Novel Strategies to Promote Nerve Regeneration

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Zusammenfassung

Schädigungen des zentralen Nervensystems (ZNS) führen oftmals zu permanenten Funktionsverlusten. Auch wenn das periphere Nervensystem (PNS) grundsätzlich in der Lage ist, verletzte Axone zu regenerieren, bleibt meist auch die PNS Regeneration unvollständig, besonders wenn die Axone zur Regeneration weite Distanzen überbrücken müssen. Bisher gibt es keine Möglichkeiten axonale und daraus resultierende funktionelle Schäden zu behandeln. Das Ziel dieser Dissertation war es, neue Behandlungsmöglichkeiten zur Nervenregeneration zu entwickeln. In den 2 Teilen der Dissertation wird der Einfluss von Interleukin-6 (IL-6), Glykogensynthase-Kinase 3 (GSK3) und der Inhibierung der Mikrotubulidetyrosinilierung auf die axonale Regeneration untersucht.

Eine inflammatorische Stimulation der Linse befördert die Regeneration des optischen Nervs nach einer Quetschung. Der molekulare Mechanismus der diesem Effekt zugrunde liegt, ist jedoch nach wie vor nicht vollständig entschlüsselt. Da eine Linsenverletzung keine geeignete Therapieoption für Nervenschädigungen ist, ist es für die Entwicklung einer Therapie wichtig, die für diesen Effekt verantwortlichen molekularen Faktoren zu finden. Daten dieser Dissertation haben gezeigt, dass das Zytokin IL-6 einer dieser Faktoren ist und etablieren es somit als vielversprechendes Therapeutikum zur Behandlung von ZNS Verletzungen.

Ein weiterer molekularer Faktor, der in der axonalen Regeneration involviert ist, ist GSK3. Es ist jedoch nach wie vor Gegenstand wissenschaftlicher Debatten, ob GSK3 Aktivität die Nervenregeneration befördert oder verschlechtert. Daten dieser Dissertation haben gezeigt, dass eine genetisch erhöhte GSK3 Aktivität die axonale Regeneration durch das Mikrotubuli assoziierte Protein 1B (MAP1B) und eine dadurch bedingte Mikrotubulityrosinierung verbessert. Auch eine pharmakologische Imitierung des Effekts einer erhöhten GSK3 Aktivität auf die Mikrotubulityrosinierung durch Parthenolid konnte die axonale Regeneration deutlich verbessern. Diese Daten sind die ersten Hinweise darauf, dass eine Aufrechterhaltung der Mikrotubulidynamik axonales Wachstum befördern kann und das Parthenolid ein vielversprechender pharmakologischer Wirkstoff zur Verbesserung der PNS Regeneration ist.

Abstract

Injuries of the central nervous system (CNS) normally cause permanent functional loss. Although the peripheral nervous system (PNS) possesses an inherent capacity to extend injured axons, also PNS regeneration often remains incomplete, particularly when axons have to regenerate over long distances. So far no treatments are available for axonal repair and subsequent functional loss after injury. The present thesis aimed to develop novel strategies to promote nerve regeneration and comprises 3 parts focusing on the role of interleukine-6 (IL-6), glycogen synthase kinase 3 (GSK3) and inhibition of microtubules detyrosination in axon regeneration.

An inflammatory stimulation of the lens promotes the regeneration of the optic nerve after injury. However, the molecular mechanisms mediating this effect are not completely understood. Since inducing a lens injury is not a suitable treatment for nerve injuries, finding the molecular factors mediating it could reveal new applicable methods to improve axon regeneration in the CNS. Data from this thesis demonstrated that the cytokine IL-6 is one of the factors mediating nerve regeneration in response to lens injury. This makes IL-6 an interesting candidate for treating CNS injuries. Another molecular target involved in axon regeneration is GSK3. However, to this point it is controversially discussed, whether GSK3 activity improves or hinders axon regeneration. Data of this thesis demonstrated that genetically increased GSK3 activity promoted axon regeneration via microtubule associated protein 1B (MAP1B) phosphorylation and a subsequent microtubule tyrosination, thus keeping microtubules in a dynamic state. Pharmacologically mimicking the effect of increased GSK3 activity on microtubule tyrosination with parthenolide substantially accelerated axon regeneration. These data demonstrate for the first time that keeping microtubules in a dynamic state is favorable for axon growth and that parthenolide is a promising pharmacological compound to improve nerve regeneration.

Table of content

1 Introduction

1.1 Implications of traumatic nerve injuries

Injuries to the nervous system often lead to irreversible damage. Particularly traumatic injuries to the central nervous system (CNS) usually cause permanent functional loss. Although the peripheral nervous system (PNS) possesses an inherent capacity to extend injured axons regeneration remains often incomplete, particularly when axons have to regenerate over long distances. Injuries can affect various regions in the nervous system and are the source of tremendous personal tragedy and socio-economic costs. For instance, injuries to the optic nerve or the spinal cord, both parts of the CNS, can lead to lifelong blindness or paraplegia. Without including any other nerve injuries, up to 500,000 people worldwide are affected by spinal cord injury every year (World Health Organization, 2013). Costs for the treatment of symptoms induced by neuronal damage in the CNS add up to € 798 billion per year in Europe, traumatic brain injuries accounting for € 33 billion alone (Olesen et al., 2012).

Also, PNS damage has severe consequences for affected patients and the society. Peripheral neuropathies caused by either trauma or disease, such as diabetes, cytostatic treatment or traumatic injuries (Azhary et al., 2010), often impair sensation, motor, gland or organ functions. It is estimated that 8% of the population aged over 55 are affected by peripheral neuropathy. One example is traumatic damage to peripheral nerves, which has an incidence of more than 5,000 people in Germany (AWMF online, 2013) and is often caused by daily activities, such as sport, traffic accidents, distensions as well as cut and burn injuries. The treatment outcome is often unpredictable and disappointing, since particularly injuries requiring long distance regeneration do not fully recover.

Obviously, the affected individual, but also the society as a whole, would greatly benefit from the development of novel therapeutic interventions for CNS and PNS traumatic nerve injuries.

1.2 Regenerative capacity after CNS injury

First evidence that central neurons remain some capacity to regenerate after injury was provided in the early 20th century by Ramon y Cajal (Lobato, 2008). However, this knowledge soon was lost and until the early 1980s, it was generally accepted that axotomized neurons of the mammalian CNS are unable to regenerate, but instead undergo apoptotic cell death (Berkelaar et al., 1994). However, David and Aguayo rediscovered Cajals pioneering experiments (David and Aguayo, 1981). Using an elegant approach, they sutured a peripheral nerve graft to the cut optic nerve of an adult rat and thereby demonstrated that central axons normally projecting into the optic nerve extended into the nerve graft. These findings demonstrated that adult mammalian CNS neurons possess at least a rudimentary capability to regenerate injured axons. The fact that the peripheral nerve graft allowed central axons to regenerate left two possible explanations. Firstly, factors present in the peripheral nerve could have stimulated axon elongation of CNS neurons or secondly, inhibitory substances specifically expressed in the central nerve were absent in the peripheral nerve graft. Years of research showed, that both hypotheses contribute to the determined regeneration of injured CNS axons into the PNS graft. The group of Martin Schwab and others showed in a number of experiments that central myelin from oligodendrocytes during traumatic injury represses axon regeneration in CNS nerves (Silver and Miller, 2004). Additionally, chondroitin sulfate proteoglycans (CSPGs) released from astrocytes into the injury site (Rudge and Silver, 1990, McKeon et al., 1991, Silver and Miller, 2004, Yiu and He, 2006), major components of the glial scar that forms after central nerve injury,

have been shown to inhibit axon regeneration by activating the RhoA/rho-associated protein kinase (ROCK) pathway after binding to their receptors (Monnier et al., 2003, Shen et al., 2009, Sharma et al., 2012). But it was also demonstrated that suppression of inhibitory signals present in the central nerve after injury was not sufficient to induce regeneration (Fischer et al., 2004a, Fischer et al., 2004b). Additionally, the intrinsic capacity of adult mammalian central neurons has to be reactivated (Goldberg, 2004). Several signaling pathways are currently known to be involved in switching such neurons into a so-called regenerative state. The phosphatidylinositide 3-kinase/protein kinase B (PI3K/AKT) and janus kinase/signal transducers and activators of transcription-3 (JAK/STAT3) signaling pathways are among the most prominent ones (Qiu et al., 2005, Park et al., 2008, Smith et al., 2009, Sun et al., 2011, Leibinger et al., 2012, Leibinger et al., 2013a).

Based on the current knowledge, successful axon regeneration in the adult CNS requires at least five prerequisites:

1.) Survival of injured neurons,

2.) Stable transformation of neurons into a regenerative state, allowing long distance growth,

3.) Overcoming inhibitory cues, such as myelin and CSPG, present at the lesion site,

4.) Reconnection with their appropriate targets and

5.) Myelination of regenerated axons to regain their physiological propagation speed.

1.2.1 Advances in promoting CNS regeneration

At the turn of the last millennium several possibilities were identified to promote axon regeneration into the growth suppressing environment of the injured CNS. One of these was discovered when an accidental lens injury (LI) protected retinal ganglion cells (RGCs) from axotomy-induced apoptotic cell death and enabled moderate axon growth into the crushed optic nerve (Fischer et al., 2000, Leon et al., 2000). Years of research have shown that LI mediates these beneficial effects by inducing an inflammatory stimulation (IS) followed by the expression and secretion of interleukin-6 (IL-6) class cytokines, such as ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) by retinal astrocytes (Müller et al., 2007, Hauk et al., 2008, Leibinger et al., 2009, Fischer, 2010). Apart from LI (Fischer et al., 2000, Leon et al., 2000, Fischer et al., 2001, Lorber et al., 2005, Pernet and Di Polo, 2006), an IS can also be induced by transplanting peripheral nerve explants into the vitreous body (Berry et al., 1996), through an intravitreal injection of toll-like receptor 2 agonists such as Pam_3Cys, Zymosan (Yin et al., 2003, Hauk et al., 2010, Gensel et al., 2015), the dectin-1 activator β-glucan (Baldwin et al., 2015) or lental β/γ-crystallins (Fischer et al., 2008). Since the discovery of the growth promoting effects of CNTF and LIF, regeneration research has focused particularly on two signaling pathways directly downstream of these molecules. Keeping the JAK/STAT3 signaling pathway active by depletion of its negative regulator suppressor of cytokine signaling 3 (SOCS3) (Smith et al., 2009), or genetically suppressing phosphatase and tensin homolog (PTEN) (Park et al., 2008, Sun et al., 2011) and thereby activating the PI3K/AKT pathway, significantly improves axon regeneration in the CNS. However, the fact that IS, in contrast to CNTF and LIF treatment, also possesses some degree of disinhibitory effects, suggests that additional factors are essentially involved in mediating IS effects on RGC growth (Ahmed et al., 2009, Leibinger et al., 2012).

1.2.2 Role of IL-6 in axon regeneration

One aim of this thesis was to investigate the involvement of IL-6 in mediating the effects of IS on RGC axon regeneration in the optic nerve, since it is another prominent member of the interleukin 6 class cytokines. All members of this cytokine family mediate their signaling via the glycoprotein 130 (gp130) receptor without binding directly to this molecule (Heinrich et al., 2003). For an interaction between the cytokine and its target cell IL-6 first needs to bind a specific α receptor at the membrane called IL-6Rα. This complex then binds to two gp130 receptor units (Heinrich et al., 2003). Alternatively, IL-6 can also bind to a soluble IL-6 receptor and subsequently bind to gp130 to induce signal transduction (Yasukawa et al., 1990, Chalaris et al., 2007). In uninjured CNS tissue, IL-6 is generally expressed at very low levels. However, upon ischemia (Acalovschi et al., 2003, Erta et al., 2012) or trauma (Hariri et al., 1994) IL-6 is upregulated in the CNS. In the peripheral nervous system (PNS) it is induced upon axotomy (Cafferty et al., 2004, Cao et al., 2006) and stimulates axon regeneration (Hakkoum et al., 2007, Yang et al., 2012).

1.3 Regenerative capability after PNS injury

In contrast to the CNS, the PNS possesses a much stronger capacity to regenerate injured axons and functional loss can therefore under certain circumstances restored. Regeneration of the PNS occurs in two main steps. In the first one, termed Wallerian degeneration, the axon distal to the lesion site rapidly degenerates within the first 24 hours upon injury and myelin is cleared initially by intact Schwann cells through phagocytosis and by macrophages at later stages. These immune cells are attracted by cytokines and chemokines which are secreted by

Schwann cells in response to injury (Vargas and Barres, 2007). In a second step, Schwann cells formerly bound to axons proliferate and form tubes, so called Bands of Bunger. These structures guide growing axons along the nerve and provide them with trophic support. These events enable axons to initially grow with a speed of about 1-2 mm/day (Sulaiman and Gordon, 2013).

1.3.1 Limitations of PNS regeneration

Although injured PNS neurons possess the intrinsic capacity and the appropriate environment to regenerate, functional recovery often remains limited. This is particularly true when axons have to grow over long distances to reinnervate their former target tissue. For example, a proximal sciatic nerve injury would need to regenerate over a distance of up to 1 meter in human beings. The reasons for incomplete recovery after damage of long nerves are manifold. Firstly, axon extension is driven by a regenerative program that depends on the induction of so-called regeneration associated genes (RAGs) through extracellular signals. Expression of most of these genes is induced within the first 3 days after injury in the rat PNS. However, expression of these RAGs in rats peaks around day 7 and then quickly declines to baseline levels after 2 months (Sulaiman and Gordon, 2013). Without the continuous expression of RAGs peripheral neurons lose their intrinsic capability for axon extension. Therefore, axons hardly grow beyond a distance of 6 cm without therapeutic intervention - a distance frequently surpassed in human nerve lesions (Hoke, 2006).

Secondly, trophic support by Schwann cells is timely limited. As mentioned before, Schwann cells distal to the injury start to proliferate, form Bands of Bunger and provide trophic support for growing axons by releasing neurotrophic factors. Thus, just as neurons, Schwann cells enter a regenerative state upon injury. However, this regenerative state also declines over time.

Secretion of neurotrophic factors by Schwann cells returns to baseline levels 2 month after injury (Sulaiman and Gordon, 2013). Additionally, Schwann cells are usually supported by axons with neuregulin (Falls, 2003, Hoke, 2006), which drives survival, proliferation and differentiation of Schwann cell as well as subsequent myelination. So, if axons fail to regenerate fast enough to get into contact with Bands of Bunger forming Schwann cells, these glial cells will eventually deteriorate.

Thirdly, regenerating axons are misguided over time and innervate inappropriate targets. Particularly the chance of false target innervation increases with regeneration distance, since this factor usually increases the number of nerve branching points between lesion site and axon target. At the site of a branching nerve, axons lack cues to reenter their original branch, which makes it likely that they will end up in the wrong one (Witzel et al., 2005). Misguidance is often induced by trophic factors supplied by Schwann cells. While these factors are important drivers of axon growth, they unfortunately also often function as chemoattractants. Therefore these trophic factors can retain axons close to the lesion site, since this is the region with their highest concentration (Witzel et al., 2005, Vargas and Barres, 2007, Scheib and Hoke, 2013).

Since the growth rate of PNS axons is one the most important factors that limits functional recovery after PNS injury, speeding up axon growth could improve the regenerative outcome. In fact, it has been demonstrated that accelerating axonal growth promotes functional recovery after peripheral nerve injury in mice (Ma et al., 2011).

1.3.2 Signaling pathways promoting PNS axon regeneration

As in the CNS, axonal regeneration in the PNS can be similarly accelerated through a so-called conditioning injury (CI) (Neumann and Woolf, 1999, Saijilafu et al., 2013). If the peripheral nerve is injured 5-7 days prior to a second injury, the nerve is already in a regenerative state and axon regeneration is dramatically increased. IL-6 is one of the responsible factors inducing the regenerative state after CI (Cafferty et al., 2004, Cao et al., 2006).

In general, axon regeneration in the PNS is driven by several signaling pathways. For example, STAT3 is known to be involved in this context (Bareyre et al., 2011). Another signal transducer required for sciatic nerve regeneration is PI3K (Saijilafu et al., 2013). PI3K/AKT-signaling affects axon elongation through several effectors. It has been shown that deletion of tuberous sclerosis complex 2 (TSC2) activating of the PI3K downstream molecule mTOR improves sciatic nerve regeneration (Abe et al., 2010). However, glycogen synthase kinase 3 (GSK3), a downstream target of AKT (Saijilafu et al., 2013) is of particular interest for further investigation, since its function in regeneration is controversially discussed.

1.3.3 GSK3 in PNS regeneration

GSK3 is a protein comprising two isoforms, GSK3α and GSK3β. Both GSK3s are serine/threonine kinases and share a homology of 85% (Hur and Zhou, 2010). GSK3 usually is highly active in resting cells. However, GSK3 activity is strongly modified by posttranslational modifications and centrally involved in various signaling cascades. AKT activation, for example by IL-6 signaling, leads to a phosphorylation of serine 21 (GSK3α) and serine 9 (GSK3β), respectively, which reduces GSK3 activity (Sutherland et al., 1993, Cross et al., 1995).

Additionally, GSK3 phosphorylation at tyrosine residues 279 (GSK3α) or 216 (GSK3β) further enhances GSK3 activity (Hughes et al., 1993). Although IL-6 signaling promotes PNS regeneration and leads to GSK3 inactivation, it is still controversially discussed whether this inactivation increases or decreases axon growth. Pharmacological inhibition of GSK3 has been shown to both positively (Alabed et al., 2010) and negatively (Dill et al., 2008) affect myelin disinhibition. Similarly, GSK3's role in axon growth promotion is also inconsistently discussed in the literature. Evidence has been provided that the inactivation of GSK3 reduces axon elongation (Krylova et al., 2002, Owen and Gordon-Weeks, 2003, Kim et al., 2006, Alabed et al., 2010). This, combined with the fact that active GSK phosphorylates microtubule-associated protein 1B (MAP1B), which transfers microtubules into a dynamic state (Lucas et al., 1998, Goold et al., 1999, Gonzalez-Billault et al., 2004), enabling an increased rate of tubulin polymerization, points towards a beneficial role of active GSK3 in embryonic axon elongation. However, GSK3 inactivation in the growth cone has been shown to improve axon elongation in DRG neurons as well (Zhou et al., 2004). Moreover, general pharmacological GSK3 inhibition can also increase axon elongation (Jiang et al., 2005, Yoshimura et al., 2005, Dill et al., 2008, Saijilafu et al., 2013). The negative influence of GSK3 on promotion of axon growth in this context has partly been attributed to its degradation of the transcription factor SMAD1 (Saijilafu et al., 2013), which has been proposed to be necessary for axon regeneration (Parikh et al., 2011).

However, so far most studies that investigated the involvement of GSK3 in axon regeneration rely on pharmacological inhibition or overexpression of GSK3. The at first glance opposing results may thus potentially be caused by pharmacological off-target effects, but also by the use of varying inhibitors. Also, overexpression of GSK3 usually results in unphysiologically increased GSK3 levels, which may affect axon regeneration but also neuron viability in general.

1.3.4 Effect of GSK3 isoforms on axon growth

Although the role of GSK3 in axon regeneration is not entirely clear, many studies show that it is involved in these processes (Krylova et al., 2002, Dill et al., 2008, Alabed et al., 2010, Hur et al., 2011a, Liz et al., 2014a). Moreover, apart from the opposing results described above, it is also unclear, whether both GSK3 isoforms play different roles or can substitute each other. While GSK3β expression is essential for the accurate development of various organ systems since a GSK3 knockout is lethal (Hoeflich et al., 2000, Liu et al., 2007) GSK3α knockout mice develop almost normal and reveal only minor behavioral irregularities (Kaidanovich-Beilin et al., 2009). These findings do not suggest a complete overlap in substrate specificity of both GSK3 isoforms (Soutar et al., 2010). The fact that GSK3β reportedly affects physiological functions associated with the nervous system to a greater extent than GSK3α may explain why GSK3β has generally received more attention in studying developmental axon growth and axon regeneration in adult animals, while the role of GSK3α in this context remains largely unconsidered. This is one of the reasons why effects of GSK3 inhibitors on axon growth have so far often been attributed solely to GSK3β, although GSK3 inhibitors affect both isoforms. So far, only few studies investigating axon growth differentiated between both GSK3 isoforms, one of which showed that shRNA knockdown of both GSK3 isoforms reduced axon formation in embryonic neurons (Garrido et al., 2007).

1.3.5 Potential downstream targets of GSK3 relevant for axon growth

Downstream of GSK3 two classes of proteins involved in axon regeneration are affected, namely transcription factors (Saijilafu et al., 2013) and microtubule associated proteins (MAPs) such as

APC, CLASP, CRMP2 and MAP1B (Lucas et al., 1998, Zhou et al., 2004, Arimura et al., 2005, Kim et al., 2006, Alabed et al., 2010, Hur et al., 2011a). Before being able to be phosphorylated through GSK3, most of these MAPs require a prior phosphorylation by other kinases, a process also known as priming (Kim et al., 2006, Seira and Del Rio, 2013). GSK3 phosphorylation usually reduces activity of these MAPs. Microtubule associated protein 1B (MAP1B), however, is activated by GSK3 phosphorylation at serine 1260 and tyrosine 1265 and requires no priming by other kinases (Lucas et al., 1998, Frame et al., 2001, Trivedi et al., 2005). MAP1B, phosphorylated by GSK3, associates with tyrosinated microtubules and thus keeps them in a dynamically unstable state. Decreasing MAP1B phosphorylation by inhibiting GSK3 significantly raises levels of detyrosinated microtubules in axon growth cones (Owen and Gordon-Weeks, 2003). Increasing microtubule detyrosination is known to stabilize microtubules, reducing their rate of tubulin polymerization. Therefore, MAP1B phosphorylated by GSK3 is suspected to promote axonal elongation in embryonic neurons by keeping microtubules in a dynamically unstable state (Lucas et al., 1998, Goold et al., 1999, Owen and Gordon-Weeks, 2003). So far, it is unknown how MAP1B mediates this microtubule detyrosination. It also remains unclear whether an inhibition of microtubule detyrosination promotes adult axon regeneration after injury.

1.4 Aim of the thesis

This thesis pursued 2 main goals:

The first one was to investigate the involvement of IL-6 in mediating the axon growth promoting effects of LI in optic nerve regeneration. The second goal was the clarification of the function of GSK3 activity and its substrates in peripheral nerve regeneration. The understanding of the underlying molecular mechanism should be used for the development of a novel pharmacological treatment strategy for peripheral nerve repair.

Since previous studies demonstrated that CNTF and LIF can only be made accountable for parts of LI induced RGC survival and axon regeneration, the first goal of this thesis was to investigate whether the related IL-6 induces parts of the LI effects, too. Since neither CNTF nor LIF display disinhibitory effects towards the inhibitory substrates myelin and CSPG, this study particularly focused on the involvement of IL-6 in the disinhibitory effects of LI.

The second aim of this thesis was to clarify the role of GSK3 activity in peripheral nerve regeneration. As mentioned above, GSK3 activity has been attributed positive as well as negative properties in the context of axon growth. These opposing findings are suspected to arise from unspecific effects of pharmacological GSK3 inhibition or from an unphysiological increase in GSK3 levels through artificial overexpression. To avoid these pitfalls, this thesis evaluated the effect of genetically modified and consequently constitutively active GSK3 on peripheral nerve regeneration. Subsequently, this study set out to determine the downstream targets of GSK3 related to axon growth and to pharmacologically interfere with these targets, thus mimicking GSK3 mediated regeneration and to potentially to introduce a new treatment option for peripheral neuropathies.

2 Discussion

2.1 IL-6 contributes to axon regeneration upon inflammatory stimulation

Since neither CNTF nor LIF are solely responsible for mediating the effect of inflammatory stimulation (IS) on optic nerve regeneration, the first part of this thesis investigated whether the cytokine IL-6 partly mediates the effects of IS. In a set of experiments this thesis demonstrated that retinal IL-6 expression was significantly upregulated after optic nerve injury and IS (Leibinger et al., 2013b). Furthermore, IL-6 promoted axon regeneration of RGCs *in vitro* and *in vivo*. IL-6 also rendered RGCs insensitive towards myelin, but not towards the chondroitin sulfate proteoglycan (CSPG) neurocan. These effects were abolished in IL-6 knockout mice. Additionally, IS induced optic nerve regeneration was reduced after genetic IL-6 depletion. These data suggest that IL-6 partially mediates the beneficial effects of IS and generally promotes axon regeneration in the CNS.

2.1.1 IL-6 expression is induced in the retina upon optic nerve crush and inflammatory stimulation

To investigate if IL-6 is potentially involved in promoting IS induced optic nerve regeneration, this thesis first tested if IL-6 and its receptor are expressed in the retina. It has been shown before that in the uninjured CNS hardly any IL-6 can be detected. However, upon ischemia (Acalovschi et al., 2003), traumatic brain injury (Hariri et al., 1994) or axotomy in the PNS (Cao et al., 2006) IL-6 is strongly induced. Expectedly, no IL-6 mRNA or protein was found in the uninjured adult rat retina (Leibinger et al., 2013b). However, upon optic nerve crush (ONC), retinal IL-6 mRNA

and protein levels were upregulated (Leibinger et al., 2013b), comparable to IL-6 induction observed before in models of glaucoma (Sappington et al., 2006, Chidlow et al., 2012) or upon sciatic nerve injury (Cao et al., 2006). Although retinal IL-6 expression was already induced upon ONC, IL-6 expression was exponentially increased after an additional IS, as detected with ELISA and quantitative realtime PCR (Leibinger et al., 2013b). Although it remains unclear which retinal cell type was responsible for the IL-6 secretion since secreted proteins pose a challenge for immunohistochemical detection, IL-6 has already been shown to be secreted by retinal astrocytes, microglia and even RGCs in response to ONC or experimental glaucoma models (Sappington and Calkins, 2006, Sappington et al., 2006, Chidlow et al., 2012). However, the fact that IL-6 mRNA and protein expression were induced upon ONC and IS in combination with the fact that RGCs also express the full length IL-6 receptor (IL-6R) (Leibinger et al., 2013b) provide a first hint that IS induced IL-6 potentially interacts with RGCs.

2.1.2 IL-6 promotes RGC neurite growth via the IL-6 receptor

To provide further evidence that the upregulated IL-6 could potentially interact with RGCs, retinal cultures were stimulated with various IL-6 concentrations and the neurite outgrowth of RGCs was determined. This experiment revealed that IL-6 induced neurite growth with the same efficacy as CNTF and LIF (Chidlow et al., 2012, Leibinger et al., 2013b). Neurite growth was increased concentration dependently and strongest growth-promoting effects were reached at concentrations exceeding 200 ng/ml, most likely due to the fact that IL-6Rs were saturated at these concentrations. This is a similar active concentration range as previously observed for rat dorsal root ganglion (DRG) neurons (Cao et al., 2006). Even *in vivo*, an intravitreal IL-6 injection enabled RGCs to extend axons beyond the lesion site after ONC to a similar extent as CNTF

injections of the same dose. To demonstrate that the IL-6 induced neurite outgrowth was actually dependent on IL-6 signaling, this thesis also showed that blockage of IL-6R by a specific antibody inhibited IL-6-mediated neurite growth. Furthermore, it was tested if inhibition of the downstream signaling cascade could also interfere with IL-6/IL-6R binding induced neurite growth. IL-6 is known to activate JAK/STAT3 and PI3K/AKT/mTOR signaling upon binding to the IL-6R. Both pathways play important roles in CNS axon regeneration (Park et al., 2008, Smith et al., 2009, Sun et al., 2011, Leibinger et al., 2012, Leibinger et al., 2013a). Consistently, RGCs quickly responded to IL-6 application by the activation of the JAK/STAT3 signaling pathway, which was abolished after co-application of the IL-6R antibody. Furthermore, IL-6 activation and pharmacological inhibition of these pathways in RGCs reduced neurite outgrowth induced by IL-6. Additionally, a combination of forskolin and IL-6 promoted even stronger neurite outgrowth (Leibinger et al., 2013b), an effect that has also been described for a combination of CNTF and forskolin (Müller et al., 2009). This is most likely due to the fact that forskolin suppresses SOCS3 upregulation. SOCS3 is known to negatively regulate JAK/STAT3 signaling, thus acting as an intrinsic brake for axon regeneration in the CNS (Park et al., 2009, Smith et al., 2009). Combined, these data strongly suggest that IL-6 promotes RGC neurite growth by binding to its receptor and activating the JAK/STAT3 and PI3K/AKT/mTOR signaling pathways.

2.1.3 IL-6 renders RGCs disinhibitory towards myelin

Since IS also renders RGCs insensitive towards inhibitors of axon regeneration, such as myelin and neurocan, this thesis also tested if IL-6 had a similar effect. Disinhibition towards inhibitors is an important prerequisite for successful CNS regeneration. Previous publications have demonstrated that despite neurite growth stimulation by CNTF axons remained sensitive

towards inhibitory myelin and neurocan (Ahmed et al., 2009, Sengottuvel et al., 2011, Leibinger et al., 2012). In contrast, IL-6 is reportedly able to desensitize various types of neurons towards inhibitory myelin (Cafferty et al., 2004, Cao et al., 2006, Yang et al., 2012). Consistently, data from this thesis showed that IL-6 renders neurite outgrowth of mature RGCs insensitive towards inhibitory myelin, an effect that was found mTOR-dependent as it was abrogated by rapamycin. Interestingly, the disinhibitory effect of IL-6 manifested itself at lower concentrations than its neurite growth promoting effect and was sufficient to induce maximal disinhibition towards myelin at a concentration of 30 ng/ml, while maximal growth promotion was reached at 200 ng/ml. This could be due to the fact that disinhibition and neurite growth promotion are dependent on different signaling pathways; and 30 ng/ml of IL-6 could be sufficient to fully activate the pathway responsible for disinhibition, but not the pathway for growth promotion.

However, although IL-6 desensitized RGCs towards myelin, IL-6 could not reduce neurite outgrowth inhibition induced by the CSPG neurocan. Therefore, it remains unclear via which pathway IL-6 exerts its disinhibitory effects. It however seems likely that IL-6 signaling influences signaling cascades upstream of RhoA/ROCK signaling, since the ROCK inhibitor Y27632 acted disinhibitory towards myelin as well as neurocan. Further investigation is required to unravel this issue.

2.1.4 IL-6 is partially involved in inducing regeneration associated genes in response to IS

IS as well as CNTF and LIF transform RGCs into a regenerative state which is accompanied by the expression of various regeneration associated genes (RAGs). To investigate if IL-6 is also sufficient to turn RGCs into a regenerative state, RAG expression after exogenous IL-6

application and ONC was evaluated. This revealed that IL-6 induced the RAGs sprr1a, gap 43 and galanin (Fischer et al., 2004b, Leibinger et al., 2013b), as detected with realtime PCR (Leibinger et al., 2013b). However, IL-6 did not appear to be the main factor responsible for initiating the transformation of RGCs into a regenerative state upon ONC and IS, since the knockout of both LIF and CNTF abolished the neuroprotective and axon regeneration promoting effect of IS (Leibinger et al., 2009). This was not observed to the same extent in IL-6 knockout mice (Leibinger et al., 2013b). The fact that RGC transformation into a regenerative state is rather induced by CNTF and LIF than IL-6, although all 3 cytokines induced regeneration associated genes, could be due to the comparatively late induction of retinal IL-6 after IS. In contrast to CNTF and LIF, whose expression is induced 1-2 days after IS (Fischer et al., 2004b, Müller et al., 2007), IL-6 expression remained close to baseline levels 3 days after IS and significant increases could only be detected 5 days after injury. While CNTF and LIF seemed to be the responsible factors that initially turn RGCs into a regenerative state, this finding indicated that IL-6 could maintain the regenerative state induced by IS. This was supported by the fact that some STAT3 was phosphorylated even in CNTF/LIF knockout mice 5 days after IS (Leibinger et al., 2009), which correlates closely with the IL-6 expression profile.

2.1.5 IL-6 is necessary for full IS-mediated optic nerve regeneration

While IL-6 does not seem to be important for initially turning of RGCs into a regenerative state after IS stimulation, its knockout reduced axon growth into the crushed optic nerve (Leibinger et al., 2013b). Similarly, RGCs from IL-6 knockout mice showed no reduction in neurite outgrowth as compared to wild type mice, further supporting the notion that IL-6 is not responsible for initiating the regenerative state. It rather appears that IL-6 promotes IS induced axon

regeneration by reducing RGC sensitivity towards myelin. IS promoted RGC neurite outgrowth was not decreased in IL-6 deficient mice on growth permissive substrates. On myelin, however, RGC neurite growth was significantly reduced in IL-6 knockout mice. These data indicate that IL-6 is an important mediator of the disinhibitory effects of IS in optic nerve regeneration. These findings and the fact that the absence of IL-6 in the respective knockout mouse resulted in reduced regeneration upon IS, indicate that IL-6 is one of the factors responsible for promoting IS mediated axon growth into the inhibitory environment of the optic nerve.

2.1.6 IL-6 does not mediate IS-induced neuroprotection

This thesis also investigated the influence of IL-6 on RGC survival after injury and its involvement in the neuroprotective effect of IS. Data presented in this thesis show that IL-6 exerted a neuroprotective effect on mature RGCs upon ONC *in vivo* and *in vitro* (Leibinger et al., 2013b). This effect, however, was weaker than the one observed after CNTF treatment. Also, the neuroprotective effect of IS was not reduced in the IL-6 knockout mouse. In contrast, previous published data showed that simultaneous knockout of CNTF and LIF reduced the neuroprotective effects of IS to wild type levels (Leibinger et al., 2009). Thus, it appears that IL-6 contributes only to a small extend to the neuroprotective effects of IS, while most of this effect is mediated by CNTF and LIF.

In conclusion, this thesis provides evidence that IL-6 is another key factor contributing to optic nerve regeneration induced by IS. Unlike CNTF, IL-6 appears to rather mediate the disinhibitory than the growth promoting and survival inducing effects of IS. These findings give further valuable insights into the molecular mechanism underlying the effects of IS, which could

potentially be utilized in the treatment of CNS injuries. IL-6R, for example, might pose a promising new target for pharmacological treatment to promote optic nerve regeneration.

2.2 Sustained GSK3 activity promotes sciatic nerve regeneration

The second part of this thesis investigated the role of GSK3 in peripheral nerve regeneration. Thus far, the influence of GSK3 activity on axon regeneration has been controversially discussed. Both active and inactive GSK3 have been described to promote but also to reduce axon growth. Although the origin of this discrepancy is not entirely clear, it is likely due to unspecific pharmacological GSK3 inhibitors or unphysiological GSK3 levels induced by GSK3 overexpression used in the respective experiments. Therefore this thesis set out to clarify the role of GSK3 in axon regeneration (Gobrecht et al., 2014) by avoiding those potential pitfalls using GSK3α/GSK3β double knock-in mice ($GSK3\alpha^{S/A}/GSK3\beta^{S/A}$). In these mice, serine 21 of GSK3α and serine 9 of GSK3β are substituted by alanine, thereby preventing GSK3 phosphorylation at this site, and thus inactivation by AKT (McManus et al., 2005). In this way, this mutation renders GSK3 constitutively active without affecting its physiological expression level. This part of the thesis shows that both GSK3α and GSK3β are phosphorylated in adult DRG neurons upon sciatic nerve injury. Prevention of Ser21/Ser9 GSK3 phosphorylation significantly promoted axon growth of cultured DRG neurons, which was kinase activity dependent. Furthermore, prevention of inhibitory GSK3 phosphorylation led to markedly accelerated sciatic nerve regeneration and functional recovery.

2.2.1 GSK3α and GSK3β are phosphorylated upon DRG axotomy

To show that GSK3 activity is altered in response to nerve injury, this thesis first investigated changes in GSK3 phosphorylation upon sciatic nerve crush, which is known to reduce GSK3 activity (Sutherland et al., 1993). Previously it has been shown that AKT activity is increased upon sciatic nerve injury (Saijilafu et al., 2013). Since activated AKT is known to phosphorylate GSK3α at serine 21 and GSK3β at serine 9, thereby inhibiting their kinase activity (Cross et al., 1995, Cohen and Frame, 2001), this thesis consistently showed that phosphorylation of both, GSKα and GSK3β was increased in DRG and sciatic nerves upon crush (Gobrecht et al., 2014). Similarly, GSK3 phosphorylation was induced in cultured DRG neurons (Gobrecht et al., 2014), most likely since the dissociation process also axotomized DRG axons. This increased GSK3 phosphorylation was reduced through pharmacological PI3K/AKT inhibition, thus providing further evidence that GSK3 phosphorylation in axotomized DRG neurons depends on AKT signaling.

2.2.2 GSK3 phosphorylation reduces DRG axon growth in vitro

After demonstrating that GSK3 was phosphorylated upon axon injury, this thesis investigated if this phosphorylation affects DRG axon growth. Previously, it has been suggested that the axotomy induced GSK3 phosphorylation is necessary for the transformation of DRG neurons into a regenerative state and consequently for axon regeneration in general (Saijilafu et al., 2013). This conclusion was drawn from experiments revealing that an inhibition of DRG axon growth by PI3K inhibition was rescued by pharmacological GSK3 inhibition (Saijilafu et al., 2013). Since some of the pharmacological inhibitors used in the above-mentioned study have

been shown to also interact with other related kinases at similar concentrations (Knockaert et al., 2002, Fischer, 2003), these findings could be based on off-target effects. A more specific genetic prevention of GSK3 phosphorylation to support this conclusion more strongly has not been performed (Saijilafu et al., 2013). To more specifically demonstrate how inhibitory GSK3 phosphorylation affected DRG axon growth, GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice were used in the current study. Surprisingly, cultured DRG neurons from these mice showed significantly increased axon growth as compared to wild type (wt) mice (Gobrecht et al., 2014). Since also regeneration associated genes, such as *gap43* and *sprr1a*, were slightly increased after injury in these knockin mice (Gobrecht et al., 2014), it has to be concluded that inhibitory phosphorylation of GSK3 is not required for DRG neurons to enter into a regenerative state and that an AKT-induced GSK3 phosphorylation rather limits peripheral nerve regeneration. Thus, GSK phosphorylation seems to act as an intrinsic brake for regeneration.

2.2.3 Axon growth induced in GSK3α$^{S/A}$/GSK3β$^{S/A}$ DRG neurons depends on GSK3 activity

Since prevention of GSK3 phosphorylation promoted axon regeneration, this thesis proceeded to investigate if this axon growth promotion is actually dependent on increased GSK3 activity. This revealed that the axon growth promoting effects observed in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice depended indeed on a higher GSK3 activity. This was demonstrated by the fact that the GSK3 inhibitor SB415286, which blocks GSK3 activity at its substrate-binding site (Coghlan et al., 2000), reduced axon growth in GSK3α$^{S/A}$/GSK3β$^{S/A}$ neurons. Lithium on the other hand, which inhibits GSK3 through AKT activation and a subsequent GSK3 phosphorylation, did not reduce axon growth of GSK3$^{S/A}$ neurons (Gobrecht et al., 2014). Also, phosphorylation of the GSK3

target MAP1B (Goold et al., 1999, Trivedi et al., 2005, Scales et al., 2009) was significantly increased in DRG neurons from GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice, further indicating that kinase activity is increased in these animals (Gobrecht et al., 2014). Additionally, these data exclude the possibility that GSK3 activity might be reduced to baseline levels in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice by so far unknown mechanisms. Furthermore, mothers against decapentaplegic homolog 1 (SMAD1) is a protein that is ubiquitinilated and subsequently degraded upon phosphorylation through active GSK3 (Saijilafu et al., 2013). Increased GSK3 activity in GSK3α$^{S/A}$/GSK3β$^{S/A}$ neurons is supported by the finding that SMAD1 levels were strongly reduced in these neurons as compared to wt neurons. Although SMAD1 presence has been shown to be essential for axon growth (Zou et al., 2009), the fact that axon growth was increased in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice indicates that only basal SMAD1 levels are required for induction of DRG axon growth. Also, although SMAD1 expression is strongly reduced in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice, SMAD1 levels were still increased after sciatic nerve crush as compared to uncrushed wild type animals.

2.2.4 Constitutively active GSK3 phosphorylates MAP1B increases microtubule dynamics by reducing microtubule detyrosination

After demonstrating that elevated GSK3 activity promoted DRG axon growth, this thesis investigated the underlying molecular mechanism. According to the literature, GSK3 can influence axon elongation via various microtubule associated proteins (MAPs) in the developing nervous system (Owen and Gordon-Weeks, 2003, Zhou et al., 2004, Zhou and Snider, 2005, Kim et al., 2006, Zhou and Snider, 2006, Scales et al., 2009, Hur et al., 2011a). Through these interactions GSK3 controls axon guidance and direction of growth. A particularly important MAP required for axon growth is MAP1B (Gonzalez-Billault et al., 2001). MAP1B is phosphorylated

via two different mechanisms (Avila et al., 1994): While mode II phosphorylation is predominantly induced by casein kinase II (Diaz-Nido et al., 1988, Avila et al., 1994) and occurs in all neuronal compartments, mode I phosphorylation of MAP1B mainly occurs in the axon tips (Ulloa et al., 1994). Mode I phosphorylation is known to turn microtubules into a dynamic state by detyrosinating α-tubulin (Gonzalez-Billault et al., 2001, Gonzalez-Billault et al., 2004) and is mainly induced by phosphorylation through active GSK3 at Serine 1260 and Threonine 1265 (Lucas et al., 1998, Trivedi et al., 2005, Scales et al., 2009). Reducing MAP1B phosphorylation through pharmacological GSK3 inhibition has been shown to reduce axon growth of DRG explants (Owen and Gordon-Weeks, 2003) and dissociated cultures (Kim et al., 2006, Alabed et al., 2010). Also, MAP1B phosphorylation by GSK3 reduces microtubule detyrosination, thus turning microtubules into a dynamic growth supporting state. While this makes axons more prone to nocodazole depolymerisation, it also promotes axon elongation of embryonic DRG neurons (Goold et al., 1999, Owen and Gordon-Weeks, 2003). This thesis demonstrated that MAP1B phosphorylation was also increased in adult DRG neurons of GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice as compared to those of wild type animals. Furthermore, nocodazole concentrations not affecting axon growth of adult wt DRG neurons reduced the axon growth promoted by constitutively active GSK3. These findings support the conclusion that constitutively active GSK3 increases axon regeneration through MAP1B phosphorylation and a subsequent increase of microtubule dynamics. Apart from interfering with microtubule dynamics, GSK3 could also improve axon regeneration of adult DRG neurons by elevating the expression of regeneration associated genes, thus positively affecting the regenerative state of these neurons. It is known that GSK3 interacts with various transcription factors associated with the regenerative state (Turenne and Price, 2001, Watcharasit et al., 2002, Watcharasit et al., 2003).

This was also supported by data from this thesis which showed that the RAGs *gap43* and *sprr1* were slightly elevated in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice (Gobrecht et al., 2014).

2.2.5 Constitutively active GSK3 accelerates sciatic nerve regeneration

After demonstrating that increased GSK3 activity promoted axon regeneration of cultured DRG neurons, likely via increasing microtubule dynamics, this thesis set out to investigate if active GSK3 also increased axon regeneration *in vivo*. This study revealed that increased GSK3 activity in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice significantly accelerated axon regeneration in the injured sciatic nerve compared with wt mice. The number of axons grown >3 mm beyond the lesion site in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice was comparable to the number found at 1.5 mm in wt animals 3 days after injury. Reestablished neuromuscular junctions in the *extensor hallucis longus* muscle (EHL) were only detected in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice after 7 days. Additionally, both motor and sensory function recovered faster after sciatic nerve injury in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice as compared to wt mice, as determined with the static sciatic index (SSI) and the von Frey test. Together, these data demonstrate that constitutively active GSK markedly accelerates axon regeneration *in vivo*.

2.2.6 Both GSK3 isoforms contribute to promoting sciatic nerve regeneration

To find further evidence that GSK3 induced axon growth promotion was in fact dependent on GSK3 activity and also which isoform is responsible for the accelerated regeneration, it was investigated whether the growth promotion induced in GSK3α$^{S/A}$/GSK3β$^{S/A}$ animals depends on a specific GSK3 isoform, since most previous studies mainly investigated GSK3β in the context of

axon regeneration. Interestingly, the single knockin of either GSK3α (GSK3α$^{S/A}$) or GSK3β (GSK3β$^{S/A}$) improved axon growth *in vitro*, *in vivo* and functional recovery to a similar extent (Gobrecht et al., 2016). This strongly suggests that the activity of both GSK3 isoforms is involved in the promotion of axon regeneration. The fact that axon regeneration was weaker in the single knockin mice as compared to the double knockin animals (Gobrecht et al., 2016) indicates that GSK3α and GSK3β might act on the same downstream targets and that total GSK3 activity is beneficial for axon regeneration in the peripheral nervous system.

Therefore, therapies specifically preventing GSK3 phosphorylation without affecting PI3K activity, for instance by drugs or gene therapy may constitute useful treatments to improve peripheral nerve regeneration. Future experiments will reveal whether the beneficial effects of constitutively active GSK3 seen in the current study are also applicable to the regeneration of the CNS.

2.3 Promotion of functional regeneration by inhibition of microtubule detyrosination

Next to demonstrating that increased GSK3 activity in GSK3α$^{S/A}$/GSK3β$^{S/A}$ knockin mice promotes sciatic nerve regeneration and functional recovery MAP1B dependently (Gobrecht et al., 2014), the third part of this thesis further investigated the molecular mechanisms underlying accelerated axon regeneration in GSK3α$^{S/A}$/GSK3β$^{S/A}$ knockin animals. From these findings a potentially clinically applicable approach was deduced by reducing microtubule detyrosination with parthenolide to improve nerve regeneration.

2.3.1 GSK3 phosphorylates MAP1B but bot CRMP2 in the sciatic nerve

To further explore the mechanism(s) underlying GSK3α$^{S/A}$/GSK3β$^{S/A}$-induced axon regeneration, this thesis investigated the impact of elevated GSK3 activity on the two selected microtubule binding proteins CRMP2 and MAP1B, which reportedly affect axon growth upon phosphorylation by GSK3 (Hur et al., 2011b). While phosphorylation of CRMP2 rather compromises axon regeneration (Hur and Zhou, 2010, Liz et al., 2014b), phosphorylation of MAP1B has been proposed to improve axon growth in embryonic neurons (Trivedi et al., 2005). Consistent with elevated GSK3 activity, phospho-CRMP2 levels were increased in DRGs of GSK3α$^{S/A}$/GSK3β$^{S/A}$ compared to wild-type mice pre- and post-sciatic nerve injury (Gobrecht et al., 2016). However, despite abundant CRMP2 protein, only very little phospho-CRMP2 was detected in normal and injured sciatic nerves of either wild-type or GSK3α$^{S/A}$/GSK3β$^{S/A}$ animals (Gobrecht et al., 2016). These results suggest a differential regulation of CRMP2 phosphorylation in different neuronal compartments. This neuronal compartment dependent CRMP2 phosphorylation could be due to the fact that CRMP2 requires a phosphorylation at serine 522 by cyclin-dependent kinase 5 (CDK5) before it can be phosphorylated and thus inactivated by GSK3 (Uchida et al., 2005). This process, known as priming, could occur differently in the axonal and in the somal compartment due to a differential expression of CDK5. Thus, CRMP2 phosphorylation appears to be irrelevant for peripheral nerve regeneration. However, phospho-CRMP2 might show different subcellular distributions in other neurons, e.g. in the CNS, which could negatively impact axon regeneration, for example in the optic nerve or spinal cord (Liz et al., 2014b). Moreover, it cannot be excluded that enhanced GSK3 activity inhibits other microtubule-binding proteins, such as APC or CLASP, which might still dampen the overall positive regenerative outcome in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice (Garrido et al., 2007, Hur et al., 2011a). In contrast to CRMP2, phosphorylation of MAP1B at Thr1265 by GSK3 does not require prior priming

(Frame et al., 2001, Cole et al., 2004, Trivedi et al., 2005) and is reportedly restricted to axons (Avila et al., 1994, Ulloa et al., 1994, Goold et al., 1999, Trivedi et al., 2005, Scales et al., 2009). Thus, axonal phospho-MAP1B levels can be considered as a more reliable indicator for GSK3 activity.

Consistently, phospho-MAP1B levels in axonal tips in culture and in pre- and post-crushed sciatic nerves correlated with increased GSK3 activity in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice and were reduced to wild-type levels by GSK3 inhibition (Gobrecht et al., 2016). Notably, phosphorylation of MAP1B was enhanced in axons of each single knockin mouse (Gobrecht et al., 2016), indicating that MAP1B is a substrate for both GSK3α and GSK3β. This observation was rather unexpected as experiments in embryonic chick CNS tissue previously proposed MAP1B as a specific substrate of GSK3β (Lucas et al., 1998, Owen and Gordon-Weeks, 2003). Whether these differences are due to different developmental stages, species or specific experimental settings still remains unknown.

2.3.2 Reduced microtubule detyrosination correlates with DRG axon growth

After having shown that GSK3 activity increases MAP1B phosphorylation, the effect of GSK3 activity on microtubule detyrosination was investigated next. This study revealed that microtubule detyrosination is closely correlated with MAP1B phosphorylation and axon growth. It has previously been proposed that increased MAP1B phosphorylation is associated with reduced microtubule detyrosination in embryonic neurons, which reportedly promotes axon extension (Lucas et al., 1998, Goold et al., 1999, Owen and Gordon-Weeks, 2003, Trivedi et al., 2005). Although the detailed enzymatic mechanism underlying this impaired microtubule detyrosination remains unclear, phospho-MAP1B-mediated modulations of a tubulin-

carboxypeptidase or a tubulin-tyrosine ligase have been discussed in this context (Goold et al., 1999, Gonzalez-Billault et al., 2002, Owen and Gordon-Weeks, 2003, Gonzalez-Billault et al., 2004). Irrespective of the underlying mechanism, this thesis demonstrates that microtubule detyrosination was indeed reduced in axonal tips of GSK3α$^{S/A}$/GSK3β$^{S/A}$ neurons. To investigate whether this modification of microtubule endings contributed to the observed enhanced axon regeneration in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice, microtubule detyrosination was pharmacologically inhibited in wild-type animals using the sesquiterpene lactone parthenolide. Similar to non-neuronal cells (Fonrose et al., 2007), parthenolide reduced the detyrosination of tubulin in axonal tips of cultured DRG neurons (Gobrecht et al., 2016). Consequently, parthenolide and cnicin promoted axonal growth concentration-dependently in DRG neuronal cultures (Gobrecht et al., 2016). At optimal concentrations, parthenolide fully mimicked the beneficial effects of GSK3α$^{S/A}$/GSK3β$^{S/A}$ on axon growth in wild-type mice (Gobrecht et al., 2014, Gobrecht et al., 2016). As parthenolide treatment did not further enhance axon growth of GSK3α$^{S/A}$/GSK3β$^{S/A}$ neurons, reduced microtubule detyrosination is likely the main mechanism underlying GSK3α$^{S/A}$/GSK3β$^{S/A}$ stimulated growth. Moreover, these data provide the first direct evidence that inhibition of microtubule detyrosination can stimulate axon growth. Although parthenolide is known to also inhibit NFκB-signaling, this is unlikely to be relevant for these findings, as approximately thousand-fold higher parthenolide concentrations are required than used in this thesis (Hehner et al., 1999, Yip-Schneider et al., 2005, Saadane et al., 2007). Consistently, this thesis demonstrated that parthenolide had no influence on NFκB activity *in vivo* and *in vitro*, using the expression of IκBα as sensitive readout (Gobrecht et al., 2016). Additionally, it was shown in experiments with two-compartment chambers that parthenolide promoted axon growth by interacting with the axon tips rather than with the neural somata (Gobrecht et al., 2016).

The concentration dependency of the sesquiterpene lactones indicates that moderate reduction of microtubule detyrosination promotes, whereas strong inhibition at high concentrations rather reduces axon growth. General toxicity was no concern in this context, as the survival of DRG neurons was unaffected by any treatment (Gobrecht et al., 2016).

Conceptually, moderate inhibition of detyrosination at plus ends transforms microtubules in a more dynamic state, which is required for optimal axonal growth (Lucas et al., 1998). Consistent with increased microtubule dynamics, parthenolide-treated axons showed higher sensitivity towards microtubule-disrupting nocodazole, similar to GSK3α$^{S/A}$/GSK3β$^{S/A}$ neurons (Gobrecht et al., 2016). Treatment with paclitaxel, which increases detyrosination and stabilizes microtubules (Erturk et al., 2007, Hammond et al., 2010) counteracted the observed growth promotion. The conclusion that increased microtubule dynamics promote axonal regeneration is consistent with a previous study showing that depletion of the injury-specific kinesin KIF3C, which destabilizes microtubules via binding to tyrosinated tubulin, compromises axon regeneration (Gumy et al., 2013). Similarly, knockdown of tubulin-tyrosine ligase reportedly increased the level of detyrosinated microtubules and impaired axon growth (Song et al., 2015). In this study, the observed effect was ascribed to impaired retrograde axonal transport of injury signals upon modification of the tubulin tyrosination cycle (Song et al., 2015). It remains to be investigated whether parthenolide treatment might additionally increase the regenerative response of neurons to axonal injury by accelerating this retrograde transport.

2.3.3 Parthenolide promotes sciatic nerve regeneration

Strikingly, accelerated sciatic nerve regeneration in GSK3α$^{S/A}$/GSK3β$^{S/A}$ mice could also be mimicked by *in vivo* parthenolide application in wild-type mice. Effective doses were identified for both intraperitoneal and intraneural applications. In agreement with data obtained *in vitro*, these *in vivo* effects of parthenolide were dose-dependent with the highest tested doses having no or

even adverse effects (Gobrecht et al., 2016). Moreover, intraneural injection of effective parthenolide doses decreased microtubule detyrosination in the sciatic nerve *in vivo*, while the phosphorylation status of CRMP2 and MAP1B remained unaffected (Gobrecht et al., 2016), thereby demonstrating that parthenolide promoted regeneration downstream of GSK3 signaling. Either one intraneural or one intraperitoneal injection of parthenolide was sufficient to promote regeneration, including accelerated motor and sensory recoveries. However, it might still be possible to further improve the functional outcome by optimizing doses and application frequencies. Whereas intraneural application is a very invasive intervention, it would be feasible to proceed with intraperitoneal injections.

It is currently unknown whether parthenolide treatment would also promote axon regeneration in the CNS. In contrast to the PNS, inhibitory molecules in CNS, such as myelin and the gliotic scar, limit axon regeneration in the CNS. Previously it has been demonstrated that axon regeneration in the CNS can be facilitated by taxol-mediated stabilization of microtubules, which decreases the sensitivity of axonal growth cones towards inhibitory molecules (Erturk et al., 2007, Hellal et al., 2011, Sengottuvel and Fischer, 2011, Sengottuvel et al., 2011). Thus, more dynamic microtubules, as induced by parthenolide, might rather elevate the inhibitory response of growth cones towards CNS inhibitors and would therefore impair CNS regeneration. Experiments addressing these questions remain to be addressed.

In conclusion, this thesis demonstrates that prevention of GSK3 phosphorylation markedly promotes axon regeneration *in vitro* and *in vivo* by compromising detyrosination of microtubules in axonal tips. These effects can be mimicked by pharmacological inhibition of tubulin detyrosination using parthenolide. Therefore, treatments aiming to reduce microtubule detyrosination in the injured peripheral nerve may be a novel and clinically feasible approach to accelerate axon regeneration and to improve functional recovery.

3 Publications

3.1 First Authorships

3.1.1 Interleunin-6 contributes to axon regeneration upon inflammatory stimulation

(Published in April 2013, (Leibinger et al., 2013b))

M Leibinger*, A. Müller*, P Gobrecht*, A Andreadaki and D Fischer

Cell Death and Disease

Impact Factor 2015: 5.378

* These authors contributed equally to this work.

I performed ca. 25% of the experiments and data analysis. I contributed to drafting and revising the figures and text of the manuscript. I also contributed to the data interpretation.

3.1.2 Sustained GSK3 activity markedly facilitates nerve regeneration

(Published in July 2014, (Gobrecht et al., 2014))

P Gobrecht, M Leibinger, A. Müller, A Andreadaki and D Fischer

Nature Communications

Impact factor 2015: 11.329

I performed ca. 90% of the experiments and data analysis. I contributed to drafting and revising the figures and the text of the manuscript. I also contributed to the interpretation of data.

3.1.3 Promotion of functional nerve regeneration by inhibition of microtubule detyrosination

(Published in April 2016, (Gobrecht et al., 2016))

P Gobrecht, A Andreadaki, H Diekmann, A Heskamp, M Leibinger and D Fischer

Journal of Neuroscience

Impact factor 2015: 6.92

I performed ca. 80% of the experiments and data analysis. I contributed to drafting and revising the figures and the text of the manuscript. I also contributed to the experimental design and data interpretation.

3.2 Co-authorships

3.2.1 CXCL12/SDF-1 facilitates optic nerve regeneration

Published in April 2013, (Heskamp et al., 2013)

A Heskamp, M Leibinger, A Andreadaki, P Gobrecht, H Diekmann and D Fischer

Neurobiology of Disease

Impact factor 2015/2016: 4.856

I performed ca. 10% of the experiments and data analysis. I also contributed to drafting and revising the figures.

3.2.2 Boosting Central Nervous System Axon Regeneration by Circumventing Limitations of Natural Cytokine Signaling

Published in October 2016, (Leibinger et al., 2016)

M Leibinger, A Andreadaki, P Gobrecht, E Levin, H Diekmann and D Fischer

Molecular Therapy

Impact factor 2015/2016: 6.938

I performed ca. 10% of the experiments and data analysis. I also contributed to drafting and revision of the figures.

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Versicherungen und Erklärungen

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

Ich habe diese Dissertation an keiner anderen Fakultät vorgelegt. Ich habe keine vorherigen erfolglosen oder erfolgreichen Promotionsversuche unternommen.

Die Disputation soll in deutscher Sprache abgelegt werden, ohne Ausschluss der Öffentlichkeit.

Bei erfolgreichem Abschluss wünsche ich den Grad „Doktor der Naturwissenschaften“ (doctor rerum naturalium - Dr. rer. nat.) verliehen zu bekommen.

Düsseldorf, den 17.03.2017 Philipp Gobrecht

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