripheral nervous system and involved in the promotion of glial cell fate, migration of Schwann cell precursors along axons, proliferation and finally differentiation into myelinating Schwann cells or non-myelinating Remak cells. (b) The amount of Nrg1 with regard to the axonal surface area determines myelin thickness. (artwork from Nave and Salzer 2006)

At the transcriptional level, a major regulator for the generation of the peripheral glial lineage is the transcription factor Sox10 (sex determining region Y (SRY)-box 10). Sox10 is involved in the regulation of ErbB3 expression in neural crest cells. Not surprisingly, Schwann cells fail to develop properly in Sox10 mutant mice, leading to neurodegeneration at later developmental stages (Britsch et al. 2001; Paratore et al. 2001). However, Sox10 is not only required for the specification of the glial cell fate,

but has more recently been identified as a critical factor for the maintenance of the myelinating phenotype in adult mice. Bremer and colleagues (2010) demonstrated demyelination to occur after Schwann cell-specific, tamoxifen-inducible conditional inactivation of Sox10. Interestingly, Sox10-depleted Schwann cells remained vital, but showed signs of dedifferentiation, indicating that Sox10 expression in the adult peripheral nerve is not essential for survival, but rather for maintaining the myelinating phenotype. In concert with the transcription factor Oct6, Sox10 initiates the expression of Krox-20, a master regulator of Schwann cell myelination (Reiprich et al. 2010; Topilko et al. 1994).

II.III. Wallerian degeneration: Degenerative events after peripheral nerve injury

Peripheral nerve injury causes a partial or total loss of motor, sensory and autonomous functions as a result of nerve degeneration distal from the point of injury. The degenerative processes following peripheral nerve injury are generally referred to as Wallerian degeneration, named after British neurophysiologist August Waller. Wallerian degeneration is characterized by Schwann cell dedifferentiation, inflammation and energy depletion leading to demyelination and axon disintegration (Coleman and Freeman 2010; Gaudet et al. 2011). Before specifying the course of events in Wallerian degeneration in further detail, it should be noted that nerve injuries are typically classified into three major groups: neurapraxia, axonotmesis and neurotmesis (Seddon 1943). Neurapraxia is the least severe condition characterized by local damage secondary to compression. It does not lead to Wallerian degeneration and full recovery is commonly achieved within days to weeks. Axonotmesis is defined as a disruption of the longitudinal continuity of axons with a varying degree of connective tissue elements – endo-, peri- and epineurium – remaining intact. Axonotmesis results in Wallerian degeneration and regeneration usually takes several months to years, with functionality being restored incompletely in many cases. Neurotmesis describes the complete disruption or transection of a nerve and therefore represents the most severe type of injury. As spontaneous recovery is negligible, surgical reconstruction of the nerve is required. However, despite modern surgical techniques, less than half of patients who undergo nerve repair fully regain motor and sensory functions (Lee & Wolfe 2000).

As incomplete injury of the axonotmesis type occurs considerably more frequently than neurotmesis, the pathophysiology of nerve injuries is commonly studied in rodents subjected to axonotmesis of the sciatic nerve, usually referred to as “sciatic nerve crush” (Bridge et al. 1994).

Wallerian degeneration involves nerve-intrinsic as well as innate and adaptive immune responses. In rodents, the initial response to injury occurs within 24 hours and is characterized by Schwann cells detaching from their associated axons accompanied by the degeneration of the insulating myelin sheaths. Myelinating Schwann cells dedifferentiate and subsequently start proliferating. Dedifferentiation of the highly specialized Schwann cells is, at least in part, mediated by transcription factors Sox-2 (sex determining region Y (SRY)-box 2), c-Jun and Egr-1/Krox-24 (early growth response protein 1) (Jessen and Mirsky, 2008; Gaudet et al. 2011).

Schwann cells and resident macrophages are among the first cells to recognize the injury via toll-like receptors (TLR) (Goethals et al. 2010). While TLRs are well known for their role in innate immune cell activation in response to microbial infections, TLRs can also be activated by endogenous ligands such as mRNA (Kariko et al. 2004) or heat shock proteins (Vabulas et al. 2001) that leak into the extracellular space after tissue injury. As a consequence of TLR stimulation, Schwann cells and resident macrophages secrete pro-inflammatory cytokines such as TNF-α or chemokines, i.e. MCP-1 (monocyte chemoattractant protein 1), leading to the recruitment of hematogenous monocytes and macrophages, respectively (Lee et al. 2006; Toews et al. 1998).

Rapidly, within 1.5 days, axons undergo fragmentation and degenerate from the distal innervating end of the nerve to the proximal site of injury (Coleman and Freeman 2010). Although significant gaps remain in the understanding of how this spatially restricted degeneration of the distal axon segment is accomplished, an emerging body of experimental evidence has narrowed the search to basically two possible mechanisms: 1) The abrogation of axonal transport causes a deprivation of critical metabolic and/or survival factors or 2) a genetically coded and active mechanism for axonal destruction is triggered (Gerdts et al. 2016). Thus far, both mechanisms appear to contribute to axon degeneration. Disintegration of the axon has been suggested to result from energetic failure and ATP depletion, leading to an imbalance in ion homeostasis with de-regulated Ca^{2+} influx and subsequent activation of calcium-dependent proteases such as calpain (Yang et al. 2013, Gerdts et al. 2016). However, the molecular trigger for this destructive cascade has long

been unknown. Just in recent years accumulating evidence has identified SARM1 (sterile α-motif-containing and armadillo-motif containing protein), a toll-like receptor adaptor family protein, as an essential factor for the rapid onset of Wallerian degeneration. In SARM1-deficient mice, morphological signs of Wallerian degeneration are delayed by 2-3 weeks. This axonoprotective effect has been linked to a preservation of axonal NAD^+ (nicotinamide adenine dinucleotide) levels (Osterloh et al. 2012, Gerdts et al. 2015). Depletion of NAD^+ after axonal injury correlates with a rapid decline in axonal ATP levels, which can be prevented by inactivation of SARM1 (Summers et al. 2016). As the exact function of SARM1 is still unknown, the question arises whether injury-induced activation of SARM1 facilitates NAD^+ depletion via an intrinsic enzymatic activity or by recruiting a yet unidentified downstream effector. Recently published work by Essuman and colleagues (2017) indeed indicates that SARM1 drives axon degeneration via an intrinsic NAD^+ cleavage activity. Nevertheless, the mechanism of SARM1 activation remains to be elucidated.

To allow for axon regrowth from the largely unaffected proximal stump, a permissive environment needs to be established. Invading and resident macrophages as well as dedifferentiated Schwann cells start to phagocytize cellular and myelin debris (Bigbee et al. 1987; Stoll et al 1989). While it was long believed that dedifferentiating Schwann cells would segregate from their own myelin sheaths and then phagocytize extracellular myelin debris, this issue has been revisited in recent years. Emerging lines of evidence suggest that the initial phase of myelin breakdown is characterized by the division of the myelin sheaths into small oval-shaped intracellular fragments. These small fragments may become degraded by a selective form of autophagy, termed myelinophagy (Jessen and Mirsky 2016; Gomez-Sanchez et al. 2015). As such, not exclusively phagocytosis, but also specific Schwann cell autophagy seems to contribute to the clearance of myelin.

The removal of myelin debris appears to be a critical step allowing for axonal sprouting, as it contains so-called ‘myelin associated inhibitors of axonal regeneration’. Three have been identified so far: Nogo, myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMgp) (Wong et al. 2002; Wang et al. 2002; Liu et al. 2002). These inhibitors are thought to at least partly function via the Nogo-p75-neurotrophin (Nogo-p75-NTR) receptor complex that is expressed on the growth cone of axons. This receptor complex has been implicated in a $G_{i/o}$ mediated inhibition of adenylate cyclase, causing a reduction of

axonal cAMP (Filbin 2003; Hannila & Filbin 2008). In addition to its substantial role in nervous system development, axonal cAMP has been demonstrated to be a major regulator of axon regeneration and is known to determine the speed of axonal regrowth in a concentration-dependent manner (Kilmer & Carlsen 1984; Pearse et al. 2004; Qiu et al. 2002). Consistently, an active transport of adenylate cyclase to axonal growth cones has been described (Carlsen 1982). Hence, effective myelin clearance is required to provide a permissive milieu for axonal sprouting and outgrowth after injury. In that regard, it was previously shown that axon regeneration is strongly delayed in B-lymphocyte-deficient JHD mice. The impairment of regenerative capacity in these mice was linked to a lack of autoantibodies against myelin debris, supporting macrophage recruitment and an efficient and timely myelin clearance (Vargas et al. 2010).

Additionally, cAMP is not only a relevant factor for axon regeneration, but is also required for redifferentiation and the induction of myelination in Schwann cells (Monje et al. 2009). However, it remains to be elucidated whether myelin debris would affect cAMP levels in Schwann cells which, despite known expression of p75-NTR in dedifferentiated Schwann cells, were found to be devoid of Nogo receptor mRNA (Josephson et al. 2002), although evidence for this is limited.

In the injured nerve, Schwann cells do not exclusively function as phagocytizing cells, but actively support regeneration by releasing trophic factors such as nerve growth factor (NGF) and by providing guidance to outgrowing axons in the bands of Büngner, tube-like Schwann cell alignments directing axons towards prospective reinnervation targets (Jessen and Mirsky 2016).

Redifferentiation of Schwann cells towards a myelinating phenotype is accomplished by expression of transcription factors such as Sox-10 and Oct-6 which concertedly induce the expression of Krox-20 (Jessen and Mirsky 2008). Schwann cells have been shown to transiently express neuregulin-1 type I. In contrast to the membrane-resident Nrg-1 type III presented on axons, type I is released from Schwann cells as a soluble factor which is thought to promote differentiation and thus remyelination in an autocrine/paracrine manner (Stassart et al. 2013).

Aside from the aforementioned emerging role of B-lymphocytes for the removal of myelin debris, the contribution of the adaptive immune system to mechanical nerve injuries with regard to T-lymphocytes is less clear and somewhat controversial. T-lymphocytes are considered to be the last type of immune cells to infiltrate the nerve after injury, reaching peak numbers between seven and 21 days post-injury,

depending on the injury model. T-lymphocytes have been reported to contribute to neuropathic pain (Moalem et al. 2004). On the other hand, autoreactive T-lymphocytes against myelin basic protein have been implicated in neuroprotection by the secretion of neurotrophins in the injured optic nerve (Moalem et al. 1999, 2000). As such, the role of T-lymphocytes in nerve de- and regeneration is not well characterized as well as the nature of specific subsets of T-lymphocytes in the injured nerve.

However, it is clearly evident that a chronologically well-regulated interplay of nerve-intrinsic and immune responses is required for the controlled and successful degeneration and regeneration of the peripheral nerve. The hallmarks of Wallerian degeneration are summarized in figure 4.

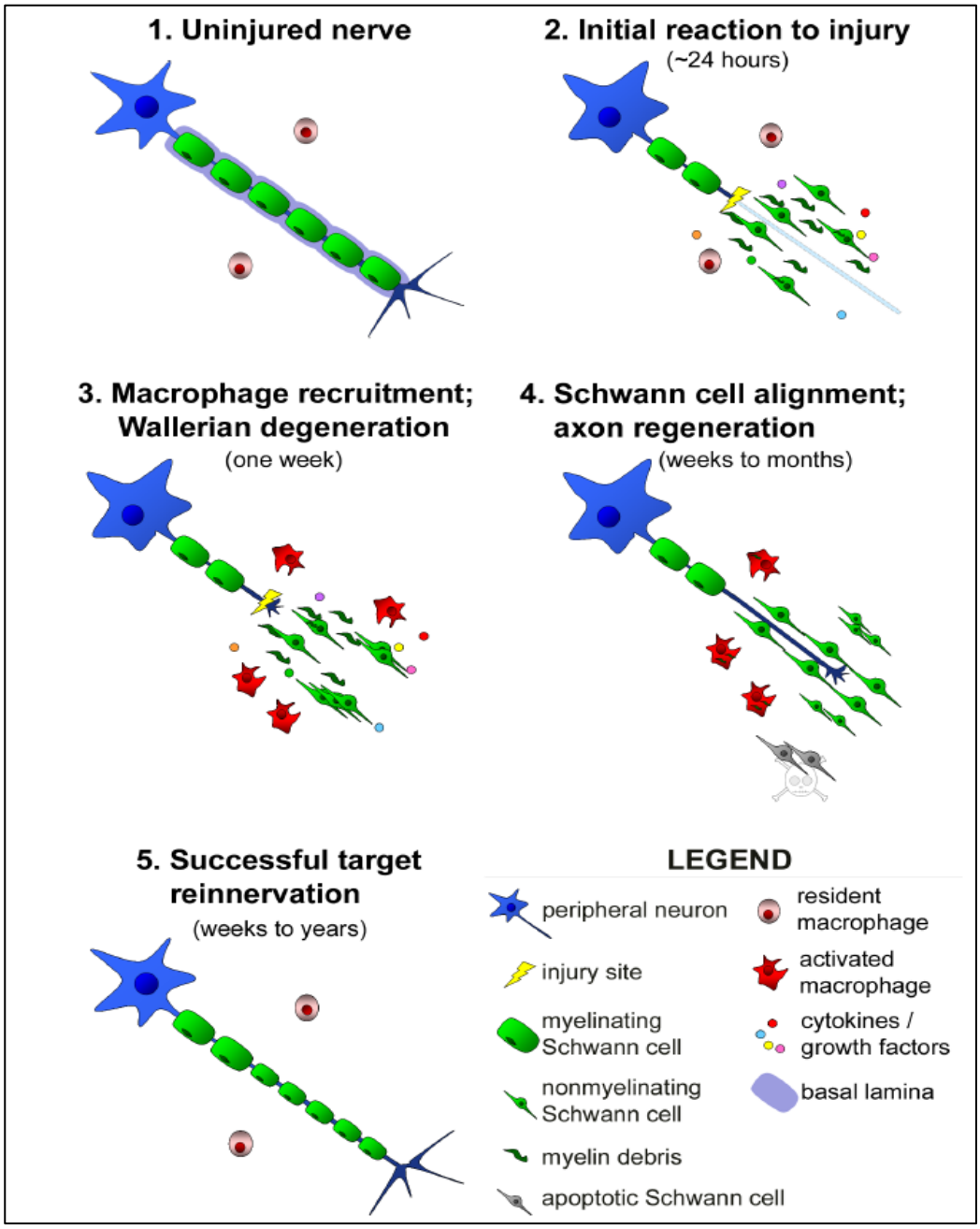


Figure 4: Course of events during Wallerian degeneration and nerve regeneration.

Injury of an intact axon (1) results in degenerative processes mainly consisting of the detachment of the axon from its target cell, breakdown of the associated myelin sheath and fragmentation of the axon distal from the injury site. Dedifferentiated Schwann cells and resident macrophages secrete cytokines to trigger an inflammatory response (2). Cellular and myelin debris is removed by macrophages and phagocytizing Schwann cells (3). Schwann cells form bands of Büngner, providing trophic support and guidance to the regenerating axon (4) which may finally lead to successful reinnervation (5). As schematically indicated (5), the myelin sheath is thinner in the distal regenerated part of the axon than in the largely unaffected proximal part (artwork from Gaudet et al. 2011).

II.IV. Neuroimmunological disorders: Immune-mediated attacks against the nervous system

Innate and adaptive immune responses are known to be involved in central and peripheral nervous system homeostasis and regeneration. However, a deregulation of immunological processes can lead to autoimmunity and neurological disorders. Although the heterogenous pathogenesis of neuroinflammatory diseases remains a matter of debate and active research, preceding infectious diseases have repeatedly been associated with the onset of neuroinflammation. It was shown that autoreactive T-lymphocytes specific to myelin or axonal antigens can cross-react with microbial and viral peptides; similarly, cross-reactivity has also been identified for B-lymphocyte responses. This similarity to foreign antigens – termed “molecular mimicry” – may provide an explanation of how an autoimmune reaction against distinct compartments of the nervous system may be initiated (Wucherpfennig et al. 1995, 1997; Kieseier et al. 2002; Hemmer et al. 2002).

One of the most prevalent and perhaps most publicly recognized inflammatory diseases of the central nervous system is multiple sclerosis, characterized by immune cell invasion, leading to chronic inflammation and demyelination, which may finally result in axonal loss and consequently to disability (Hemmer et al. 2002).

In analogy to multiple sclerosis, activation and infiltration of immune cells specific to peripheral nerve antigens can lead to the development of polyneuropathies. Immune-mediated polyneuropathies represent a heterogeneous group of diseases comprising both acute forms, such as Guillain-Barré-Syndrome (GBS), and chronic forms, i.e. chronic inflammatory demyelinating polyneuropathy (CIDP). Several variants and subforms exist (Hughes and Cornblath 2005; Köller et al. 2005).

The incidence of GBS ranges from 0.6 to 4 cases per 100,000 individuals per year throughout the world. First symptoms typically include pain, weakness in the limbs, numbness and paresthesia. Involvement of the autonomic nervous system is commonly observed, causing cardiac arrhythmia, hypertension and urine retention, inter alia. Up to 25 % of patients require mechanical ventilation and between 4 - 15 % succumb to the disease. After 2-4 weeks, nearly all patients experience a variable plateau phase followed by a recovery phase that may last weeks to several months. Approximately 20 % of patients remain disabled after a year. Even in the case of good recovery, long-lasting residual weakness is recognized in a significant number of patients (Hughes and Cornblath 2005).

The prevalence of CIDP is considered to be 1-2 of 100,000 individuals, characterized by a progressive worsening of symptoms for more than two months. The beneficial effects of immunomodulatory therapies in both GBS and CIDP support the idea of an immune-mediated pathogenesis for either condition (Köller et al. 2005).

Despite a separation of the PNS from the systemic immune compartment by the blood-nerve-barrier (BNB), the BNB appears fragmentary at the most proximal and distal sites of the nerve: nerve roots, dorsal root ganglia and nerve terminals are considered permeable structures enabling the entrance of autoreactive lymphocytes as well as macrophages. BNB transmigration is further promoted by the expression of specific adhesion molecules as well as immune cells secreting matrix metalloproteinases, catalyzing the degradation of extracellular matrix (Kieseier et al. 1998, 2012).

Classically, CD4-positive T-lymphocytes have been categorized into T-helper lymphocytes type I (T_H1), which mediate cellular immunity, and T-helper lymphocytes type II (T_H2) necessary for humoral immune responses. Both of these orchestrate the inflammatory process (Fig. 5) (Meyer zu Hörste et al. 2007). Pro-inflammatory T_H1 cells are responsible for the activation of macrophages as effector cells of peripheral neuroinflammation. T_H1 cells produce cytokines such as TNF-α and their signature cytokine interferon-gamma (IFN-γ) which activates macrophages and Schwann cells to release inflammatory mediators by themselves and counteracting the development of T_H2 cells (Zhang et al. 2013_a; Murwani et al. 1996). In endothelial cells, IFN-γ increases the expression of the adhesion molecule VCAM-1, a critical step in the process of vascular transmigration of invading immune cells expressing α4-integrin (Enders et al. 1998; Leussink et al. 2002). Serum levels of IFN-γ and TNF-α have been recognized to be elevated in GBS and TNF-α levels correlate with the severity and clinical course of the disease (Zhang et al. 2013_a).

The T_H2 signature cytokine interleukin 4 (IL-4) inhibits the differentiation of T_H1 cells and reduces the secretion of IFN-γ and TNF-α. Contrary to T_H1 cytokines, IL-4 has been associated with the remission phase of GBS (Nyati et al. 2011). IL-4 contributes to the release of IL-10 from T_H2 cells, partly in an autocrine manner, and both IL-4 and IL-10 dampen cytokine production in activated macrophages (Paul and Zhu 2010; Saraiva and O'Garra 2010). Two subtypes of macrophages can be distinguished: While T_H1 cells promote the activation of macrophages towards the M1 phenotype, inducing phagocytic activity, pro-inflammatory cytokines and cytotoxic mediators such as reactive oxygen and nitrogen species, T_H2 cells contribute to the

induction of the protective M2 phenotype. M2 macrophages show high expression of anti-inflammatory cytokines such as IL-10 and may be involved in the termination of the inflammatory process (Meyer zu Hörste et al. 2007; Zhang et al. 2013a).
However, T_H2 cells may also contribute to the chronic progressive character of CIDP by stimulating persistent autoantibody production in B-lymphocytes (Horiuchi et al. 2001).

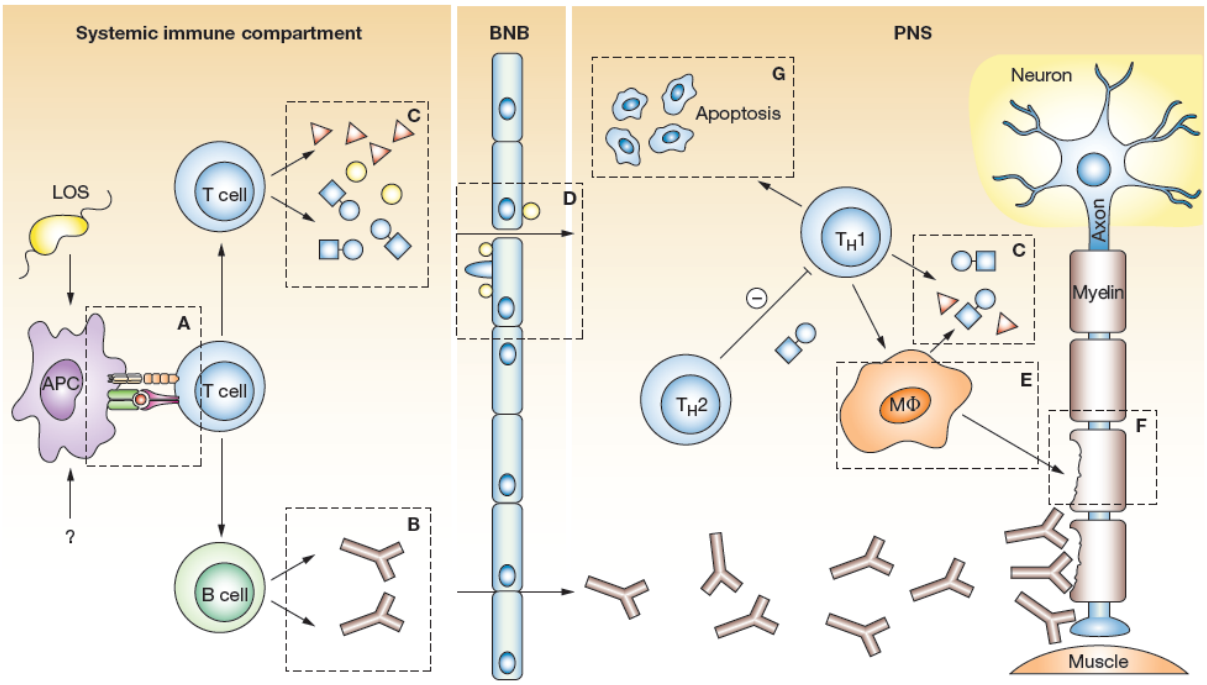


Fig. 5: Pathogenetic mechanisms of peripheral nerve inflammation. (A) Following an infectious disease, antigen presenting cells (APC) may activate T-lymphocytes via the presentation of microbial or viral epitopes resembling endogenous antigens of the peripheral nerve. Activated T-lymphocytes (B) stimulate B-lymphocytes to release autoantibodies and (C) secrete pro-inflammatory cytokines and chemokines. (D) T-lymphocytes invade the nerve via expression of adhesion molecules and matrix metalloproteinases degrading extracellular matrix. (E) Attracted macrophages (Mϕ) directly phagocytize myelin and release cytokines as well as cytotoxic mediators which cause further damage to Schwann cells and possibly axons. (F) Loss of myelin and Schwann cell trophic support may lead to axonal disintegration. (G) The inflammatory response may eventually be terminated by an altered balance of pro-inflammatory T-helper lymphocytes type I (T_H2) and anti-inflammatory T-helper lymphocytes type II (T_H2) and the initiation of T-lymphocyte apoptosis. (artwork adapted from Meyer zu Hörste et al. 2007)

The question arises whether and to what extent chronic inflammation and demyelination may lead to Wallerian degeneration. Although a physical separation of the proximal and distal nerve stumps does not usually occur in inflammatory

conditions, the distal segment can become functionally isolated by a significant impairment of axonal transport. Thus, the term Wallerian-like degeneration has been coined (Freeman and Coleman 2010). Neuroinflammation has been demonstrated to impede axonal transport and increase the structural complexity of mitochondria, suggestive of a transient compensatory mechanism to combat energetic failure that is thought to result in axonal damage (Errea et al. 2015). In support of this idea, energy restriction by mitochondrial uncoupling has been shown to trigger Wallerian degeneration (Alvarez et al. 2008). Finally, genetic models of altered mitochondrial dynamics display axonal damage and degeneration (Misko et al. 2010, 2012). In addition, the secretion of pro-inflammatory cytokines from macrophages or T-lymphocytes may forward Schwann cell dedifferentiation (Stettner et al. 2014). Accordingly, Schwann cell dedifferentiation has recently been demonstrated to occur in NOD/B7-2 knockout mice, a spontaneous autoimmune peripheral neuropathy model that shares several pathological features with CIDP (Jang et al. 2017). These findings imply that different kinds of nerve diseases and injuries may share mechanistic commonalities that contribute to the onset of Wallerian degeneration.

II.V. Current therapeutic options for the injured or inflamed peripheral nerve

Despite an emerging understanding of the cellular and molecular processes underlying the pathophysiology of mechanical nerve injuries as well as immune-mediated neuropathies, the available treatment options are clearly limited. For inflammatory neuropathies, first-line therapeutic options are intravenous immunoglobulins (IVIg), plasma exchange and corticosteroids. Additionally, antibody therapy with rituximab (anti-CD20) may show effectiveness in a subgroup of patients by facilitating B-lymphocyte depletion (Kieseier 2012). However, especially for most cases of chronic inflammation, long-term recovery cannot be achieved by current treatment options, underlining the need for therapeutic approaches that, in addition to dampening pathological immune reactions, directly target regenerative processes.

This is essentially true for mechanical nerve injuries. Despite the remarkable ability of peripheral nerves to regenerate, in many cases functionality is not restored completely as a consequence of excess inflammation and cellular damage, leading to insufficient reinnervation or remyelination. Hence, injury may result in sequelae such as neuropathic pain. At present, surgical reconstruction of severely damaged

nerves after disruption or transection (neurotmesis) is the only available treatment option; as such, patients suffering from neurotmesis or axonotmesis largely depend on a sufficient extent of self-recovery, which is poor in a significant number of the affected individuals (Lee and Wolfe 2000).

In light of an enormous demand, researchers have increasingly focused on the identification of therapeutic targets to support or even accelerate axon regrowth and remyelination. However, despite those efforts, no such therapy has been introduced to clinical practice thus far.

II.VI. Objective

Animal models of chronic immune-driven neuropathies display several pathological aspects of human disease. However, the spontaneous onset of neuroinflammation at an advanced age and the heterogeneous disease progression in these models makes the assessment of potential regenerative therapies a challenging task. In contrast, standardized injury models such as the sciatic nerve crush represent a valuable tool to study nerve regeneration. Since the response to injury with regard to the time course of Wallerian degeneration and subsequent regeneration are well-characterized, the regenerative potential of genetic and pharmacological strategies can be reliably evaluated. As outlined above, nerve degeneration as observed after injury and in inflammatory neuropathies is likely to share mechanistic commonalities: Schwann cell dedifferentiation, demyelination, axonal energy depletion and disintegration are major hallmarks of both pathologies. Therefore, the exploration of therapeutic approaches in a mechanical injury model may not only have relevance for this particular condition, but also pave the way for novel regenerative strategies in the inflamed PNS. To this end, the thesis in hand aims at investigating the regenerative potency of immunomodulatory compounds in the sciatic nerve crush model.

III. Publications

III.I. Dimethyl fumarate accelerates peripheral nerve regeneration via activation of the anti‐inflammatory and cytoprotective Nrf2/HO−1 signaling pathway (Szepanowski et al. 2017)

Fabian Szepanowski, Daniel M. Donaldson, Hans-Peter Hartung, Anne K. Mausberg, Christoph Kleinschnitz, Bernd C. Kieseier and Mark Stettner

Abstract

Dimethyl fumarate represents an established treatment option for relapsing-remitting multiple sclerosis. Its clinical efficacy has been ascribed to the activation of the NF-E2 related factor 2 (Nrf2) transcription factor that has been associated with an upregulation of anti-oxidative and anti-inflammatory enzymes.

We investigated whether DMF was able to promote peripheral nerve regeneration following axonotmesis of the sciatic nerve.

DMF treatment significantly improved grip strength and nerve conduction at three weeks after injury which was paralleled by a dramatic acceleration of remyelination, as confirmed by analysis of semi-thin sections and electron microscopy of the distal injured nerve stump. While nuclear Nrf2 immunofluorescence was markedly increased by DMF, we could neither find the lipid peroxidation marker malondialdehyde to be significantly reduced nor glutathione levels to be elevated. However, expression of the cytoprotective and anti-inflammatory enzyme HO-1 was considerably upregulated in DMF treated mice.

These data indicate that DMF may exert neuroregenerative effects in the peripheral nervous system *in vivo* by upregulation of HO-1 expression, possibly via activation of Nrf2. Although Nrf2 has been reported to exert anti-oxidative effects by activating anti-oxidant response element (ARE) gene expression, only a tendency towards reduced lipid peroxidation and no elevation of glutathione could be observed. Therefore, our data suggest that DMF may support nerve regeneration primarily via its anti-inflammatory potency, rather than significantly modulating anti-oxidative mechanisms *in vivo*.

Personal contribution: approx. 80 % (study design: 95 %; performance of experiments: 80 %; data analysis: 65 %; preparation of figures: 100 %; writing of manuscript: 100 %)

FS wrote the manuscript, prepared all figures and contributed to all experiments as well as data analysis.
DMD contributed to immunohistochemical stainings and morphometric analyses.
MS contributed to preparation of electron microscopic images.
FS and BCK conceived the study. All authors were involved in discussion of results and critically reviewed the manuscript.

Status: Published (January 20th 2017)

Journal: *Acta Neuropathologica (Acta Neuropathol.)*

Journal statistics from Journal Citation Reports (Thomson Reuters):
Impact Factor (2016): 12.213

5-year Impact Factor (2016): 11.297

Journal Ranking: 9 / 258 (Neurosciences); 3 / 194 (Clinical Neurology);
2 / 79 (Pathology)

DOI: 10.1007/s00401-017-1676-z

Dimethyl fumarate accelerates peripheral nerve regeneration via activation of the anti-inflammatory and cytoprotective Nrf2/HO-1 signaling pathway

Fabian Szepanowski[1], Daniel M. Donaldson[1], Hans-Peter Hartung[1], Anne K. Mausberg[1,2], Christoph Kleinschnitz[2], Bernd C. Kieseier[1] and Mark Stettner[1,2]

[1]Department of Neurology, Medical Faculty, Heinrich-Heine-University, Duesseldorf, Germany
[2]Department of Neurology, Medical Faculty, University Duisburg-Essen, Essen, Germany

Correspondence address: Fabian Szepanowski, Department of Neurology, Medical Faculty, Heinrich-Heine-University, Moorenstrasse 5, 40225 Düsseldorf, Germany.
fabian.szepanowski@uni-duesseldorf.de

Keywords: Dimethyl Fumarate; Heme Oxygenase 1; Nrf2; Nerve Regeneration; Oxidative Stress

Peripheral nerves exhibit a remarkable ability to regenerate, however, there is an unmet need to better understand relevant pathways that could support or even accelerate this process. Fumaric acid esters, especially its dimethyl ester (DMF), are an established treatment option for autoimmune diseases [7]. DMF is known to activate the NF-E2-related factor 2 (Nrf2) transcription factor [8] which is ubiquitously and constitutively expressed and primarily localized in the cytoplasm. Its suppressor, Kelch-like ECH-associated protein 1 (Keap1), prevents Nrf2 from entering the nucleus and acts as an adaptor protein for Nrf2 ubiquitinylation. In case of oxidative or electrophilic stress, Nrf2 is released from Keap1 and translocated into the nucleus in order to induce antioxidant response element (ARE) gene expression [5]. Additionally, Nrf2 has been demonstrated to induce the expression of heme oxygenase 1 (HO-1) [1], a potent cytoprotective and anti-inflammatory enzyme [9; 10]. An emerging body of experimental evidence suggests that DMF, targeting Keap1 and interfering with the Nrf2/Keap1 interaction, indirectly activates Nrf2 by enabling its translocation into the nucleus [3]. The upregulation of HO-1 expression or activation of anti-oxidative mechanisms has been suggested to account for the efficacy of DMF [2; 4; 6; 8]. To date it remains unclear whether DMF-mediated anti-inflammatory, anti-oxidative or a synergism of both effects may be sufficient to propagate nerve regeneration in a primary mechanical injury model.

To evaluate the efficacy of DMF during Wallerian degeneration, we performed sciatic nerve crush in C57BL/6 mice, which were treated daily with 100 mg/kg DMF over the course of twelve days, starting two days before crush injury until nine days post-crush. Nerve functionality was assessed via grip strength analysis of both the injured and the contralateral non-injured hindlimbs (fig. 1a). Following a strong impairment of grip strength at seven days post-crush, 14 days after injury we observed a similar extent of recovery in vehicle as well as DMF treated mice. However, at 21 days post-crush, recovery of grip strength in DMF-treated mice was significantly improved compared to vehicle treated mice. To confirm our finding for this clinical parameter, we performed nerve conduction tests at 14 and 21 days post-crush (fig. 1b; supplementary fig. 1), revealing a significant elevation of nerve conduction velocity in DMF-treated mice at the latter stage. In order to complement these data with histological measures, we investigated the impact of DMF on myelin thickness via g-ratio measurements

(the numerical ratio between axonal and whole myelinated fibre diameter) from semi-thin sections (fig. 1c). Between 14 and 21 days post-crush, we observed a gradual improvement of myelination in DMF treated mice, whereas myelin thickness remained significantly reduced in control mice (fig. 1 d, e; supplementary fig. 2). To decipher whether DMF treatment would modulate protective pathways during Wallerian degeneration, we investigated the localization of Nrf2 on sciatic nerve sections by immunohistochemistry at six days post-crush. We recognized a greater extent in the co-localization of Nrf2 and nuclear staining and found nuclear Nrf2 immunofluorescence intensity to be significantly increased in response to injury; DMF treatment further enhanced nuclear Nrf2 fluorescence intensity (fig. 2 a+b). A considerable number of Nrf2 positive cells appeared to be Schwann cells (supplementary fig. 3). As a potential downstream target of Nrf2, we next studied HO-1 protein expression from sciatic nerve homogenates by ELISA. HO-1 was markedly upregulated after injury, which was additionally amplified by DMF treatment (fig. 2 c). The number of neither endoneurial T-lymphocytes nor macrophages was found to be altered by DMF (supplementary fig. 4).

Finally, we quantified sciatic nerve levels of the lipid peroxidation marker malondialdehyde (MDA) using a thiobarbituric acid based assay. We found lipid peroxidation to be significantly elevated in crushed nerves of both control as well as DMF-treated mice, albeit overall MDA levels were slightly but not significantly decreased under DMF treatment (fig. 2 d). In line with this, DMF did not affect glutathione levels after injury (fig. 2 e). Collectively, our data suggest that DMF may hold a yet underestimated neuroregenerative potential which may be primarily mediated by an upregulation of HO-1 in Schwann cells and possibly motor neurons, rather than a modulation of antioxidative mechanisms. Given the cytoprotective and anti-inflammatory potency of HO-1 in animal models of neuroinflammation and -trauma, DMF may not only be clinically effective in autoimmune diseases, but could possibly represent an interesting therapeutic candidate for propagating repair of the peripheral nerve.

Conflict of Interest

FS, DMD and AKM have nothing to disclose. HPH has received honoraria for consulting, lecturing, travel expenses for attending meetings, and financial support for research from Bayer Health Care, Biogen Idec, Genzyme, GeNeuro, Merck Serono, Novartis, Roche, Sanofi Aventis and TEVA; CK from Ablynx, Bayer Health Care, Boehringer Ingelheim, Biogen Idec, Biotronik, Daiichi Sankyo, Eisai, Ever Pharma, Genzyme, Merck Serono, Mylan, Novartis, Roche, Sanofi Aventis, Siemens and TEVA; BCK from Bayer Health Care, Biogen Idec, Merck Serono, Novartis, Genzyme and TEVA. BCK is currently also employee of Biogen. MS has received honoraria for consulting, lecturing, travel expenses for attending meetings or financial support for research from Bayer Health Care, Biogen Idec, Genzyme, Novartis, Sanofi Aventis, Grifols and TEVA.

References

1. Alam J, Stewart D, Touchard C, Boinapally S, Choi AMK, Cook JL (1999) Nrf2, a Cap'n'Collar Transcription Factor, Regulates Induction of the Heme Oxygenase-1 Gene. *J. Biol. Chem.* 274:26071-26078

2. Albrecht P, Bouchachia I, Goebels N, Henke N, Hofstetter HH, Issberner A et al. (2012) Effects of dimethyl fumarate on neuroprotection and immunomodulation. *J. Neuroinflammation* 9:163

3. Brennan MS, Matos MF, Li B, Hronowski X, Gao, Juhasz P et al. (2015) Dimethyl Fumarate and Monoethyl Fumarate Exhibit Differential Effects on KEAP1, NRF2 Activation, and Glutathione Depletion In Vitro. *PLOS ONE* 10(3):e0120254

4. Han R, Xiao J, Zhai H, Hao J (2016) Dimethyl fumarate attenuates experimental autoimmune neuritis through the nuclear factor erythroid-derived 2-related factor 2/hemoxygenase-1 pathway by altering the balance of M1/M2 macrophages. *J. Neuroinflammation* 13:97

5. Lee JM, Li J, Johnson DA, Stein TD, Kraft AD, Calkins MJ et al. (2005) Nrf2, a multi-organ protector? FASEB J. 19(9):1061-1066

6. Lin SX, Lisi L, Russo CD, Polak PE, Sharp A, Weinberg G et al. (2011) The anti-inflammatory effects of dimethyl fumarate in astrocytes involve glutathione and haem oxygenase-1. *ASN NEURO 3(2):art:e00055.doi:10.1042/AN20100033*

7. Linker RA, Haghikia A (2016) Dimethyl fumarate in multiple sclerosis: latest developments, evidence and place in therapy. Ther. Adv. Chronic Dis. 7(4):198-207

8. Linker RA, Lee DH, Ryan S, van Dam AM, Conrad R, Bista P et al. (2011) Fumaric acid esters exert neuroprotective effects in neuroinflammation via activation of the Nrf2 antioxidant pathway. *Brain* 134(3): 678-692

9. Otterbein LE, Soares MP, Yamashita K, Bach FH (2003) Heme oxygenase-1: Unleashing the protective properties of heme. *Trends Immunol.* 24(8):449-455

10. Soares MP, Bach FH (2009) Heme oxygenase-1: From Biology to Therapeutic Potential. *Trends Mol. Med.* 15(2):50-58

Figures

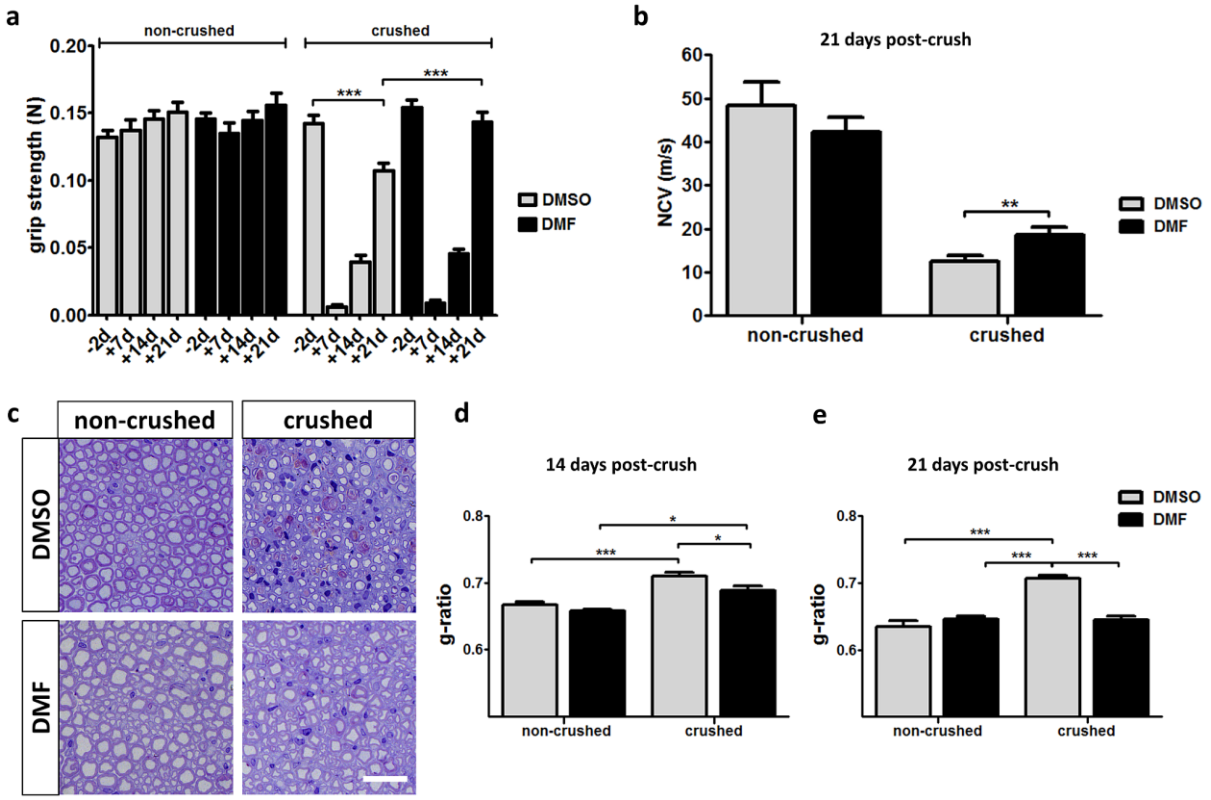


Figure 1: DMF ameliorates nerve regeneration as shown by clinical, electrophysiological and histological measures. (A) Grip strength in crushed and contralateral non-crushed hindlimbs two days before crush (-2d) and seven (+7d), 14 (+14d) and 21 (+21d) days post-crush. *N* = 17 minimum for each column. Statistical analysis was performed by one way ANOVA followed by Newman-Keuls post-hoc test. B) Nerve conduction velocities (NCV) at 21 days post-crush. *N* = 16 minimum for each column. Statistical analysis was performed by Student's t-test, 2-tailed. C) Toluidine blue stained semi-thin sections of the distal injured nerve stump at 21 days post-crush. Scale bar indicates 25 µm. D) g-ratio measurements at 14 days (*N* = 3/4/3/4 from left to right) and E) 21 days post-crush (*N* = 4/5/4/5 from left to right). Statistical analysis was performed by one way ANOVA followed by Newman-Keuls post-hoc test. Data represent mean ± s.e.m. *P≤0.05*, P≤0.01*** and *P≤0.001***.*

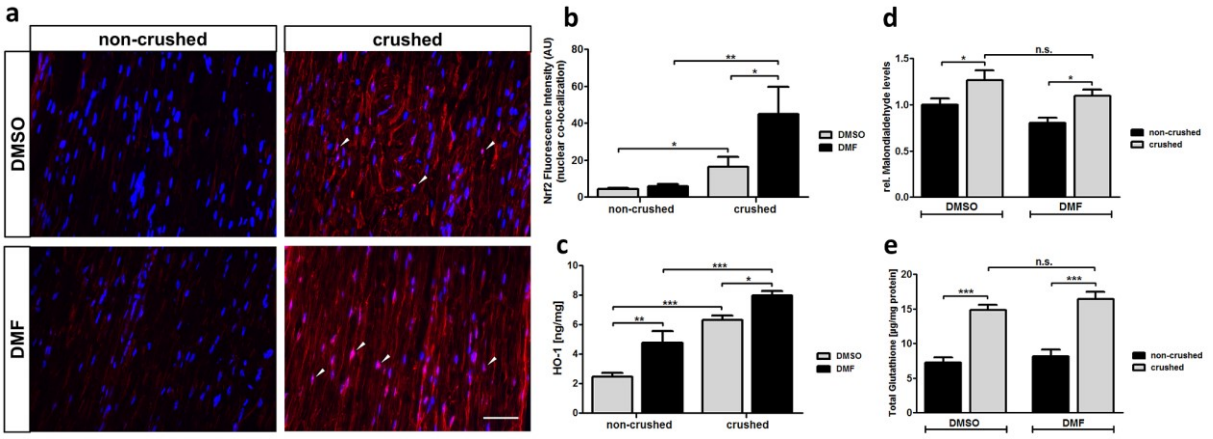
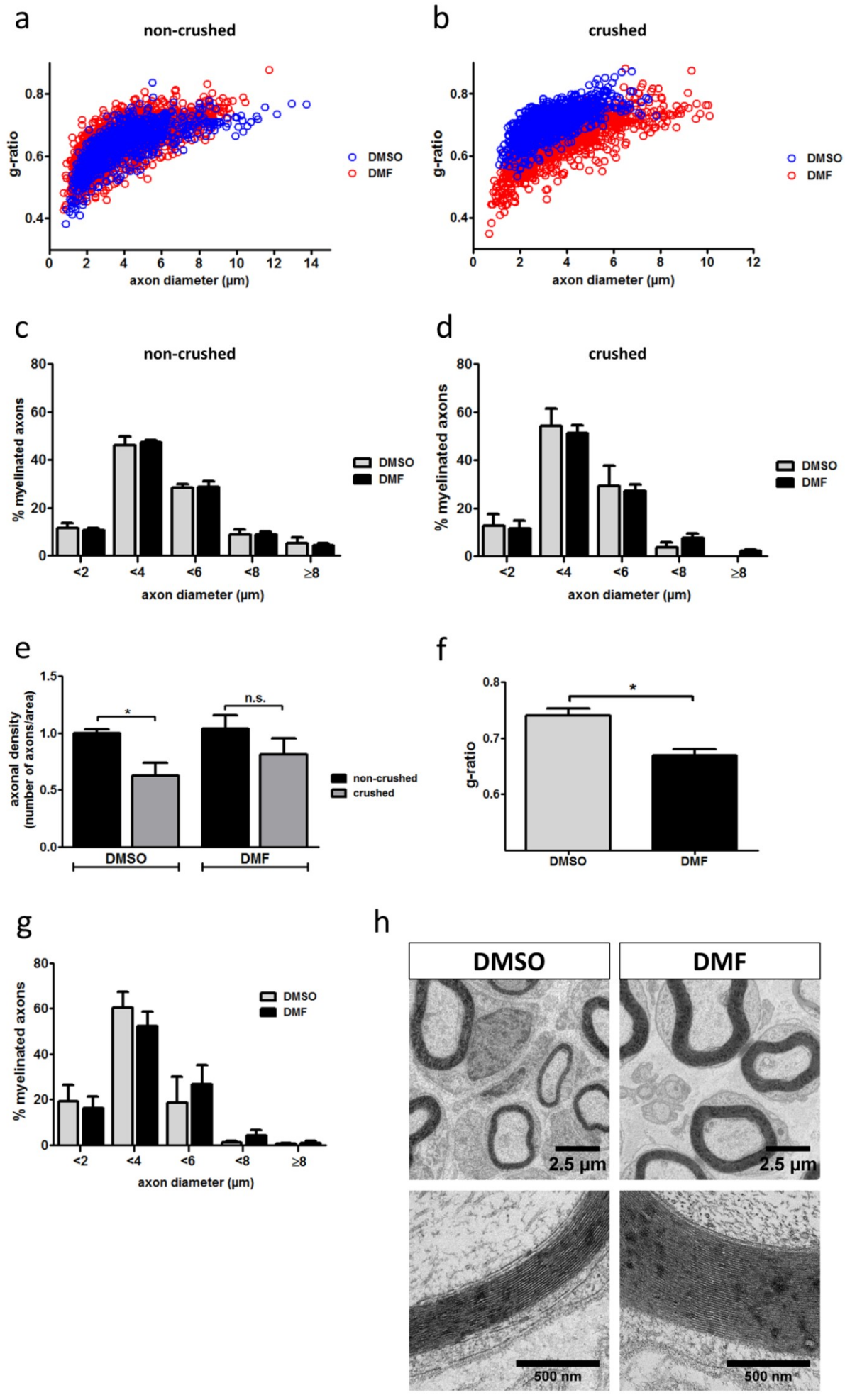


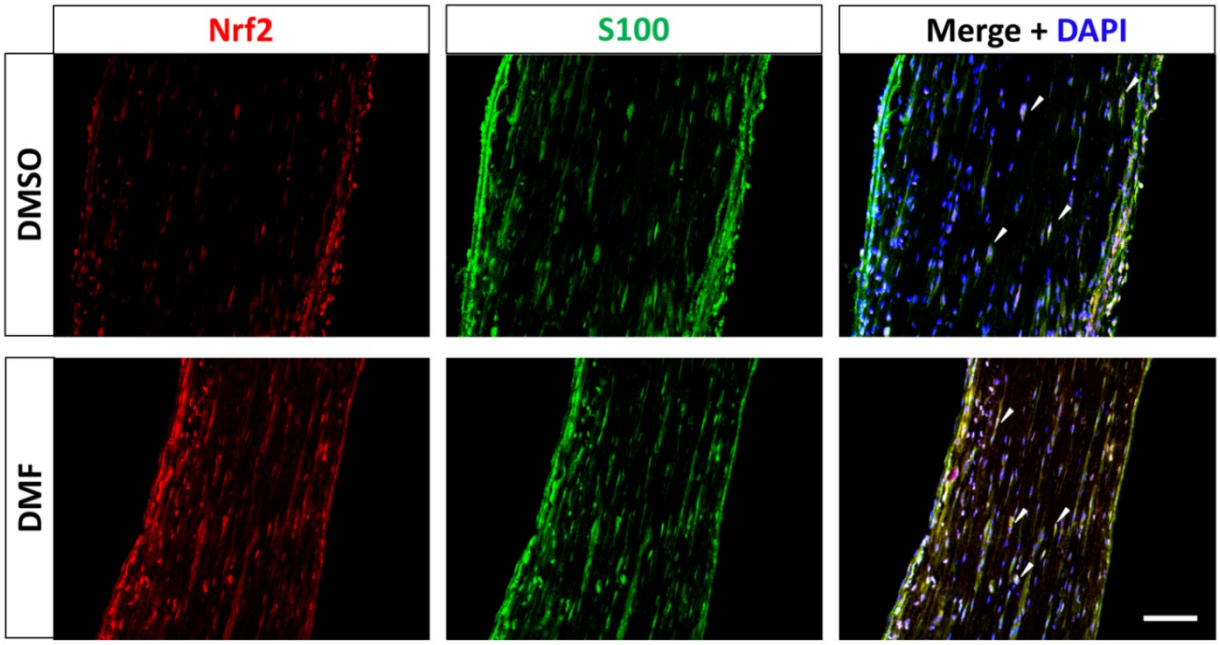
Figure 2: DMF enhances HO-1 expression after injury possibly via activation of Nrf2. (a) Immunohistochemical detection of Nrf2 on sciatic nerve sections at six days post-crush. Arrows indicate Nrf2 and DAPI co-localization. Scale bar represents 100 µm. (b) Nuclear Nrf2 fluorescence intensity. *N* = 3/5/6/7 from left to right. Statistical analysis was done by Kruskal Wallis test and Mann-Whitney U test. (c) Quantification of HO-1 via ELISA. *N* = 8 for each column. (d) Sciatic nerve malondialdehyde content (*N* = 11 for each column) and (e) total glutathione levels. *N* = 6/6/5/5 from left to right. Data represent mean ± s.e.m. Statistical analysis was done by one way ANOVA and Newman-Keuls post-hoc test. *P≤0.05*, P≤0.01*** and *P≤0.001***, n.s.* indicates no significant difference.

Supplementary Figures

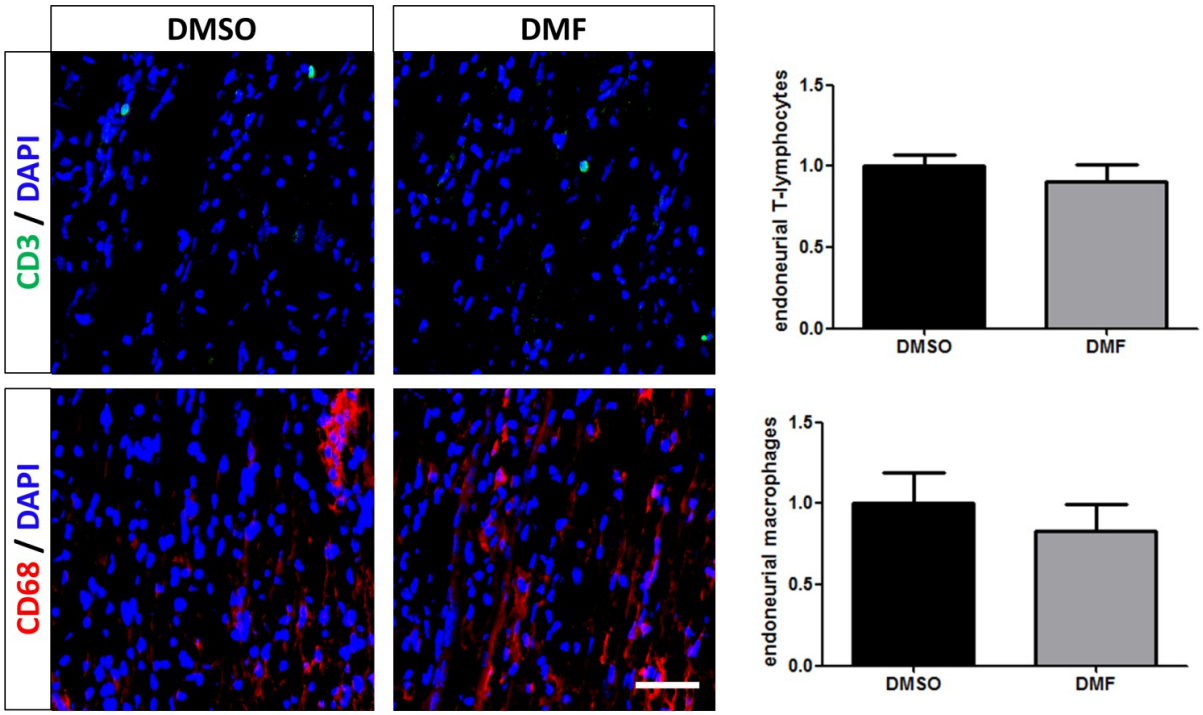
Supplementary Fig. 1: Nerve conduction test at 14 days post-crush.
Whereas nerve conduction velocity in contralateral non-crushed nerves was normal, no reliable response to stimulation could be recorded in crushed nerves, indicating comparability and completeness of the crush injury. N = 6/5/6/5 from left to right. Data represent mean ± s.e.m.


a
non-crushed
g-ratio
axon diameter (µm)
DMSO
DMF
b
crushed
g-ratio
axon diameter (µm)
DMSO
DMF
c
non-crushed
% myelinated axons
axon diameter (µm)
<2
<4
<6
<8
≥8
DMSO
DMF
d
crushed
% myelinated axons
axon diameter (µm)
DMSO
DMF
e
axonal density (number of axons/area)
*
n.s.
DMSO
DMF
non-crushed
crushed
f
g-ratio
*
DMSO
DMF
g
% myelinated axons
axon diameter (µm)
DMSO
DMF
h
DMSO
DMF
2.5 µm
2.5 µm
500 nm
500 nm

Supplementary Fig. 2: Morphometric assessment of nerve regeneration at 21 days post-crush.
(a, b) G-ratios were plotted against axon diameters, indicating a shift towards increased myelin thickness regardless of axon diameter in crushed nerves of DMF treated mice. (c, d) Axon diameter histograms indicate no differences in the percentage of small caliber myelinated axons in crushed nerves between the treatment groups. Conversely, only DMF treated mice display a small portion of large caliber myelinated axons (≥ 8 µm diameter). (e) Consistently, whereas axonal density (number of axons per fascicle, normalized to area) was significantly reduced in control mice, DMF treated mice did not show a significant difference from contralateral non-crushed nerves for this measure. To further confirm these findings, (f) g-ratios and (g) axon diameter distributions were analyzed from electron microscopic images, yielding comparable results to the measurements from semi-thin sections. (h) Representative electron microscopic images of comparably sized myelinated axons in crushed nerves. Statistical analysis was done by Student's t-test, 2-tailed. N = 4 (DMSO)/5 (DMF) for a-e (semi-thin) and N = 3/3 for f, g (electron microscopy). Data represent mean ± s.e.m. *P≤0.05*, P≤0.01*** and *P≤0.001***, n.s.* indicates no significant difference.



Supplementary Figure 3: Nrf2 and S100 co-staining on sciatic nerve sections at six days post-crush. Double labeling experiments using Nrf2 and S100 antibodies point to an activation of Nrf2 in Schwann cells. Co-localization is indicated by arrows. Scale bar represents 100 µm.



Supplementary Fig. 4: Assessment of endoneurial T-lymphocytes (CD3) and macrophages (CD68) in crushed sciatic nerves at six days post-crush. No significant differences were observed in the number of neither for $CD3^+$ T-lymphocytes nor $CD68^+$ macrophages. Scale bar indicates 50 µm. N = 3 / 4 from left to right, both graphs. Data represent mean ± s.e.m.

Supplementary Information

Materials and Methods

Animals

C57BL/6 mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France). Animal use and experiments were approved by local authorities (LANUV North Rhine-Westphalia, Germany / application number 84.02.04.2012.A376).

Sciatic Nerve Crush

Male, age-matched (3-4 months) wildtype C57BL/6 were anesthetized for surgery via intraperitoneal injection of a mixture of xylazine (Rompun; Bayer, Leverkusen, Germany) (10 mg/kg) and ketamine (Actavis, Munich, Germany) (100 mg/kg) and placed on a heating plate (37°C) to maintain constant body temperature. The fur of the lower back was removed with an electric razor and the skin was disinfected using 70 % ethanol. All instruments were sterilized. A small incision (1 cm) was made in the skin above the right hindlimb between the mm. gluteus maximus and biceps femoris. Opening the facial plane between both muscles revealed the sciatic nerve which was carefully lifted using bent forceps and crushed right before its distal branches using a non-serrated clamp at maximum intensity for 30 seconds. The nerve was replaced under the muscle and the incision was closed using non-absorbable suture material. The contralateral nerve was left intact to serve as control.

Administration of DMF

Mice received DMF (Dimethyl fumarate, Sigma-Aldrich, Munich, Germany) dissolved in Hybri-Max™ DMSO (Sigma-Aldrich) via intraperitoneal injection at a concentration of 100 mg/kg once daily over the course of 12 days, starting two days before crush until 9 days post-crush. Controls received an equal volume of vehicle.

Assessment of nerve functionality by grip strength analysis

Nerve functionality was evaluated via grip strength analysis of the right (crushed) and left (non-crushed) hindlimbs using a grip strength meter at 2 days before crush injury and 7, 14 and 21 days post-injury. Mice were tested three times in succession and data were averaged for each mouse and time point.

Electrophysiology

Nerve conduction velocities and compound muscle action potentials were determined at 14 and 21 days post-crush. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and immediately placed on a heating plate (37°C) to maintain constant body temperature. Stimulation of the sciatic nerve was performed by repetitively generated single pulses using monopolar 30 G needle electrodes until supramaximal stimulation was achieved. Compound muscle action potential was recorded at the plantar foot muscle with a needle electrode using a portable electrodiagnostic system (KeyPoint 4, Medtronic, Meerbusch, Germany). Nerve conduction velocity was calculated from the distance and the motor latency differences between proximal and distal stimulations.

Tissue preparation for immunohistochemistry

Sciatic nerves were carefully removed by only handling the most proximal end with forceps and cutting the nerve at its most distal end using scissors. Nerves were placed in suitable cryomolds, covered with a cryo-embedding compound and placed on dry ice. Embedded nerves were stored at -80°C. Longitudinal sections of 7 µm thickness were prepared in a cryostat chamber and slides were air-dried for at least one hour before further processing or stored at -20°C.

Immunofluorescence

Sciatic nerve sections were post-fixed in 4 % paraformaldehyde for 20 min. After fixation, slides were washed 5 min in PBS and twice for 5 min in PBT (PBS + 0.1 % Triton X-100). Slides were incubated with blocking solution (10 % normal goat serum (v/v) in PBT) for 30 min at room temperature. For detection of Nrf2, primary antibody (rabbit polyclonal anti-Nrf2 antibody; Thermo Fisher Scientific; Waltham, MA, USA) was applied and slides were incubated at 4°C for 16 hours. Slides were washed twice for 5 min in PBT and biotinylated secondary antibody (biotinylated goat anti-rabbit IgG; Vector Laboratories; Peterborough, UK) was applied and incubated at room temperature for one hour. Slides were washed twice for 5 min in PBT and DyLight594-conjugated streptavidin (Vector Laboratories) and DAPI were applied. For the detection of Schwann cells rabbit anti-S100A1 polyclonal antibody (Sigma-Aldrich) was used. For detection of T-lymphocytes polyclonal rabbit anti-human CD3 antibody (Dako) and for macrophages mouse anti-human CD68 clone KP1 (Dako) was used, which both cross-react with mouse.

Sciatic nerve histology

Nerves were fixed in 0.1 M cacodylate buffer containing 2,5 % glutaraldehyde and kept at 4°C overnight. The fixative was discarded and replaced by washing buffer (0.1 M cacodylate + 3 % sucrose). Nerves were washed for four days at 4°C. Washing was followed by incubation in an osmium tetroxide reagent for 3 hours. Osmium tetroxide reagent was composed of one part 5 % potassium dichromate solution (pH 7,4), one part 3,4% NaCl solution and two parts 2 % osmium tetroxide solution (Sigma-Aldrich). Afterwards, samples were briefly washed in 0.1 M cacodylate buffer. Samples were dehydrated in an ascending ethanol series (70 %; 96 %; ≥ 99,8 % undenatured ethanol) for one hour each. Following dehydration, samples were incubated in 250 µl propylene oxide (Sigma-Aldrich) in tightly closed containers for one hour at room temperature, then one hour in a 1:1 mixture of propylene oxide/epon (Epoxy embedding medium kit; Sigma-Aldrich) and finally kept at 4°C in epon only overnight. Samples were placed in silicone molds and covered with epon embedding mixture. Embedded samples were incubated at 37°C for 6 h, at 47°C for 15 h and finally at 60°C for 28 h until epon was completely hardened. Sectioning was performed approximately 3 mm distal from the crush site. Transverse sections were prepared at a thickness of 1 µm at a Reichert-Jung Ultracut Microtome and immediately stained with toluidine blue (1 % toluidine blue (w/v) dissolved in a 1 % disodium tetraborate (w/v) solution), washed in distilled H_2O (approximately 10 ml) containing 1-2 drops of acid ethanol (0.01 % HCl in absolute ethanol), placed on a microscope slide, dried on a heating plate and mounted with Roti Histokitt II (Roth). Sections were photographed on a Zeiss Axioplan 2 (Zeiss, Jena, Germany) microscope. Transmission electron microscopy (EM) was performed on a Zeiss EM 9-02 with ultra-thin sections (50-70 nm) stained with 1 % uranyl acetate and 0.4 % lead citrate.

Assessment of morphometric data

Morphometric analysis was performed by a blinded investigator using ImageJ (National Institutes of Health, Bethesda, MA, USA). Axon numbers of whole fascicles were measured manually by marking each individual axon. Axon numbers were normalized to fascicle area. For the evaluation of g-ratios and axonal diameters, the circumference of axons and their respective myelin sheaths was measured within randomly selected fields. A minimum of 200 axons per nerve were evaluated. For the calculation of g-ratios, axonal circumference was divided by the circumference of the respective myelin sheath. Axonal diameters were calculated from the axonal circumference. For morphometric analysis of EM images, at least 80 axons per nerve were examined within randomly selected fields.

Quantification of MDA levels

Sciatic nerve MDA levels were determined by the thiobarbituric acid method using the commercially available Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich).

Sciatic nerve samples were homogenized in assay buffer. Detection of MDA was performed as recommended by the manufacturer. Sciatic nerve MDA content was normalized to the respective protein content of the sample.

Quantification of Glutathione levels

Total glutathione levels were determined by Glutathione Colorimetric Assay Kit (BioVision, Milpitas, CA, USA). Sciatic nerves were homogenized in reaction buffer and detection of glutathione was performed as recommended by the manufacturer. Quantification was performed by the pseudo-end-point method. Glutathione levels were normalized the respective protein content of the sample.

Quantification of HO-1 expression via ELISA

Sciatic nerve HO-1 protein expression was quantified using the ImmunoSet HO-1 (mouse) ELISA set (Enzo Life Sciences, Loerrach, Germany). Briefly, sciatic nerves were homogenized in ice-cold PBS containing cOmplete™ Mini (Roche, Basel, Switzerland) proteinase inhibitor cocktail. Homogenates were centrifuged for 10 min at 11,000 rpm and supernatants were transferred to new sample tubes, diluted 1:2 with assay buffer (PBS containing 1 % BSA and 0.1 % Tween20) and vortexed rigorously. Detection of HO-1 was performed as recommended by the manufacturer. HO-1 content was normalized to the respective protein content of the sample.

Image and data analysis

Analysis of images was performed using ImageJ (National Institutes of Health, Bethesda, MA, USA). Data analysis and compilation of graphs was performed using Microsoft (Redmond, WA, USA) Excel and GraphPad (La Jolla, CA, USA) Prism 5. Statistical analysis was done by Student's t-test, multiple comparisons were performed by one way ANOVA followed by Newman-Keuls post-hoc test or Kruskal Wallis test and Mann-Whitney U test. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$** and $P \leq 0.001$***.

III.II. Fingolimod promotes peripheral nerve regneration via modulation of lysophospholipid signaling (Szepanowski et al. 2016)

Fabian Szepanowski, Angelika Derksen, Irina Steiner, Gerd Meyer zu Hörste, Thomas Daldrup, Hans-Peter Hartung and Bernd C. Kieseier

Abstract

The lysophospholipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are pleiotropic signaling molecules with a broad range of physiological functions. Targeting the S1P1 receptor on lymphocytes with the immunomodulatory drug fingolimod has proven effective in the treatment of multiple sclerosis. An emerging body of experimental evidence points to additional direct effects on cells of the central and peripheral nervous system. Furthermore, fingolimod has been reported to reduce LPA synthesis via inhibition of the lysophospholipase autotaxin. Here we investigated whether modulation of particular signaling aspects of S1P as well as LPA by fingolimod might propagate peripheral nerve regeneration in vivo and independent of its anti-inflammatory potency.

Sciatic nerve crush was performed in wildtype C57BL/6, in immunodeficient *Rag1*$^{-/-}$ and *Foxn1*$^{-/-}$ mice. Analyses were based on walking track analysis and electrophysiology, histology, and cAMP formation. Quantification of different LPA species was performed by liquid chromatography coupled to tandem mass spectrometry. Furthermore, functional consequences of autotaxin inhibition by the specific inhibitor PF-8380 and the impact of fingolimod on early cytokine release in the injured sciatic nerve were investigated.

Clinical and electrophysiological measures indicated an improvement of nerve regeneration under fingolimod treatment that is partly independent of its anti-inflammatory properties. Fingolimod treatment correlated with a significant elevation of axonal cAMP, a crucial factor for axonal outgrowth. Additionally, fingolimod significantly reduced LPA levels in the injured nerve. PF-8380 treatment correlated with improved myelin thickness. Sciatic nerve cytokine levels were not found to be significantly altered by fingolimod treatment.

Our findings provide in vivo evidence for direct effects of fingolimod on cells of the peripheral nervous system that may propagate nerve regeneration via a dual mode of action, differentially affecting axonal outgrowth and myelination by modulating relevant aspects of S1P and LPA signaling.

Personal contribution: approx. 80 % (study design: 60%, performance of experiments: 70 %; data analysis: 90 %; preparation of figures: 100 %; writing of manuscript: 100 %)

An ‘authors’ contributions’ section is provided at the end of the manuscript.

Status: Published (July 10th 2016)

Journal: *Journal of Neuroinflammation* (*J. Neuroinflammation*)

Journal statistics from Journal Citation Reports (Thomson Reuters):
Impact Factor (2016): 5.102

5-year Impact Factor (2016): 5.717

Journal Ranking: 40 / 258 (Neurosciences); 31 / 150 (Immunology)

DOI: 10.1186/s12974-016-0612-9

Szepanowski et al. Journal of Neuroinflammation (2016) 13:143
DOI 10.1186/s12974-016-0612-9

Journal of Neuroinflammation

RESEARCH Open Access

Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling

Fabian Szepanowski[1*], Angelika Derksen[1], Irina Steiner[2], Gerd Meyer zu Hörste[1,3,4], Thomas Daldrup[2], Hans-Peter Hartung[1] and Bernd C. Kieseier[1]

Abstract

Background: The lysophospholipids sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) are pleiotropic signaling molecules with a broad range of physiological functions. Targeting the $S1P_1$ receptor on lymphocytes with the immunomodulatory drug fingolimod has proven effective in the treatment of multiple sclerosis. An emerging body of experimental evidence points to additional direct effects on cells of the central and peripheral nervous system. Furthermore, fingolimod has been reported to reduce LPA synthesis via inhibition of the lysophospholipase autotaxin. Here we investigated whether modulation of particular signaling aspects of S1P as well as LPA by fingolimod might propagate peripheral nerve regeneration in vivo and independent of its anti-inflammatory potency.

Methods: Sciatic nerve crush was performed in wildtype C57BL/6, in immunodeficient *Rag1*$^{-/-}$ and *Foxn1*$^{-/-}$ mice. Analyses were based on walking track analysis and electrophysiology, histology, and cAMP formation. Quantification of different LPA species was performed by liquid chromatography coupled to tandem mass spectrometry. Furthermore, functional consequences of autotaxin inhibition by the specific inhibitor PF-8380 and the impact of fingolimod on early cytokine release in the injured sciatic nerve were investigated.

Results: Clinical and electrophysiological measures indicated an improvement of nerve regeneration under fingolimod treatment that is partly independent of its anti-inflammatory properties. Fingolimod treatment correlated with a significant elevation of axonal cAMP, a crucial factor for axonal outgrowth. Additionally, fingolimod significantly reduced LPA levels in the injured nerve. PF-8380 treatment correlated with improved myelin thickness. Sciatic nerve cytokine levels were not found to be significantly altered by fingolimod treatment.

Conclusions: Our findings provide in vivo evidence for direct effects of fingolimod on cells of the peripheral nervous system that may propagate nerve regeneration via a dual mode of action, differentially affecting axonal outgrowth and myelination by modulating relevant aspects of S1P and LPA signaling.

Keywords: Fingolimod, Sphingosine-1-phosphate, Lysophosphatidic acid, PF-8380, Peripheral nerve regeneration

* Correspondence: fabian.szepanowski@uni-duesseldorf.de
[1]Department of Neurology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany
Full list of author information is available at the end of the article

Background

Lysophospholipids are metabolites of glycerophospholipids and sphingolipids that are commonly found as lipid constituents of cell membranes. Besides being a structural membrane component, certain members of the lysophospholipid family have considerable cell signaling properties [1]. Among these, sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) and their signaling pathways have been best characterized. Lysophospholipid signaling appears to have a large range of physiological and pathophysiological functions [2, 3] in the adult organism and also plays important roles during embryonic development, especially in the development of the nervous system and vascular development [4–6].

S1P acts as a ligand for five G-protein-coupled receptors [7–11], generally referred to as $S1P_{1–5}$. Since S1P receptors couple to a variety of G-proteins, stimulation of S1P receptors can impact several signal transduction pathways and cellular processes. In contrast to $S1P_{2–5}$, $S1P_1$ is thought to exclusively couple with $G_{i/o}$, affecting cyclic adenosine monophosphate (cAMP) levels and Ca^{2+} mobilization, among others [10, 12].

Fingolimod (also named FTY720) is a first-in-class S1P receptor agonist that has been approved for the treatment of remitting-relapsing multiple sclerosis due to its immunomodulatory properties [13]. Structurally, a sphingosine analogue, fingolimod becomes phosphorylated by sphingosine kinases [14] and binds to four of the five S1P receptors [15], except for $S1P_2$. Its immunomodulatory effect is presumably based on its "functionally antagonistic" effect on the $S1P_1$ receptor expressed on lymphocytes, causing $S1P_1$ receptor internalization and subsequently abrogation of $S1P_1$-mediated signaling. It thereby prevents lymphocyte egress [16, 17]. In addition, phosphorylated fingolimod (fingolimod-P) has been reported to act as an inhibitor of autotaxin, an ectonucleotide pyrophosphatase/phosphodiesterase with lysophospholipase D activity that generates LPA from more complex lysophospholipids [18]. LPA has been implicated in dorsal root demyelination after partial sciatic nerve injury, most likely by activation of LPA_1 receptor signaling [19], and demyelination has been found to be significantly reduced in heterozygous autotaxin knockout mice [20]. Furthermore, traumatic brain injury has been reported to induce significant increases of various LPA species in cerebrospinal fluid already 3 hours after injury in mice, and administration of LPA-antibody improves brain tissue damage outcomes [21]. Additionally, recent publications point to a significant role of LPA and LPA_1 receptor signaling in the pathophysiology of spinal cord injuries [22, 23].

As S1P receptors are known to be widely expressed in the nervous system [24], there is an emerging body of experimental evidence pointing to direct effects of fingolimod on cells of the central nervous system [25], including neuroprotection from excitotoxic death and the promotion of remyelination. Additionally, fingolimod has been reported to promote functional recovery in spinal cord injury models [26, 27]. However, the effects of fingolimod on cells of the peripheral nerve in vivo have not been investigated so far. To address the question whether fingolimod may promote peripheral axon regeneration or remyelination and to differentiate between potential direct PNS-specific and its established immunomodulatory effects, we performed sciatic nerve crush in wildtype C57BL/6 as well as in two immunodeficient mouse strains: *Rag1*$^{-/-}$ mice lacking mature B- and T-lymphocytes and in athymic *Foxn1*$^{-/-}$ mice devoid of T-lymphocytes. To further explore the role of LPA during sciatic nerve de- and regeneration as well as to distinguish the significance of S1P receptor modulation from LPA mediated effects, we treated animals with the autotaxin inhibitor PF-8380 [28] and assessed the effect of fingolimod on LPA formation after injury in sciatic nerve around the crush site.

Methods

Animals

Rag1$^{-/-}$ mice (B6.129S7-*Rag1*^{tm1Mom}/J) were obtained from Jackson Laboratories (Bar Harbor, ME, USA), *Foxn1*$^{-/-}$ mice (B6.Cg/NTac-*Foxn1*^{nu}) from Taconic (Hudson, NY, USA), and control C57BL/6 mice from Janvier Labs (Le Genest-Saint-Isle, France). Animal use and experiments were approved by local authorities (LANUV North Rhine-Westphalia, Germany).

Sciatic nerve crush

Male, age-matched (3–4 months) wildtype C57BL/6, *Rag1*$^{-/-}$, and *Foxn1*$^{-/-}$ mice were anesthetized for surgery via intraperitoneal injection of a mixture of xylazine (Rompun; Bayer, Leverkusen, Germany) (10 mg/kg) and ketamine (Actavis, Munich, Germany) (100 mg/kg) and placed on a heating plate (37 °C) to maintain constant body temperature. The fur of the lower back was removed with an electric razor, and the skin was disinfected using 70 % ethanol. All instruments were sterilized. A small incision (1 cm) was made in the skin above the right hindlimb between the mm. gluteus maximus and biceps femoris. Opening the facial plane between both muscles revealed the sciatic nerve which was carefully lifted using bent forceps and crushed right before its distal branches using a non-serrated clamp at maximum intensity for 30 s. The nerve was replaced under the muscle, and the incision was closed using non-absorbable suture material. The contralateral nerve was left intact to serve as control.

Administration of fingolimod and PF-8380

Mice received non-phosphorylated fingolimod (FTY720, Cayman Europe, Tallinn, Estonia) dissolved in a solution of 10 % DMSO in PBS (Sigma-Aldrich, Munich, Germany) via intraperitoneal injection at a concentration of 1 mg/kg once daily over the course of 16 days, starting 2 days before crush until 14 days post-crush. PF-8380 (Sigma-Aldrich) was dissolved in DMSO and administered at a concentration of 10 mg/kg via intraperitoneal injection once daily, as well starting 2 days before until 14 days post-crush. Controls received an equal volume of solvent.

Clinical assessment of nerve functionality by walking track analysis

Nerve functionality was assessed 2 days before as well as 7 and 14 days post-crush by walking track analysis and calculation of the sciatic functional index (SFI) as described elsewhere [29].

Electrophysiology

Electrophysiology was essentially performed as described previously [30]. Nerve conduction velocities and compound muscle action potentials were determined at 14 days post-crush. Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and immediately placed on a heating plate (37 °C) to maintain constant body temperature. Stimulation of the sciatic nerve was performed by repetitively generated single pulses using monopolar 30 G needle electrodes until supramaximal stimulation was achieved. Compound muscle action potential was recorded at the plantar foot muscle with a needle electrode using a portable electrodiagnostic system (KeyPoint 4, Medtronic, Meerbusch, Germany). Nerve conduction velocity was calculated from the distance and the motor latency differences between proximal and distal stimulations.

Tissue preparation

Following electrophysiology, mice were sacrificed via cervical dislocation. Sciatic nerves were carefully removed by only handling the most proximal end with forceps and cutting the nerve at its most distal end using scissors. For immunohistochemical applications, the nerves were immediately dipped in an isopentane bath immersed in liquid nitrogen for approximately 5 s. Then frozen nerves were placed in suitable cryomolds, covered with a cryo-embedding compound and placed on dry ice. Embedded nerves were stored at −80 °C. Longitudinal sections of 7-μm thickness were prepared in a cryostat chamber, and slides were air-dried for at least 1 hour before further processing or stored at −20 °C.

Antibodies

The following antibodies were used and diluted in Antibody Diluent (Dako, Hamburg, Germany) as indicated: Rabbit anti-cAMP polyclonal antibody—1:50 (Merck-Millipore, Darmstadt, Germany); biotinylated goat anti-rabbit IgG—1:200 (Vector Laboratories, Peterborough, UK); DyLight 594 Streptavidin—1:200 (Vector Laboratories); rabbit anti-neurofilament L (NF-L)—1:1000 (Merck-Millipore); and Alexa Fluor 488 Goat Anti-Rabbit IgG—1:200 (Life Technologies).

Immunofluorescence

Sections were post-fixed in 4 % paraformaldehyde for 20 min. After fixation, slides were washed 5 min in PBS and twice for 5 min in PBT (PBS + 0.1 % Triton X-100). Slides were incubated with blocking solution (10 % normal goat serum (*v*/*v*) in PBT) for 30 min at room temperature. For the detection of cAMP, primary antibody was applied and slides were incubated at 4 °C for 16 h. Slides were washed twice for 5 min in PBT, and biotinylated secondary antibody was applied and incubated at room temperature for 1 hour. Slides were washed twice for 5 min in PBT, and DyLight594-conjugated streptavidin was applied. If desired, co-staining with NF-L was performed. Slides were washed 2 × 5 min in PBT and incubated with anti-NF-L antibody at room temperature for 1 hour. Slides were washed twice for 5 min and incubated with Alexa Fluor 488-conjugated secondary antibody. Slides were washed 5 min in PBT and 5 min in PBS and mounted with Vectashield Hardset mounting medium containing DAPI (Vector Laboratories).

Preparation of semi-thin sections

Nerves were fixed in 0.1 M cacodylate buffer containing 2.5 % glutaraldehyde and kept at 4 °C overnight. The fixative was discarded and replaced by washing buffer (0.1 M cacodylate + 3 % sucrose). Nerves were washed for 4 days at 4 °C. Washing was followed by incubation in an osmium tetroxide reagent for 3 h. Osmium tetroxide reagent was composed of one part 5 % potassium dichromate solution (pH 7.4), one part 3.4 % NaCl solution, and two parts 2 % osmium tetroxide solution (Sigma-Aldrich). Afterwards, samples were briefly washed in 0.1 M cacodylate buffer.

Samples were dehydrated in an ascending ethanol series (70 %; 96 %; ≥99.8 % undenatured ethanol) for 1 hour each. Following dehydration, samples were incubated in 250 μl propylene oxide (Sigma-Aldrich) in tightly closed containers for 1 hour at room temperature, then 1 hour in a 1:1 mixture of propylene oxide/epon (epoxy embedding medium kit; Sigma-Aldrich), and finally kept at 4 °C in epon only overnight. Samples were placed in silicone molds and covered with epon embedding mixture.

Embedded samples were incubated at 37 °C for 6 h, at 47 °C for 15 h, and finally at 60 °C for 28 h until epon was completely hardened. Transverse sections were prepared at a thickness of 1 μm at a Reichert-Jung Ultracut Microtome and immediately stained with toluidine blue (1 % toluidine blue (*w*/*v*) dissolved in a 1 % disodium tetraborate (*w*/*v*) solution), washed in distilled H_2O (approximately 10 ml) containing 1–2 drops of acid ethanol (0.01 % HCl in absolute ethanol), placed on a microscope slide, dried on a heating plate, and mounted with Roti Histokitt II (Roth).

Quantification of LPA by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)

Sciatic nerve homogenates were extracted using 800 μl of a mixture methanol:chloroform (2:1) (both VWR International, Darmstadt, Germany) after addition of 20 μl LPA 17:1 (1 μg/ml) (Avanti Polar Lipids, Alabaster, AL, USA) as internal standard (IS), 50 μl deionized water (VWR), and 40 μl 6 M HCl (Merck, Darmstadt, Germany). Samples were vortexed for 5 min and incubated 20 min at -20 °C. After centrifugation (10 min, 14,000×*g*), the upper phases were transferred to new sample tubes and extracted with 200 μl chloroform (Merck) and 250 μl deionized water. Samples were vortexed rigorously and centrifuged for 10 min at 14,000×*g*. The lower organic phases were dried under nitrogen gas flow at 60 °C. The dried residues were dissolved in 50 μl LC-MS grade methanol (mobile phase B). Analysis was performed on a Waters/Acquity ultra performance LC-MS system controlled with MassLynx software. Analysis was operated with electrospray ionization (ESI) probe in negative ESI employing multiple reaction monitoring (mrm) mode. LPA species for the establishment of mrm experiments were obtained from Avanti Polar Lipids. The following transitions were used: m/z 435 [M-H-] to 153 (LPA 18:1), m/z 409 [M-H-] to 153 (LPA 16:0), m/z 437 [M-H-] to 153 (LPA 18:0), and m/z 421 [M-H-] to 153 for the internal standard (LPA 17:1). Collision energy (ce) and cone voltage (cv) were both set to 30 V for all mrm experiments. The column used for chromatography was a Thermo Scientific Hypersil Gold, 1.9 μm, 50 × 2.1 mm column. A total flow rate of 0.4 ml/min was applied with an injection volume of 10 μl. The mobile phases consisted of 5 mM ammonium formate (Sigma-Aldrich) buffered water (A) and methanol (B), both containing 0.1 % formic acid (Sigma-Aldrich). Gradient elution was performed starting at 50 % mobile phase B applying isocratic conditions for 2.5 min and then progressing to 100 % B over 3 min. 100 % B was held up for 2 min. Within 1 min, the gradient increased linear to initial conditions and was held up to 10 min for re-equilibration. Column temperature was adjusted at room temperature (25 °C ± 5 °C). The autosampler operated at 10 °C. Run time was set to 10 min. Retention times of LPA species were 2.99 min (LPA 18:1), 2.95 min (LPA 18:0), 3.10 min (LPA 16:0), and 2.17 min (LPA 17:1 IS), respectively. Data analysis was performed using peak-area-ratios of analytes to internal standard.

Determination of nerve cytokine levels by ELISA

For the detection of cytokines, commercially available ELISA kits (Duo Set mouse TNF-α, R&D Systems, Minneapolis, MN, USA; Mouse IL-10 ELISA MAX Standard Set, Biolegend, San Diego, CA, USA) were used essentially as instructed. Briefly, sciatic nerves were homogenized in 250 μl ice-cold PBS containing cOmplete™ Mini (Roche, Basel, Switzerland) proteinase inhibitor cocktail. Homogenates were centrifuged for 10 min at 11,000 rpm, and supernatants were transferred to new sample tubes, diluted 1:2 with assay buffer (PBS containing 1 % BSA and 0.1 % Tween20) and vortexed rigorously. Detection of cytokines was performed as recommended by the manufacturers. Sciatic nerve cytokine content was normalized to the respective protein content of the sample.

Image and data analysis

Analysis of images was performed using ImageJ (National Institutes of Health, Bethesda, MA, USA). Data analysis and compilation of graphs was performed using Microsoft (Redmond, WA, USA) Excel and GraphPad (La Jolla, CA, USA) Prism 5. Statistical analysis was done by Student's *t* test, multiple comparisons were performed by one-way ANOVA followed by the Newman-Keuls post hoc test or Kruskal-Wallis test and Mann-Whitney *U* test for non-Gaussian distributions. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***.

Results

Impact of fingolimod on electrophysiological and clinical measures after injury

We performed sciatic nerve crush in immunocompetent wildtype and in immunodeficient T- and B-lymphocyte deficient *Rag1*$^{-/-}$ as well as in T-lymphocyte deficient *Foxn1*$^{-/-}$ mice to identify lymphocyte-independent effects of fingolimod on peripheral nervous system regeneration.

Fingolimod treatment resulted in significantly increased nerve conduction at 14 days post-crush in wildtype C57BL/6 mice (Fig. 1a). This finding is consistent with significantly improved functional recovery as determined by walking track analysis and SFI (Fig. 1c). In contrast, *Rag1*$^{-/-}$ mice did not show an improvement of nerve regeneration by clinical or electrophysiological measures neither with fingolimod nor DMSO-only treatment (Fig. 1a, c), and DMSO-treated control Rag1$^{-/-}$ mice even exhibited some additional mean, however, non-significant decline in SFI (Fig. 1c). However, *Foxn1*$^{-/-}$ mice, which

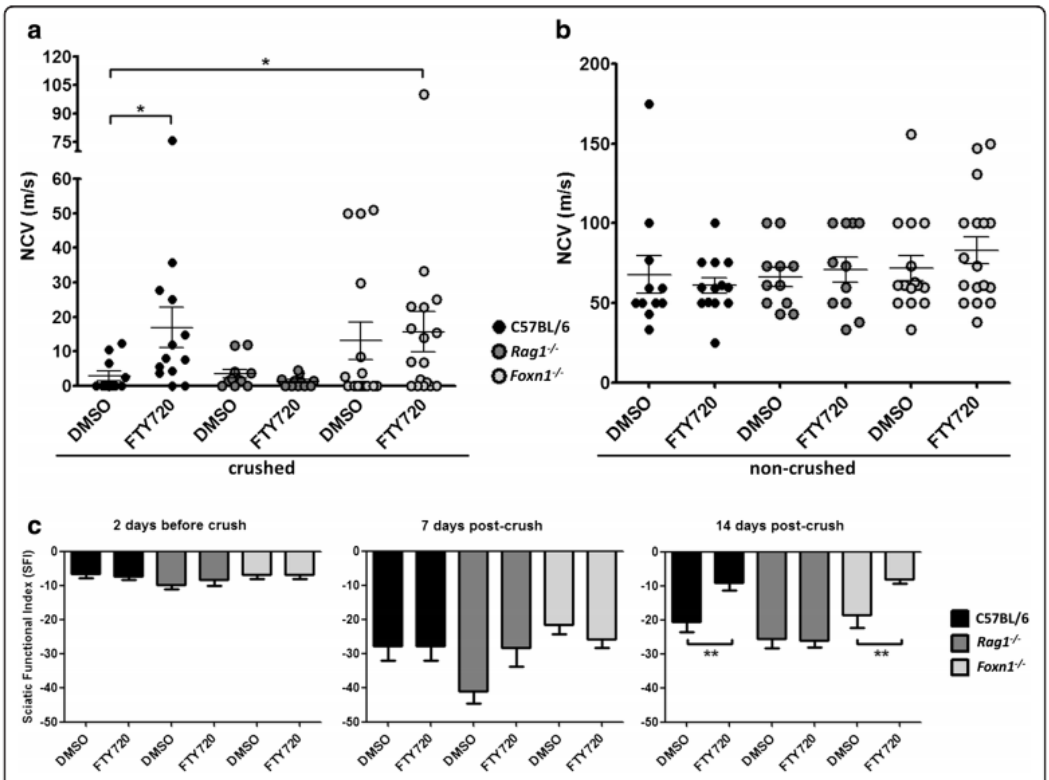


Fig. 1 Fingolimod (FTY720) improves peripheral nerve regeneration by electrophysiological and clinical measures. **a** C57BL/6 as well as *Foxn1*$^{-/-}$ mice treated with fingolimod were found to display significantly improved nerve conduction velocities (NCV) compared to wildtype controls. *Rag1*$^{-/-}$ mice did not show an improvement of nerve regeneration by this measure. Statistical analysis was done by Kruskal-Wallis test and Man-Whitney *U* test. $N = 11/13/11/11/15/17$ animals (from left to right). **b** Nerve conduction was normal in contralateral non-crushed nerves of the same animals. **c** Assessment of functional recovery was determined by walking track analysis via sciatic functional indices (SFI). Two days before crush sciatic nerve function was normal in all three mouse strains. Seven days post-crush nerve function was significantly impaired in all three mouse strains ($P \leq 0.01$) as indicated by a decline in SFI. Note that *Rag1*$^{-/-}$ control mice showed an additional mean, but non-significant worsening of nerve function. At 14 days post-crush, significantly improved nerve conduction is consistent with significantly improved SFI in fingolimod-treated C57BL/6 and *Foxn1*$^{-/-}$ animals. No significant difference in SFI was observed in *Rag1*$^{-/-}$ mice at this stage. $N = 8/9/7/6/9/14$ animals minimum for each graph (from left to right). Statistical analysis was done by Student's *t* test, two-tailed. Data represent mean ± s.e.m. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***

are devoid of T- but not B-lymphocytes, did show an improvement of nerve regeneration under fingolimod treatment (Fig. 1a, c). Although the mean increase in nerve conduction velocity in both fingolimod-treated and control *Foxn1*$^{-/-}$ mice implies a potentially positive role of T-lymphocyte deficiency on nerve regeneration, only fingolimod-treated *Foxn1*$^{-/-}$ mice showed a significant improvement compared to C57BL/6 controls and performed better in the functional analysis (Fig. 1a, c).

Effect of fingolimod on axonal cAMP levels

To better understand potentially relevant molecular mechanisms by which fingolimod may exert the observed neuroregenerative effects, we investigated cAMP, an axonal outgrowth enhancing factor that accumulates proximal from and around the injury site during axonal regeneration [31–34]. We hypothesized that fingolimod causes abrogation of $S1P_1$-mediated signaling through $S1P_1$ receptor internalization in cells of the peripheral nerve, thereby indirectly increasing cAMP levels by reduced inhibition of adenylate cyclase. Quantification of cAMP immunofluorescence proximal from and around the injury site at 14 days post-crush revealed significantly increased cAMP levels under fingolimod treatment in C57BL/6 and *Foxn1*$^{-/-}$ mice (Fig. 2a–c), and this increase in cAMP paralleled significantly improved clinical and

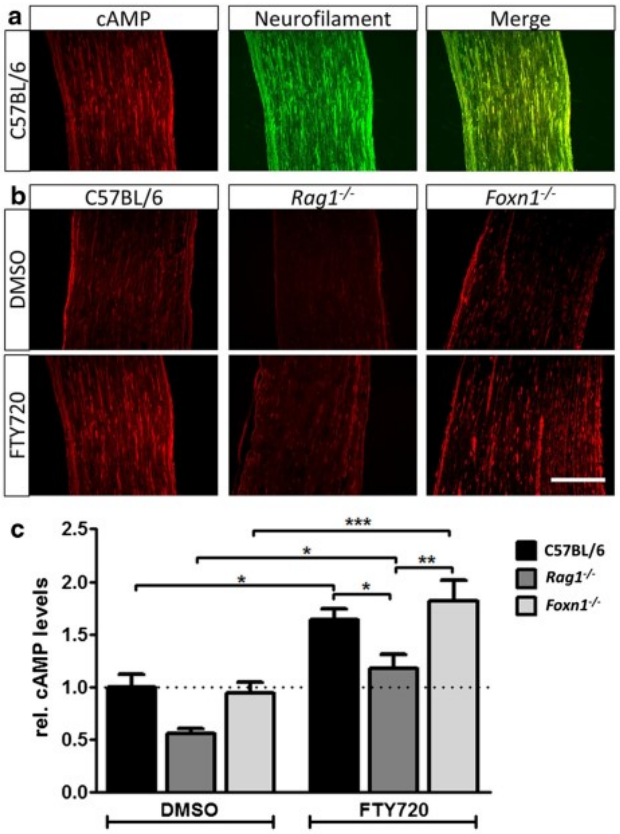


Fig. 2 Fingolimod (FTY720) significantly elevates cAMP levels in the regenerating nerve. **a** Fourteen-day post-crush, colocalization of cAMP and neurofilament light-chain immunohistochemistry indicates an accumulation of axonal cAMP around and proximal from the injury site. **b** Representative images of cAMP immunofluorescence. *Scale bar* indicates 100 μm. **c** Quantification of cAMP immunofluorescence revealed significantly increased axonal cAMP levels in crushed nerves under fingolimod treatment in wildtype mice and both immunodeficient mouse strains. Note that although fingolimod induced a significant cAMP increase in *Rag1*$^{-/-}$ mice compared to *Rag1*$^{-/-}$ controls, overall cAMP levels were reduced in *Rag1*$^{-/-}$ mice, which correlates with the impairment of regenerative capacity in these mice. $N = 4/4/6/4/4/6$ animals (from left to right; quantification of one representative slide per animal). Statistical analysis was done by one-way ANOVA and Newman-Keuls post hoc test. Data represent mean ± s.e.m. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***

electrophysiological measures in these animals (Fig. 1). In contrast, *Rag1*$^{-/-}$ mice did not show a relevant elevation of cAMP levels under fingolimod treatment when compared to wildtype or *Foxn1*$^{-/-}$ controls and cAMP levels were even significantly reduced in fingolimod-treated *Rag1*$^{-/-}$ compared to fingolimod-treated C57BL/6 and *Foxn1*$^{-/-}$ mice. Strikingly, overall reduced axonal cAMP correlates with impaired regeneration in fingolimod-treated *Rag1*$^{-/-}$ mice.

Myelin thickness after injury and inhibition of autotaxin

Following peripheral nerve injury, myelin sheaths of regenerated axons are commonly found to be significantly thinner compared to those of contralateral control nerves [35]. Therefore, to assess the effect of fingolimod on myelin thickness after injury, we performed g-ratio (the numerical ratio between the diameter of the axon and the diameter of the outer myelinated fiber) measurements from semi-thin sections prepared from the distal stump of injured and control nerves at 14 days post-crush (Fig. 3a). Analysis of g-ratios revealed significantly decreased myelin thickness in crushed nerves of control animals compared to myelin thickness in contralateral non-crushed nerves whereas myelin thickness was significantly increased in crushed nerves of fingolimod-treated mice (Fig. 3b). This was also the case in *Rag1*$^{-/-}$ mice.

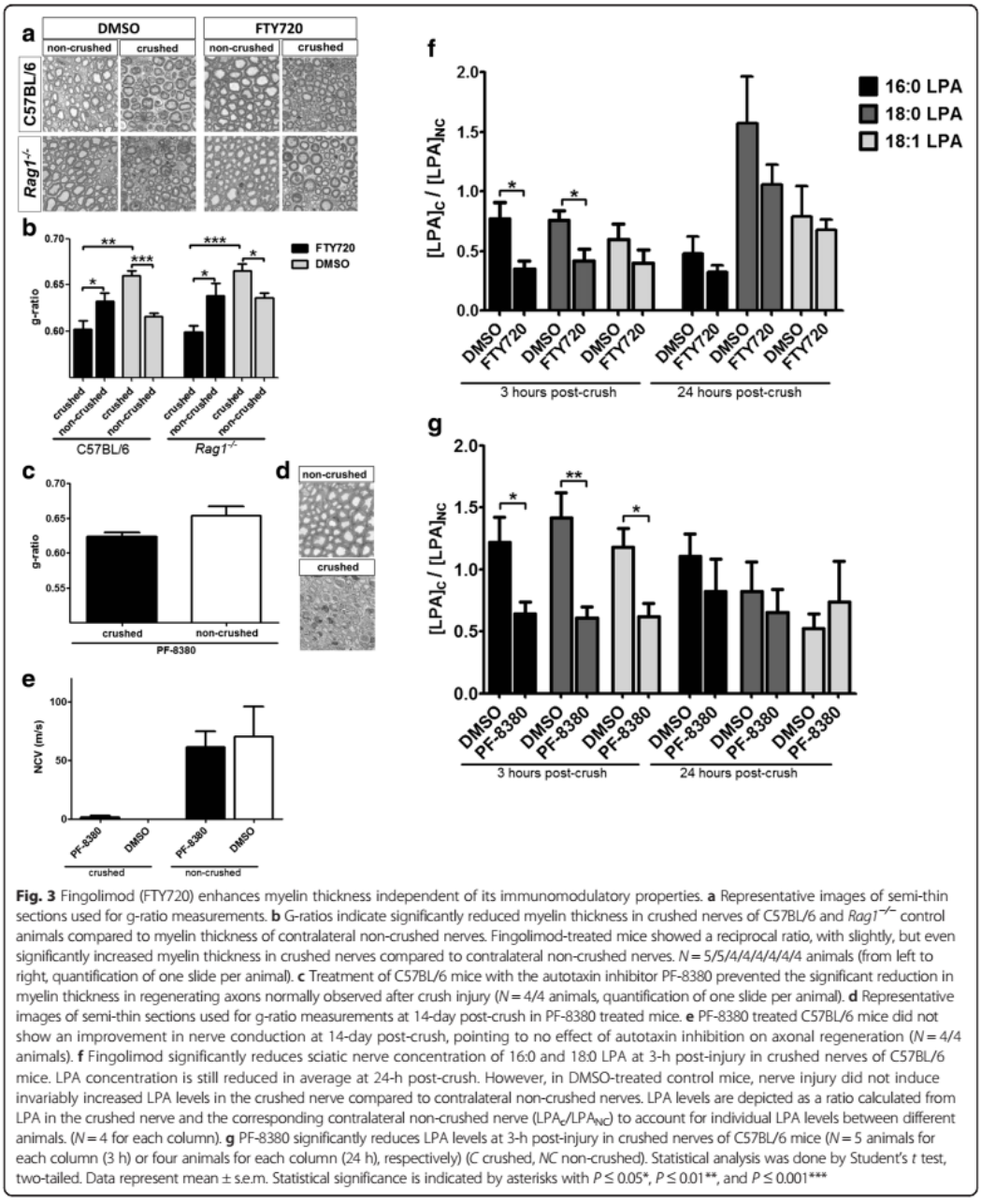


Fig. 3 Fingolimod (FTY720) enhances myelin thickness independent of its immunomodulatory properties. **a** Representative images of semi-thin sections used for g-ratio measurements. **b** G-ratios indicate significantly reduced myelin thickness in crushed nerves of C57BL/6 and *Rag1*$^{-/-}$ control animals compared to myelin thickness of contralateral non-crushed nerves. Fingolimod-treated mice showed a reciprocal ratio, with slightly, but even significantly increased myelin thickness in crushed nerves compared to contralateral non-crushed nerves. $N = 5/5/4/4/4/4/4/4$ animals (from left to right, quantification of one slide per animal). **c** Treatment of C57BL/6 mice with the autotaxin inhibitor PF-8380 prevented the significant reduction in myelin thickness in regenerating axons normally observed after crush injury ($N = 4/4$ animals, quantification of one slide per animal). **d** Representative images of semi-thin sections used for g-ratio measurements at 14-day post-crush in PF-8380 treated mice. **e** PF-8380 treated C57BL/6 mice did not show an improvement in nerve conduction at 14-day post-crush, pointing to no effect of autotaxin inhibition on axonal regeneration ($N = 4/4$ animals). **f** Fingolimod significantly reduces sciatic nerve concentration of 16:0 and 18:0 LPA at 3-h post-injury in crushed nerves of C57BL/6 mice. LPA concentration is still reduced in average at 24-h post-crush. However, in DMSO-treated control mice, nerve injury did not induce invariably increased LPA levels in the crushed nerve compared to contralateral non-crushed nerves. LPA levels are depicted as a ratio calculated from LPA in the crushed nerve and the corresponding contralateral non-crushed nerve (LPA_C/LPA_{NC}) to account for individual LPA levels between different animals. ($N = 4$ for each column). **g** PF-8380 significantly reduces LPA levels at 3-h post-injury in crushed nerves of C57BL/6 mice ($N = 5$ animals for each column (3 h) or four animals for each column (24 h), respectively) (*C* crushed, *NC* non-crushed). Statistical analysis was done by Student's *t* test, two-tailed. Data represent mean ± s.e.m. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***

Therefore, we hypothesized that fingolimod may improve myelin thickness by diminishing LPA levels in the injured nerve via inhibition of autotaxin. We assessed LPA levels in the sciatic nerve of C57BL/6 mice at 3 and 24 h post-crush under DMSO and fingolimod treatment. Analysis of sciatic nerve LPA content by LC-MS/MS

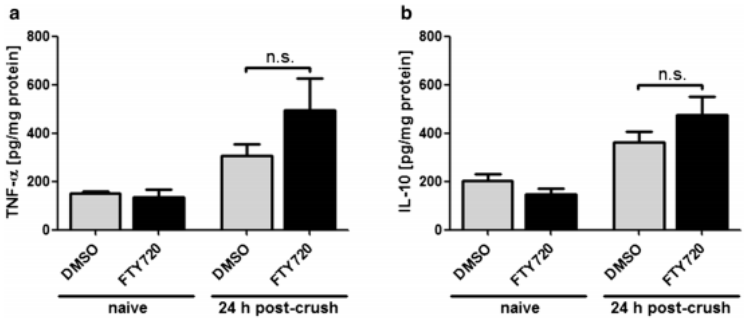


Fig. 4 Fingolimod (FTY720) does not alter pro- and anti-inflammatory cytokine levels after injury. Neither sciatic nerve levels of TNF-α (**a**) nor IL-10 (**b**) were found to be significantly affected by fingolimod treatment at 24 h post-crush. Baseline sciatic nerve cytokine levels were determined in naive mice. *N* = 3/3/11/9 animals for each graph, from left to right. Statistical analysis was done by Student's *t* test, two-tailed; n.s. indicates no significant difference. Data represent mean ± s.e.m

revealed significantly reduced LPA levels at 3 h post-crush in fingolimod-treated mice (Fig. 3f). Twenty four hours post-crush LPA levels were still lower in average, though not significantly. However, LPA levels were not found to be invariably increased in the crushed nerves of DMSO-treated control mice, indicating that physiological LPA levels produced by autotaxin may be sufficient to induce demyelination after injury. To evaluate the functional consequence of LPA reduction, we assessed myelin thickness in C57BL/6 mice treated with the specific autotaxin inhibitor PF-8380. G-ratio measurements from semi-thin sections obtained at 14 days post-crush revealed normal and strikingly not significantly reduced myelin thickness in distal regenerating axons (Fig. 3c), pointing to a role of LPA in demyelination or downregulation of myelin proteins, respectively. Finally, to distinguish LPA from S1P-mediated effects on axonal regeneration, we performed electrophysiology with animals treated with PF-8380 at 14 days post-crush. We could not detect any signs of improved nerve conduction in these mice, suggesting that inhibition of autotaxin and lowering LPA levels, respectively, may primarily affect myelination, but not peripheral axon regeneration (Fig. 3e).

Sciatic nerve cytokine levels after injury

Since cytokines are known to potentially impact nerve regeneration [36, 37], we finally tested whether fingolimod affects sciatic nerve levels of the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) and the anti-inflammatory cytokine interleukin 10 (IL-10), which are both known to be highly expressed at 24-h post-injury [38]. We recognized a significant increase of both TNF-α and IL-10 levels after injury but could not find fingolimod to significantly affect this cytokine response (Fig. 4).

Discussion

In this work, we provide in vivo evidence for direct effects of the S1P receptor agonist fingolimod on the peripheral nervous system that may result in the promotion of peripheral nerve regeneration. Although certain positive effects of fingolimod-induced T-lymphocyte sequestration on nerve regeneration cannot be ruled out completely, our data strongly indicate that direct effects of fingolimod on neurons and Schwann cells are responsible for improved nerve regeneration. Fingolimod treatment was associated with a significant elevation of axonal cAMP, a crucial factor for central and peripheral axon regeneration [31–34]. The observed increase in cAMP may most likely be the result of fingolimod-induced $S1P_1$ receptor internalization in cells of the peripheral nerve that may cause the abrogation of $S1P_1$-mediated inhibition of adenylate cyclase. It has been proposed that the $S1P_1$-G_i-adenylate cyclase system may become internalized as a ternary complex, leading to sustained inhibition of adenylate cyclase as long as the ligand fingolimod-P is bound [39]. However, we propose differences between "short-term" (several hours) and "long-term" (several days to weeks) administration of fingolimod as, in contrast to inhibition of cAMP formation in cell culture experiments by sustained signaling from intracellular compartments [40], our data demonstrate that long-term administration of fingolimod in vivo may result in an elevation of cAMP. The internalized $S1P_1$-G_i-adenylate cyclase system is likely to become degraded over time, and constantly high concentrations of fingolimod-P may prevent the localization of de novo synthesized $S1P_1$ to the plasma membrane. Hence, the pivotal difference in the long-term situation may be the spatial segregation of $S1P_1$ and adenylate cyclase, consequently allowing for an increased cAMP response in the regenerating

nerve. Clearly, further studies are needed to corroborate this assumption.

In contrast to T-lymphocyte-deficient *Foxn1*$^{-/-}$ mice which exhibited signs of significantly improved nerve regeneration under fingolimod treatment, B- and T-lymphocyte deficient *Rag1*$^{-/-}$ mice did not show an improvement of nerve regeneration, neither with fingolimod nor vehicle treatment. Although one recent study has reported evidence for improved nerve regeneration after femoral nerve injury in *Rag2*$^{-/-}$ mice which lack functional T- and B-lymphocytes as well [41], others have found nerve regeneration and motor neuron survival to be reduced in *Rag2*$^{-/-}$ mice [42], which is in line with our observations in *Rag1*$^{-/-}$ mice. The idea of an overall reduced regenerative capacity of *Rag1*$^{-/-}$ mice is further supported by the finding that B-lymphocytes appear to be required for the production of autoantibodies against myelin debris containing inhibitors of axonal outgrowth, consequently allowing for normal axonal regeneration after nerve injury [43]. Myelin-associated inhibitors of axonal outgrowth have been implicated in reducing neuronal cAMP in a G_i-dependent manner via activation of the Nogo receptor-p75 neurotrophin receptor complex [44], providing a likely explanation for significantly reduced axonal cAMP in response to fingolimod treatment and, consistently, the impaired regenerative capacity observed in *Rag1*$^{-/-}$ mice due to the lack of B-lymphocytes. Although fingolimod is known to also reduce the number of circulating B-lymphocytes, it has recently been shown to increase the proportion of regulatory B-lymphocytes producing IL-10. While we could not detect a significant elevation of IL-10 nor a decrease in TNF-α levels in the sciatic nerve of fingolimod-treated mice at 24-h post-crush (Fig. 4), an increase in IL-10 secreting B-lymphocytes may occur at later stages and could potentially contribute to accelerated nerve regeneration [37, 45].

However, despite decreased cAMP, *Rag1*$^{-/-}$ mice still showed significantly improved myelin thickness. Another member of the lysophospholipid family, LPA, was found to be significantly reduced in the crushed nerve in fingolimod-treated mice shortly after injury. Strikingly, inhibition of autotaxin by the specific inhibitor PF-8380 prevented the significant reduction of myelin thickness normally observed after peripheral nerve injury. Although LPA levels were not invariably and not significantly increased in control mice, it appears unlikely that a sole increase in LPA would account for demyelination, but rather an elevated expression of the LPA_1 receptor after injury. In this context, it has been reported that the LPA_1 receptor is upregulated within the first days after sciatic nerve injury in the distal nerve stump, and this upregulation is accompanied by an abrupt downregulation of the myelin gene *P0* [46]. In line with this, a single intrathecal injection of LPA or nerve injury was found to cause a decrease in the expression of the myelin proteins PMP22 and MBP, but the demyelinating effect was largely abolished in LPA_1-null mice [19]. Furthermore, antagonism of the LPA_1 receptor has recently been demonstrated to reduce demyelination after spinal cord injury [21]. Therefore, improved myelination could be a consequence of both fingolimod as well as PF-8380-mediated inhibition of autotaxin, attenuating LPA_1 receptor activation via reduced LPA synthesis. Although autotaxin represents only one of multiple metabolic pathways to produce LPA, autotaxin appears to be one major contributor to LPA synthesis after injury, as it was previously shown that injury-induced dorsal root demyelination is significantly reduced in heterozygous autotaxin knockout mice [19]. However, since PF-8380 treatment did not lead to signs of improved axonal regeneration, our results might suggest a dual mode of action for fingolimod in peripheral nerve regeneration—fingolimod may promote axonal regeneration by indirectly elevating cAMP formation as a consequence of $S1P_1$ receptor internalization and may prevent LPA-induced downregulation of myelin gene expression in the regenerating nerve. Further studies are warranted to corroborate this assumption.

Conclusions

Collectively, our data presented here demonstrate that modulation of lysophospholipid signaling in the peripheral nervous system by fingolimod may enhance nerve regeneration by acting on multiple molecular and cellular levels and partly independent of its anti-inflammatory effects. In addition to the established role of fingolimod as a modulator of S1P receptor-mediated signaling, the inhibition of LPA synthesis after injury may represent a yet unrecognized mechanism that contributes to the presumptive remyelinating effect of fingolimod.

Abbreviations

cAMP, cyclic adenosine monophosphate; *Foxn1*, forkhead box protein N1; IL-10, interleukin 10; LPA, lysophosphatidic acid; LPA_1, lysophosphatidic acid receptor 1; NF-L, neurofilament light chain; *Rag1*, recombination activating gene 1; S1P, sphingosine-1-phosphate; $S1P_1$, sphingosine-1-phosphate receptor 1; SFI, sciatic functional index; TNF-α, tumor necrosis factor alpha.

Acknowledgements

We like to thank Zippora Kohne for the excellent technical assistance.

Funding

GMzH was funded in part by a grant from the Deutsche Forschungsgemeinschaft (DFG grant number ME4050/1-1) and a National Multiple Sclerosis Society (NMSS) postdoctoral fellowship. There was no role of funding in the conception of this study, analysis, or interpretation of data or preparation of the manuscript.

Availability of data and materials

All methods and data supporting the conclusions of this work have been included in the article.

Authors' contributions
FS, AD, and GMzH conceived the study and designed the experiments. FS and AD performed the experiments. IS designed and performed the LC-MS/MS experiments. FS wrote the manuscript. All authors were involved in the discussion of results and reviewed the manuscript. All authors read and approved the final manuscript.

Competing interests
FS, AD, IS, GMzH, and TD have no competing interests. HPH received honoraria for consulting, speaking, and serving on steering committees from Bayer, Biogen, GeNeuro, Genzyme, MedImmune, Merck Serono, Novartis, Octapharma, Opexa, Receptos, Sanofi, and Teva with approval by the Rector of Heinrich-Heine-University. BCK has received honoraria for lecturing, travel expenses for attending meetings, and financial support for research from Bayer Health Care, Biogen Idec, Genzyme/Sanofi Aventis, Grifols, Merck Serono, Mitsubishi Europe, Novartis, Roche, Talecris, and TEVA.

Consent for publication
Not applicable.

Ethics approval and consent to participate
Animal use and experiments were approved by local authorities (LANUV North Rhine-Westphalia, Germany/application number 84.02.04.2012.A376).

Author details
[1]Department of Neurology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany. [2]Department of Forensic Toxicology, Institute of Legal Medicine, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany. [3]Evergrande Center for Immunologic Diseases, Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, Boston, MA, USA. [4]Department of Neurology, University of Münster, Münster, Germany.

Received: 26 October 2015 Accepted: 6 June 2016
Published online: 10 June 2016

References

1. Ishii I, Fukushima N, Ye X, Chun J. Lysophospholipid receptors: signaling and biology. Annu Rev Biochem. 2004;73:321–54.
2. Hla T. Physiological and pathological actions of sphingosine 1-phosphate. Semin Cell Dev Biol. 2004;15(5):513–20.
3. Sengupta S, Wang Z, Tipps R, Xu Y. Biology of LPA in health and disease. Semin Cell Dev Biol. 2004;15(5):503–12.
4. Mizugishi K, Yamashita T, Olivera A, et al. Essential role for sphingosine kinases in neural and vascular development. Mol Cell Biol. 2005;25(24):11113–21.
5. Meng H, Lee VM. Differential expression of sphingosine-1-phosphate receptors 1–5 in the developing nervous system. Dev Dyn. 2009;238(2):487–500.
6. Kono M, Mi Y, Liu Y, Sasaki T, et al. The sphingosine-1-phosphate receptors S1P1 S1P2 and S1P3 function coordinately during embryonic angiogenesis. J Biol Chem. 2004;279(28):29367–73.
7. Lee M, Brocklyn JR, Thangada S, et al. Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. Science. 1998;279(5356):1552–5.
8. Im DS, Heise CE, Ancellin N, et al. Characterization of a novel sphingosine 1-phosphate receptor Edg-8. J Biol Chem. 2000;275(19):14281–6.
9. Van Brocklyn JR, Gräler MH, Bernhardt G, et al. Sphingosine-1-phosphate is a ligand for the G protein-coupled receptor EDG-6. Blood. 2000;95(8):2624–9.
10. Windh RT, Lee M-J, Hla T, et al. Differential coupling of the sphingosine 1-phosphate receptors Edg-1 Edg-3 and H218/Edg-5 to the Gi Gq and G12 families of heterotrimeric G proteins. J Biol Chem. 1999;274(39):27351–8.
11. Glickman M, Malek RL, Kwitek-Black AE, et al. Molecular cloning tissue-specific expression and chromosomal localization of a novel nerve growth factor-regulated G-protein-coupled receptor nrg-1. Mol Cell Neurosci. 1999;14:141–52.
12. Okamoto H, Takuwa N, Gonda K, et al. EDG1 is a functional sphingosine-1-phosphate receptor that is linked via a Gi/o to multiple signaling pathways including phospholipase C activation Ca2+ mobilization Ras-mitogen-activated protein kinase activation and adenylate cyclase inhibition. J Biol Chem. 1998;273(42):27104–10.
13. Brinkmann V, Billich A, Baumruker T, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov. 2010;9:883–97.
14. Billich A, Bornancin F, Dévay P, et al. Phosphorylation of the immunomodulatory drug FTY720 by sphingosine kinases. J Biol Chem. 2003;278(48):47408–15.
15. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. J Biol Chem. 2002;277(24):21453–7.
16. Oo ML, Thangada S, Wu M-T, et al. Immunosuppressive and anti-angiogenic sphingosine 1-phosphate receptor-1 agonists induce ubiquitinylation and proteasomal degradation of the receptor. J Biol Chem. 2007;282(12):9082–9.
17. Matloubian M, Lo CG, Cinamon G, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature. 2004;427(6972):355–60.
18. van Meeteren LA, Brinkmann V, Saulnier-Blache JS, et al. Anticancer activity of FTY720: phosphorylated FTY720 inhibits autotaxin a metastasis-enhancing and angiogenic lysophospholipase D. Cancer Lett. 2008;266(2):203–8.
19. Inoue M, Rashid MH, Fujita R, et al. Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling. Nat Med. 2004;10(7):712–8.
20. Nagai J, Uchida H, Matsushita Y, et al. Autotaxin and lysophosphatidic acid 1 receptor-mediated demyelination of dorsal root fibers by sciatic nerve injury and intrathecal lysophosphatidylcholine. Mol Pain. 2010;6:78.
21. Crack JC, Moses Z, Morganti-Kossmann MC, et al. Anti-lysophosphatidic acid antibodies improve traumatic brain injury outcomes. J Neuroinflammation. 2014;11:37.
22. Santos-Nogueira E, López-Serrano C, Hernández J, et al. Activation of lysophosphatidic acid receptor type 1 contributes to pathophysiology of spinal cord injury. J Neurosci. 2015;35(28):10224–35.
23. Goldshmit Y, Matteo R, Sztal T, et al. Blockage of lysophosphatidic acid signaling improves spinal cord injury outcomes. Am J Pathol. 2012;181(3):978–92.
24. Toman R, Sarah S. Lysophospholipid receptors in the nervous system. Neurochem Res. 2002;27(7–8):619–27.
25. Groves A, Kihara Y, Chun J. Fingolimod: direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. J Neurol Sci. 2013;328(1–2):9–18.
26. Norimatsu Y, Ohmori T, Kimura A, et al. FTY720 improves functional recovery after spinal cord injury by primarily nonimmunomodulatory mechanisms. Am J Pathol. 2012;180:1625–35.
27. Lee KD, Chow WN, Sato-Bigbee C, et al. FTY720 reduces inflammation and promotes functional recovery after spinal cord injury. J Neurotrauma. 2009;26(12):2335–44.
28. Gierse J, Thorarensen A, Beltey K, et al. A novel autotaxin inhibitor reduces lysophosphatidic acid levels in plasma and the site of inflammation. J Pharm Exp Ther. 2010;334:310–7.
29. Inserra MM, Bloch DA, Terris DJ. Functional indices for sciatic peroneal and posterior tibial nerve lesions in the mouse. Microsurgery. 1998;18(2):119–24.
30. Meyer Zu Horste G, Prukop T, Liebetanz D, et al. Antiprogesterone therapy uncouples axonal loss from demyelination in a transgenic rat model of CMT1A neuropathy. Ann Neurol. 2007;61:61–72.
31. Carlsen RC. Axonal transport of adenylate cyclase activity in normal and axotomized frog sciatic nerve. Brain Res. 1982;232:413–24.
32. Kilmer SL, Carlsen RC. Forskolin activation of adenylate cyclase in vivo stimulates nerve regeneration. Nature. 1984;307(5950):455–7.
33. Pearse DD, Pereira FC, Marcillo AE. cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury Nat. Med. 2004;10(6):610–6.
34. Qiu J, Cai D, Dai H. Spinal axon regeneration induced by elevation of cyclic AMP. Neuron. 2002;34(6):895–903.
35. Schröder JM. Altered ratio between axon diameter and myelin sheath thickness in regenerated nerve fibers. Brain Res. 1972;45(1):49–65.
36. Kato K, Liu H, Kikuchi SI, et al. Immediate anti-tumor necrosis factor-α (etanercept) therapy enhances axonal regeneration after sciatic nerve crush. J Neurosci Res. 2010;88(2):360–6.
37. Atkins S, Loescher AR, Boissonade FM, et al. Interleukin-10 reduces scarring and enhances regeneration at a site of sciatic nerve repair. J Peripher Nerv Syst. 2007;12:269–76.
38. Taskinen HS, Olsson T, Bucht A, et al. Peripheral nerve injury induces endoneurial expression of IFN-γ, IL-10 and TNF-α mRNA. J Neuroimmunol. 2000;102(1):17–25.
39. Jalink K, Moolenaar WH. G protein-coupled receptors: the inside story. Bioessays. 2010;32:13–6.
40. Mullershausen F, Zecri F, Cetin C, et al. Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. Nat Chem Biol. 2009;5(6):428–34.

41. Mehanna A, Szpotowicz E, Schachner M, Jakovcevski I. Improved regeneration after femoral nerve injury in mice lacking functional T- and B-lymphocytes. Exp Neurol. 2014;261:147–55.
42. Beahrs T, Tanzer L, Sanders VM, Jones KJ. Functional recovery and facial motoneuron survival are influenced by immunodeficiency in crush-axotomized mice. Exp Neurol. 2010;221:225–30.
43. Vargas ME, Watanabe J, Singh SJ, et al. Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. Proc Natl Acad Sci USA. 2010;107(26):11993–8.
44. Filbin MT. Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat Rev Neurosci. 2003;4(9):703–13.
45. Grützke B, Hucke S, Gross CC, et al. Fingolimod treatment promotes regulatory phenotype and function of B cells. Ann Clin Transl Neurol. 2015;2(2):119–30.
46. Weiner J, Fukushima N, Contos JJA, et al. Regulation of Schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling. J Neurosci. 2001;21(18):7069–78.

III.III. Targeting lysophospholipid signaling as a therapeutic approach towards improved peripheral nerve regeneration (Szepanowski and Kieseier 2016)

Fabian Szepanowski and Bernd C. Kieseier

In this perspective article, the findings from the related paper “Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling” are summarized and discussed. On this basis, a hypothetical mechanism of action for fingolimod in peripheral nerve regeneration is proposed.

Personal contribution: 95 % (manuscript preparation: 90 %; figure preparation: 100 %)

Status: Published (December 9th 2016)

Journal: *Neural Regeneration Research (Neural. Regen. Res.)*

Journal statistics from Journal Citation Reports (Thomson Reuters):

Impact Factor (2016): 1.769

5-year Impact Factor (2016): 0.984

Journal Ranking: 202 / 258 (Neurosciences), 153 / 189 (Cell Biology)

DOI: 10.4103/1673-5374.194720

PERSPECTIVE

Targeting lysophospholipid signaling as a therapeutic approach towards improved peripheral nerve regeneration

Peripheral nerve injury causes a partial or total loss of motor and sensory functions as a result of axonal disruption and subsequent axonal disintegration as well as denervation distal from the point of injury. Although peripheral nerves are, in contrast to the central nervous system, able to regenerate and reinnervate, functionality is not always restored completely due to insufficient reinnervation or remyelination, and injury may result in sequelae such as neuropathic pain. The degenerative processes following peripheral nerve injury are generally referred to as Wallerian degeneration (Gaudet et al., 2011).

In rodents, the initial response to injury occurs within 24 hours and is characterized by Schwann cells detaching from their associated axons accompanied by degeneration of the insulating myelin sheaths and a subsequent breakdown of axonal integrity; Schwann cells rapidly dedifferentiate and start proliferating. These dedifferentiated Schwann cells and resident macrophages are among the first cells to recognize the injury and secrete pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and chemokines, *e.g.*, monocyte chemoattractant protein 1 (MCP-1), both of which propagate the recruitment of hematogenous monocytes and macrophages, respectively (Meyer zu Hörste et al., 2007; Gaudet et al., 2011). This well-orchestrated cellular response to injury allows for the timely clearace of cellular and myelin debris in order to enable axon regeneration from the largely unaffected proximal stump. One crucial factor that is known to determine the speed of axonal regrowth is cyclic adenosine monophosphate (cAMP) (Hannila and Filbin, 2008).

We have recently investigated whether modulation of lysophospholipid signaling using the immunomodulatory drug fingolimod (also named FTY720) may propagate nerve regeneration in a mechanical injury model of the peripheral nervous system (Szepanowski et al., 2016). Fingolimod is a first-in-class sphingosine-1-phosphate (S1P) receptor agonist that is thought to exert a "functionally antagonistic" effect on the $S1P_1$ receptor subtype by facilitating its internalization. It thereby prevents the egress of $S1P_1$ expressing activated lymphocytes from lymph nodes (Brinkmann et al., 2010). Additionally, fingolimod has been reported to act as an inhibitor of the lysophospholipase autotaxin, thereby reducing lysophosphatidic acid (LPA) biosynthesis (van Meeteren et al., 2008). Both S1P and LPA are bioactive lysophospholipids that address specific G-protein coupled receptors. S1P and LPA receptors have been recognized to be widely expressed in the nervous system and have been associated with a variety of physiological and pathophysiological processes. Not surprisingly, there have been numerous studies indicating direct effects of fingolimod on cells of the central nervous system, including neuroprotective and remyelinating properties (Groves et al., 2013). To evaluate the regenerative potential of fingolimod and to distinguish its immunosuppressive from potential direct effects on the peripheral nervous system *in vivo*, sciatic nerve crush injury was performed in wildtype as well as in immunodeficient mice. It was demonstrated that fingolimod treatment improved nerve regeneration by electrophysiological and clinical measures not only in immunocompetent mice, but also independent of its effect on T-lymphocyte sequestration. Interestingly, in combined B- and T-lymphocyte deficient mice, fingolimod treatment failed to cause any improvements by these measures. In order to identify the molecular mechanism underlying this discrepant response to fingolimod treatment, axonal cAMP levels were studied. A significant elevation of axonal cAMP in wildtype and in T-lymphocyte deficient mice was recognizable, whereas combined T- and B-lymphocyte deficient mice displayed overall reduced cAMP. Consistent with these findings, an impairment of regeneration after nerve injury has previously been described for B-lymphocyte deficient mice (Vargas et al., 2010). Vargas and colleagues demonstrated that a timely onset of axonal regrowth is dependent on B lymphocytes producing autoantibodies against myelin debris; the absence of B-lymphocytes resulted in delayed axonal recovery. Myelin debris contains inhibitors of axonal regeneration which are thought to at least partly act through the Nogo-p75 neurotrophin receptor complex, inhibiting axonal cAMP formation in a G_i dependent manner (Hannila and Filbin, 2008). Our finding of significantly reduced cAMP in combined immunodeficient mice, but not in exclusively T-lymphocyte deficient mice, may accentuate the importance of B-lymphocytes for peripheral nerve regeneration, and may provide a plausible explanation for the lack of effectiveness of fingolimod in combined immunodeficient mice.

Another beneficial effect of fingolimod treatment comprised an improvement of myelin thickness in regenerating axons, which surprisingly also occurred in B- and T-lymphocyte deficient mice. Thus, we considered this finding unlikely to be cAMP related, leading us to investigate LPA, which has been linked to demyelination in numerous nerve injury models (Yung et al., 2015). Quantification of LPA from sciatic nerve homogenates *via* liquid chromatography coupled to tandem mass spectrometry revealed that fingolimod reduces LPA shortly after injury. Although 24 hours post-injury no significant difference in LPA levels between control and fingolimod treated mice was evident anymore, a transient attenuation of LPA signaling may be sufficient to ameliorate tissue damage outcomes and demyelination (Crack et al., 2014). Since we hypothesized the reduction of LPA to be a consequence of fingolimod mediated autotaxin inhibition, mice were treated with the specific autotaxin inhibitor PF-8380 to differentiate between S1P and LPA mediated effects on myelination. The effect of PF-8380 on myelination resembled that of fingolimod, but did not affect axon regeneration, confirming a supportive effect of autotaxin inhibition on myelin integrity.

A previous study investigating the regenerative potential of fingolimod in the peripheral nervous system *in vitro* proposed a different mode of action (Heinen et al., 2015). Heinen and colleagues suggest that fingolimod may not support axon outgrowth or myelination *via* direct actions on neurons or Schwann cells, but may induce the secretion of neurotrophic factors from Schwann cells which in turn promote axonal sprouting. The authors report that the cAMP inducible expression of a positive regulator of myelination, Krox-20, was counteracted by fingolimod in forskolin treated Schwann cells. While $S1P_1$ receptor signaling is known to reduce intracellular cAMP levels *via* inhibition of adenylate cyclase in a G_i dependent manner, the antagonistic effect of fingolimod on $S1P_1$ would be expected to increase cAMP production. Interestingly, it was shown for cell culture experiments involving $S1P_1$ receptor expressing CHO cells that short-term incubation with fingolimod causes persistent S1P signaling from intracellular compartments, leading to sustained inhibition of cAMP formation (Mullershausen et al., 2009). In this context, it has been suggested that the $S1P_1$-G_i-adenylate cyclase system might be internalized as a ternary complex, thereby suppressing enzymatic activity of adenylate cyclase as long as the ligand fingolimod is bound (Jalink and Moleenaar, 2010). In contrast to inhibition of cAMP formation *in vitro*, the increase in axonal cAMP observed in our recent study may be the result of a long-term treatment regime with fingolimod for more than two weeks, where constantly high concentrations of fingolimod may potentially affect the dynamics of receptor internalization, leading to a spatial segregation of $S1P_1$ and adenylate cyclase by "trapping" *de novo* synthesized $S1P_1$ in intracellular compartments and allowing for an increased activation of membrane-associated adenylate cyclase during the course of axonal regeneration (**Figure 1**).

As such, potentially beneficial effects of fingolimod may be

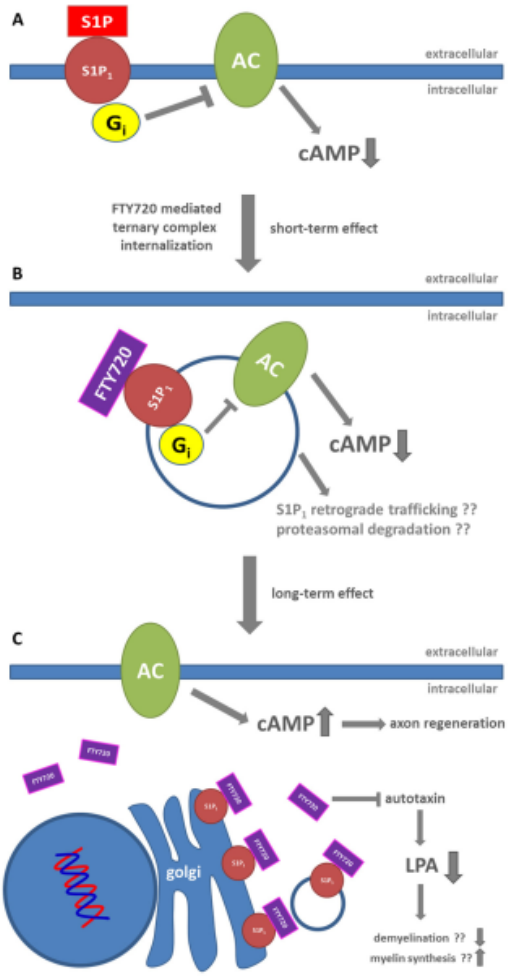


Figure 1 Possible mode of action for fingolimod (FTY720) mediated improvement of nerve regeneration.
In the presence of the natural ligand sphingosine-1-phosphate (S1P), activation of the $S1P_1$ receptor leads to inhibition of adenylate cyclase (AC) through G_i. (A) Binding of phosphorylated FTY720 to $S1P_1$ may lead to internalization of the $S1P_1$-G_i-adenylate cyclase system as ternary complex causing sustained inhibition of cyclic adenosine monophosphate (cAMP) formation. The ternary complex may directly undergo proteasomal degradation or retrograde transport to the Golgi. (B) In the presence of constantly high concentrations of FTY720, *de novo* synthesized $S1P_1$ may be 'trapped' in intracellular compartments, possibly the Golgi, preventing re-localization of $S1P_1$ to the plasma membrane and thus formation of the presumptive $S1P_1$-G_i-adenylate cyclase complex. This allows for increased generation of cAMP due to a reduction of adenylate cyclase inhibition. Additionally, FTY720 may attenuate demyelination *via* inhibition of autotaxin catalyzed LPA formation (C).

based on an early stimulation of axonal sprouting *via* neurotrophic factors released by Schwann cells as well as an attenuation of LPA signaling. At later stages, fingolimod may support axon outgrowth *via* an abrogation of S1P signaling, allowing for an increased cAMP response in the regenerating nerve.

Certainly, there is a need for future studies to further elucidate the molecular mechanisms underlying the presumptive neuroregenerative effects of fingolimod. The current development of novel S1P receptor agonists with greater specificity to S1P receptor subtypes may dramatically expand our understanding of the role of lysophospholipid signaling in physiological and pathophysiological conditions of the nervous system. However, given the emerging body of evidence so far, modulation of lysophospholipid signaling appears not only to be a highly relevant therapeutic target for immunomodulation, but could possibly also represent a promising target for inducing clinically meaningful improvements after primary and secondary nerve damage.

Fabian Szepanowski*, Bernd C. Kieseier

Department of Neurology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany

***Correspondence to:** Fabian Szepanowski, M.Sc., fabian.szepanowski@uni-duesseldorf.de.*
***Accepted:** 2016-11-11*
***orcid:** 0000-0003-2744-5416 (Fabian Szepanowski)*
***doi:** 10.4103/1673-5374.194720*
***How to cite this article:** Szepanowski F, Kieseier BC (2016) Targeting lysophospholipid signaling as a therapeutic approach towards improved peripheral nerve regeneration. Neural Regen Res 11(11):1754-1755.*

References

Brinkmann V, Billich A, Baumruker T, Heining P, Schmouder R, Francis G, Aradhye S, Burtin P (2010) Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. Nat Rev Drug Discov 9:883-897.
Crack PJ, Zhang M, Morganti-Kossmann MC, Morris AJ, Wojciak JM, Fleming JK, Karve I, Wright D, Sashindranath M, Goldshmit Y, Conquest A, Daglas M, Johnston LA, Medcalf RL, Sabbadini RA, Pébay A (2014) Anti-lysophosphatidic acid antibodies improve traumatic brain injury outcomes. J Neuroinflammation 11:37.
Gaudet AD, Popovich PG, Ramer MS (2011) Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. J Neuroinflammation 8:110.
Groves A, Kihara Y, Chun J (2013) Fingolimod: direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. J Neurol Sci 328:9-18.
Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp Neurol 209:321-332.
Heinen A, Beyer F, Tzekova N, Hartung HP, Küry P (2015) Fingolimod induces the transition to a nerve regeneration promoting Schwann cell phenotype. Exp Neurol 271:25-35.
Jalink K, Moolenaar WH (2010) G protein-coupled receptors: the inside story. Bioessays 32:13-16.
Meyer zu Hörste G, Hartung HP, Kieseier BC (2007) From bench to bedside--experimental rationale for immune-specific therapies in the inflamed peripheral nerve. Nat Clin Pract Neurol 3:198-211.
Mullershausen F, Zecri F, Cetin C, Billich A, Guerini D, Seuwen K (2009) Persistent signaling induced by FTY720-phosphate is mediated by internalized S1P1 receptors. Nat Chem Biol 5:428-434.
Szepanowski F, Derksen A, Steiner I, Meyer Zu Hörste G, Daldrup T, Hartung HP, Kieseier BC (2016) Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling. J Neuroinflammation 13:143.
van Meeteren LA, Brinkmann V, Saulnier-Blache JS, Lynch KR, Moolenaar WH (2008) Anticancer activity of FTY720: phosphorylated FTY720 inhibits autotaxin, a metastasis-enhancing and angiogenic lysophospholipase D. Cancer Lett 266:203-208.
Vargas ME, Watanabe J, Singh SJ, Robinson WH, Barres BA (2010) Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. Proc Natl Acad Sci U S A 107:11993-11998.
Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic acid signaling in the nervous system. Neuron 85:669-682.

III.IV. Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling (Szepanowski et al. 2018)

Fabian Szepanowski, Leon-Phillip Szepanowski, Anne K. Mausberg, Christoph Kleinschnitz, Bernd C. Kieseier and Mark Stettner

Lysophosphatidic acid (LPA) is a pleiotropic signaling lipid that acts as ligand for at least six specific G-protein coupled receptors. Schwann cells (SC) are known to mainly express the LPA1 receptor subtype. An emerging body of evidence has linked LPA with injury-induced peripheral nerve demyelination as well as neuropathic pain. However, the molecular mechanisms underlying its demyelinating effect have not been conclusively elucidated.

We aimed to decipher the demyelinating effect *in vitro* as well as *in vivo* by studying markers of SC differentiation and dedifferentiation: Myelinated dorsal root ganglia (DRG) cultures were treated either with LPA, LPA plus AM095 (LPA1 antagonist) or vehicle. Myelin content was subsequently investigated by Sudan Black staining and immunocytochemistry. *In vivo*, we performed sciatic nerve crush in C57BL/6 mice treated with AM095 at 10 mg/kg.

In DRG cultures, LPA caused a significant reduction of myelin as demonstrated by both Sudan Black staining and immunocytochemical analysis of myelin basic protein. Demyelination was paralleled by an upregulation of TNF-alpha as well as downregulation of Sox10, a marker for SC differentiation. LPA mediated effects were largely blocked by the addition of the LPA1 receptor antagonist AM095. In the *in vivo* model, AM095 treatment prior to crush injury increased Sox10 expression in SCs in the distal nerve stump while reducing the number of cells expressing the SC dedifferentiation marker Sox2. Additionally, overall TNF-alpha immunofluorescence was found to be reduced. These data indicate that LPA may be a critical factor that shifts SCs towards a post-injury phenotype and contributes to the onset of Wallerian degeneration.

Personal contribution: approx. 80 % (study design: 95 %; performance of experiments: 75 %; data analysis: 80 %; figure preparation: 100 %; writing of manuscript: 100 %)

An ‘authors’ contributions’ section is provided at the end of the manuscript.

Status: Published – online first (October 16th 2017)

Journal: *Neuroscience Letters (Neurosci. Lett.)*

Journal statistics from Journal Citation Reports (Thomson Reuters):
Impact Factor (2016): 2.180

5-year Impact Factor (2016): 2.110

Journal Ranking: 179 / 258 (Neurosciences)

DOI: 10.1016/j.neulet.2017.10.023

Neuroscience Letters 662 (2018) 136–141

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling

Fabian Szepanowski[a,b,*], Leon-Phillip Szepanowski[b], Anne K. Mausberg[b], Christoph Kleinschnitz[b], Bernd C. Kieseier[a], Mark Stettner[b]

[a] Department of Neurology, Medical Faculty, Heinrich-Heine-University, Düsseldorf, Germany
[b] Department of Neurology, Medical Faculty, University Duisburg-Essen, Essen, Germany

ARTICLE INFO

Keywords:
Lysophosphatidic acid
AM095
Schwann cell differentiation
Nerve injury
Demyelination
Neuropathy

ABSTRACT

Lysophosphatidic acid (LPA) is a pleiotropic signaling lipid that acts as ligand for at least six specific G-protein coupled receptors. Schwann cells (SC) are known to mainly express the LPA_1 receptor subtype. An emerging body of evidence has linked LPA with injury-induced peripheral nerve demyelination as well as neuropathic pain. However, the molecular mechanisms underlying its demyelinating effect have not been conclusively elucidated.

We aimed to decipher the demyelinating effect *in vitro* as well as *in vivo* by studying markers of SC differentiation and dedifferentiation: Myelinated dorsal root ganglia (DRG) cultures were treated either with LPA, LPA plus AM095 (LPA_1 antagonist) or vehicle. Myelin content was subsequently investigated by Sudan Black staining and immunocytochemistry. *In vivo*, we performed sciatic nerve crush in C57BL/6 mice treated with AM095 at 10 mg/kg.

In DRG cultures, LPA caused a significant reduction of myelin as demonstrated by both Sudan Black staining and immunocytochemical analysis of myelin basic protein. Demyelination was paralleled by an upregulation of TNF-alpha as well as downregulation of Sox10, a marker for SC differentiation. LPA mediated effects were largely blocked by the addition of the LPA_1 receptor antagonist AM095. In the *in vivo* model, AM095 treatment prior to crush injury increased Sox10 expression in SCs in the distal nerve stump while reducing the number of cells expressing the SC dedifferentiation marker Sox2. Additionally, TNF-alpha immunofluorescence was reduced in CD11b-positive cells. These data indicate that LPA may be a critical factor that shifts SCs towards a post-injury phenotype and contributes to the onset of Wallerian degeneration.

1. Introduction

Sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) represent the two major bioactive members of the lysophospholipid family. Both lipids have been ascribed significant physiological and pathophysiological roles in the developing and adult nervous, vascular and immune system by acting as ligands for specific G-protein coupled receptors [4]. While the clinical efficacy of S1P receptor modulation has been established for multiple sclerosis [9], LPA receptors represent an emerging therapeutic target for a variety of inflammatory diseases, with phase II clinical trials being completed for systemic sclerosis and idiopathic pulmonary fibrosis [15].

In the peripheral nerve, myelinating Schwann cells are known to be responsive to LPA mainly via expression of the LPA receptor 1 (LPA_1) [3,6]. Activation of LPA_1 has been associated with neuropathic pain after partial sciatic nerve injury and a single intrathecal injection of LPA was found to be sufficient to trigger demyelination in dorsal roots [12]. In this context, LPA treatment was shown to result in the downregulation of myelin basic protein (MBP) and myelin protein zero (P_0) [12]. Additionally, upregulation of LPA_1 has been recognized to occur in the distal nerve stump following injury [6]. However, significant gaps in our understanding of the mechanism by which LPA facilitates demyelination in the peripheral nervous system have remained.

Interestingly, it was previously reported that LPA induces proliferation in Schwann cells isolated from axotomized sciatic nerves [17]. The induction of proliferation in otherwise quiescent adult Schwann cells is a major hallmark of Wallerian degeneration [1]. In response to injury, Schwann cells rapidly (within 24 h) undergo dedifferentiation and start to proliferate. Furthermore, Schwann cells are involved in the initial cytokine/chemokine response by secreting cues

* Corresponding author at: Department of Neurology, Moorenstrasse 5, 40225 Düsseldorf, Germany.
E-mail address: fabian.szepanowski@uni-duesseldorf.de (F. Szepanowski).

http://dx.doi.org/10.1016/j.neulet.2017.10.023
Received 5 July 2017; Received in revised form 27 September 2017; Accepted 15 October 2017
Available online 16 October 2017
0304-3940/

such as tumor necrosis factor alpha (TNF-alpha) and monocyte chemoattractant protein 1 (MCP-1) [1]. Given that LPA has been demonstrated to induce cytokine expression in a variety of cell types [21], we hypothesized that LPA may not only promote Schwann cell proliferation, but may actually contribute to Schwann cell dedifferentiation and the emergence of a post-injury phenotype that is typically associated with peripheral nerve injury.

2. Materials and methods

2.1. Preparation of murine dorsal root ganglia (DRG) cultures

Undissociated DRG cultures were prepared essentially as previously described [14]. DRGs were harvested from E 15.5 C57BL/6 embryos (Janvier Labs, Le Genest-Saint-Isle, France) by opening the cutis and subcutis along the spine and removing the spinal cord not completely enclosed by the vertebral column. DRGs were collected in Leibovitz's L-15 medium (Thermo Fisher Scientific/Gibco, Waltham, MA, USA) stored on wet ice, centrifuged at 800 rpm for 5 min and carefully resuspended in Neurobasal medium (NBM). NBM consisted of Dulbecco's modified eagle's medium (DMEM, low glucose; Lonza, Basel Switzerland) containing 10% horse serum (Invitrogen, Carlsbad, CA, USA), 50 U/ml penicillin/streptomycin (Sigma-Aldrich, Munich, Germany), 10 ng/ml nerve growth factor beta (Sigma-Aldrich), 4 g/l D-glucose (Sigma-Aldrich) and 2 mM L-glutamine (Gibco). DRGs were plated on collagen-coated 24-well plates. Following incubation in NBM for seven days, cultures were incubated with mouse myelination medium (MMM). MMM consisted of minimal essential medium (MEM; Invitrogen) containing 10% horse serum, 4 g/l D-glucose, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 5 ng/ml nerve growth factor beta, 0.02 mg/ml bovine pituitary extract (Merck Millipore, Billerica, MA, USA), 0.005% ascorbic acid (Sigma-Aldrich), 500 nM forskolin (Sigma-Aldrich) and N2 supplement (Invitrogen). MMM was renewed every 3-4 days. Cultures were kept at 37 °C and 10 % CO_2 atmosphere.

2.2. Treatment of cultures with LPA and AM095

LPA (1-oleoyl-2-hydroxy-sn-glycero-3-phosphate) was obtained from Avanti Polar Lipids (Alabaster, AL, USA) and the LPA_1 antagonist AM095 from ApexBio (Boston, MA, USA). LPA was dissolved in PBS containing 0.1% bovine serum albumin (BSA, fatty acid free; Sigma Aldrich). AM095 was dissolved in DMSO (Sigma-Aldrich). After 10 days incubation in MMM, cultures were treated with forskolin-omitted MMM containing either vehicle (0.01% BSA and 0.1% DMSO), 10 µM LPA + 0.1% DMSO or 10 µM LPA + 10 µM AM095 for 24 h.

2.3. Sciatic nerve crush and administration of AM095

Male, age-matched (3–4 months) C57BL/6 mice (Janvier Labs) were anesthetized for surgery via intraperitoneal injection of a mixture of xylazine (Rompun; Bayer, Leverkusen, Germany) (10 mg/kg) and ketamine (Actavis, Munich, Germany) (100 mg/kg) and placed on a heating plate (37 °C) to maintain constant body temperature. All instruments were sterilized. The fur of the lower back was removed with an electric razor, and the skin was disinfected using 70% ethanol. A small incision (1 cm) was made in the skin above the right hindlimb between the mm. gluteus maximus and biceps femoris. Opening the facial plane between both muscles revealed the sciatic nerve which was carefully lifted using bent forceps and crushed right before its distal branches using a lockable non-serrated clamp at maximum intensity for 30 s. The nerve was replaced under the muscle, and the incision was closed using non-absorbable suture material.

AM095 was administered at a concentration of 10 mg/kg [7] via intraperitoneal injection immediately before surgery. Controls received an equal volume of vehicle.

2.4. Tissue preparation and immunohistochemistry

Mice were sacrificed 24 h post-crush via cervical dislocation. Preparation of sciatic nerves and immunohistochemical procedures were performed essentially as described previously [2]. Images were taken using a Zeiss Axioplan 2 microscope.

2.5. Antibodies

The following antibodies were used and diluted in Antibody Diluent (Dako) as indicated: mouse anti-MBP monoclonal SMI-94 (Biolegend, San Diego, CA, USA) 1:500; rabbit anti-neurofilament L polyclonal (Merck-Millipore) 1:800; rat anti-TNF-alpha monoclonal MP6-XT22 (Acris, Herford, Germany) 1:100; rabbit anti-Sox2 polyclonal (abcam, Cambridge, UK) 1:1000; rabbit anti-Sox10 polyclonal (abcam) 1:1000; rat anti-mouse CD11b clone 5C6 (Serotec) 1:200; Alexa Fluor 488/594 goat anti-rabbit IgG (Life Technologies, Carlsbad, CA, USA) 1:200; Alexa Fluor 594 goat anti-rat IgG (Life Technologies) 1:200; biotinylated goat anti-rabbit IgG (Vector Laboratories) 1:200; DyLight 594 Streptavidin (Vector Laboratories) 1:200.

2.6. Immunocytochemistry

Cultures were fixed with 4% PFA for 20 min, washed and incubated with blocking solution (PBS containing 10% normal goat serum and 0.1% Triton X-100) for 1 h at room temperature (RT). Cultures were incubated with primary antibodies for 1 h at RT. Following two washing steps in PBST (PBS containing 0.1% Triton X-100), secondary antibodies were applied and incubated for 1 h at RT. Following three washing steps in PBST, PBS was applied to each well and fluorescence images were immediately taken in randomly selected fields by a blinded investigator using a Nikon Eclipse TE200 microscope.

2.7. Sudan black staining

Cultures were stained with Sudan Black dye as described previously [14] to assess the extent of myelination. Myelin quantification was performed by counting the total number of internodes and correlating them to the total number of neurons within the individual culture wells. Images were taken on a Zeiss LSM510 microscope.

2.8. Image and data analysis

Image analysis was performed using ImageJ (NIH, Bethesda, MA, USA). Data analysis and compilation of graphs was performed using Microsoft Excel 2010 and GraphPad Prism 5. Statistical analysis was done by Student's *t*-test, for multiple comparisons one-way ANOVA followed by Newman-Keuls post hoc test was performed. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$**, and $P \leq 0.001$***.

3. Results

3.1. LPA causes demyelination of DRG cultures in an LPA_1 dependent manner

To study the effect of LPA on demyelination *in vitro*, myelinated DRG cultures were treated with LPA, a combination of LPA and the LPA_1 receptor antagonist AM095 or vehicle for 24 h and subsequently stained with Sudan Black (Fig. 1A). LPA caused a reduction of myelin as determined by measuring the number of internodes per neurons (Fig. 1A, B). A significant reduction of myelin in response to LPA treatment was not observed when applied in combination with AM095, suggesting that the demyelinating effect of LPA is dependent on the LPA_1 receptor.

To substantiate these findings, we assessed myelination via

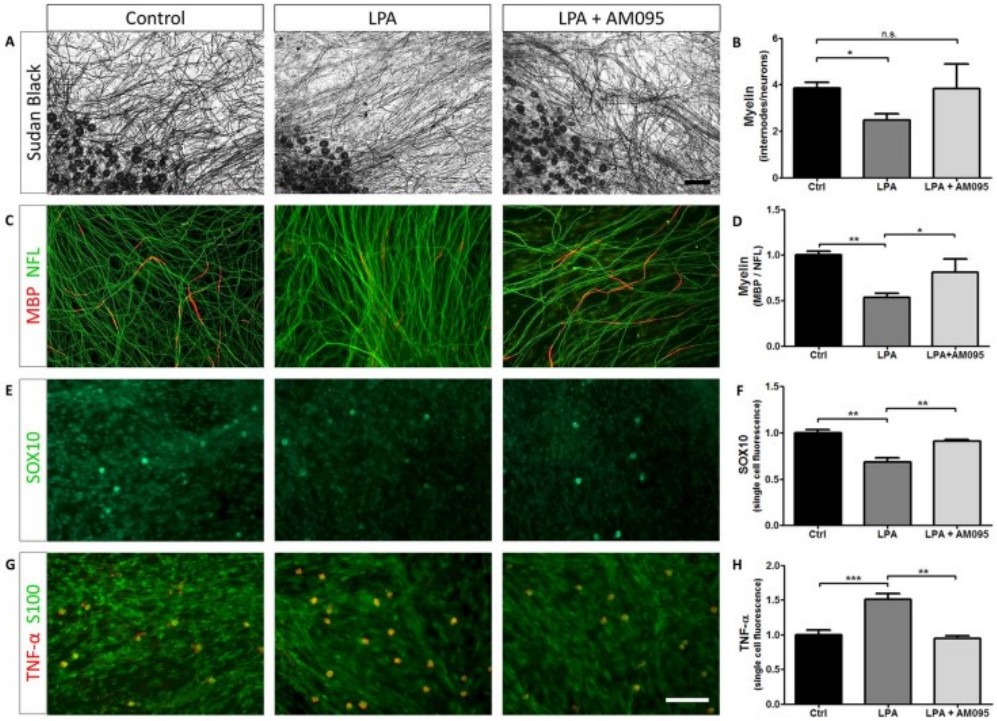


Fig. 1. LPA_1 signaling in SCs induces demyelination in DRG cultures. Myelinated DRG cultures were treated with vehicle (control), LPA or a combination of LPA + AM095. Myelination was assessed by Sudan Black staining by measuring the number of internodes per neurons (A, B) and by immunocytochemical analysis of the ratio of myelin basic protein (MBP) and neurofilament (NFL) fluorescence (C, D). Sox10 (E, F) and TNF-alpha (G, H) expression was analyzed by immunocytochemistry by measuring single cell fluorescence. Data were normalized to the respective controls (D, F, H). Scale bars indicate 100 µm. $N = 3$ minimum for each condition. Data are presented as mean ± s.e.m. Statistical significance is indicated by asterisks with $P \leq 0.05$*, $P \leq 0.01$** and $P \leq 0.001$***, n.s. indicates no significant difference.

immunocytochemistry by determining the ratio of myelin basic protein (MBP) fluorescence normalized to neurofilament (NFL) fluorescence intensity. MBP was significantly reduced with regard to NFL in LPA treated cultures and this effect was suppressed in combination with AM095 (Fig. 1C, D). AM095 treatment without additional LPA did not affect myelin content (supplementary Fig. 1). In line with this, LPA induced a significant downregulation of the differentiation marker Sox10 in SCs from LPA treated cultures (Fig. 1E, F). These effects were paralleled by a significant induction of TNF-alpha in S100-positive SCs, but not in combination with AM095 (Fig. 1G, H).

3.2. LPA contributes to downregulation of Sox10 expression in SCs after nerve injury

To better understand the relevance of our *in vitro* findings in the context of peripheral nerve injury, we performed sciatic nerve crush in C57BL/6 mice treated with a single injection of AM095 or vehicle prior to injury. At 24 h post-crush, we assessed Sox10 expression in S100-positive SCs in the proximal and distal nerve stumps by immunohistochemistry (Fig. 2A, B). While Sox10 was markedly downregulated in the distal nerve stump of vehicle treated mice, AM095 treatment preserved Sox10 expression in a significant portion of SCs (C).

3.3. LPA_1 antagonism reduces the number of Sox2-positive cells and alters TNF-alpha levels in the distal nerve stump

As the impact of AM095 on Sox10 expression implicated an involvement of LPA in SC dedifferentiation, we investigated the expression of the dedifferentiation marker Sox2 and TNF-alpha in the distal nerve stump (Fig. 3A, B). AM095 treatment significantly reduced the number of Sox2-positive cells, albeit the number of TNF-alpha positive or TNF-alpha/Sox2 double positive cells was not significantly altered.

However, as overall TNF-alpha fluorescence intensity appeared to be reduced on nerve sections from AM095 treated mice, we hypothesized that LPA may be involved in cytokine expression in myeloid cells. Analysis of TNF-alpha single cell fluorescence was subsequently performed in CD11b-positive cells located around the crush site, revealing a significant reduction of TNF-alpha in these cells (Fig. 3C, D).

4. Discussion

Numerous transcriptional regulators have been associated with the process of SC dedifferentiation that results in a phenotype commonly associated with peripheral nerve injury, broadly resembling that of immature SCs [8]. However, in addition to the downregulation of myelination associated genes, dedifferentiated SCs in the injured peripheral nerve display features such as inflammatory cytokine expression which are not necessarily characteristic for immature SCs. Vice versa, inflammatory cytokines such as interleukin-17 can induce dedifferentiation in SCs [13]. Despite a rapidly expanding understanding of positive and negative regulators of myelination, evidence for molecular cues that actually trigger SC dedifferentiation in the context of Wallerian degeneration has remained relatively sparse. In this study, we provide both *in vitro* and *in vivo* evidence for a role of LPA as a

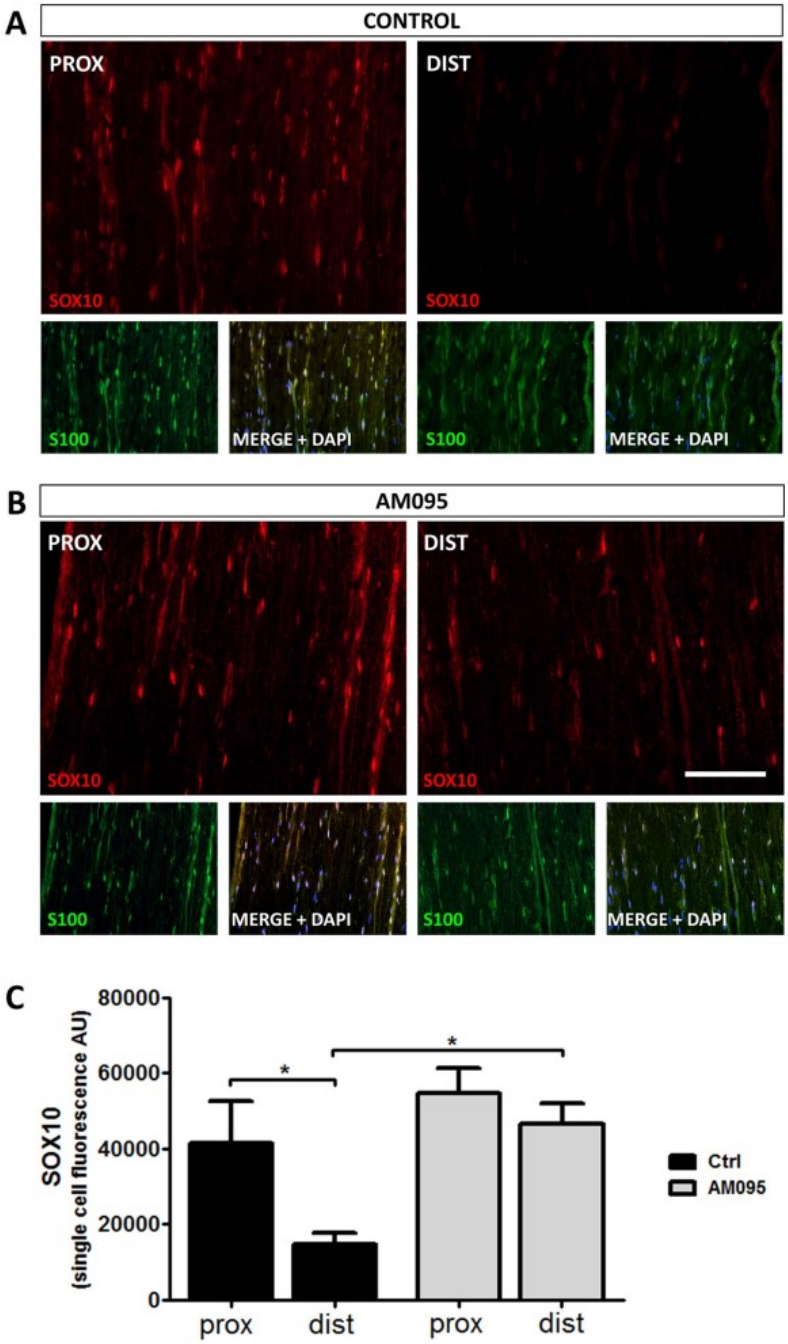


Fig. 2. LPA_1 signaling contributes to injury-induced downregulation of Sox10 in the distal nerve stump. Sox10 expression in S100-positive SCs was investigated by immunohistochemistry in the proximal (prox) and distal (dist) nerve stumps 24 h after crush injury and administration of a single dose of vehicle (A) or AM095 (B). Sox10 was quantified by single cell fluorescence measurements (C). Scale bar indicates 50 µm. Data are presented as mean ± s.e.m. $N = 3$ for each column. Statistical significance is indicated by asterisks with $P \leq 0.05$*.

contributor to injury-induced Schwann cell dedifferentiation. In DRG cultures, LPA induced demyelination was paralleled by a downregulation of the transcription factor Sox10, which has previously been demonstrated to be vital for maintaining a myelinating SC phenotype [11]. In support of these findings, Tsukahara and Ueda [18] recently reported on the concomitant downregulation of MBP and Sox10 mRNA in response to LPA treatment in the S16 Schwann cell line. The downregulation of these genes was reversed by treatment with a specific LPA_1;LPA_3 receptor antagonist. As LPA_3 is apparently neither expressed in S16 cells nor dorsal root, the authors suggest LPA_1 to be

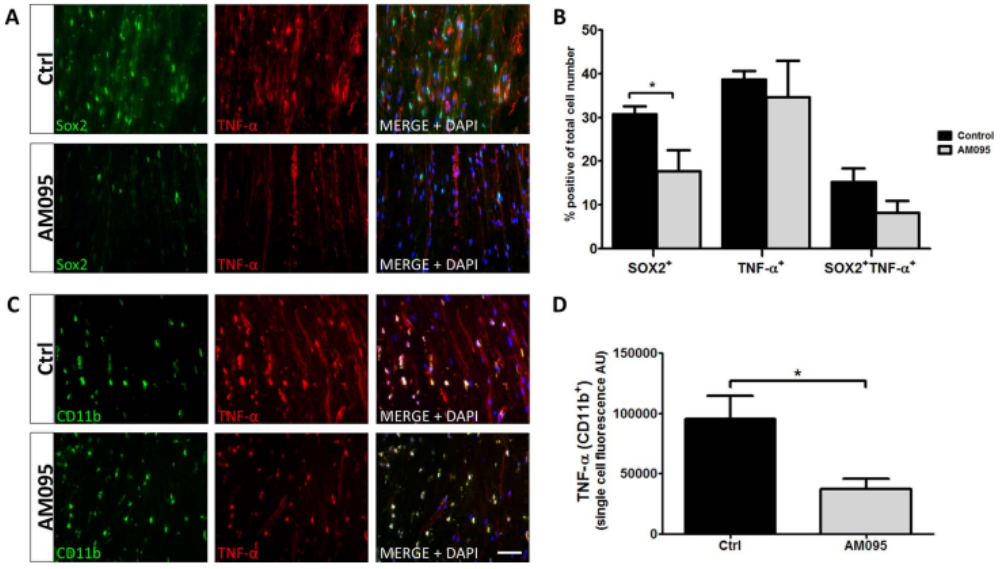


Fig. 3. Antagonism of LPA_1 reduces the number of Sox2-positive cells and alleviates TNF-alpha in myeloid cells. In the distal nerve stump, Sox2 and TNF-alpha were analyzed by immunohistochemistry (A) and the number of Sox2-positive ($Sox2^+$), TNF-alpha-positive ($TNF\text{-}\alpha^+$) as well as double positive cells ($Sox10^+TNF\text{-}\alpha^+$) was evaluated (B). In CD11b-positive ($CD11b^+$) cells, TNF-alpha single cell fluorescence was significantly reduced in AM095 treated mice (C, D). Scale bar indicates 50 µm. Data are presented as mean ± s.e.m. $N = 3$ minimum for each column. Statistical significance is indicated by asterisks with $P \leq 0.05$*.

required for LPA mediated demyelination. In this regard, downstream effects of LPA_1 activation may include Rho/ROCK and acetylation of NFκB [18]. Increased NFκB acetylation may inhibit myelination-associated differentiation factors such as Sox10, Oct6 and Krox20 [20].

Notably, LPA treatment also caused an upregulation of TNF-alpha, reflecting a SC behavior that has typically been associated with Wallerian degeneration. The requirement of LPA_1 signaling for the downregulation of Sox10 expression could subsequently be confirmed *in vivo* in mice treated with the specific antagonist AM095. Strikingly, the preservation of Sox10 expression in the distal nerve stump was paralleled by a significant reduction in the number of Sox2-positive cells. Sox2 has previously been identified as a negative regulator of myelinating SC differentiation and implicated in congenital hypomyelinating neuropathy [16] and appears to be involved in SC sorting after peripheral nerve injury [19]. As Sox2 is thought to drive Schwann cell proliferation, the regulation of Sox2 by LPA_1 may explain the proliferative effect of LPA on Schwann cells after injury [16,17]. As such, LPA signaling may be a relevant factor for the determination of SC differentiation states by modulating the expression of key transcription factors. Whether LPA also affects dedifferentiation of non-myelinating Schwann cells remains an open question. It is not known if non-myelinating Schwann cells express LPA receptors [22]. However, as non-myelinating Schwann cells are widely considered to adopt a post-injury phenotype and contribute to nerve repair as well, LPA may also forward dedifferentiation and proliferation in non-myelinating Schwann cells, possibly via the induction of Sox2.

However, the impact of LPA_1 antagonism *in vivo* with regard to TNF-alpha did not appear to be exclusively limited to SCs. While the number of Sox2-positive cells was decreased, the overall number of TNF-alpha positive cells was not significantly altered, albeit overall immunoreactivity on sciatic nerve sections appeared to be reduced. This prompted us to investigate TNF-alpha immunofluorescence in CD11b-positive cells. We recognized a significant decrease of TNF-alpha in these cells, suggesting a contribution of LPA to inflammatory cytokine release from resident or invading myeloid cells. In support of this idea, LPA has previously been implicated in pro-inflammatory cytokine release from microglia [5] as well as several other cell types [20]. While the initial source of LPA after injury may be a transiently increased expression of the enzyme autotaxin, generating LPA from lysophosphatidylcholine, LPA signaling may become amplified by a feed-forward loop [10,22]. It was shown that LPA by itself can trigger the activation of LPA production via a LPA_3 receptor dependent mechanism [10]. Although the cell type responsible for LPA_3 dependent LPA production has not been conclusively identified so far, microglia have been implicated in this process [22]. A similar mechanism may apply to macrophages in the peripheral nerve, forwarding the inflammatory response by potentiating cytokine release via the production of LPA and the subsequent auto- and paracrine activation of LPA_1 signaling.

With regard to therapeutic implications, the question arises whether interfering with LPA_1 signaling after injury would have beneficial or rather detrimental effects. SCs fulfill well-established orchestrational and effector roles in the initiation of the inflammatory response and phagocytic activity after mechanical nerve damage [1]. Given the requirement of a timely clearance of myelin and cellular debris for axon regeneration, preventing the transition towards the post-injury SC phenotype may actually hinder nerve regeneration. In this context, inhibition of LPA biosynthesis after sciatic nerve crush appears to affect myelin thickness, but does not seem to promote nerve regeneration in terms of nerve conduction velocity [2]. However, for demyelinating neuropathies the prevention of SC dedifferentiation and maintenance of the myelinating phenotype represents a desirable, yet unachieved therapeutic goal. While LPA signaling has been implicated in several inflammatory conditions [21], a possible contribution to the pathophysiology of inflammatory and demyelinating neuropathies remains to be elucidated. As such, further studies are warranted to corroborate these assumptions.

5. Conclusion

Collectively, our data provide evidence for a role of LPA in the dynamic regulation of SC dedifferentiation and activation in response to mechanical injuries in the peripheral nervous system. Improving the

understanding of LPA in the context of SC differentiation may not only hold implications for the treatment of nerve injuries, but may also contribute to a better understanding of the pathogenesis of distinct demyelinating neuropathies.

Authors' contribution

FS and MS conceived the study and designed experiments. FS, LPS and AKM performed the experiments and analyzed data. FS wrote the manuscript. All authors were involved in discussion of results, reviewed and approved the final manuscript.

Conflict of interest

FS, LPS and AKM have nothing to declare. CK has received honoraria for consulting, lecturing, travel expenses for attending meetings or financial support for research from Ablynx, Bayer Health Care, Boehringer Ingelheim, Biogen Idec, Biotronik, Daiichi Sankyo, Eisai, Ever Pharma, Genzyme, Merck Serono, Mylan, Novartis, Roche, Sanofi Aventis, Siemens and TEVA; BCK from Bayer Health Care, Biogen Idec, Merck Serono, Novartis, Genzyme and TEVA. BCK is currently also employee of Biogen. MS has received honoraria for consulting, lecturing, travel expenses for attending meetings or financial support for research from Bayer Health Care, Biogen Idec, Genzyme, Novartis, Sanofi Aventis, Grifols and TEVA.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2017.10.023.

References

[1] A.D. Gaudet, P.G. Popovich, M.S. Ramer, Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury, J. Neuroinflammation 8 (2011) 110.

[2] F. Szepanowski, A. Derksen, I. Steiner, G. MeyerzuHörste, T. Daldrup, H.P. Hartung, B.C. Kieseier, Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling, J. Neuroinflammation 13 (2016) 143.

[3] H. Kobashi, T. Yaoi, R. Oda, S. Okajima, H. Fujiwara, T. Kubo, S. Fushiki, Lysophospholipid receptors are differentially expressed in rat terminal schwann cells, as revealed by a single cell RT-PCR and In situ hybridization, Acta Histochem. Cytochem. 39 (2) (2006) 55–60.

[4] I. Ishii, N. Fukushima, X. Ye, J. Chun, Lysophospholipid receptors: signaling and biology, Annu. Rev. Biochem. 73 (2004) 321–354.

[5] I. Plastira, E. Bernhart, M. Goeritzer, H. Reicher, V.B. Kumble, N. Kogelnik, A. Wintersperger, A. Hammer, S. Schlager, K. Jandl, A. Heinemann, D. Kratky, E. Malle, W. Sattler, 1-Oleyl-lysophosphatidic acid (LPA) promotes polarization of BV-2 and primary murine microglia towards an M1-like phenotype, J. Neuroinflammation 13 (2016) 205.

[6] J.A. Weiner, N. Fukushima, J.J.A. Contos, S.S. Scherer, J. Chun, Regulation of schwann cell morphology and adhesion by receptor-mediated lysophosphatidic acid signaling, J. Neurosci. 21 (18) (2001) 7069–7078.

[7] J.S. Swaney, C. Chapman, L.D. Correa, K.J. Stebbins, A.R. Broadhead, G. Bain, A.M. Santini, J. Darlington, C.D. King, C.S. Baccei, C. Lee, T.A. Parr, J.R. Roppe, T.J. Seiders, J. Ziff, P. Prasit, J.H. Hutchinson, J.F. Evans, D.S. Lorrain, Pharmacokinetic and pharmacodynamic characterization of an oral lysophosphatidic acid type 1 receptor-selective antagonist, J. Pharm. Exp. Ther. 336 (3) (2011) 693–700.

[8] K.R. Jessen, R. Mirsky, Negative regulation of myelination: relevance for development, injury, and demyelinating disease, Glia 56 (14) (2008) 1552–1565.

[9] L. Kappos, E.W. Radue, P. O'Connor, C. Polman, R. Hohlfeld, P. Calabresi, K. Selmaj, C. Agoropoulou, M. Leyk, L. Zhang-Auberson, P. Burtin, A placebo-controlled trial of oral fingolimod in relapsing multiple sclerosis, N. Engl. J. Med. 362 (2010) 387–401.

[10] L. Ma, H. Uchida, J. Nagai, M. Inoue, J. Chun, J. Aoki, H. Ueda, Lysophosphatidic acid-3 receptor-mediated feed-forward production of lysophosphatidic acid: an initiator of nerve injury-induced neuropathic pain, Mol. Pain 5 (2009) 64.

[11] M. Bremer, F. Fröb, T. Kichko, P. Reeh, E.R. Tamm, U. Suter, M. Wegner, Sox10 is required for Schwann-cell homeostasis and myelin maintenance in the adult peripheral nerve, Glia 59 (7) (2011) 1022–1032.

[12] M. Inoue, M.H. Rashid, R. Fujita, J.J.A. Contos, J. Chun, H. Ueda, Initiation of neuropathic pain requires lysophosphatidic acid receptor signaling, Nat. Med. 10 (2004) 712–718.

[13] M. Stettner, B. Lohmann, K. Wolffram, J.P. Weinberger, T. Dehmel, H.P. Hartung, A.K. Mausberg, B.C. Kieseier, Interleukin-17 impedes Schwann cell-mediated myelination, J. Neuroinflammation 11 (2014) 63.

[14] M. Stettner, K. Wolffram, A.K. Mausberg, C. Wolf, S. Heikaus, A. Derksen, T. Dehmel, B.C. Kieseier, A reliable in vitro model for studying peripheral nerve myelination in mouse, J. Neurosci. Methods 214 (1) (2013) 69–79.

[15] M. Velasco, C. O'Sullivan, G.K. Sheridan, Lysophosphatidic acid receptors (LPARs): Potential targets for the treatment of neuropathic pain, Neuropharmacol 113 (2017) 608–617.

[16] N. Le, R. Nagarajan, J.Y.T. Wang, T. Araki, R.E. Schmidt, J. Milbrandt, Analysis of congenital hypomyelinating Egr2Lo/Lo nerves identifies Sox2 as an inhibitor of Schwann cell differentiation and myelination, Proc. Natl. Acad. Sci. U.S.A. 102 (7) (2005) 2596–2601.

[17] P.W. Frohnert, M.S. Stonecypher, S.L. Carroll, Lysophosphatidic acid promotes the proliferation of adult schwann cells isolated from axotomized sciatic nerve, J. Neuropathol. Exp. Neurol. 62 (5) (2003) 520–529.

[18] R. Tsukahara, H. Ueda, Myelin-related gene silencing mediated by LPA1 – Rho/ROCK signaling is correlated to acetylation of NFκB in S16 Schwann cells, J. Pharmacol. Sci. 132 (2) (2016) 162–165.

[19] S. Parrinello, I. Napoli, S. Ribeiro, P.W. Digby, M. Fedorova, D.B. Parkinson, R.D.S. Doddrell, M. Nakayama, R.H. Adams, A.C. Lloyd, EphB signaling directs peripheral nerve regeneration through Sox2-dependent schwann cell sorting, Cell 143 (1) (2010) 145–155.

[20] Y. Chen, H. Wang, S.O. Yoon, X. Xu, M.O. Hottiger, J. Svaren, K.A. Nave, H.A. Kim, E.N. Olson, Q.R. Lu, HDAC-mediated deacetylation of NF-κB is critical for Schwann cell myelination, Nat. Neurosci. 14 (4) (2011) 437–441.

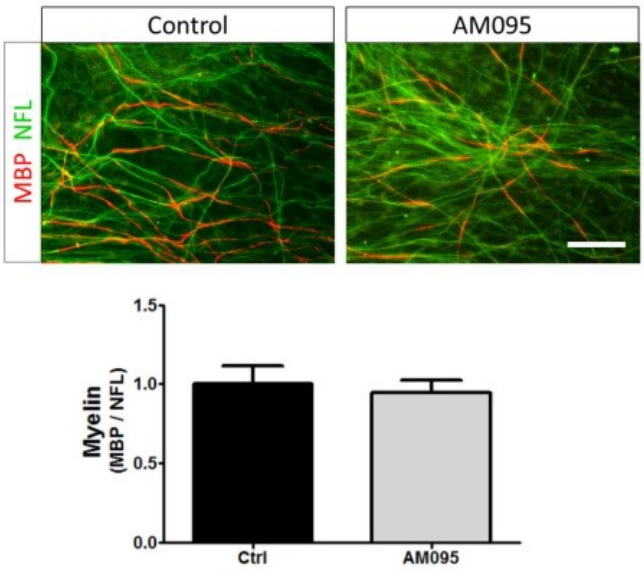
[21] Y.C. Yung, N.C. Stoddard, J. Chun, LPA receptor signaling: pharmacology physiology, and pathophysiology, J. Lipid Res. 55 (2014) 1192–1214.

[22] Y.C. Yung, N.C. Stoddard, H. Mirendil, J. Chun, Lysophosphatidic Acid signaling in the nervous system, Neuron 85 (4) (2015) 669–682.

Szepanowski et al.

Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling

Supplementary Figure 1



Supplementary figure 1: AM095 does not impact myelin content.

Myelinated DRG cultures were treated with vehicle or AM095 (10 µM) for 24 hours. AM095 without additional LPA did not affect myelin content as revealed by immunocytochemical analysis of the ratio of myelin basic protein (MBP) and neurofilament (NFL) fluorescence. Data were normalized to the control group. Scale bar indicates 100 µm. *N = 3* for each column. Data are presented as mean ± s.e.m.

IV. Concluding remarks

The amelioration of neuroinflammation and the propagation of regeneration in the peripheral nervous system represent major challenges in the fields of basic and clinical neuroscience. While a narrow repertoire of immunomodulatory drugs is available for the treatment of inflammatory neuropathies, these compounds do at best marginally affect regenerative processes. Despite the identification of numerous therapeutic targets for the promotion of nerve regeneration in preclinical studies, none has ever been translated into clinical practice.
This prompted us to study the neuroregenerative potency of clinically established and emerging classes of immunomodulatory drugs with pleiotropic effects on cells of the nervous and immune system.

In III.I. (Szepanowski et al. 2017) we provide data suggesting that dimethyl fumarate (DMF), an established drug for the treatment of multiple sclerosis, may facilitate improved nerve regeneration after mechanical nerve injury primarily via an Nrf2-dependent upregulation of the anti-inflammatory, cytoprotective and anti-apoptotic enzyme heme oxygenase 1 (HO-1).
HO-1 is a stress-inducible factor that catalyzes the degradation of free heme, resulting in an increase of its catabolic products carbon monoxide and biliverdin, both of which have been demonstrated to be exceptionally potent in the restoration of cellular and tissue homeostasis during inflammatory and oxidative conditions (Soares and Bach 2009). The enormous relevance of this seemingly simple enzymatic activity is highlighted by the finding that HO-1 is a downstream effector of the anti-inflammatory cytokine IL-10 (Lee and Chau 2002).
The protective and regenerative potential of anti-inflammatory strategies such as IL-10 treatment has been demonstrated in peripheral nerve and spinal cord injury models (Atkins et al. 2007; Wagner et al. 1998; Bethea et al. 1999) as well as experimental autoimmune neuritis (EAN), a rodent model of Guillain-Barré-Syndrome (Bai et al. 1997). Furthermore, IL-10 can support neuronal survival from glutamate-induced cytotoxicity *in vitro* (Zhou et al. 2009).
The upregulation of HO-1 has been reported in numerous injury and disease models, including EAN (Schluesener and Seid 2000), traumatic brain (Fukuda et al. 1996) and spinal cord injury (Mautes et al. 1998) as well as experimental cerebral malaria (Pamplona et al. 2007). Consistently, deletion of the HO-1 encoding gene *Hmox1*

exacerbates pathological outcomes in several disease models (Soares and Bach 2009) and stimulation or overexpression of HO-1 has been found protective under neuroinflammatory or ischemic conditions (Chora et al. 2007; Panahian et al. 1999). Moreover, an involvement of HO-1 in human pathophysiology has been demonstrated in traumatic brain injury and focal cerebral infarctions (Beschorner et al. 2000).

As a downstream effector of IL-10, pharmacological modulation of HO-1 expression may shift the T_H1/T_H2 and M1/M2 balance towards the protective T_H2 and M2 phenotypes, respectively, limiting pro-inflammatory immune responses. In this regard, DMF was recently reported to ameliorate the clinical course of EAN by causing M2 macrophage polarization via an Nrf2/HO-1 dependent mechanism (Han et al. 2016).

HO-1 may also play a crucial role in the phagocytosis of myelin debris by Schwann cells. Schwann cells have been shown to induce HO-1 expression in parallel to the onset of their phagocytic activity between 2-3 days post-injury (Hirata et al. 2000). Coherently, Nrf2 knockout mice display reduced myelin clearance in line with impaired axonal outgrowth, remyelination and functional recovery (Zhang et al. 2013_b), suggesting that Nrf2/HO-1 signaling contributes to the adaptation of Schwann cells to degenerative conditions. Interestingly, the majority of Nrf2-positive cells targeted by DMF in our work appeared to be Schwann cells. This is additionally supported by the fact that DMF was applied over the course of Wallerian degeneration until nine days post-crush, but lead to significantly improved functional and histopathological outcomes at much later time points (14 and 21 days post-injury). As such, Nrf2/HO-1 signaling may prevent early excess inflammation and render neurons and glial cells resistant to the hostile microenvironment that emerges from nerve damage and might therefore improve the starting conditions for axon outgrowth and remyelination.

In III.II. (Szepanowski et al. 2016) we demonstrated that the modulation of sphingosine-1-phosphate (S1P) and lysophosphatidic acid (LPA) signaling with the immunomodulatory drug fingolimod significantly enhances nerve regeneration.

A body of experimental evidence from both *in vitro* and *in vivo* studies had previously suggested that fingolimod, aside from its effect on lymphocyte sequestration, may have direct effects on neuronal and glial cells, including neuroprotection and the

promotion of oligodendrocyte-mediated remyelination (Groves et al. 2013). However, the *in vivo* effects of fingolimod on peripheral nervous system regeneration had not been investigated.

In order to distinguish direct effects on neurons and Schwann cells from the established immunosuppressive action of fingolimod by preventing lymphocyte egress, we performed sciatic nerve crush in immunocompetent C57BL/6 mice and two immunodeficient mouse strains: *Foxn1*-deficient and *Rag1*-deficient mice, displaying an exclusive T-lymphocyte or a combined T- and B-lymphocyte deficiency, respectively.

As reported and extensively discussed in the original research paper (Szepanowski et al. 2016) and the perspective article (Szepanowski and Kieseier 2016), we found fingolimod to significantly improve nerve regeneration by means of accelerated axon growth via increased axonal cAMP production. Surprisingly, this effect was only seen in wildtype and *Foxn1*$^{-/-}$ mice, but not in *Rag1*$^{-/-}$ mice. This outcome was proposed to result from a delayed clearance of myelin associated inhibitors of axonal outgrowth in combined T- and B-lymphocyte deficiency.

Nevertheless, similar to fingolimod-treated wildtype mice, *Rag1*$^{-/-}$ mice displayed significantly improved myelin thickness in regenerating nerves. This finding was linked to an inhibition of the enzyme autotaxin early after injury, reducing the biosynthesis of the lipid mediator LPA, which is implicated in peripheral and central nervous system demyelination (Yung et al. 2015). While we did not find sciatic nerve LPA content to be elevated following nerve crush, fingolimod treatment caused a reduction of LPA in the injured nerve at 3 hours, but not at 24 hours post-crush. These findings suggest that autotaxin may be the primary source of LPA shortly after injury, but not at later stages. This idea was confirmed by use of the specific autotaxin inhibitor PF-8380, yielding similar results with regard to myelin thickness and sciatic nerve LPA concentration.

Up to this point, it was however not clear by which mechanism LPA facilitates demyelination and why a transient reduction of LPA may have such impact on myelin thickness. As our data were rather correlative, we were excited to gain a better insight into the mechanism behind the reported demyelinating effect of LPA.

In III.IV. (Szepanowski et al. 2018) we report that LPA contributes to post-injury Schwann cell dedifferentiation via the LPA_1 receptor.

To improve our understanding of the role of LPA in Schwann cell physiology, we initially investigated LPA as well as LPA_1 antagonism in an *in vitro* model of the peripheral nervous system. Myelinated dorsal root ganglia were prepared and treated with LPA alone or in combination with the specific LPA_1 receptor antagonist AM095. Within 24 hours, LPA treatment resulted in a significant extent of demyelination paralleled by a downregulation of the differentiation factor Sox10 and upregulation of the pro-inflammatory cytokine TNF-α, suggestive of Schwann cell dedifferentiation from the myelinating towards a “Wallerian” phenotype. These effects were largely abolished in combination with the antagonist AM095.

To confirm the requirement of LPA for Schwann cell dedifferentiation *in vivo*, sciatic nerve crush was performed in mice that had received a single dose of AM095 before surgery. In accordance with our cell culture findings, LPA_1 antagonism prevented the downregulation of Sox10 in the distal degenerating nerve stump. Moreover, the number of cells expressing Sox2, a marker for Schwann cell dedifferentiation, and TNF-α immunofluorescence in myeloid cells were found to be reduced.

Given the prominent role of Schwann cells for a timely removal of myelin debris and the subsequent support of axonal regrowth and guidance, it appears questionable whether the prevention or delay of Schwann cell dedifferentiation by inhibition of LPA signaling would have beneficial effects on nerve regeneration in the context of mechanical injury. In contrast to fingolimod treatment, sole administration of the autotaxin inhibitor PF-8380 over the course of 14 days failed to improve nerve conduction velocity (Szepanowski et al. 2016), suggesting that an exclusive attenuation of LPA signaling may not represent a promising approach for the improvement of nerve regeneration. The requirement of appropriate Schwann cell adaptation to nerve injury for the establishment of a regenerative milieu is further underlined by studies in MCP-1 receptor deficient mice suggesting that macrophage recruitment to the injured nerve may be dispensable for the clearance of myelin debris. This indicates that Schwann cell phagocytic activity can partly compensate for the lack of macrophages (Lindborg et al. 2017).

Nevertheless, the modulation of LPA signaling may have significant relevance for the treatment of inflammatory and demyelinating neuropathies. Until recently, a contribution of Schwann cell dedifferentiation to the pathophysiology of inflammatory neuropathies was suspected, but had been dramatically under-researched for decades. Jang and colleagues (2017) finally took the effort to compare Schwann cell behavior during Wallerian degeneration in C57BL/6 mice with inflammatory

demyelinating neuropathy in NOD/B7-2 knockout mice, a model for CIDP. The authors report an upregulation of dedifferentiation associated genes in both the nerve injury and the neuroinflammation model. Strikingly, it is demonstrated that both models show an inverse regulation of Krox20/c-Jun to a remarkably similar degree; downregulation of Krox20, a master regulator of myelination, was accompanied by a marked increase in c-Jun expression, a transcription factor involved in Schwann cell dedifferentiation. Interestingly, a pilot study had previously investigated the expression of c-Jun in nerve and skin biopsies from neuropathy patients by immunohistochemistry. Whereas c-Jun expression was barely detectable in nerves of healthy controls, nerves from patients with different neuropathies, including GBS and CIDP, displayed notable immunoreactivity mostly confined to Schwann cells (Hutton et al. 2011). These findings support the idea of a shared mechanism involved in demyelination between mechanical injuries and demyelinating neuropathies.

Probably owing to the prominence of fingolimod and thus the focus of researchers on S1P signaling, the role of LPA in immunity may have fallen from view for some while. However, in recent years the LPA/autotaxin axis has repeatedly been associated with inflammatory and autoimmune disorders, including systemic sclerosis, asthma and arthritis (Yung et al. 2014). LPA receptors are expressed in cells of the innate and adaptive immune system, including macrophages, dendritic cells and lymphocytes, as well as in lymphoid tissues. In unstimulated Jurkat T-lymphocytes, LPA stimulates the expression of matrix metalloproteinases and promotes chemotaxis via LPA_2 signaling (Zheng et al. 2001). In activated human $CD4^+$ T-lymphocytes, LPA_1 is upregulated and facilitates IL-2 secretion in an LPA concentration dependent manner. Conversely, LPA_2 inhibits IL-2 release (Zheng et al. 2000). Moreover, LPA_1 may promote the differentiation of T_H17 cells, a subset of pro-inflammatory T helper cells characterized by their production of interleukin-17 (IL-17) (Miyabe et al. 2013).
In dendritic cells, LPA may contribute to maturation and cytokine release as well as augment their ability to activate T-lymphocytes and amplify the release of IFN-γ (Chen et al. 2006). However, the role of LPA in the regulation of dendritic cells appears complex and depends on LPA receptor expression patterns at various stages of differentiation and maturation (Panther et al. 2002; Emo et al. 2012).
Interestingly, LPA may complement the role of S1P in lymphocyte trafficking. While $S1P_1$ signaling is required for lymphocyte egress from lymphoid tissues, the

LPA/autotaxin axis has been demonstrated to regulate lymphocyte homing (Bai et al. 2013; Kanda et al. 2008).

Although the role of LPA in the pathophysiology of inflammatory demyelinating neuropathies has not been investigated so far, it is well conceivable that LPA may fuel and aggravate neuroinflammation in the peripheral nerve. Here, LPA may trigger the production of pro-inflammatory cytokines released from resident and invading macrophages and possibly even Schwann cells (Szepanowski et al. 2018). LPA by itself may further stimulate LPA production via LPA_3 signaling (Ma et al. 2009), resulting in a deteriorating feed-forward loop. Moreover, LPA itself may function as a chemokine recruiting macrophages and possibly lymphocytes into the nerve (Gustin et al. 2008). Finally, our data suggest that LPA directly promotes Schwann cell dedifferentiation, a critical step towards demyelination, which may additionally be forwarded by elevated cytokine levels. Here, TNF-α and IL-17 may play substantial roles, especially if the differentiating effect of LPA on T_H17 cells holds true (Stettner et al. 2014; Miyabe et al. 2013).

While drugs targeting LPA receptors or its biosynthesis have not yet been approved, the efficacy of LPA receptor antagonists for the treatment of idiopathic pulmonary fibrosis (LPA_1) and systemic sclerosis (LPA_1; LPA_3) is currently being investigated in phase 2 clinical trials (Velasco et al. 2017). The pleiotropic actions of LPA potentially covering nerve-intrinsic and immunological aspects of peripheral nerve disease warrant investigation of LPA receptor modulators in animal models of acute and chronic neuroinflammation.

In summary, this thesis has identified two distinct classes of signaling pathways, namely Nrf2 and lysophospholipid signaling, as potential therapeutic targets for the promotion of peripheral nerve regeneration. The established clinical efficacy and safety profiles in multiple sclerosis and, most importantly, availability of dimethyl fumarate and fingolimod to health care providers may facilitate and simplify the initiation of clinical trials to assess the efficacy of these compounds in patients suffering from primary or secondary nerve damage.

V. Appendix

V.I. Additional publications

V.I.I. The Role of Peripheral Myelin Protein 2 in Remyelination

Stettner M, Zenker J, Klingler F, Szepanowski F, Hartung HP, Mausberg AK, Kleinschnitz C, Chrast R, Kieseier BC (2017) *Cell. Mol. Neurobiol.* (online-first)

Abstract

The protein component of the myelin layer is essential for all aspects of peripheral nerves, and its deficiency can lead to structural and functional impairment. The presence of peripheral myelin protein 2 (P2, PMP2, FABP8, M-FABP) in Schwann cells has been known for decades and shown recently to be involved in the lipid homeostasis in the peripheral neural system. However, its precise role during de- and remyelination has yet to be elucidated. To this end, we assessed remyelination after sciatic nerve crush injury *in vivo*, and in an experimental de/remyelination *ex vivo* myelinating culture model in P2-deficient *($P2^{-/-}$)* and wild-type (*WT*) animals. In vivo, the nerve crush paradigm revealed temporal structural and functional changes in *$P2^{-/-}$* mice as compared to *WT* animals. Concomitantly, *$P2^{-/-}$* DRG cultures demonstrated the presence of shorter internodes and enlarged nodes after *ex vivo* de/remyelination. Together, these data indicate that P2 may play a role in remyelination of the injured peripheral nervous system, presumably by affecting the nodal and internodal configuration.

Journal: *Cellular and Molecular Neurobiology (Cell. Mol. Neurobiol.)*

Journal statistics from Journal Citation Reports (Thomson Reuters):

Impact Factor (2016): 2.939

5-year Impact Factor (2016): 2.859

Journal Ranking: 128 / 258 (Neurosciences); 107 / 189 (Cell Biology)

DOI: 10.1007/s10571-017-0494-0

V.I.II. Neuronal ADAM10 Promotes Outgrowth of Small-Caliber Myelinated Axons in the Peripheral Nervous System

Meyer zu Hörste G, Derksen A, Stassart R, Szepanowski F, Thanos M, Stettner M, Boettcher C, Lehmann HC, Hartung HP, Kieseier BC (2015) *J. Neuropathol. Exp. Neurol.* 74(11):1077-1085

Abstract

The regulation of myelination and axonal outgrowth in the peripheral nervous system is controlled by a complex signaling network involving various signaling pathways. Members of the A Disintegrin And Metalloproteinase (ADAM) family are membrane-anchored proteinases with both proteolytic and disintegrin characteristics that modulate the function of signaling molecules. One family member, ADAM17, is known to influence myelination by cleaving and thus regulating one of the key signals, neuregulin-1, which controls peripheral nervous system myelination. A similar function for ADAM10 had been suggested by previous in vitro studies. Here, we assessed whether ADAM10 exerts a similar function in vivo and deleted ADAM10 in a cell type–specific manner in either neurons or Schwann cells. We found that ADAM10 is not required in either Schwann cells or neurons for normal myelination during development or for remyelination after injury. Instead, ADAM10 is required specifically in neurons for the outgrowth of myelinated small-fiber axons *in vitro* and after injury *in vivo*. Thus, we report for the first time a neuron-intrinsic function of ADAM10 in axonal regeneration that is distinct from that of the related protein family member ADAM17 and that may have implications for targeting ADAM function in nervous system diseases.

Journal: *Journal of Neuropathology and Experimental Neurology*
(J. Neuropathol. Exp. Neurol.)

Journal statistics from Journal Citation Reports (Thomson Reuters):

Impact Factor (2016): 3.503

5-year Impact Factor (2016): 3.351

Journal Ranking: 88 / 258 (Neurosciences); 53 / 194 (Clinical Neurology);
16 / 79 (Pathology)

DOI: 10.1097/NEN.0000000000000253

V.II. Poster presentations

Szepanowski F, Szepanowski LP, Kleinschnitz C, Kieseier BC, Stettner M (2017) Lysophosphatidic acid contributes to a Schwann cell phenotype associated with peripheral nerve injury. Congress of the Deutsche Gesellschaft für Neurologie (DGN), Leipzig, Germany.

Szepanowski LP, Szepanowski F, Kleinschnitz C, Stettner M (2017) In vitro effects of pure glyphosate vs. Glyphosate-based herbicide on peripheral nervous system myelination. Congress of the Deutsche Gesellschaft für Neurologie (DGN), Leipzig, Germany.

Szepanowski F, Szepanowski LP, Kleinschnitz C, Kieseier BC, Stettner M (2017) Lysophosphatidic acid contributes to a Schwann cell phenotype associated with peripheral nerve injury. Annual meeting of the Peripheral Nerve Society (PNS), Sitges, Spain.

Szepanowski LP, Szepanowski F, Kleinschnitz C, Stettner M (2017) In vitro effects of pure glyphosate vs. Glyphosate-based herbicide on peripheral nervous system myelination. Annual meeting of the Peripheral Nerve Society (PNS), Sitges, Spain.

Szepanowski F, Szepanowski LP, Kleinschnitz C, Kieseier BC, Stettner M (2017) Lysophosphatidic acid contributes to a Schwann cell phenotype associated with peripheral nerve injury. Congress of the European Academy of Neurology (EAN), Amsterdam, Netherlands.

Szepanowski F, Donaldson DM, Hartung H-P, Warnke C, Kieseier BC, Stettner M (2016) Dimethyl fumarate accelerates peripheral nerve regeneration via induction of the Nrf2/HO-1 signaling pathway. Congress of the Deutsche Gesellschaft für Neurologie (DGN), Mannheim, Germany.

Szepanowski F, Donaldson DM, Hartung H-P, Warnke C, Kieseier BC, Stettner M (2016) Dimethyl fumarate accelerates peripheral nerve regeneration via induction of the Nrf2/HO-1 signaling pathway. Meeting of the Inflammatory Neuropathy Consortium (INC) of the Peripheral Nerve Society (PNS), Glasgow, Scotland.

Szepanowski F, Steiner I, Derksen A, Meyer zu Hörste G, Daldrup T, Hartung HP, Kieseier BC (2015) Dual mode of action for FTY720 in peripheral nerve regeneration. Congress of the Deutsche Gesellschaft für Neurologie (DGN), Düsseldorf, Germany.

Szepanowski F, Derksen A, Meyer zu Hörste G, Hartung HP, Kieseier BC (2014) The sphingosine-1-phosphate receptor agonist FTY720 promotes peripheral nerve regeneration. Congress of the International Society for Neuroimmunology (ISNI), Mainz, Germany.

Szepanowski F, Derksen A, Meyer zu Hörste G, Hartung HP, Kieseier BC (2014) The sphingosine-1-phosphate receptor agonist FTY720 promotes peripheral nerve regeneration. Congress of the Deutsche Gesellschaft für Neurologie (DGN), München, Germany.
Poster prize in the category “Peripheral and autonomic nervous system I”

Szepanowski F, Derksen A, Meyer zu Hörste G, Hartung HP, Kieseier BC (2014) The sphingosine-1-phosphate receptor agonist FTY720 promotes peripheral nerve regeneration. Meeting of the Inflammatory Neuropathy Consortium (INC) of the Peripheral Nerve Society (PNS), Düsseldorf, Germany.

VI. References

Alvarez S, Moldovan M, Krarup C (2008) Acute energy restriction triggers Wallerian degeneration in mouse. Exp. Neurol. 212(1):166-78

Atkins S, Loescher AR, Boissonade FM, Smith KG, Occleston N, O'Kane S, Ferguson MW, Robinson PP (2007) Interleukin-10 reduces scarring and enhances regeneration at a site of sciatic nerve repair. J. Peripher. Nerv. Syst. 12(4):269-76

Bai XF, Zhu J, Zhang GX, Kaponides G, Höjeberg B, van der Meide PH, Link H (1997) IL-10 suppresses experimental autoimmune neuritis and down-regulates TH1-type immune responses. Clin. Immunol. Immunopathol. 83(2):117-26

Bai Z, Cai L, Umemoto E, Takeda A, Tohya K, Komai Y, Veeraveedu PT, Hata E, Sugiura Y, Kubo A, Suematsu M, Hayasaka H, Okudaira S, Aoki J, Tanaka T, Albers HM, Ovaa H, Miyasaka M (2013) Constitutive lymphocyte transmigration across the basal lamina of high endothelial venules is regulated by the autotaxin/lysophosphatidic acid axis. J. Immunol. 190(5):2036-48

Beschorner R, Adjodah D, Schwab JM, Mittelbronn M, Pedal I, Mattern R, Schluesener HJ, Meyermann R (2000) Long-term expression of heme oxygenase-1 (HO-1, HSP-32) following focal cerebral infarctions and traumatic brain injury in humans. Acta Neuropathol. 100(4):377-84

Bethea JR, Nagashima H, Acosta MC, Briceno C, Gomez F, Marcillo AE, Loor K, Green J, Dietrich WD (1999) Systemically administered interleukin-10 reduces tumor necrosis factor-alpha production and significantly improves functional recovery following traumatic spinal cord injury in rats. J. Neurotrauma. 16(10):851-63

Bigbee JW, Yoshino JE, DeVries GH (1987) Morphological and proliferative responses of cultured Schwann cells following rapid phagocytosis of a myelin-enriched fraction. J. Neurocytol. 1987 16(4):487-96

Birchmeier C (2009) ErbB receptors and the development of the nervous system. Exp. Cell Res. 315(4):611-618

Bremer M, Fröb F, Kichko T, Reeh P, Tamm ER, Suter U, Wegner M (2010) Sox10 is required for Schwann-cell homeostasis and myelin maintenance in the adult peripheral nerve. Glia 59(7):1022-32

Bridge PM, Ball DJ, Mackinnon SE, Nakao Y, Brandt K, Hunter DA, Hertl C (1994) Nerve crush injuries--a model for axonotmesis. Exp Neurol. 127(2):284-90

Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA, Birchmeier C, Wegner M (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev. 15(1):66-78

Carlsen RC (1982) Axonal transport of adenylate cyclase activity in normal and axotomized frog sciatic nerve. Brain Res. 232(2):413-24

Chora AA, Fontoura P, Cunha A, Pais TF, Cardoso S, Ho PP, Lee LY, Sobel RA, Steinman L, Soares MP (2007) Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation. J. Clin. Invest. 117(2):438-47

Coleman MP, Freeman MR (2010) Wallerian degeneration, wld(s), and nmnat. Annu. Rev. Neurosci. 33:245-67

Dickson BJ (2002) Molecular Mechanisms of Axon Guidance. Science 298(5600):1959-1964
Emo J, Meednu N, Chapman TJ, Rezaee F, Balys M, Randall T, Rangasamy T, Georas SN (2012) Lpa2 is a negative regulator of both dendritic cell activation and murine models of allergic lung inflammation. J. Immunol. 188(8):3784-90

Enders U, Lobb R, Pepinsky RB, Hartung HP, Toyka KV, Gold R (1998) The role of the very late antigen-4 and its counterligand vascular cell adhesion molecule-1 in the pathogenesis of experimental autoimmune neuritis of the Lewis rat. Brain 121(7):1257-66

Errea O, Moreno B, Gonzalez-Franquesa A, Garcia-Roves PM, Villoslada P (2015) The disruption of mitochondrial axonal transport is an early event in neuroinflammation. J Neuroinflammation 12:152

Essuman K, Summers DW, Sasaki Y, Mao X, DiAntonio A, Milbrandt J (2017) The SARM1 Toll/Interleukin-1 Receptor Domain Possesses Intrinsic NAD+ Cleavage Activity that Promotes Pathological Axonal Degeneration. Neuron 93(6):1334-1343

Filbin MT (2003) Myelin-associated inhibitors of axonal regeneration in the adult mammalian CNS. Nat. Rev. Neurosci. 4(9):703-13

Fukuda K, Richmon JD, Sato M, Sharp FR, Panter SS, Noble LJ (1996) Induction of heme oxygenase-1 (HO-1) in glia after traumatic brain injury. Brain Res. 736(1-2):68-75

Gaudet AD, Popovich PG, Ramer MS (2011) Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury. J. Neuroinflammation 8:110

Gerdts J, Brace EJ, Sasaki Y, DiAntonio A, Milbrandt J (2015) SARM1 activation triggers axon degeneration locally via NAD^+ destruction. Science 348(6233):453-7

Gerdts J, Summers DW, Milbrandt J, DiAntonio A (2016) Axon Self-Destruction: New Links among SARM1, MAPKs, and NAD+ Metabolism. Neuron 89(3):449-60

Goethals S, Ydens E, Timmerman V, Janssens S (2010) Toll-like receptor expression in the peripheral nerve. Glia 58(14):1701-9

Gomez-Sanchez JA, Carty L, Iruarrizaga-Lejarreta M, Palomo-Irigoyen M, Varela-Rey M, Griffith M, Hantke J, Macias-Camara N, Azkargorta M, Aurrekoetxea I, De Juan VG, Jefferies HB, Aspichueta P, Elortza F, Aransay AM, Martínez-Chantar ML, Baas F, Mato JM, Mirsky R, Woodhoo A, Jessen KR (2015) Schwann cell autophagy, myelinophagy, initiates myelin clearance from injured nerves. J. Cell Biol. 210(1):153-68

Groves A, Kihara Y, Chun J (2013) Fingolimod: direct CNS effects of sphingosine 1-phosphate (S1P) receptor modulation and implications in multiple sclerosis therapy. J. Neurol. Sci. 328(1-2):9-18

Gustin C, Van Steenbrugge M, Raes M (2008) LPA modulates monocyte migration directly and via LPA-stimulated endothelial cells. Am. J. Physiol. Cell Physiol. 295(4):C905-14

Han R, Xiao J, Zhai H, Hao J (2016) Dimethyl fumarate attenuates experimental autoimmune neuritis through the nuclear factor erythroid-derived 2-related factor 2/hemoxygenase-1 pathway by altering the balance of M1/M2 macrophages. J. Neuroinflammation. 13(1):97

Hannila SS, Filbin MT (2008) The role of cyclic AMP signaling in promoting axonal regeneration after spinal cord injury. Exp. Neurol. 209(2):321-32

Hemmer B, Nessler S, Zhou D, Kieseier B, Hartung HP (2002) Immunopathogenesis and immunotherapy of multiple sclerosis. Nat. Clin. Pract. Neurol. 2(4):201-11

Hirata K, He JW, Kuraoka A, Omata Y, Hirata M, Islam AT, Noguchi M, Kawabuchi M (2000) Heme oxygenase1 (HSP-32) is induced in myelin-phagocytosing Schwann cells of injured sciatic nerves in the rat. Eur. J. Neurosci. 12(11):4147-52

Horiuchi I1, Ochi H, Murai H, Osoegawa M, Minohara M, Furuya H, Kira J (2001) Th2 shift in mononeuritis multiplex and increase of Th2 cells in chronic inflammatory demyelinating polyneuropathy: an intracellular cytokine analysis. J Neurol. Sci. 193(1):49-52

Hong K, Hinck L, Nishiyama M, Poo M, Tessier-Lavigne M, Stein E (1999) A Ligand-Gated Association between Cytoplasmic Domains of UNC5 and DCC Family Receptors Converts Netrin-Induced Growth Cone Attraction to Repulsion. Cell 97(7): 927-941

Hughes RA, Cornblath DR (2005) Guillain-Barré syndrome. Lancet 366(9497):1653-66

Hutton EJ, Carty L, Laurá M, Houlden H, Lunn MP, Brandner S, Mirsky R, Jessen K, Reilly MM (2011) c-Jun expression in human neuropathies: a pilot study. J. Peripher. Nerv. Syst. 16(4):295-303

Jang SY, Yoon BA, Shin YK, Yun SH, Jo YR, Choi YY, Ahn M, Shin T, Park JI, Kim JK, Park HT (2017) Schwann cell dedifferentiation-associated demyelination leads to exocytotic myelin clearance in inflammatory segmental demyelination. Glia. 65(11):1848-1862

Jessen KR, Mirsky R (2008) Negative regulation of myelination: relevance for development, injury, and demyelinating disease. Glia 56(14):1552-65

Jessen KR, Mirsky R (2016) The repair Schwann cell and its function in regenerating nerves. J. Physiol. 594(13):3521-31

Josephson A, Trifunovski A, Widmer HR, Widenfalk J, Olson L, Spenger C (2002) Nogo-receptor gene activity: cellular localization and developmental regulation of mRNA in mice and humans. J. Comp. Neurol. 453(3):292-304

Kanda H, Newton R, Klein R, Morita Y, Gunn MD, Rosen SD (2008) Autotaxin, an ectoenzyme that produces lysophosphatidic acid, promotes the entry of lymphocytes into secondary lymphoid organs. Nat. Immunol. 9(4):415-23

Karikó K, Ni H, Capodici J, Lamphier M, Weissman D (2004) mRNA is an endogenous ligand for Toll-like receptor 3. J. Biol. Chem. 279(13):12542-50

Kieseier BC, Clements JM, Pischel HB, Wells GM, Miller K, Gearing AJ, Hartung HP (1998) Matrix metalloproteinases MMP-9 and MMP-7 are expressed in experimental autoimmune neuritis and the Guillain-Barré syndrome. Ann. Neurol. 43(4):427-34

Kieseier BC, Dalakas MC, Hartung HP (2002) Immune mechanisms in chronic inflammatory demyelinating neuropathy. Neurology 2002 59(12 Suppl 6):S7-12

Kieseier BC, Lehmann HC, Meyer Zu Hörste G (2012) Autoimmune diseases of the peripheral nervous system. Autoimmun. Rev. 11(3):191-5

Kilmer SL, Carlsen RC (1984) Forskolin activation of adenylate cyclase in vivo stimulates nerve regeneration. Nature 307(5950):455-7

Köller H, Kieseier BC, Jander S, Hartung HP (2005) Chronic inflammatory demyelinating polyneuropathy. N. Engl. J. Med. 352(13):1343-56

Lee H, Jo EK, Choi SY, Oh SB, Park K, Kim JS, Lee SJ (2006) Necrotic neuronal cells induce inflammatory Schwann cell activation via TLR2 and TLR3: implication in Wallerian degeneration. Biochem. Biophys. Res. Commun. 350(3):742-7

Lee SK, Wolfe SW (2000) Peripheral nerve injury and repair. J. Am. Acad. Orthop. Surg. 8(4):243-52

Lee TS, Chau LY (2002) Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat. Med. 8(3):240-6

Leussink VI, Zettl UK, Jander S, Pepinsky RB, Lobb RR, Stoll G, Toyka KV, Gold R (2002) Blockade of signaling via the very late antigen (VLA-4) and its counterligand vascular cell adhesion molecule-1 (VCAM-1) causes increased T cell apoptosis in experimental autoimmune neuritis. Acta Neuropathol. 103(2):131-6

Lindborg JA, Mack M, Zigmond RE (2017) Neutrophils are critical for myelin removal in a peripheral nerve injury model of Wallerian degeneration. J. Neurosci. 37(43):10258-10277

Liu BP, Fournier A, GrandPré T, Strittmatter SM (2002) Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. Science 297(5584):1190-3

Ma L, Uchida H, Nagai J, Inoue M, Chun J, Aoki J, Ueda H (2009) Lysophosphatidic acid-3 receptor-mediated feed-forward production of lysophosphatidic acid: an initiator of nerve injury-induced neuropathic pain. Mol. Pain 5:64

Mautes AE, Kim DH, Sharp FR, Panter S, Sato M, Maida N, Bergeron M, Guenther K, Noble LJ (1998) Induction of heme oxygenase-1 (HO-1) in the contused spinal cord of the rat. Brain Res. 795(1-2):17-24

Meyer zu Hörste G, Hartung HP, Kieseier BC (2007) From bench to bedside--experimental rationale for immune-specific therapies in the inflamed peripheral nerve. Nat. Clin. Pract. Neurol. 3(4):198-211

Michailov GV, Sereda MW, Brinkmann BG, Fischer TM, Haug B, Birchmeier C, Role L, Lai C, Schwab MH, Nave KA (2004) Axonal neuregulin-1 regulates myelin sheath thickness. Science 304(5671):700-703

Ming G, H Song, Berninger B, Holt CE, Tessier-Lavigne M, Poo M (1997) cAMP-Dependent Growth Cone Guidance by Netrin-1. Neuron 19(6): 1225-1235

Misko A, Jiang S, Wegorzewska I, Milbrandt J, Baloh RH (2010) Mitofusin 2 is necessary for transport of axonal mitochondria and interacts with the Miro/Milton complex. J. Neurosci. 30(12):4232-40

Misko AL, Sasaki Y, Tuck E, Milbrandt J, Baloh RH (2012) Mitofusin2 mutations disrupt axonal mitochondrial positioning and promote axon degeneration. J. Neurosci. 32(12):4145-55

Miyabe Y, Miyabe C, Iwai Y, Takayasu A, Fukuda S, Yokoyama W, Nagai J, Jona M, Tokuhara Y, Ohkawa R, Albers HM, Ovaa H, Aoki J, Chun J, Yatomi Y, Ueda H, Miyasaka M, Miyasaka N, Nanki T (2013) Necessity of lysophosphatidic acid receptor 1 for development of arthritis. Arthritis Rheum. 65(8):2037-47

Moalem G, Gdalyahu A, Shani Y, Otten U, Lazarovici P, Cohen IR, Schwartz M (2000) Production of neurotrophins by activated T cells: implications for neuroprotective autoimmunity. J. Autoimmun. 15(3):331-45

Moalem G, Leibowitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M (1999) Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. Nat. Med. 5(1):49-55

Moalem G, Xu K, Yu L (2004) T lymphocytes play a role in neuropathic pain following peripheral nerve injury in rats. Neuroscience 129(3):767-77

Monje PV, Rendon S, Athauda G, Bates M, Wood PM, Bunge MB (2009) Non-antagonistic relationship between mitogenic factors and cAMP in adult Schwann cell re-differentiation. Glia 57(9):947-61

Murwani R, Hodgkinson S, Armati P (1996) Tumor necrosis factor alpha and interleukin-6 mRNA expression in neonatal Lewis rat Schwann cells and a neonatal rat Schwann cell line following interferon gamma stimulation. J. Neuroimmunol. 71(1-2):65-71

Nave KA (2010_a) Myelination and support of axonal integrity by glia. Nature 468(7321):244-252

Nave KA (2010_b) Myelination and the trophic support of long axons. Nat. Rev. Neurosci. 11(4):275-283

Nave KA, Salzer JL (2006) Axonal regulation of myelination by neuregulin 1. Curr. Opin. Neurobiol. 16(5): 492-500

Nyati KK, Prasad KN, Rizwan A, Verma A, Paliwal VK (2011) TH1 and TH2 response to Campylobacter jejuni antigen in Guillain-Barre syndrome. Arch. Neurol. 68(4):445-52

Osterloh JM, Yang J, Rooney TM, Fox AN, Adalbert R, Powell EH, Sheehan AE, Avery MA, Hackett R, Logan MA, MacDonald JM, Ziegenfuss JS, Milde S, Hou YJ, Nathan C, Ding A, Brown RH Jr, Conforti L, Coleman M, Tessier-Lavigne M, Züchner S, Freeman MR (2012) dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. Science 337(6093):481-4

Pamplona A, Ferreira A, Balla J, Jeney V, Balla G, Epiphanio S, Chora A, Rodrigues CD, Gregoire IP, Cunha-Rodrigues M, Portugal S, Soares MP, Mota MM (2007) Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. Nat. Med. 13(6):703-10

Panahian N, Yoshiura M, Maines MD (1999) Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J. Neurochem. 72(3):1187-203

Panther E, Idzko M, Corinti S, Ferrari D, Herouy Y, Mockenhaupt M, Dichmann S, Gebicke-Haerter P, Di Virgilio F, Girolomoni G, Norgauer J (2002) The influence of lysophosphatidic acid on the functions of human dendritic cells. J. Immunol. 169(8):4129-35

Paratore C, Goerich DE, Suter U, Wegner M, Sommer L (2001) Survival and glial fate acquisition of neural crest cells are regulated by an interplay between the transcription factor Sox10 and extrinsic combinatorial signaling. Development 128(20):3949-61

Paul WE, Zhu J (2010) How are T(H)2-type immune responses initiated and amplified? Nat. Rev. Immunol. 10(4):225-35

Pearse DD, Pereira FC, Marcillo AE, Bates ML, Berrocal YA, Filbin MT, Bunge MB (2004) cAMP and Schwann cells promote axonal growth and functional recovery after spinal cord injury. Nat. Med. 10(6):610-6

Poliak S, Peles E (2003) The local differentiation of myelinated axons at nodes of Ranvier. Nat. Rev. Neurosci. 4:968-980

Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT (2002) Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34(6):895-903

Reiprich S, Kriesch J, Schreiner S, Wegner M (2010) Activation of Krox20 gene expression by Sox10 in myelinating Schwann cells. J. Neurochem. 112(3):744-54

Saraiva M, O'Garra A (2010) The regulation of IL-10 production by immune cells. Nat. Rev. Immunol. 10(3):170-81

Schluesener HJ, Seid K (2000) Heme oxygenase-1 in lesions of rat experimental autoimmune encephalomyelitis and neuritis. J. Neuroimmunol. 110(1-2):114-20

Seddon HJ (1943) Three types of nerve injury. Brain 66(4):237-288

Shelly M, Lim BK,Cancedda L, Heilshorn SC, Gao H, Poo M (2010) Local and Long-Range Reciprocal Regulation of cAMP and cGMP in Axon/Dendrite Formation. Science 327(5965):547-552

Soares MP, Bach FH (2009) Heme oxygenase-1: from biology to therapeutic potential. Trends Mol. Med. 15(2):50-8

Stassart RM, Fledrich R, Velanac V, Brinkmann BG, Schwab MH, Meijer D, Sereda MW, Nave KA (2013) A role for Schwann cell-derived neuregulin-1 in remyelination. Nat. Neurosci. 2013 16(1):48-54

Stettner M, Lohmann B, Wolffram K, Weinberger JP, Dehmel T, Hartung HP, Mausberg AK, Kieseier BC (2014) Interleukin-17 impedes Schwann cell-mediated myelination. J. Neuroinflammation 11:63

Stewart JD (2003) Peripheral nerve fascicles: Anatomy and clinical relevance. Muscle Nerve 28(5): 525–541

Stoll G, Griffin JW, Li CY, Trapp BD (1989) Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. J. Neurocytol. 18(5):671-83

Summers DW, Gibson DA, DiAntonio A, Milbrandt J (2016) SARM1-specific motifs in the TIR domain enable NAD+ loss and regulate injury-induced SARM1 activation. Proc. Natl. Acad. Sci. U.S.A. 113(41):E6271-E6280

Sunderland S (1990) The anatomy and physiology of nerve injury. Muscle Nerve 13(9):771–784

Szepanowski F, Derksen A, Steiner I, Meyer zu Hörste G, Daldrup T, Hartung HP, Kieseier BC (2016) Fingolimod promotes peripheral nerve regeneration via modulation of lysophospholipid signaling. J. Neuroinflammation 13:143

Szepanowski F, Donaldson DM, Hartung HP, Mausberg AK, Kleinschnitz C, Kieseier BC, Stettner M (2017) Dimethyl fumarate accelerates peripheral nerve regeneration via activation of the anti-inflammatory and cytoprotective Nrf2/HO-1 signaling pathway. Acta Neuropathol. 133(3):489-491

Szepanowski F, Kieseier BC (2016) Targeting lysophospholipid signaling as a therapeutic approach towards improved peripheral nerve regeneration. Neural. Regen. Res. 11(11):1754-1755

Szepanowski F, Szepanowski LP, Mausberg AK, Kleinschnitz C, Kieseier BC, Stettner M (2018) Lysophosphatidic acid propagates post-injury Schwann cell dedifferentiation through LPA_1 signaling. Neurosci. Lett. 662:136–141

Taveggia C, Zanazzi G, Petrylak A, Yano H, Rosenbluth J, Einheber S, Xu X, Esper RM, Loeb JA, Shrager P, Chao MV, Falls DL, Role L, Salzer JL (2005) Neuregulin-1 type III determines the ensheathment fate of axons. Neuron 47(5):681-694

Toews AD, Barrett C, Morell P (1998) Monocyte chemoattractant protein 1 is responsible for macrophage recruitment following injury to sciatic nerve. J. Neurosci. Res. 53(2):260-7

Topilko P, Schneider-Maunoury S, Levi G, Baron-Van Evercooren A, Chennoufi AB, Seitanidou T, Babinet C, Charnay P (1994) Krox-20 controls myelination in the peripheral nervous system. Nature 371(6500):796-9

Tuck KL, Meyer M, Barde YA (2001) Neurotrophins are required for nerve growth during development. Nat. Neurosci. 4(1):29-37

Vabulas RM, Ahmad-Nejad P, da Costa C, Miethke T, Kirschning CJ, Häcker H, Wagner H (2001) Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. J. Biol. Chem. 276(33):31332-9

Vargas ME, Watanabe J, Singh SJ, Robinson WH, Barres BA (2010) Endogenous antibodies promote rapid myelin clearance and effective axon regeneration after nerve injury. Proc. Natl. Acad. Sci. U.S.A. 107(26):11993-8

Velasco M, O'Sullivan C, Sheridan GK (2017) Lysophosphatidic acid receptors (LPARs): Potential targets for the treatment of neuropathic pain. Neuropharmacology 113(Pt B):608-617

Wagner R, Janjigian M, Myers RR (1998) Anti-inflammatory interleukin-10 therapy in CCI neuropathy decreases thermal hyperalgesia, macrophage recruitment, and endoneurial TNF-alpha expression. Pain 74(1):35-42

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420(6911):74-8

White FA, Banghoo SK, Miller RJ (2005) Chemokines: Integrators of pain and inflammation. Nat. Rev. Drug Discov. 4(10):834–844

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci. 5(12):1302-8

Wucherpfennig KW, Catz I, Hausmann S, Strominger JL, Steinman L, Warren KG (1997) Recognition of the immunodominant myelin basic protein peptide by autoantibodies and HLA-DR2-restricted T cell clones from multiple sclerosis patients. Identity of key contact residues in the B-cell and T-cell epitopes. J. Clin. Invest. 1997 100(5):1114-22

Wucherpfennig KW, Strominger JL (1995) Molecular mimicry in T cell-mediated autoimmunity: viral peptides activate human T cell clones specific for myelin basic protein. Cell 80(5):695-705

Yung YC, Stoddard NC, Chun J (2014) LPA receptor signaling: pharmacology, physiology, and pathophysiology. J. Lipid Res. 55(7):1192-214

Yung YC, Stoddard NC, Mirendil H, Chun J (2015) Lysophosphatidic Acid signaling in the nervous system. Neuron 85(4):669-82

Zhang HL, Zheng XY, Zhu J (2013_a) Th1/Th2/Th17/Treg cytokines in Guillain-Barré syndrome and experimental autoimmune neuritis. Cytokine Growth Factor Rev. 24(5):443-53

Zhang L, Johnson D, Johnson JA (2013_b) Deletion of Nrf2 impairs functional recovery, reduces clearance of myelin debris and decreases axonal remyelination after peripheral nerve injury. Neurobiol. Dis. 54:329-38

Zheng Y, Kong Y, Goetzl EJ (2001) Lysophosphatidic acid receptor-selective effects on Jurkat T cell migration through a Matrigel model basement membrane. J. Immunol. 166(4):2317-22

Zheng Y, Voice JK, Kong Y, Goetzl EJ (2000) Altered expression and functional profile of lysophosphatidic acid receptors in mitogen-activated human blood T lymphocytes. FASEB J. 14(15):2387-9

Zhou Z, Peng X, Insolera R, Fink DJ, Mata M (2009) Interleukin-10 provides direct trophic support to neurons. J. Neurochem. 110(5):1617-27

VII. List of abbreviations

ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
ATP	adenosine triphosphate
B7-2	CD86
BDNF	brain-derived neurotrophic factor
Ca^{2+}	calcium ions
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
cGMP	cyclic guanosine monophosphate
CIDP	chronic inflammatory demyelinating polyneuropathy
CNS	central nervous system
DCC	deleted in colorectal cancer
DMF	dimethyl fumarate
DRG	dorsal root ganglia
EAN	experimental autoimmune neuritis
ErbB2/3	erb-b2 receptor tyrosine kinase 2/3
Foxn1	forkhead box protein N1
GBS	Guillain-Barre-Syndrome
HO-1	heme oxygenase 1
IL	interleukin
IVIg	intravenous immunoglobulins
Krox20	early growth response 2
Krox24	early growth response 1
LPA	lysophosphatidic acid
LPA_1	lysophosphatidic acid receptor 1
M1	classically activated macrophage
M2	alternatively activated macrophage
MAG	myelin associated glycoprotein
MBP	myelin basic protein
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
NGF	nerve growth factor
NOD	non-obese diabetic
Nrf2	NF-E2 related factor 2
Nrg-1	neuregulin 1
NT-3	neurotrophin 3
Oct6	POU domain class 3, transcription factor 1
OMgp	oligodendrocyte myelin glycoprotein
P2	peripheral myelin protein 2
p75-NTR	low-affinity nerve growth factor receptor
PNS	peripheral nervous system
Rag1	recombination activating gene 1
S1P	sphingosine-1-phosphate
$S1P_1$	sphingosine-1-phosphate receptor 1
SARM1	sterile alpha and TIR motif containing 1
Sox10	SRY-related HMG-box 10

Sox2	SRY-related HMG-box 2
T_H1	type 1 helper T-lymphocyte
T_H2	type 2 helper T-lymphocyte
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
VCAM-1	vascular cell adhesion molecule 1

VIII. List of figures

IX. Acknowledgement

An erster Stelle möchte ich mich ganz herzlich bei meinem Doktorvater Herrn Prof. Dr. Bernd C. Kieseier bedanken. Eine sehr lange Leine sowie die ungezwungene, konstruktive Betreuung waren genau das richtige für mich und haben diese Arbeit gelingen lassen.

Herrn Prof. Dr. Ulrich Rüther, der mir schon im Bachelorstudium die Grundlagen des wissenschaftlichen Arbeitens mit auf den Weg gab, danke ich für die Übernahme des Korreferats.

Ganz besonders möchte ich Herrn PD Dr. Dr. Mark Stettner für die großartige Unterstützung, vor allem im vergangen Jahr, danken. An dieser Stelle sei auch Frau Dr. Anne Mausberg erwähnt, die immer bereit war, ihre Expertise weiterzugeben und es bis heute nicht aufgegeben hat, mir doch noch ein wenig Immunologie beizubringen. Ich freue mich, dass die Reise mit Euch beiden weitergeht.

Die Erinnerungen an die vergangenen Jahre wären um einiges farbloser, hätten wir sie nicht mit Thomas Dehmel verbringen dürfen. Eine detaillierte Aufzählung würde hier den Rahmen sprengen, aber nicht zuletzt danke ich für die ausführliche Nachhilfe in der Wacholderkunde.

Ich möchte mich außerdem bei allen (ehemaligen) Mitgliedern der Arbeitsgruppe bedanken: Zippora Kohne, Marcia Gasis, Annette Heß, Kathleen Ingenhoven, Maximilian Heininger, Dr. Angelika Derksen und PD Dr. Clemens Warnke.

Meiner Freundin Irina Steiner danke ich nicht nur für eine fantastische akademische Zusammenarbeit, sondern vielmehr dafür, dass sie es die längste Zeit unseres Studiums mit mir ausgehalten hat.

Abschließend möchte ich mich bei Freunden und Familie bedanken, insbesondere bei meinen Eltern, die mich auf meinem Weg immer unterstützt haben und denen diese Arbeit gewidmet ist.

X. Affidavit (Eidesstattliche Erklärung)

Ich versichere an Eides statt, dass die Dissertation von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsatze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.

Einzelne Teile des Textes können Übereinstimmungen mit den von mir verfassten und in diese Arbeit eingepflegten Originalpublikationen aufweisen.

Diese Arbeit wurde weder bei einer anderen akademischen Institution eingereicht noch habe ich erfolglose Promotionsversuche unternommen.

Düsseldorf,

Fabian Szepanowski