Doctoral Thesis

The role of glial versus neuronal nogo-a in axonal regeneration in the mouse central nervous system

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THE ROLE OF GLIAL VERSUS NEURONAL NOGO-A IN AXONAL REGENERATION IN THE MOUSE CENTRAL NERVOUS SYSTEM

A thesis submitted to attain the degree of

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(Dr. sc. ETH Zurich)

presented by

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# Table of Contents

**Summary** .................................................................................................................. - 4 -

**Zusammenfassung** ...................................................................................................... - 6 -

**Context and Aims** ...................................................................................................... - 8 -

**Chapter 1** .................................................................................................................. - 9 -

Introduction: Extracellular and intracellular limitations of axonal regeneration in the central nervous system – special focus on Nogo-A and the visual system .............................................. - 9 -

1 Introduction: Myelin associated neurite growth inhibitors - focus on Nogo-A .......... - 10 -

1.1 The central nervous system is a hostile environment for axonal regeneration:
history of discovery........................................................................................................... - 10 -

1.2 The neurite growth inhibitory protein Nogo-A: description of Nogo signalling and possible pharmacological interventions ........................................................................... - 11 -

1.3 Physiological functions of Nogo-A ........................................................................ - 15 -

1.4 Nogo-A in CNS diseases .......................................................................................... - 17 -

2 Regeneration and plasticity after CNS injury ............................................................... - 17 -

2.1 Stimulation of regeneration by up-regulating intrinsic neuronal growth mechanisms ................................................................................................................... - 18 -

2.2 Nogo-A neutralization improves regeneration and promotes plasticity in animal models of spinal cord injury ................................................................................... - 19 -

2.3 Suppression of the Nogo-A pathway in animal models of spinal cord injury ....... - 21 -

2.3.1 Nogo-A neutralization by antibodies ................................................................ - 21 -

2.3.2 Blockade of Nogo-A receptors and signalling ................................................... - 22 -

2.3.3 Preparing translation: preclinical studies for Nogo-A blocking agents ............ - 26 -

2.3.4 Interventions blocking Nogo-A signalling in clinical trials for spinal cord injury - 28 -

2.4 Regeneration after genetic deletion of Nogo-A and NgR1 ......................................... - 29 -

2.5 Regeneration in the visual system ........................................................................... - 33 -

2.5.1 The optic nerve crush as an excellent model system to study axonal regeneration in the CNS .............................................................................................................. - 33 -

2.5.2 Regeneration studies in the visual system ........................................................... - 36 -

2.5.3 From the optic nerve to the spinal cord .............................................................. - 39 -

3 Expression pattern of Nogo-A in the visual system: implications on intracellular and extracellular cell-type specific functions .............................................................. - 39 -

4 Conclusions ................................................................................................................ - 42 -

References ..................................................................................................................... - 43 -

**Chapter 2** .................................................................................................................. - 57 -

Cell type-specific Nogo-A gene ablation promotes axonal regeneration in the injured adult optic nerve ................................................................................................................... - 57 -

1 Abstract ...................................................................................................................... - 58 -
2 Introduction.............................................................................................................................................. - 60 -
3 Results ............................................................................................................................................................. - 61 -
  3.1 Targeted deletion of Nogo-A in oligodendrocytes ...................................................................................... - 61 -
  3.2 Cnp-Cre-driven ablation of Nogo-A increases regenerative axonal sprouting after optic nerve crush injury .............................................................................................................................................. - 64 -
  3.3 Nogo-A deletion in Cnp-Cre+/xRtn4flox/flox mice enhances inflammation-induced axonal regeneration after optic nerve injury .............................................................................................................................................. - 64 -
  3.4 Three-dimensional analysis of the growth pattern of regenerating axons in the transparent optic nerve .............................................................................................................................................. - 66 -
  3.5 Compensatory up-regulation of EphrinA3 and EphA4 in Cnp-Cre+/xRtn4flox/flox mice - 66 -
  3.6 Nogo-A distribution and neuronal survival in the retinae of Cnp-Cre+/xRtn4flox/flox mice .............................................................................................................................................. - 69 -
  3.7 Nogo-A expression is required in retinal ganglion cells for optic axon regeneration - 69 -
  3.8 The immunohistochemical signal for the Nogo receptor NgR1 is increased in Cnp-Cre+/xRtn4flox/flox retinae .............................................................................................................................................. - 73 -
4 Discussion ......................................................................................................................................................... - 80 -
  4.1 Axonal regeneration in conventional versus cell type-specific Nogo-A KO lines - 80 -
  4.2 The limitations of axonal regeneration by Nogo-A and guidance molecules in the injured optic nerve .............................................................................................................................................. - 80 -
  4.3 The role of Nogo-A in neuronal survival ........................................................................................................... - 81 -
  4.4 Nogo-A and Nogo receptors in retinal ganglion cells: trans and cis-interaction? - 82 -
5 Materials and Methods .................................................................................................................................... - 83 -
References .............................................................................................................................................................. - 88 -

Chapter 3 ......................................................................................................................................................... - 93 -
Contribution of glial Nogo-A to molecular mechanisms underlying axonal regeneration and cell survival in the visual system .............................................................................................................................................. - 93 -
1 Abstract .............................................................................................................................................................. - 93 -
2 Introduction ........................................................................................................................................................... - 94 -
3 Results .................................................................................................................................................................... - 96 -
  3.1 Müller cells injury response in glial Nogo-A KOs ............................................................................................. - 97 -
  3.2 Regulation of ER stress-related genes in the retina of Cnp-Cre+/xRtn4flox/flox mice - 99 -
  3.3 The expression of autophagy related genes is not modified by glial deletion of Nogo-A .............................................................................................................................................. - 99 -
  3.4 Regulation of Nogo-A receptors in the retina of Cnp-Cre+/xRtn4flox/flox mice - 102 -
  3.5 Model systems: Mog-Cre+/xRtn4flox/flox vs. Cnp-Cre+/xRtn4flox/flox .............................................................................................................................................. - 105 -
4 Discussion ........................................................................................................................................................... - 108 -
  4.1 Nogo-A and Müller cell reaction ........................................................................................................................ - 108 -
  4.2 Possible neuro-protective mechanisms of Nogo-A in the retina ........................................................................ - 109 -
  4.3 NgR receptor complex changes upon glial deletion of Nogo-A ....................................................................... - 110 -
  4.4 Lack of significant Nogo-A down-regulation in Mog-Cre+/xRtn4flox/flox mice - 111 -
5 Materials and methods ........................................................................................................ - 112 -
References ............................................................................................................................. - 115 -

Chapter 4 ............................................................................................................................ - 119 -
In vitro investigation of the potential hindrance of Nogo-A receptor signaling by Nogo-A expressed in cis position ........................................................................................................ - 119 -
1 Abstract ............................................................................................................................. - 120 -
2 Introduction ....................................................................................................................... - 122 -
3 Results ................................................................................................................................ - 124 -
  3.1 Expression of Nogo-A and Nogo-A receptors in the F11 neuronal cell-type .......... - 124 -
  3.2 Down-regulation or deletion of Nogo-A: effect on neurite outgrowth and cell spreading ................................................................................................................................. - 126 -
  3.3 Protein expression changes following Nogo-A up and down-regulation .......... - 126 -
4 Discussion ......................................................................................................................... - 129 -
5 Materials and methods ..................................................................................................... - 130 -
References ........................................................................................................................... - 133 -

Chapter 5 ............................................................................................................................ - 135 -
Concluding remarks ............................................................................................................. - 135 -
1 Conclusions and Outlook ................................................................................................. - 136 -
  1.1 Nogo-A as an extrinsic and intrinsic growth, synapse and survival regulator .... - 136 -
  1.2 Contribution of guidance molecules and myelin inhibitory proteins to the blockade of CNS axonal regeneration ........................................................................................................ - 137 -
  1.3 Model systems to study axonal regeneration in the CNS .................................... - 139 -
  1.4 Implications for clinical therapies targeting Nogo-A signaling ......................... - 140 -
  1.5 Final conclusions ......................................................................................................... - 143 -
References ........................................................................................................................... - 144 -

Acknowledgements ........................................................................................................... - 147 -
Curriculum vitae .................................................................................................................. - 148 -
Summary

In the central nervous system (CNS), Nogo-A has been well described as a myelin-associated inhibitory protein for axonal regeneration after injury. However, Nogo-A is also expressed in neurons, especially in regions of the nervous system where plasticity is high, such as the hippocampus, the cortex and dorsal root ganglia (DRGs). In addition to playing a major role in the blockade of axon growth in the adult CNS, Nogo-A has been shown to modulate neuronal progenitor migration, axon guidance and fasciculation, dendritic branching, synaptic plasticity and even cell survival.

Our recent studies suggested that not only glial, but also neuronal Nogo-A could influence axonal growth and that this neuronal counterpart may be beneficial for axonal regeneration. Besides function-blocking antibodies and pharmacological inhibitors, so far only conventional knock-out (KO) mouse lines have been available to study the functions of Nogo-A. In these complete KO models, systemic gene deletion affected Nogo-A expression in glial and neuronal cell populations at the same time, and led to modest axonal regrowth in the injured CNS. We therefore hypothesized that the selective blockade of Nogo-A in oligodendrocytes may increase axonal regeneration to a higher level than previously observed in full KO mice.

To study the separate role of glial and neuronal Nogo-A/RTN4 on axonal regeneration, we have generated cell-type specific conditional Nogo-A KO animals by using the Cre-lox recombination system. Targeted Nogo-A deletion in oligodendrocytes and in neurons was obtained by crossing a floxed Nogo-A mouse line (Rtn4\textsubscript{flox/flox}) with mice expressing Cre-recombinase under the control of the oligodendrocyte-specific 2',3'-cyclic nucleotide 3'-phosphodiesterase promoter (Cnp-Cre\textsuperscript{+/-}) or the neuron-specific Thy1 promoter (Thy1-Cre\textsuperscript{tg+}), respectively. Acute excision of the Nogo-A gene in neurons was carried out by adeno-associated virus serotype 2 (AAV2). Cre virus injections in Rtn4\textsubscript{flox/flox} animals. We found that Nogo-A was down-regulated to different extents in glia and neuron-specific knock-out mice, in a manner consistent with the proportion of Nogo-A-expressing glial and neuronal cells in the CNS. Axonal regeneration in the optic nerve after crush injury was increased in the glia-specific Nogo-A knock-out mice, suggesting that the targeted deletion of glial Nogo-A improves neuronal growth capacity more than previously observed after systemic Nogo-A gene ablation. The increased axonal growth was associated with the up-regulation of growth associated genes, such as GAP-43 and SPRR1A. Inversely, deletion of Nogo-A from retinal ganglion cells (RGCs) impaired the regenerative growth response. We found that neuronal Nogo-A in RGCs could participate in enhancing axonal sprouting, possibly by cis-interaction with Nogo receptors at the cell membrane that may counteract trans Nogo-A signaling and...
Rho-A pathway activation. In addition, neuronal survival was also significantly improved in the retinas of glial Nogo-A KO mice. Major cellular and molecular events that could contribute to the mechanisms of axonal regeneration and cell survival were also investigated. In these experiments, Nogo-A did not contribute to Müller cell gliosis, ER-stress or autophagic processes. Although Nogo-A is mostly accounted as an extracellular inhibitor of axonal regeneration by acting on the cytoskeleton through the Rho-A/ROCK pathway, oligodendroglial Nogo-A in *trans* has additionally been implicated to suppress the neuronal growth program by modulating CREB, mTOR and ERK1/2 activation.

The results presented in this thesis show that oligodendroglial and neuronal Nogo-A play distinct roles in axonal regeneration in the central nervous system. While myelin Nogo-A presented in *trans* leads to growth inhibition, Nogo-A expressed extra- or intracellularly in neurons might exert a positive function possibly by interacting with its receptors in *cis* position. Therefore, inactivating Nogo-A in glia while preserving neuronal Nogo-A expression may be the most successful strategy to promote axonal regeneration in the CNS. The pioneer work presented in this thesis was carried out in the visual system and may be followed by studies in other parts of the CNS, where traumatic lesions, such as spinal cord injury, lead to permanent neurological impairments.
Zusammenfassung


beobachtet wurde. Das erhöhte axonale Wachstums war zusätzlich mit der Hochregulierung Wachstums-assoziiierter Gene wie GAP-43 und SPRR1A verbunden.


**Context and Aims**

The membrane protein Nogo-A is a well-known myelin inhibitory factor for axonal regeneration after injury, however, several studies demonstrated that Nogo-A also plays a major role in regulating neuronal development and plasticity in the central nervous system. The fact that Nogo-A is not only expressed by oligodendrocytes, but also by neuronal cell populations further complicates our understanding on the physiological role of Nogo-A. **Chapter 1** of this thesis summarizes the discovery, structure and function of Nogo-A, and provides an overview on the therapeutic potential of Nogo-A neutralization in improving axonal regeneration and functional recovery after spinal cord injury in clinically relevant and knock-out mouse studies. Furthermore, **Chapter 1** introduces the visual system and the optic nerve crush injury as a fundamental *in vivo* model to understand the molecular mechanisms underlying regenerative growth inhibition in the central nervous system.

The main goal of my thesis was to investigate cell type specific functions of Nogo-A in axonal regeneration, and to this aim we generated glial and neuronal conditional Nogo-A knock-out mouse lines in our laboratory. In **Chapter 2**, the characterization of these mouse lines revealed successful, cell-type dependent deletion of Nogo-A in various parts of the CNS. Axonal regeneration and neuronal survival was evaluated in the optic nerve and retina, respectively. To determine both the amount and growth pattern of regenerating axons, three-dimensional analysis of whole mounted, transparent optic nerves was carried out. In addition to the genetic deletion of Nogo-A, the effect of adeno-associated virus-mediated recombination of the Nogo-A gene in neurons on axonal regeneration was also tested. The changes in growth/regeneration and cell survival related signaling pathways were investigated by Western blotting, qRT-PCR and biochemical assays and revealed a possible mechanism by which neuronal Nogo-A exerts a positive function on neurite outgrowth. Following the deletion of Nogo-A from glial cells, the compensatory up-regulation of related myelin inhibitory proteins and guidance molecules was also investigated. In **Chapter 3**, further functions, such as gliosis/inflammation, Nogo-A receptor expression, autophagy and ER stress were screened to detect possible alterations explaining the regeneration and survival phenotype we observed in the glial conditional knock-outs. Finally another alternative myelin/oligodendrocyte-specific Nogo-A conditional knock-out is introduced in this chapter.

In order to further investigate a possible cell-autonomous role for Nogo-A, multiple *in vitro* experiments were carried out in fibroblasts and a neuronal cell line with the aid of gain- and loss-of-function approaches. These studies are presented in **Chapter 4**.
Chapter 1

Introduction: Extracellular and intracellular limitations of axonal regeneration in the central nervous system – special focus on Nogo-A and the visual system

by Flóra Vajda

Further contributions by:

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Contributions:
F.V. wrote the manuscript (revised by V.P. and M.E.S.). F.V. and A.GK. prepared the figures.

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1 Introduction: Myelin associated neurite growth inhibitors - focus on Nogo-A

1.1 The central nervous system is a hostile environment for axonal regeneration: history of discovery

Already the early 1900s, Ramón y Cajal described that lesioned axons in the CNS do not regenerate but retract and form dystrophic end-bulbs after an injury; only occasional sprouting over short distances can be observed (Ramón y Cajal et al., 1991). This lack of axonal regeneration within the CNS after large lesions leads to irreversible functional deficits, e.g. to impaired locomotion or hand function in spinal cord injured or stroke patients.

In the peripheral nervous system (PNS), a high level of regenerative capacity can enable the reinnervation of target organs, which in turns leads to functional recovery. In 1911, a pupil of Ramón y Cajal, Tello, showed massive ingrowth of silver stained fibers of cortical origin into a peripheral nerve graft implanted into the forebrain cortex of rabbits. Further experiments in the 1980s also demonstrated that PNS axons (that otherwise regenerate) fail to grow in the CNS milieu, but CNS axons elongated for unprecedented distances when the CNS environment was replaced by peripheral nerves (David and Aguayo, 1981). Therefore these studies demonstrated that under favourable conditions CNS neurons are able to regenerate and opened the avenue for research on determining the molecular differences between the growth-permissive PNS and hostile CNS environment.

Several components of the CNS myelin were discovered to inhibit axonal growth after an injury. Experiments in the late 1980s that compared outgrowth inhibition properties of CNS and PNS myelin extracts (Caroni and Schwab, 1988) lead to the discovery of Nogo-A (initially named ‘IN-250’) (Spillmann et al., 1998, Chen et al., 2000), a myelin membrane protein contributing to the regeneration failure in the CNS after an injury (Schnell and Schwab, 1990). Further components of CNS myelin have been shown to have inhibitory properties: myelin-associated glycoprotein (MAG) (McKerracher et al., 1994, Mukhopadhyay et al., 1994) and oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002b) are also located in the myelin sheaths of oligodendrocyte and play a role in inhibiting regrowth of injured neurons, at least in vitro. Versican V2 and brevican, two chondroitin sulphate proteoglycans (CSPG) are present in the CNS myelin and also suppress neurite outgrowth (Niederost et al., 1999, Schmalfeldt et al., 2000). CSPGs in the extracellular matrix (ECM) contribute to the formation of a growth inhibitory glial scar around the injury site, which together with the aggregation of infiltrating immune and activated CNS glial cells form a barrier for regenerating axons (Morgenstern et al., 2002).
addition, sulfatide, a major lipid constituent of CNS myelin was recently identified as a novel myelin-associated inhibitor of neurite outgrowth (Winzeler et al., 2011).

Some axonal guidance cues that are inevitable during development for correct path finding of growing axons have been shown to be inhibitors of axonal regeneration when expressed by oligodendrocytes in adulthood. A well described example is EphrinB3, known for its developmental midline repellent function, which strongly inhibits axonal growth (Benson et al., 2005). Furthermore, EphA4, a receptor for several Ephrin ligands, has been shown to limit axonal regeneration in the adult CNS (Goldshmit et al., 2004, Goldshmit et al., 2011, Kempf et al., 2013, Joly et al., 2014). Semaphorin 4D is also present in myelinating oligodendrocytes and inhibits axon growth (Moreau-Fauvarque et al., 2003).

A cascade of molecular and cellular events is triggered by an injury in the CNS that leads to the formation of the glial scar. The infiltration of the CNS by peripheral inflammatory and immune cells (neutrophils, macrophages and eventually T cells) together with the activation of resident microglia cells will lead to the secretion of multiple factors (cytokines such as TNFα, IL-1, IL-8 or prostaglandins) that can support or hamper neurite growth (Jones et al., 2005). At the same time, both activated astrocytes and oligodendrocyte precursor cells start producing CSPGs and release them in the extracellular matrix (ECM), contributing to the formation of the glial scar around the injury site together with the aggregation of the above mentioned immune and CNS cells (Morgenstern et al., 2002). The increased expression of CSPGs contributes to the non-permissive nature of the glial scar.

The wide spectrum of myelin inhibitors and ECM molecules that restrict axonal growth raises questions about their physiological function. The onset of myelination correlates with the decline of neuronal growth in the maturing CNS (Kapfhammer and Schwab, 1994), suggesting myelin and myelin associated inhibitors might serve as a signal terminating the growth phase leading to the consolidation of neuronal circuits during CNS development (Schwab and Strittmatter, 2014).

1.2 The neurite growth inhibitory protein Nogo-A: description of Nogo signalling and possible pharmacological interventions

Nogo-A (RTN4A) is a 1200 amino acid (aa)-long transmembrane protein containing a C-terminal reticulon homology domain (RHD). Alternative splicing and alternative promoter usage of the Rtn4 gene give rise to two further proteins, Nogo-B (expressed in many tissue of the body) and Nogo-C (expressed mostly in muscles) (Chen et al., 2000, GrandPre et al., 2000, Prinjha et al., 2000). All three Nogo isoforms share the common C-terminus of 180aas, which consists of two hydrophobic, membrane anchored regions and a short (60-70aa-long)
hydrophilic region, known as the growth limiting Nogo-66 domain (Fournier et al., 2001). Nogo-A contains a long 800aa unique sequence that contains a second neurite growth inhibitory region, the Nogo-A-Δ20 domain (rat aa544-725) (Oertle et al., 2003).

Nogo-A is enriched in the membrane of the endoplasmatic reticulum (ER), where it has been shown to be involved in the formation and maintenance of the tubular ER morphology (Voeltz et al., 2006, Kiseleva et al., 2007, Jozsef et al., 2014). However, a functionally active fraction of Nogo-A is localized in the cell surface membrane of oligodendrocytes and neurons (Dodd et al., 2005).

The two highly active inhibitory domains of Nogo-A inducing growth cone collapse and neurite outgrowth inhibition, Nogo-66 and Nogo-A-Δ20, signal through separate receptors, the Nogo-66 receptor1 (NgR1) receptor complex (Fournier et al., 2001), and the sphingosine-1-phosphate receptor 2 (S1PR2) (Kempf et al., 2014), respectively (Figure 1). Both receptors activate the small GTPase RhoA and its effector, Rho-associated protein kinase (ROCK) (Schwab, 2010) (Figure 1A).

Nogo-66 binds to the glycoprotein receptor NgR1 (Nogo-66 receptor 1) (Fournier et al, 2001) and also directly interacts with the paired immunoglobulin-like receptor B (PirB) (Atwal et al., 2008). As NgR1 is a glycoposphatidylinositol (GPI)-anchored cell surface protein lacking an intracellular signaling domain, it interacts with p75NTR (low affinity neurotrophin receptor) (Wang et al., 2002a, Wong et al., 2002) or TROY (tumor necrosis factor - TNFα - receptor superfamily member 19) (Park et al., 2005, Shao et al., 2005) to transduce signals intracellularly. Both receptors are transmembrane proteins of the TNF receptor family, but as the expression of p75 is only detectable in subpopulations of mature neurons, TROY can act as a functional co-receptor of NgR1 in neurons lacking p75NTR. Another co-receptor, LINGO-1 (leucine rich repeat - LRR - and IgG domain containing Nogo receptor interacting protein 1) functions as an adaptor protein for Nogo-66 signaling through NgR1/p75 signaling complex (Mi et al., 2004).

Interestingly, the two other structurally unrelated myelin associated inhibitors, MAG and OMgp, share the same NgR1 receptor complex with Nogo-A (Domeniconi et al., 2002, Liu et al., 2002, Wang et al., 2002b), therefore share a common intracellular signaling pathway. The additional NgR family members, namely NgR2 and NgR3, have also been implicated in binding growth inhibitory ligands, MAG (Venkatesh et al., 2005) and CSPGs (Dickendesher et al., 2012, Baumer et al., 2014) with high affinity. NgR1 has also been described to interact with two secreted proteins, leucine-rich glioma inactivated (LGI1) and olfactomedin-1, which were proposed to antagonized Nogo-66 binding and myelin induced growth inhibition (Thomas et al., 2010, Nakaya et al., 2012).
Chapter 1: Introduction

Figure 1. Nogo-A signalling pathways and functional blockers. Nogo-A exerts its inhibitory function mainly through two domains, the Nogo-66 (red) and the Nogo-A-Δ20 region (green). Nogo-66 binds to the NgR1 receptor. As NgR1 is a GPI-anchored protein, it forms a complex with LINGO-1 and p75 or Troy, co-receptors that enable signal transduction. The NgR1 signalling complex activates Rho-A-GTPase which regulates cytoskeleton dynamics and mediates growth inhibitory action of Nogo-A. The Nogo-A-Δ20 domain binds to sphingosine 1-phosphate receptor 2 (S1PR2) activating G13 and LARG, which in turn also activates Rho-A. (A) Therefore, signalling from both Nogo-A inhibitory domains converges on Rho-A and its effector, Rho-associated protein kinase (ROCK), leading to F-actin depolymerization, microtubule disassembly and myosin II contractility. (B) When internalized, the Nogo-A-Δ20 fragment can form signalling endosomes and modulate growth related gene expression by decreasing CREB phosphorylation. (C) The Nogo-A-Δ20 fragment has also been shown to mediate growth cone collapse in a protein synthesis dependent way involving the mTOR pathway. Additionally, the Δ20 region has also been shown to indirectly inactivate integrin signalling. The inhibitors and blocking agents against different molecules involved in Nogo-A, MAG and OMgp and CSPG signalling are depicted on the figure in red: function blocking antibodies against Nogo-A (IN-1, 11C7, 7B12, human anti-human Nogo-A antibody (Novartis Pharma, AT355), humanized anti-Nogo-A antibody (GlaxoSmithKline, GSK1223249, Ozanezumab)); NgR1 blocker (NEP1-40); NgR1 or LINGO-1 decoy proteins (NgR1(310)ecto-Fc, LINGO-1-Fc); S1PR2 blocker (JTE-013); CSPG digestion (ChABC); pharmacological Rho or ROCK blockers (C3 transferase, Cethrin (BA-210), Y-27632, fasudil or KD025). (The figure is as seen in Translational Neuroscience, Chapter 25.)
Chapter 1: Introduction

Functional receptors for Nogo-A-Δ20 have long been searched for, and the mechanisms of how this N-terminal region of Nogo-A exerts its inhibitory function have been unclear till recently. One study demonstrated that Nogo-A-Δ20 inhibits the function of certain integrins and its action mechanism depends on the composition of the ECM (Hu and Strittmatter, 2008). An orphan G-protein coupled receptor, GPR50 has also been found to interact with Nogo-A-Δ20, although exerting opposite functional effects on neurite outgrowth (Grunewald et al., 2009). However, recently a high-affinity functional receptor has been discovered for Nogo-A-Δ20: the sphingosine-1-phosphate receptor 2 (S1PR2) (Kempf et al., 2014). S1PR2 shows high expression levels in the developing nervous system and interestingly, after activation by its well described natural ligand sphingosine-1-phosphate, the S1PR2 receptor signals through Rho-A (Pyne and Pyne, 2010). Nogo-A-Δ20 binding to S1PR2 triggers the activation of G protein G13, the Rho GEF LARG, and finally also Rho-A, the common operator leading to growth cone collapse and outgrowth inhibition (Figure 1A).

CNS neurons respond to Nogo-A and other myelin inhibitors with growth cone collapse and neurite outgrowth arrest, which suggests that the underlying intracellular mechanisms must involve altered dynamics of the cytoskeleton. Rho-A activation as a consequence of both NgR1 and S1PR2 signaling is one of the key signaling mechanisms for Nogo-A mediated growth inhibition (Alabed et al., 2006). GTPases, such as Rho-A, Rac1 and Cdc42 trigger downstream cytoskeletal rearrangements; actin depolymerization and actomyosin contraction for retraction in case of Rho-A and actin polymerization for growth in case of Rac1. The signaling cascade activated by Nogo-A binding does not only activate Rho-A, but also decrease the activity of Rac-1 (Niederost et al., 2002), putting a double break on actin cytoskeleton dynamics. Downstream signaling from Rho-A and ROCK involves the phosphorylation of cofilin by LIM kinase (LIMK) and activation of myosin light chain (MLC) II, leading to F-actin depolymerization and myosin II contractility, respectively (Nash et al., 2009). Besides affecting the actin cytoskeleton, myelin derived inhibitors also destabilize the microtubule assembly by ROCK dependent phosphorylation of the collapsing response mediator protein-2 (CRMP-2) (Mimura et al., 2006).

Nogo-A also initiates an intracellular Ca\textsuperscript{2+} rise mediating the collapse of neuronal growth cones (Bandtlow et al., 1993). Other signaling components of Nogo-A involve protein kinase C (PKC) (Sivasankaran et al., 2004) and epidermal growth factor receptor (EGFR) (Koprivica et al., 2005), pathways which may act in concert with RhoA. Subsequent to the local cytoskeleton reorganization Nogo-A evokes, Nogo-A-Δ20 has been shown to be internalized by a Pincher dependent mechanism and to be retrogradely transported to the neuronal cell bodies, leading to Rho-A activation and deactivation of the cAMP response.
element (CREB) (Joset et al., 2010) (Figure 1B). This pathway resembles the ones neurotrophic factors utilize to elicit growth and neuronal plasticity, opposite functions than Nogo-A-Δ20 (Shao et al., 2002). Along this line, it had been shown that up-regulation of cAMP and phospho-CREB by neurotrophic factor treatment helps neurons to overcome myelin growth inhibition (Cai et al., 2002, Gao et al., 2004). Interestingly, signaling from negative and positive growth regulators converge on the same transcription factor and this observation might be generalized for the regulation of other growth controllers, such as the mammalian target of rapamycin (mTOR), the synaptic expression of which was observed to be decreased by Nogo-A (Baldwin et al., 2011). Additionally, recently, Nogo-A-Δ20 but not Nogo-66 mediated growth cone collapse has been shown to be dependent on the mTOR pathway-activated protein translation (Manns et al., 2014) (Figure 1C).

Multiple ways have been developed to suppress Nogo-A/Nogo receptor interactions and the underlying signalling pathways (Gonzenbach and Schwab, 2008). Neutralizing Nogo-A either by function blocking antibodies (IN-1 (Schnell and Schwab, 1990), 11C7 and 7B12 (Liebscher et al., 2005), human anti-human Nogo-A antibody (Novartis, ATI355), humanized anti-Nogo-A antibody (GlaxoSmithKline, GSK1223249, Ozanezumab)) or by interfering with Nogo-A receptors (NEP1-40 (GrandPre et al., 2002), NgR(310)ecto-Fc (Li et al., 2004), Lingo-Fc (Ji et al., 2006), JTE-013) and underlying signalling pathways (pharmacological Rho or ROCK blocker: C3 transferase, Cethrin, Y-27632, fasudil, KD025) have been shown to reduce the inhibitory effects of CNS myelin on neurite growth, cell spreading and cell migration in vitro, and to boost regenerating, compensatory sprouting and functional recovery after CNS injury in vivo (Figure 1; red blocking arrows).

1.3 Physiological functions of Nogo-A

In the adult CNS, the bulk of Nogo-A protein is found in myelin (Huber et al., 2002) and its inhibitory function has been well described for axonal growth, plasticity and regeneration after CNS injury; reviewed in (Schwab, 2004, Pernet and Schwab, 2012). In oligodendrocytes, Nogo-A localizes mainly to the outer and innermost adaxonal myelin sheath (to the axon-oligodendrocyte contact zones) and to synaptic sites (Wang et al., 2002c). The developmental expression of Nogo-A in oligodendrocytes correlates with the course of myelination, and Nogo-A has been proposed to stabilize neuronal circuits at the end of CNS maturation (Schwab, 2010). By controlling spatial segregation and myelin extent Nogo-A was shown to be a key factor for precise myelination of the developing CNS (Pernet et al., 2008, Chong et al., 2012).
However, in addition to oligodendrocytes and myelin, Nogo-A is highly expressed in growing, immature neurons and also in subpopulations of mature neurons (Huber et al., 2002, Wang et al., 2002c). During development, Nogo-A is down-regulated in immature neurons at the time of synaptogenesis (Aloy et al., 2006, Petrinovic et al., 2013), however in plastic brain regions such as the hippocampus, Nogo-A remains expressed and is located at synapses (Wang et al., 2002c) where it was shown to negatively regulate synaptic plasticity (Mironova and Giger, 2013). Several studies investigated further potential neuronal Nogo-A functions. During development, neuronal Nogo-A influenced neuronal migration (Mingorance et al., 2004, Mathis et al., 2010), survival (Mi et al., 2012, Guo et al., 2013), cell spreading and neurite growth (Oertle et al., 2003, Petrinovic et al., 2010).

Recently a new concept emerged regarding the physiological role of myelin associated inhibitors. A number of repulsive axon guidance cues and myelin inhibitory molecules have been shown to also play a role in circuitry stabilization, synapse formation, function and plasticity (Shen and Cowan, 2010, Mironova and Giger, 2013). The expression and localization of Nogo-A first in neurons then in glial cells, in close proximity to synaptic contacts (Wang et al., 2002c) suggests a developmental function in synaptogenesis and later in synaptic transmission. Oligodendroglial Nogo-A has been shown to restrict the plasticity of the ocular dominance columns in the visual cortex by an NgR and PirB - Nogo-A receptor-dependent manner (McGee et al., 2005, Syken et al., 2006), possibly contributing to ending the so-called “critical-period”. In the hippocampus, pre and post-synaptically expressed Nogo-A and NgR1 has been shown to regulate synaptic plasticity (Lee et al., 2008, Delekate et al., 2011). Antibody-mediated neutralization of Nogo-A or NgR1 induces sprouting of CA1/3 dendritic arbors and CA3 axons (Zagrebelsky et al., 2010), and increases long-term potentiation (LTP) on acute hippocampal slices (Delekate et al., 2011). Accordingly, Nogo-66 or OMgp application attenuated LTP (Raiker et al., 2010). Although genetic deletion of Nogo-A, NgR1 or PirB have not lead to substantial increase in LTP (Karlen et al., 2009, Raiker et al., 2010) probably due to compensatory mechanisms in the mutant mice, NgR1 has been shown to limit the number of excitatory synapses during brain development (Wills et al., 2012). In the hippocampus and motor cortex of Nogo-A down-regulating transgenic rats increased LTP was observed (Tews et al., 2013). Acute treatment of brain slices with function-blocking antibodies against Nogo-A or against NgR1 increased LTP in the intact motor cortex, whereas in vivo, intrathecal application of Nogo-A-blocking antibodies resulted in a higher dendritic spine density and improved motor learning of skilled forelimb-reaching tasks (Zemmar et al., 2014). Likewise, NgR1 knock-out animals exhibited increased plasticity in the somatosensory cortex (Akbik et al., 2013).
These studies suggest that although the observed morphological and electrophysiological changes are more likely to rely on altered receptor/ligand interactions on the cells surface, cell autonomous effects of Nogo-A cannot be completely excluded. After injury, the effects of neuronal Nogo-A are not yet well understood. The presence of Nogo-A in plastic brain regions and its up-regulation in neurons after CNS injuries (Cheatwood et al., 2008, Pernet et al., 2011) suggest that neuronal Nogo-A possesses a distinct, potentially positive role for plasticity.

1.4 Nogo-A in CNS diseases

Interestingly, the expression of Nogo-A is altered in multiple neurodegenerative diseases of the CNS, changes that have been implicated in influencing the disease progression. In amyotrophic lateral sclerosis (ALS), also referred to as motor neuron disease (MND), Nogo-A is up-regulated in spinal cord motoneurons and muscles, suggesting its possible involvement in the pathophysiology of the disease (Dupuis et al., 2002, Jokic et al., 2006, Pradat et al., 2007). In patients with multiple sclerosis (MS), soluble Nogo-A fragments are found in the cerebrospinal fluid (CSF) that may correlate with the disease progression (Reindl et al., 2003, Jurewicz et al., 2007). Both in motoneuron (ALS) and myelin (MS) diseases therefore Nogo-A might serve as a marker for diagnosis. Furthermore, the findings that NgR1 might bind the amyloid precursor protein APP suggests the involvement of Nogo-A in Alzheimer's disease (Park et al., 2006, Zhou et al., 2011), but further data are required in order to understand the possible roles Nogo-A and NgR1 might play in APP and β-amyloid (Aβ) processing.

2 Regeneration and plasticity after CNS injury

Depending on the lesion size and localization, the consequences of CNS injuries are complex. Patients and experimental animals with large lesions have to cope with permanent and severe functional deficits. Smaller lesions of the spinal cord or the motor cortex have a relatively good prognosis due to adaptive changes in the neuronal circuitries of the spinal cord and brain allowing spontaneous functional improvements (Bareyre et al., 2004, Starkey and Schwab, 2014). In animal models, injured and spared fibers can sprout and grow collaterals, initiating the remodelling of intraspinal circuits e.g. by forming ‘detour’ pathways (Raineteau and Schwab, 2001, Bareyre et al., 2004). The distances covered by sprouting fibers are short in general, and the growth response of the neurons is down-
regulated within a few days, possibly because of the presence of growth inhibitory factors like Nogo-A, CSPGs or scar-associated molecules (Schwab and Bartholdi, 1996).

Two key questions and aims in the field of CNS repair are therefore to enhance the growth capacity of the neurons, and overcome the inhibitory properties of the CNS environment to allow regeneration of fibers over long distances and around large spinal cord or brain lesion sites.

2.1 Stimulation of regeneration by up-regulating intrinsic neuronal growth mechanisms

Immature CNS neurons in early developmental stages possess robust axon growth and regenerative ability, which is down regulated at adult stages in most types of CNS neurons, possibly through a decrease in trophic support and the influence of inhibition of growth (Tuszynski and Gage, 1995, Blesch et al., 2012, Schwab and Strittmatter, 2014). One way neurite growth and regeneration can be stimulated is the up-regulation of the intrinsic growth machinery of neurons, in particular via stimulating the mTOR (mammalian target of rapamycin) and STAT3 (signal transducer and activator of transcription 3) pathways (Liu et al., 2011). Eliminating the gene encoding for the tumour suppressor phosphatase and tensin homolog (PTEN) promoted robust extension of retinal ganglion cell axons and injured corticospinal tract fibers, an effect that was dependent on the mTOR pathway (Park et al., 2008, Liu et al., 2010). Although the manipulation of these intracellular growth regulators induces powerful long-distance axonal regeneration in the optic nerve and to a lesser extent in the spinal cord after injury, only few reports on the functional consequences of this neurite growth stimulation exist so far (de Lima et al., 2012b). However, excessive stimulation of mTOR can lead to epileptic seizures and tumour formation, suggesting that over-stimulated neurite growth may lead to the formation aberrant neuronal connections, or that the growth machinery might get out of control and this would results in the over-proliferation of glial cells (Akhavan et al., 2010, Pun et al., 2012). The down-regulation or the deletion of the growth repressor Krüppel-like factor 4 (KLF4), a zinc-finger transcription factor, also increased the number and length of regenerating retinal ganglion cell axons after optic nerve crush (Moore et al., 2009). Up-regulation of growth-activating neurotrophic factors such as ciliary neurotrophic factor (CNTF) leads to the activation of the JAK/STAT3 pathway and thereby to increased axonal regeneration (Leaver et al., 2006, Muller et al., 2007, Pernet et al., 2013a). Up-regulation of STAT3 or down-regulation of suppressor of cytokine signalling 3 (SOCS3), the negative feedback controller of this pathway, both elicited
increased axonal regeneration in the lesioned rodent optic nerve (Smith et al., 2009, Pernet et al., 2013b).

Two extracellular growth inhibitory factors, MAG and Nogo-A, were recently shown to negatively affect the mTOR pathway suggesting that the final growth response is a result of interconnected intrinsic and extrinsic signalling pathways (Figure 1C) (Peng et al., 2011, Manns et al., 2014).

An important consideration for stimulated axonal growth in the injured adult CNS is that these fibers have to find functionally meaningful targets where they should form synapses, avoiding synapse formation on targets that would lead to malfunctions. Thus, in addition to a growth permissive extracellular environment, guidance and positional cues as well as target recognition signals should be provided to the regenerating axons. At present, however, almost no information is available in the literature on these mechanisms in adult, injured model systems.

2.2 Nogo-A neutralization improves regeneration and promotes plasticity in animal models of spinal cord injury

After blockade of the Nogo-A pathways, two types of anatomical repair phenomena were observed: enhanced axonal sprouting and increased regeneration (Figure 2B). Regenerative sprouting can frequently be observed after fiber tract lesions, as collateral sprouting close to the lesion site or from the injury site directly. If these fibers elongate over longer distances (beyond 1 mm) they are considered as regenerating axons (regenerative growth). In the injured spinal cord such axons can grow around the lesion and scar area and extend beyond the lesion site towards potential targets in the lower spinal cord. Fiber numbers often drop off at >2-5mm, but regenerating axons covering >10 mm could also be observed. Corticospinal tract (CST) and serotonergic fibers were studied most extensively. Additionally to injured axons, the non-injured, spared fibers can respond to the injury with sprouting, a phenomenon often called compensatory sprouting (Figure 2B). In experimental stroke, recovery is predominantly mediated by compensatory collateral sprouting of the uninjured tracts.

Depending on the type of CNS injury, complete or incomplete, long distance regeneration or local sprouting could be more relevant for the functional recovery.
Figure 2. Anatomical and behavioral aspects of Nogo-A signalling inhibition in spinal cord injury. (A) Scheme of a rat spinal cord cross-section depicting localization of the dorsal, dorsolateral and ventral corticospinal tracts and a T-shaped transection (grey shaded area) which interrupts all of those tracts and is a widely used model in experimental spinal cord injury research. (B) Scheme illustrating axonal regeneration versus sprouting after spinal cord injury: regenerating axons originate from the cut end of injured axons (red); however the shafts of damaged axons and pre-existing, non-injured, spared fibers sprout also in response to the injury (green). (C) After spinal cord injury, the irregular horizontal ladder test is widely used for the assessment of locomotion and fine motor control in rats. (D) Camera lucida reconstruction of the spinal hemicord with regenerating corticospinal fibers rostral and caudal to the lesion site (light area) in control IgG and anti-Nogo-A antibody (mAb 11C7 and mAb 7B12) treated rats. In the anti-Nogo-A antibody treated rats corticospinal fibers regenerated over ventrolateral tissue bridges into the caudal spinal cord. This regenerative growth was absent in the control antibody treated animals. Reprinted with permission from Ann Neurol. (Liebscher et al., 2005). (The figure is as seen in Translational Neuroscience, Chapter 25.)
2.3 Suppression of the Nogo-A pathway in animal models of spinal cord injury

2.3.1 Nogo-A neutralization by antibodies

As reviewed in Table 1, multiple in vivo studies were carried out in spinal cord injury (SCI) animal models to explore the future possibility of clinical studies with Nogo-A/NgR1 receptor blocking agents.

In the early 1990s, Schnell and Schwab (Schnell and Schwab, 1990) reported that the delivery of the monoclonal antibody called IN-1, an IgM recognizing and neutralizing Nogo-A, through implantation of antibody producing hybridoma cells in the brain increased the regeneration of cut axons past the lesion site in an incomplete rat SCI model. This experiment with IN-1 treatment has been repeated in multiple species (rat, marmoset monkey) and lesion paradigms, and demonstrated increased functional recovery as assessed by various locomotor tests (Basso, Beattie and Bresnahan (BBB), narrow beam, horizontal ladder (Figure 2C), etc.) (Bregman et al., 1995, Thallmair et al., 1998, Brosamle et al., 2000, Merkler et al., 2001, Raineteau et al., 2001, Raineteau et al., 2002, Fouad et al., 2004). Highly purified new monoclonal antibodies directed against the Nogo-A specific domains of the rat protein (11C7 and 7B12 antibodies) allowed repeating these experiments in clinically more relevant settings. These antibodies were delivered intrathecally via an osmotic minipump for two weeks, starting immediately after the injury. After mid-thoracic T-lesion (Figure 2A), anti-Nogo-A antibody treated animals had a much higher number of regenerating CST and serotonergic fibers (Figure 2D) and consistently showed substantial functional recovery associated to this regenerative improvement in rats and macaque monkeys (Liebscher et al., 2005, Freund et al., 2006, Freund et al., 2007, Mullner et al., 2008, Freund et al., 2009).

As most of these in vivo studies were performed with acute antibody delivery (starting at the time of the SCI), delayed anti-Nogo-A antibody treatment regimens were also tested. These experiments showed that delaying the antibody delivery in rats by one week was still efficient in increasing CST axon regeneration and hindlimb recovery, whereas a delay of two weeks until treatment start led to poorer outcomes (Gonzenbach et al., 2010, Gonzenbach et al., 2012). Combination of Nogo-A neutralization with several pharmacological treatments led to even more enhanced anatomical reorganization and functional improvements. The effects of anti-Nogo antibody treatment were strengthened by simultaneous neurotrophin-3 (NT-3) and NMDA-receptor 2d (NR2d) subunit delivery, which promotes neuronal growth capacity, survival and synaptic plasticity, respectively (Schnell et al., 1994, Schnell et al., 2011). CSPGs and peri-neural net proteins exert additional inhibition on axonal growth.
Combined treatments reducing CSPGs by chondroitinase ABC (ChABC) and applying anti-Nogo-A antibodies were found to be beneficial for CST sprouting, regeneration and for the recovery of forelimb functions after high cervical contusion lesions (Zhao et al., 2013).

Anti-Nogo-A antibodies bind to cell membrane Nogo-A and may lead to internalization of the protein-antibody complex eventually leading to down-regulation of Nogo-A tissue levels (Weinmann et al., 2006). Additionally, due to the large size of the antibodies, steric blockade of the Nogo-A-Nogo receptor interaction may be a mechanism for Nogo-A action neutralization.

### 2.3.2 Blockade of Nogo-A receptors and signalling

Another approach to block Nogo-A function is to block the activation of the NgR1 receptor complex with a function-blocking peptide (NEP1-40) or soluble NgR1-Fc proteins. One advantage of this approach is the possible simultaneous blockade of several myelin associated inhibitory NgR1 ligands, such as Nogo-A, MAG and OMgp, eventually even CSPGs (Dickendesher et al., 2012). Both acute and delayed intrathecal, subcutaneous or intraperitoneal delivery of NEP1-40 was reported to increase the regeneration of both corticospinal and raphespinal fibers and to lead to increased BBB scores and grid-walk performance (GrandPre et al., 2002, Li and Strittmatter, 2003, Steward et al., 2008). Experiments applying the NgR1(310)ecto-Fc (soluble function-blocking NgR ectodomain) intrathecally or intracerebroventricularly led to a similar increase of regeneration and sprouting of corticospinal and serotoninergic fibers together with increased locomotor recovery (Li et al., 2004, Wang et al., 2011, Wang et al., 2014). The studies by Wang et al. in 2011 and 2014 were performed with spinal cord contused rats, applying NgR1(310)ecto-Fc treatment in a close-to-reality SCI model. Triple therapy combining NgR1(310)ecto-Fc, ChABC for degrading CSPGs and peripheral nerve preconditioning increased the intrinsic growth potential of dorsal root ganglion neurons and allowed axons to regenerate millimetres past the spinal cord injury site (Wang et al., 2012b).

Although only shown by a single study, blocking LINGO-1, a co-receptor of NgR1, by soluble LINGO-1-Fc fragments also resulted in elevated rubrospinal and corticospinal fiber sprouting and led to improved functional recovery in the cylinder test and in BBB scores (Ji et al., 2006).

Multiple studies showed that inhibition of Rho-A/ROCK signalling leads to increased axonal regeneration and improved functional recovery. The C3 transferase or Y-27632 (Dergham et al., 2002), Cethrin (BA-210) (Lord-Fontaine et al., 2008, McKerracher and
Guertin, 2013), or fasudil (Sung et al., 2003) treatments led to similar enhancement of anatomical reorganization and behavioral improvements.

### Table 1. Key in vivo studies on Nogo-A signalling inhibition in spinal cord injury

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Study</th>
<th>Species age</th>
<th>SCI model</th>
<th>Route of administration</th>
<th>Anatomical readout and effect</th>
<th>Functional readout and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-Nogo-A mAb IN-1</td>
<td>(Schnell and Schwab, 1990)</td>
<td>Rats, 14-47 days old Lewis</td>
<td>Half to two-third dorsal, T5-7, complete transection</td>
<td>Hybridoma cells secreting IN-1 Ab into the dorsal frontoparietal cortex, unilaterally, 7-10 days before the SCI.</td>
<td>2-3 wks after SCI, labelling of the CST with wheat germ agglutinin-HRP. Increased sprouting and regeneration up to 7-11 mm past the lesion site.</td>
<td>N.A.</td>
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<tr>
<td>Anti-Nogo-A mAb IN-1</td>
<td>(Bregman et al., 1995)</td>
<td>Rats, 6-8 wks Lewis (150-200g)</td>
<td>Overhemisection (right), T6</td>
<td>Hybridoma cells secreting IN-1 Ab into the CSF at the time of the SCI.</td>
<td>Up to 9 wks post SCI, increased CST (up to 7-11 mm, BDA tr.) serotoninergic and noradrenergic fiber growth (immunostaining).</td>
<td>After 4-6 wks recovery, 80% of IN-1 treated rats recovered contact placing responses, and stride length (foot print analysis).</td>
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<tr>
<td>Anti-Nogo-A mAb IN-1</td>
<td>(Thallmaier et al., 1998)</td>
<td>Rats, 2-3 months Lewis</td>
<td>Unilateral pyramidotomy</td>
<td>Hybridoma cells secreting IN-1 Ab into the cortex contralateral to the lesion at the time of the pyramidotomy.</td>
<td>2 wks post-lesion, increased sprouting of intact contralateral CST fibers in different brainstem nuclei and in the SC (BDA tracing).</td>
<td>Close to full recovery (at 42 days) in skilled forelimb (single pellet reaching task), further motor (rope climbing) and sensory tests (sticky paper).</td>
</tr>
<tr>
<td>rIN-1 Fab (humanized)</td>
<td>(Brosamle et al., 2000)</td>
<td>Rat, 6-8 wks female Lewis</td>
<td>Dorsolateral hemisection, T8</td>
<td>Intrathecal rIN-1 Fab delivery by osmotic minipump for 2wks at the time/site of SCI</td>
<td>rIN-1 Fab treatment promoted long-distance CST axonal re-growth within 2wks (BDA tracing).</td>
<td>N.A.</td>
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<tr>
<td>Anti-Nogo-A mAb IN-1</td>
<td>(Merker et al., 2001)</td>
<td>Rats, Lewis (200-250g)</td>
<td>Dorsal overhemisection, T8</td>
<td>Hybridoma cells secreting IN-1 Ab into the hippocampus unilaterally, at the time of the SCI.</td>
<td>N.A. Lesion sizes are comparable between treatment groups.</td>
<td>5 wks after the injury increased performance in locomotor tests: open field locomotor score, grid walk, misstep withdrawal response, narrow-beam crossing.</td>
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<tr>
<td>Anti-Nogo-A mAb IN-1</td>
<td>(Raineteau et al., 2001, Raineteau et al., 2002)</td>
<td>Rats, adult Lewis (175-275g)</td>
<td>Bilateral pyramidotomy</td>
<td>Hybridoma cells secreting IN-1 Ab into the left hippocampus, at the time of the SCI.</td>
<td>2-4wks past injury rubrospinal tract (RST) tracing from red nucleus and CST tracing from cortex by BDA. 2-fold increase in the number of collaterals emerging from the RST.</td>
<td>1-4wks past injury precision movements of the forelimb (single pellet reaching task) almost completely recovered. Intracortical or red nucleus microstimulation induced forelimb response and confirmed the reorganization of the RST system, respectively.</td>
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<td>NEP1-40 (Nogo-66 antagonistic peptide)</td>
<td>(GrandPre et al., 2002)</td>
<td>Rats, female Sprague-Dawley (175-250g)</td>
<td>Dorsal hemisection, T6-7</td>
<td>Intrathecal NEP1-40 delivery by osmotic minipump for 4wks at the time/site of SCI</td>
<td>4 wks after SCI both corticospinal (BDA tracing) and raphespinal axon (immunostaining) regeneration is promoted.</td>
<td>Significantly higher BBB scored in the NEP1-40 treated group 14-28 days after SCI.</td>
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<td>NEP1-40 (Nogo-66 antagonistic peptide)</td>
<td>(Li and Strittmatter, 2003)</td>
<td>Mouse, 8-10 wks C57BL/6</td>
<td>Dorsal overhemisection, T6-7</td>
<td>Subcutaneous NEP1-40 treatment at the time. 4 hours or 7 days after SCI.</td>
<td>3-4 wks past SCI dCST sprouting (BDA tracing) significantly increased both in acute and delayed NEP1-40</td>
<td>BBB score increase at 17 or 28 days post SCI (acute or delayed respectively). Additional foot-print and grid-walk.</td>
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<tr>
<td><strong>Anti-Nogo-A mAb IN-1</strong></td>
<td>Rats, female Sprague-Dawley (190–250g)</td>
<td>Dorsal over-hemisection, T6-7</td>
<td>Intra-thecal delivery by osmotic minipump for 4wks.</td>
<td>Increased axonal sprouting of dCST (BDA tracing) and raphespinal fibers (immunostaining). Increased occurrence of BDA or 5-HT–synaptophysin double-labeled varicosities. Improved spinal cord electrical conduction and BBB scores at 14 and 28 days past SCI. A single foot-print and grid-walk improvement.</td>
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<td><strong>Anti-Nogo-A mAb 11C7 and mAb 7B12</strong></td>
<td>Marmoset monkeys of either sex (280–440g)</td>
<td>Hemisection, T8</td>
<td>Hyrbridoma cells secreting IN-1 Ab into the hippocampus unilaterally, at the time of the SCI. 2wks post lesion, in four out of five mAb IN-1 treated animals fine labeled neurites had grown into, through and around the lesion site.</td>
<td>NA.</td>
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<td><strong>Anti-Nogo-A mAb 7B12 and mAb h-Nogo-A</strong></td>
<td>Macaque monkeys of either sex</td>
<td>Dorsolateral transsection, C7-8</td>
<td>Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 4wks at the time/site of SCI.</td>
<td>Increased axonal sprouting of CST (BDA tracing) and raphespinal fibers (immunostaining). Increased occurrence of BDA or 5-HT–synaptophysin double-labeled varicosities. Improved spinal cord electrical conduction and BBB scores at 14 and 28 days past SCI. A single foot-print and grid-walk improvement.</td>
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<td><strong>NEP1-40</strong></td>
<td>Mouse, 8–10 wks C57BL/6</td>
<td>Dorsal over-hemisection, T6-7</td>
<td>Intraperitonal NEP1-40 treatment 5 hours or 7 days after SCI. 46days post SCI no significant differences in the density of CST axon arbors or in the density of serotonergic axons caudal to the injury.</td>
<td>Increased BBB scores at 7-14 days, but not during further assessments or by foot print and grid walk analysis.</td>
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<td><strong>Anti-Nogo-A mAb 11C7 and mAb 7B12</strong></td>
<td>Rats, female Sprague-Dawley (200–250g)</td>
<td>Dorsolateral transsection, C7-8</td>
<td>Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 4wks at the time/site of SCI.</td>
<td>Both trained and non-trained 11C7 treated groups displayed increased CST fiber number caudal to the lesion (BDA tracing) and higher 5-HT fiber density in lamina VII. Tested by bipedal stepping, 9wks post SCI, non-trained 11C7 treated rats demonstrated consistent step cycles, low paw drags and improved coordination. Parallel Ab treatment and treadmill training.</td>
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<td>Anti-Nogo-A mAb 11C7 and mAb 7B12</td>
<td>(Gonzenbach et al., 2010) Rats, female Lewis (180-300g) T8</td>
<td>Intraocular anti-Nogo-A Ab delivery by osmotic minipump for 2wks at the time of treatment groups.</td>
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<td>Half dorsal transaction (T-lesion), 8</td>
<td>N.A. Lesion sizes were compared between treatment groups.</td>
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<td>As locomotor training, anti-Nogo-A antibody treatment had a preventive effect on the development of muscle spasms.</td>
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<td>NGR-Fc decoy protein (AA-Ngr(310)ecto-Fc)</td>
<td>(Wang et al., 2011) Rats, adult female Sprague-Dawley (250-270g) T1</td>
<td>Intracerebroventricular infusion from SCI, ChABC + mAb + Fc therapy</td>
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<td>Moderate contusion injury, T7 (10g, 25mm) T9</td>
<td>Induced raphespinal axons (5-10x) immunostaining.</td>
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<td>Recovery of weight bearing and BBB scores in chronic conditions - 90-180 days post contusion SCI.</td>
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<td>Anti-Nogo mAb (11C7) + Neurtrophin-3</td>
<td>(Schnell et al., 2011) Rats, adult female Sprague-Dawley</td>
<td>Intrathecal 11C7 (2w) application, NT-3 producing fibroblasts on the lesion.</td>
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<td>(NT-3) + NMDA-receptor 2d (NR2d)</td>
<td>Hemisection, T10</td>
<td>Increased numbers midline-crossing fibers below the lesion in the Nogo-A + NT-3 + NR2d group.</td>
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<td>subunit</td>
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<td>Probably polysynaptic responses recorded slightly better motor function in the absence of adverse effects (e.g. pain).</td>
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<td>Anti-Nogo-A mAb 11C7</td>
<td>(Gonzenbacher et al., 2012) Rats, 8-10 wks, female Lewis (180-200g) T9</td>
<td>Intrathecal anti-Nogo-A Ab delivery by osmotic minipump for 2wks at the time of treatment groups.</td>
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<td>Half dorsal transaction (T-lesion), 8</td>
<td>10wks post SCI higher numbers of labeled CST fibers (BDA staining) in the acute and 1wk delayed treatment groups, but not in the 2wk delayed Ab application.</td>
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<td>Increased BBB scores, swimming and narrow beam recovery in acute and 1wk treatment group, but not in the 2wk-delayed group. Reduction of muscle spasms.</td>
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<tr>
<td>NGR1(310)ecto-Fc decoy protein +</td>
<td>(Wang et al., 2012b) Rats, 11-12wks female Sprague-Dawley (250-270g) T7</td>
<td>Intrathecal NGR1(310)ecto-Fc (for 28days from SCI), intracerebroventricularly ChABC (6x), with or without sciatric nerve injury (7days pre or 3days post SCI).</td>
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<tr>
<td>chondroitinase ABC (ChABC) +</td>
<td>Dorsal crush SCI, T7</td>
<td>5wks after SCI, triple therapy combining NGR1 decoy, ChABC and preconditioning, allows sensory afferent axons to regenerate mm's past the SCI site (CTB retrograde tracing in the sciatric nerve).</td>
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<tr>
<td>preconditioning peripheral sciatic</td>
<td></td>
<td>Increased forelimb function in skilled paw reaching task (Montoya staircase) in the combinational therapy group.</td>
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<tr>
<td>nerve axotomy</td>
<td></td>
<td>N.A.</td>
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<tr>
<td>Anti-Nogo-A mAb 11C7 +</td>
<td>(Zhao et al., 2013) Rats, adult male Lister hooded (150-200g) C4</td>
<td>Acutely intrathecally 11C7 (2wks), delayed ChABC injection and infusion from 3wks (10days), training from 4wks.</td>
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<tr>
<td>chondroitinase ABC (ChABC)</td>
<td>Dorsal column lesion, C4</td>
<td>11C7: &gt;3 µm diameter axons, ChABC: finer axons with varicosities, combinational treatment: highest sprouting and regeneration (BDA tracing).</td>
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<td></td>
<td></td>
<td>Increased forelimb function in skilled paw reaching task (Montoya staircase) in the combinational therapy group.</td>
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<tr>
<td>Human</td>
<td>(Wang et al., 2014) Rats, 10-11wks female Sprague-Dawley (220-240g) T7</td>
<td>Intracerebroventricular infusion (continuous) or bolus delivery to the lumbar intrathecal space (once every 4 days).</td>
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<tr>
<td>NGR1(310)-Fc</td>
<td>Moderate contusion injury, T7 (10g, 25mm) T9</td>
<td>8wks post injury, increased the fiber density of raphespinal axons caudal to the SCI (5-HT immunostaining).</td>
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<td></td>
<td></td>
<td>Lumbar bolus dosing schedule promoted locomotor recovery from SCI as effective as continuous infusion in open field (increased BBB scores) and grid walking tasks.</td>
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</table>

Abbreviations: BBB Basso, Beattie and Bresnahan locomotor activity test; BDA tr., biotinylated dextran amine tracing; CST, corticospinal tract; ChABC, chondroitinase ABC; CTB, cholera toxin-β-subunit; mAb, monoclonal antibody; N.A. not assessed; SCI, spinal cord injury, wk week
2.3.3 Preparing translation: preclinical studies for Nogo-A blocking agents

The significant number of studies with Nogo-A/NgR1 signalling suppression paved the path to clinical studies that are currently being carried out or planned in CNS injured, MS and ALS patients (Hug and Weidner, 2012).

Pre-clinical spinal cord and cortical lesion studies with anti-Nogo-A antibodies were conducted both on rodents and on primates. As the motor system organization, the tissue reaction in response to injury and the behaviour of non-human primates resemble the human situation, these studies were an important step for the clinical translation (Courtine et al., 2007). The efficacy of the anti-Nogo-A antibody treatment was confirmed by studies on macaque monkeys and showed substantial recovery of hand dexterity as well as sprouting and regeneration of CST axons without signs of pain or other side effects (Freund et al., 2006, 2009). Toxicological studies with the clinical, human anti-Nogo-A antibody were also carried out on two species, rats and primates.

The severity, location and extent of spinal cord injury or stroke define the possible treatment options and their efficacy. In complete spinal cord injured patients, for example, in addition to growth promoting interventions, bridges would be required to allow axotomized fibers to cross the lesion site (Bunge, 2001, Tetzlaff et al., 2011). In cases of anatomically incomplete lesions, regenerating fibers are able to bypass the injury site when supported by tissue bridges and pharmacological treatments, e.g. anti-Nogo-A, CSPG degradation or stimulation of intrinsic neuronal growth mechanisms. The damage after spinal cord injury in humans often occurs in the ventral spinal cord frequently affecting more than one spinal segment, whereas in the experimental models the spinal cord is lesioned by a dorsal approach with a well-defined transection or small contusion. Studies with clinically more relevant lesions such as large compressions and contusions would be valuable for direct comparison with clinical cases (Filli and Schwab, 2012). A recent study in spinal cord contused rats treated with humanized NgR1(310)-Fc showed that regeneration and locomotor recovery was promoted (Wang et al., 2014). Optimal treatments may need to be defined depending on whether long distance regeneration or local sprouting of injured or intact compensatory fibers are more desirable in a lesion with given location and extent.

For many of the animal experiments with Nogo pathway blockers acute treatment regimens were applied. Delayed delivery of both Nogo-A and NgR1 neutralizing agents were tested in rat models of spinal cord injury; the efficiency of the treatments was good with treatment start at one week after injury, but declined at two weeks after injury (Gonzenbach et al., 2012, Wang et al., 2014). Interestingly, in stroke models delayed treatment was effective even when started nine weeks after the infarct (Lee et al., 2004, Tsai...
et al., 2011). For combined pharmacological and rehabilitative training therapy the timing and the optimal time windows need to be taken into account. Parallel intensive treadmill training together with anti-Nogo-A antibody treatment in the first two weeks after a spinal cord lesion resulted in very poor functional recovery despite of a strong regenerative fiber response (Maier et al., 2009). A similar result was seen after stroke. However, a sequential treatment, first with the antibody followed in time by rehabilitative training was strongly beneficial (Wahl et al., 2014). It is probable that the injured CNS goes through an initial phase of sprouting and plasticity which can be enhanced by Nogo-A neutralization. Subsequently, the newly formed connections have to be stabilized and functionally meaningless connections have to be pruned by activity-dependent processes which can be enhanced by intense rehabilitative training.

An important point concerns the best route of application of a drug. The blood-brain-barrier prevents or severely restricts access of many compounds, including antibodies, to the intact CNS (Pardridge, 2002). At lesion or inflammatory sites, however, the blood-brain-barrier is open temporarily, allowing antibodies to penetrate into the surrounding CNS tissue. In most of the spinal cord injury and stroke preclinical experiments, a direct intrathecal way of application of antibodies, peptides or fusion proteins was chosen. By lumbar subdural catheter infusion with osmotic minipumps, high levels of drugs can be reached over 2-4 wks in the spinal cord. Distributed by the CSF circulation, antibodies can also reach the brain in rats and monkeys (Weinmann et al., 2006).

Translating in vivo experiments to clinical studies also faces the challenge of comparable and relevant functional outcome measures. For rats and higher vertebrates, a detailed kinematic gait analysis, which is modelled after the human analysis techniques, (Zorner et al., 2010) should replace the current simple, widely used, but subjective and non-linear locomotor scores (Basso et al., 1995). Objective and quantitative assessments for hand function, balance or bladder function are being developed for experimental animals. Clinically, neurophysiological assessments (motor and somatosensory evoked potentials, fMRI, TMS) also play important roles; many of these testing methods have been successfully used in animals too (Curt et al., 2004). Additionally, the standardization of diagnosis and functional assessment protocols is of high importance. Clinical data are collected in databases (EMSCI, European Multicentre Study about Spinal Cord Injury; NACTN, North American Clinical Trial Network) and serve as valuable data bases for the design and as historical controls for future clinical studies.

The deficits of the bladder, bowel, and sexual function, and complications like chronic pain or spasticity have a strong impact on the life quality of spinal cord injured patients. These functions have not been extensively addressed in the preclinical studies. Anti-Nogo-A
antibody-treated rats with partial spinal cord transections regained autonomous bladder function seven to nine days earlier than control antibody-administered rats. Furthermore, in these animals the pain threshold was not altered (Liebscher et al., 2005), and the occurrence and severity of spastic cramps were decreased (Gonzenbach et al., 2010, Gonzenbach et al., 2012), suggesting that the treatment led to the stabilization of functionally correct circuits for these functions. Importantly, none of the studies with Nogo-A suppressing treatments reported pain, increased spasticity, discomfort or behavioural disturbances associated to the therapy (Gonzenbach and Schwab, 2008).

In the long run and for the repair of very large lesions, multiple approaches are going to be required in order to optimize regeneration and functional recovery. Alongside treatments to overcome growth inhibition by myelin and the glial scar, well balanced and timed stimulation of the neuronal growth program and implantation of artificial or cellular bridges could be used. These pharmacological treatments then should be coordinated with rehabilitative training to support the plastic changes in the CNS leading to functional recovery.

2.3.4 Interventions blocking Nogo-A signalling in clinical trials for spinal cord injury

The first in-human anti-Nogo-A antibody phase I clinical trial was conducted with 52 acutely spinal cord injured patients suffering of severe para- and tetraplegia in several centres in Europe and Canada and was completed in September 2011 (Abel R et al., 2011) (http://clinicaltrials.gov/ct2/show/NCT00406016). This study assessed the technical feasibility, safety and pharmacokinetics of administering the fully human anti-Nogo-A human antibody ATI355 (Novartis Pharma) intrathecally to patients with acute spinal cord injury and confirmed AIS-A and B clinical classification. The antibody administration started at 4-28 days after the injury by either continuous intrathecal infusion for up to 28 day or in 6 intrathecal bolus injections over 4 weeks. Infusions and injections were made into the lumbar liquor space. The ATI355 antibody was very well tolerated and no serious adverse events were reported. The bolus application had increased safety in comparison with catheter-mediated infusions from external pumps. A multinational, multicentre phase II placebo-controlled clinical trial is currently in preparation to assess the efficacy of the treatment. This trial, which will be conducted in severe incomplete spinal cord injured patents, requires optimized early diagnosis and sensitive, well standardized outcome measures in all participating centres.
2.4 Regeneration after genetic deletion of Nogo-A and NgR1

Besides antibodies and pharmacological blockers aiming at Nogo-A signaling inhibition, multiple genetically modified mouse lines lacking Nogo or its receptors have been generated to study axonal regeneration in vivo (Table 2).

In 2003, three independent laboratories generated different Nogo-A deficient mice in order to study the role of Nogo-A in axonal regeneration (Kim et al., 2003, Simonen et al., 2003, Zheng et al., 2003). In Nogo-A knock-outs (KO), in which Nogo-B or –C expression is preserved, in addition to enhanced regeneration of lesioned fibers, collateral sprouting of intact nerve fibers was observed (Simonen et al., 2003). Extensive sprouting and improved regeneration was only found in young mice lacking Nogo-A/B used by the Strittmatter laboratory, which was associated with increased locomotor recovery (BBB scores) (Kim et al., 2003). In these two studies, axonal regeneration could be increased to variable extents by genetic deletion if Nogo-A in mice. In the third study, where a different line of Nogo-A/B KOs and mice lacking Nogo-A/B/C were studied, the mice showed some CST sprouting, but no longer distance regeneration (Zheng et al., 2003). The contradictory results obtained in these Nogo KO studies were attributed to the age, genetic background and gene targeting method of the mouse lines used, to the applied lesion paradigm and to the compensatory up-regulation of other Nogo or growth inhibitory proteins (Woolf, 2003). Back-crossing experiments indicated a major regeneration-enhancing effect of Nogo-A deletion in mice and a strong influence of the genetic background (Dimou et al., 2006). Inhibitory guidance cues (EphrinA3, EphA4, Sema4D and 3F and plexinB2) were shown to be up-regulated after the loss of Nogo-A and are likely to contribute to the blockade of regeneration, as suggested by the increased sprouting in Nogo-A/EphA4 double KO mice (Kempf et al., 2013). Additionally, as the other common ligands, MAG and OMgp, for NgR1 are still present upon deletion of Nogo-A, they, too, could hinder the initiated increased growth. To test this hypothesis, Nogo-A, OMgp and MAG triple mutants were generated and were found to exhibit greater axonal growth and improved locomotion, consistent with a principal role of Nogo-A and overlapping actions of MAG and OMgp through their shared receptors (Cafferty et al., 2010). However, a parallel study with independently generated Nogo-A, OMgp and MAG triple KOs failed to show the synergistic effect of the three inhibitors in axonal sprouting and functional recovery.

The relatively weak growth improvement in conventional Nogo-A KOs was attributed to the ablation of the Nogo-A gene in both glial and neuronal cells. In the optic nerve, Nogo-A KOs showed no regenerative improvement, however inflammation induced retinal ganglion cell (RGC) growth was enhanced when Nogo-A was overexpressed in the RGCs of Nogo-A
KOs. Silencing Nogo-A in RGCs on the other hand, reduced axonal growth (Pernet et al., 2011). The limited enhancement of axonal regeneration observed after CNS injury in conventional, full KO mice therefore may partially be due to the lack of neuronal Nogo-A expression that was shown to exert a positive function on the growth of RGC neurons.

Several studies with genetic NgR1 modulations were carried out in parallel to the work on Nogo-A KOs. Studies with both conventional and conditional NgR1 gene deletions show increased growth of corticospinal or raphespinal axons in the spinal cord (Kim et al., 2004, Wang et al., 2011) and increased regeneration of RGCs after optic nerve crush injury (Fischer et al., 2004a, Wang et al., 2012b). Other NgR family members have also been reported to bind inhibitory molecules (NgR1 and 2: OMgp and MAG, NgR1, 2 and 3: CSPG) (Dickendesher et al., 2012); Nogo receptor triple mutants (NgR1/2/3 KO) or NgR1 and NgR3 double mutants showed enhanced axonal regeneration in the optic nerve.

Interestingly, the acute neutralization of Nogo-A or NgR1 consistently promoted stronger regeneration of axons, suggesting that compensatory expression changes of other inhibitory factors might play an important role in repressing axonal growth in the genetically modified animals. Overall, a high degree of similarity is observed in acute blocking treatments, irrespectively of the ways used to disrupt Nogo-A signaling (ligand, receptor, intracellular signaling cascade). In light of a potential clinical application of these interventions, however, it is encouraging that neutralization of Nogo-A and the underlying signaling cascades leads to increased regeneration and improved functional recovery without any adverse effects such as aberrant outgrowth from intact axons and hyperalgesia.
Table 2. Key in vivo studies with Nogo-A/NgR1 modified transgenic mouse lines

<table>
<thead>
<tr>
<th>Knock-out mouse line</th>
<th>Study</th>
<th>Age, gender, genetics</th>
<th>Injury model</th>
<th>Verification of the model, anatomical readout and effect</th>
<th>Functional readout and effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nogo-A KO</td>
<td>(Simonen et al., 2003)</td>
<td>8-17 wks, C57BL/6 and 129/SvJ crossed mice, both genders</td>
<td>SCI: dorsal hemisection, T8</td>
<td>Complete Nogo-A KO. Nogo-B strongly up-regulated. Decreased inhibitory effect of spinal cord extracts, 2wks post SCI increased CST (BDA tracing) regrowth towards and in lesion, more frequent caudal sprouts from intact fibers.</td>
<td>Only intact mice, no major obvious behavioral alterations.</td>
</tr>
<tr>
<td>Nogo-A/B KO (atg allele) and Nogo-A/B/C KO</td>
<td>(Fischer et al., 2004a)</td>
<td>AAV mediated NgR1(^{wt}) or WT NgR1 transduction in female Sprague Dawley rats (160-180g)</td>
<td>ONC + lens injury</td>
<td>2wks after ONC, NgR(^{wt})-transfected optic nerves increased axon regeneration (GAP-43 staining) several-fold but only along with growth program activation. Overexpression of WT NgR1 blocked almost all regeneration even in lens-injured animals.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Nogo-A/B KO (atg allele)</td>
<td>(Cafferty and Strittmatter, 2006)</td>
<td>7-9 wks, backcrossed to C57BL/6</td>
<td>SCI: unilateral pyramidotomy (PyX)</td>
<td>4wks post PyX, pm nounced collateral sprouting of intact CST fibers across the midline and into denervated spinal cord in both Nogo-A/B (atg allele) and NgR1 KO mice.</td>
<td>4wks post PyX, in both KO's recovery of paw preference in the food pellet retrieval task.</td>
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<tr>
<td>Nogo-A/B KO (atg allele)</td>
<td>(Dimou et al., 2006)</td>
<td>6-7 wks, C57BL/6 or 129X1/SvJ mice</td>
<td>SCI: dorsal bilateral lesion, T8</td>
<td>2wks after SCI, 129X1/SvJ Nogo-A KO mice had 2-4 times more regenerating CST fibers (BDA tracing) in the caudal SC than C57BL/6 Nogo-A KO mice.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Nogo-A/B KO (trap and atg allele)</td>
<td>(Steward et al., 2007)</td>
<td>Both lines 8-10 wks, backcrossed to C57BL/6</td>
<td>SCI: dorsal hemisection, T8</td>
<td>Dramatic pattern of ectopic axonal labeling (with BDA tracing at the time of injury, possibly by BDA leakage into the caudal ventricle) in both knockout and control mice, that is absent</td>
<td>N.A.</td>
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</table>
**Chapter 1: Introduction**

| Nogo-A/B KO (trap and atg allele) | (Cafferty et al., 2007) | 8 or 14wks, backcrossed to C57BL/6 | SCL: bilateral dorsal hemisection, T7 | 3wks post SCL, 14wk-old Nogo-A/B KO<sup>xtrap</sup> have significantly less regenerating CST and raphespinal fibers than the 8wk-old KO's. Nogo-A/B KO<sup>xtrap</sup> KO's have significantly more axons caudal to the lesion than do Nogo-A/B KO<sup>xtrp</sup> or Nogo-A/B KO<sup>xtrp</sup> mice. | 3 wks post SCL, 14wk-old Nogo-A/B KO<sup>xtrp</sup> mice recovered less well in BBB scores than did the 8wk-old mice of the same genotype. |
| Nogo-A, MAG, and OMgp triple KOs | (Cafferty et al., 2010) | 2-4months, backcrossed to C57BL/6 | SCL: dorsal hemisection, T6 | Myelin lacking all three inhibitors is less inhibitory than Nogo-A-deficient myelin. Also, 4wks post SCI, rostral CST sprouting, CST regeneration (BDA tracing) and 5-HT fiber growth is the most enhanced in the Nogo-A, MAG, and OMgp triple KOs. | The triple-mutant mice exhibit greater improved locomotion (BBB scores). |
| Nogo-A, MAG, and OMgp triple KOs | (Lee et al., 2010) | 8-10wks, C57BL/6 or 129X1/SvJ mice | SCL: T8 dorsal or lateral hemisection, complete transection or pyramidotomy | Deleting any of the three inhibitors in mice enhanced sprouting of corticospinal or raphespinal serotonergic axons (Nogo KO; CST, MAG or OM gp; serotonergic axon sprouting), but no synergistic effect was observed in the triple KOs. | No behavioral improvement associated to regeneration. |
| Nogo-A KO | (Pernet et al., 2011) | 2-4month, male C57BL/6 mice | ONC + AAV2 virus manipulations (overexpression/silencing Nogo-A) | Nogo-A KO's show no regenerative improvement, however inflammation induced RGC growth is enhanced when Nogo-A is overexpressed in RGCs in Nogo-A KOs. Silencing Nogo-A reduced axonal growth capacity (CTB tracing). | N.A. |
| NgR1<sup>xtrp</sup>-Actin-Cre, inducible CKO | (Wang et al., 2011) | 11-12wks, C57BL/6, NgR1 deletion before ONC or 75-225 days post SCI. | ONC and SCI: dorsal hemisection | Increased RGC regeneration in the NgR1 CKOs 2wks after ONC (GAP-43 staining). Delayed deletion of NgR1 75 days post-SCI increased 5HT fiber density in the lumbar cord. | Deletion of NgR1 in chronic SCI condition leads to improved stepping performance, motor behavior. Strong correlation of 5HT axon density with the parameters measured kinematically. |
| NgR1, NgR2, NgR3 triple KOs | (Dickender and EphA4, 2012) | 6-8wks, C57BL/6 | ONC +/- Zymosan | Nogo receptor triple mutant or NgR1 and NgR3 double mutant, but not single mutants showed enhanced axonal regeneration (GAP-43 staining). | N.A. |
| NgR1 KO | (Wang et al., 2012) | 11-12wks, C57BL/6 | ONC with Zymosan at the time of the injury | NgR1<sup>xtrp</sup> mice show substantial optic nerve regeneration, and NgR1 disruption synergizes with macrophage activation (Zymosan). >2mm regenerating fibers (GAP-43 stained) past the ONC site. | N.A. |
| Nogo-A, EphA4 double KOs | (Kemp et al., 2013) | 20wks, C57BL/6 | SCL: (bilateral) dorsal hemisection, T8 | Nogo-A/EphA4 double KO mice showed increased axonal sprouting and regeneration 24 days after SCI (BDA tracing). | N.A. |

Abbreviations: AAV, adeno-associated virus; BDA, biotinylated dextran amine; CKO, conditional knock-out; CST, corticospinal tract; CTB, cholera toxin-β-subunit; KO, knock-out; N.A. not assessed; ONC, optic nerve crush; RGC, retinal ganglion cell; SCI, spinal cord injury; wk, week.
2.5 Regeneration in the visual system

2.5.1 The optic nerve crush as an excellent model system to study axonal regeneration in the CNS

The retina and the optic nerve are developmentally derived from the anterior part of the neural tube, from the diencephalon, and therefore are part of the CNS. The retina is composed of layers of specialized neurons, and axons in the optic nerve stem from a single cell population or projection neurons, the retinal ganglion cells (RGCs). These axons are myelinated by brain-derived oligodendrocytes (Ono et al., 1997). In rodents, there are no oligodendrocytes in the retina, only in the optic nerve, and no neuronal cell bodies are present in the optic nerve. The retina and optic nerve therefore provide a well compartmentalized system to separately study the role of neurons and oligodendrocytes in axonal regeneration.

The retina and optic nerve offer a number of advantages to investigate the mechanisms underlying several CNS pathologies (London et al., 2013). The eye shares many anatomical, functional and immunological features with other CNS regions; much of our understanding regarding the processes of neuroprotection, cell renewal and axonal regeneration has emerged from studies performed in the retina and optic nerve.

Spontaneous axonal regeneration after an injury is, as in other CNS regions, relatively poor in the adult optic nerve. This lack of RGC axonal regrowth is due to their insufficient intrinsic neuronal growth potential, the axotomy-induced apoptotic cell death and the growth-inhibiting environment composed of the myelin, the glial scar formed by reactive astrocytes and the repulsive extracellular matrix. Early studies from Cajal’s student, F. Tello, demonstrated that some RGCs can extend axons into peripheral nerve grafts attached to the cut optic nerve of rabbits (Ramón y Cajal et al., 1991). This observation was the first hint showing that mature CNS neurons retain some capacity to regenerate their axons. Further systematic experiments by Aguayo and his colleagues confirmed this finding (Aguayo et al., 1987), using the regrowth of optic nerve axons through peripheral nerve grafts as bridges up to visual targets such as the superior colliculus.

The popularity of the optic nerve crush (ONC) injury model in the orbit (Figure 3A) stems from the easy accessibility of the optic nerve and from the simple way RGCs can be experimentally manipulated by intra-ocular injections. Detailed knowledge can be acquired from this system; comprehensive, general conclusions regarding regeneration and neuronal survival in the CNS can be drawn from experiments performed in the visual system. The advantages using the ONC model are threefold:
Figure 3. Optic nerve crush (ONC) as model to study axonal regeneration and cell survival after CNS injury. (A) In order to perform retro-bulbar optic nerve crush, the optic nerve is exposed intra-orbitally and then crushed by tying a knot with a 9-0 suture at ~0.5mm from the back of the eye. Careful removal of the suture ensures a well-defined lesion site that disrupts all RGC axons and a consequent fundus examination controls the integrity of the ophthalmic artery. (B) By intraocular injection RGCs are readily accessible for pharmacological treatments and gene therapy. After ONC, the optic axons can be anterogradely traced by intraocularly injecting cholera toxin β-Alexa594 (CTB-A594). (C) CTB-A594-positive regenerating axons can be observed on longitudinal optic nerve sections (D) or on whole-mounted, transparent optic nerves. (E) RGC survival can be examined on retinal flat-mount preparations stained for a neuronal marker, βIII-Tubulin.
1) RGCs are readily accessible for pharmacological treatments and gene therapy by intraocular injection (Figure 3B). Sustained expression of growth factors can be required to reach significant levels of axon growth stimulation after injury. For example, depending on the half-life of cytokines and neurotrophic factors, their injection into the eyeball result in high concentrations in the vitreous liquid and due to their anatomical proximity, RGCs can be directly targeted. In addition, recently a new serotype of the adeno-associated virus (AAV), AAV2 has been shown to specifically infect RGCs with high rate, allowing targeted manipulation of signaling pathways involved in cell survival and regeneration (Cheng et al., 2002, Pernet et al., 2005). However, other AAV variants have been shown to efficiently and selectively infect Müller cells, thereby opening new possible ways for drug delivery (Klimczak et al., 2009, Pernet et al., 2013a). Additionally, RGCs take up and anterogradely transport cholera toxin B (in many cases conjugated with Alexa594) with high efficiency and this provides an excellent tracing method labelling almost 100% of RGC axons in the optic nerve.

2) The optic nerve consists of a long, continuous tract of axons that are not interrupted by interneurons, rendering the optic nerve ideal for regeneration studies. Both local sprouting after the lesion site and long distance regeneration to the targets can be addressed in this system on longitudinal tissue sections, or recently also in whole mounted transparent optic nerves (Figure 3C and D). Shortly after the lesion site, local arborization and branching of regenerating axons can be studied in the complex environment composed by CNS myelin, the glial scar and the surrounding extracellular matrix (ECM). In the retrobulbar ONC model, re-growing axons need to regenerate several millimeters towards the brain for successful target innervation. By overcoming the intrinsic limitations and extrinsic hurdles affecting regeneration, recent studies reported full regrowth of a few RGC axons to the brain (de Lima et al., 2012a). However, there are new questions such as path finding and guidance, target recognition, synapse formation, pruning and formation of functional circuits for these re-growing axons; they can be addressed by newly developed 3-dimensional tracing techniques of individual axons in the transparent optic nerve and brain (Luo et al., 2013, Pernet and Schwab, 2014).

3) Besides axonal regeneration, neuronal survival can also be studied after an optic nerve injury. More than 80% of the RGCs undergo apoptosis 2 weeks after the injury (Berkelaar et al., 1994). RGCs start dying 5-6 days after intraorbital optic nerve injury, and this distance-dependent onset of apoptosis may be due to the lack of target-derived neurotrophic factors. Neuronal survival can be investigated on flat-mount preparations of injured retinae (Figure 3E). Interestingly, although promoting neuronal survival should logically lead to increased regenerative potential, as survival of RGCs is a prerequisite for
axonal regeneration, the two phenomena can involve distinct and sometimes antagonistic mechanisms (Goldberg and Barres, 2000, Pernet and Di Polo, 2006, Huntwork-Rodriguez et al., 2013, Watkins et al., 2013).

2.5.2 Regeneration studies in the visual system

In the 1980s, the optic nerve injury paradigm became a model of choice and early experiments reported improved survival and/or regeneration after peripheral nerve graft implantation or by intraocular neurotrophic factor injection (Bray et al., 1991, Mansour-Robaey et al., 1994). Since then important milestones has been reached by using this model, demonstrating that under certain conditions mature RGCs can be transformed into a regenerative state allowing cell survival and axonal regrowth (Figure 4).

During development, RGCs are in an active growth state and express axon growth associated genes, but this growth rate dramatically decreases by postnatal day 2 (Goldberg et al., 2002). The first robust regeneration results in adult RGCs were reported after experimental lens injury (Fischer et al., 2000, Leon et al., 2000) and this intervention led to the up-regulation of SPRR1A and GAP43, two important growth-associated genes (Bonilla et al., 2002, Fischer et al., 2004b). The same regeneration phenotype was observed after intravitreal injection of Toll-like receptor agonists (Zymosan or Pam3Cys) leading to experimental inflammation (Yin et al., 2003, Hauk et al., 2008, Hauk et al., 2010, Pernet et al., 2011). The underlying mechanisms of lens injury and Zymosan induced regeneration were shown to be the infiltration of activated macrophages and neutrophils and the expression of multiple factors promoting cell survival and regeneration, such as oncomodulin (Yin et al., 2006, Yin et al., 2009, Kurimoto et al., 2013). Moreover, retinal astrocytes and Müller cells also undergo activation and secrete ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) for RGCs under these inflammatory conditions (Muller et al., 2007, Leibinger et al., 2009) (Figure 4A). However, after ONC injury, the loss of target-derived neurotrophic support, mainly the lack of brain-derived neurotrophic factor (BDNF) leads to progressive RGC apoptosis (Liu et al., 2011) (Figure 4A).

Switching adult RGCs into a growth state by modulating transcription factors such as KLFs (Moore et al., 2009, Moore et al., 2011, Steketee et al., 2014) or by the blockade/release of signaling pathways strongly promoted axonal regeneration (Liu et al., 2011) (Figure 4A). Activation of a major growth-regulator, mTOR, shed light on the importance of the PI3K/AKT/mTOR pathway in axonal regeneration, first in RGCs and subsequently in the spinal cord. Activation of mTOR by genetic deletion of PTEN, its negative regulator, induced axonal regeneration and enhanced neuroprotection in RGCs.
Factors that limit axonal regeneration are present both in the retina and in the optic nerve. (A) Besides the intrinsic growth potential of RGCs defining their regenerative capacity, activated Müller cells and infiltrating immune cells secrete pro or anti-regeneration/survival factors such as CNTF, BDNF, LIF, oncomodulin or IL-6. (B) The combination of reactive astrocytes and infiltrating macrophages contribute to the formation of glial scar around the optic nerve lesion site that expresses CSPGs and represent a first line of barrier for re-growing axons. Additionally, the myelin debris, myelin associated inhibitory proteins (Nogo-A, MAG, OMgp) and repulsive axon guidance molecules (Ephrins, Semaphorins) render the adult optic nerve a hostile environment for regeneration. (C) Following intense growth program activation, a number of RGC axons reach the optic chiasm and follow normal trajectories in the contralateral optic tract (green). However, some of
these axons regenerating to long distances misroute to the contralateral optic nerve (blue) or ipsilateral optic tract (purple). In the optic nerve, past the lesion site, several aberrant growth phenotypes such as axonal branching (red) or axonal U-turn (yellow) can be observed.

(Park et al., 2008) and was further potentiated by the co-deletion of SOCS3, a feedback inhibitor of the JAK/STAT3 pathway (Sun et al., 2011). Likewise, the activation of STAT3 was found to be important in initiating axonal growth (Muller et al., 2009, Pernet et al., 2013b). In another line of research, elevating the intracellular levels of cyclic adenosine monophosphate (cAMP) in combination with CNTF treatment was shown to be sufficient to switch RGCs into growth state and helped them overcoming inhibition by the myelin environment (Cai et al., 2002, Cui et al., 2003, Gao et al., 2004).

In order to promote long distance and abundant regeneration of RGCs reaching the target regions, many laboratories applied combinational treatment regimens. Deletion of PTEN along with SOCS3 yielded lengthy regeneration with newly grown axons reaching the optic chiasm (Sun et al., 2011). Even more impressive regeneration was observed when along with PTEN deletion, inflammatory molecules (oncomodulin) and cell permeable cAMP (CPT-cAMP) were administered into the eyeball at the time of optic nerve injury (Kurimoto et al., 2010). By applying a similar treatment regimen (PTEN deletion, Zymosan, CPT-cAMP), Lima et al. found that some regenerating RGC axons extended all the way till their targets (the lateral geniculate nucleus in the thalamus and superior colliculus in the midbrain) and that this regeneration led to partial visual recovery (de Lima et al., 2012a). As promising as these results are, so far these findings could not be reproduced (Diekmann et al., 2013, Luo et al., 2013).

Beside the intrinsic insufficiency of RGCs to regrow their axons, the previously described extracellular inhibitory environment represents a great barrier for long distance regeneration (Figure 4B). Not surprisingly, acting on the myelin inhibitory environment (by NgR or Nogo-A deletion) in combination with boosting the intrinsic growth program (by Zymosan or Pam3Cys induced inflammation) yielded synergetic effects on axonal regeneration (Pernet et al., 2011, Wang et al., 2012a). Combined application of anti-Nogo-A antibody (IN-1) and CNTF had a synergistic effect in the intraorbital ON crush paradigm (Cui et al., 2004). Additionally, Stat3 overexpression together with the application of a ROCK blocker leading to Rho-A signaling inhibition also led to further increased regeneration (Pernet et al., 2013b). In this and further studies, the importance of not only the distance but also the directionality and guidance of regenerating axons arose. Few laboratories reported that although the number regrowing axons can be successfully promoted, these fibers very often follow irregular trajectories, show abnormal morphologies and misrouting at decision points, such as the optic chiasm (Giger et al., 2010, Diekmann et al., 2013, Luo et al., 2013,
Pernet et al., 2013a, Pernet et al., 2013b) (Figure 4C). These observations were enabled by the improved tissue clearing methods and 3D analysis of regenerating axons. Further research should be carried out with detailed growth analysis in order to understand how we can provide regenerating RGC axons the correct guidance cues, and enable them to re-establish functionally relevant connections in the visual targets.

2.5.3 From the optic nerve to the spinal cord

In summary, the ONC injury paradigm serves as a model on its own providing valuable insights for finding the best treatment promoting axonal regeneration. Importantly, it was also proved to provide a link to spinal cord injury research. Target innervation and functional recovery is a challenging goal in the regenerating optic nerve, where axons need to regenerate for long distances in a hostile environment composed of mostly myelinated tracts. In the spinal cord, however, regenerating or sprouting axons can extend towards intact neuronal circuits that are in their close proximity and can form new connections with these neurons. Additionally, the pre-existing, non-injured, spared fibers respond to the injury with intensive sprouting and compensate for the lost connections. Another point of difference is the substantial cell death that follows a retrobulbar optic nerve injury (given the fact that the RGC cell bodies are in direct vicinity to the injury), but is absent from other CNS areas, as the neurons projecting to the injured tracts are mostly located further away. Even though the two systems possess different anatomical and neuronal survival features, transferability of the results from the optic nerve to the spinal cord helped the progress of finding a cure for devastating CNS injuries.

3 Expression pattern of Nogo-A in the visual system: implications on intracellular and extracellular cell-type specific functions

Possible neuronal functions of Nogo-A are the subject of increasing interest, especially those fulfilled during development, in plastic CNS regions and after injury, when the expression level of the protein suggests crucial roles in neurite growth. On the cellular level Nogo-A is particularly abundant in the endoplasmic reticulum (ER), however, it is also present on the cell surface, both on the axon shafts and growth cones of neurons (Dodd et al., 2005).

Neutralization of neuronal Nogo-A by a function-blocking antibody, genetic ablation of Nogo-A, or function blocking antibodies against the Nogo receptor NgR1 have been shown to increase fasciculation and decrease branching of cultured dorsal root ganglion axons (Petrinovic et al., 2010). Localization of Nogo-A to the axon shaft might render
unmyelinated neurite surfaces repulsive to each other, suggesting that neuron-to-neuron inhibitory Nogo-A signaling takes places during development. Another study suggested that Nogo-A can self-limit axon outgrowth in a cell autonomous manner in primary cortical neurons (Dickson et al., 2010).

However, Nogo-A is also present in the axonal growth cones where the relation of the protein to the growth machinery needs to be further investigated. Several recent studies have suggested that in contrast to myelin Nogo-A, neuronal Nogo-A might be beneficial for regeneration in injured conditions. Firstly, Nogo-A expression was found to be increased in retinal ganglion cells (RGCs) after optic nerve crush injury, and the up-regulation of Nogo-A was not involved in injury-induced cell death (Pernet et al., 2011). Secondly, silencing Nogo-A selectively in RGCs resulted in marked reduction of regenerative sprouting and decreased the expression growth-associated molecules. Thirdly, axonal regeneration was not notably enhanced in conventional Nogo-A knock-out animals, in which both glial and neuronal Nogo-A is deleted.

The expression pattern of Nogo-A in the retina and optic nerve also suggests cell-type specific, segregated functions for this protein. In the optic nerve, Nogo-A is present in oligodendrocytes and activates Nogo-A signaling leading to the Rho-A pathway activation in injured neurons eventually leading to growth cone collapse and the blockade of axonal regeneration (Figure 5A). In intact retinae of adult mice, the bulk of Nogo-A is expressed by Müller glial cells, localized to the end-feet surrounding retinal ganglion cell bodies (Figure 5B). Even though the function of Nogo-A in Müller cells is not yet understood, this close proximity of the Nogo receptor expressing RGCs suggests close interaction between the two cell types. Nogo-A is up-regulated in about 50% of RGCs following optic nerve crush. Both Nogo-A receptors, NgR1 and S1PR2 receptors are also expressed (Wang et al., 2002c) in injured, Nogo-A overexpressing RGCs (Figure 5B) that showed increased regeneration in the optic nerve. These findings led us to the hypothesis that the Nogo-A ligand may bind to its receptors in cis position (intracellularly or on the neuronal cell surface) and thereby prevent inhibitory signaling originating from the extracellular, myelin Nogo-A presented in trans. This phenomenon has previously been described for axonal guidance molecules such as Ephrins and Semaphorins.

Similarly to Nogo-A and its receptors, members of the ephrin and Eph ligand-receptor family fulfil repulsive cell surface functions (Klein, 2004) via forward signaling through Ephs or reverse signaling through ephrins (Egea and Klein, 2007). Expression mapping of these molecules in several nervous system regions revealed that ephrins and Ephs are coexpressed in the same neurons during the period of axon outgrowth. This finding has further complicated the bidirectional signaling system, raising the possibility of cis-
Figure 5. Nogo-A expression and function in the retina and optic nerve. (A) In the optic nerve, Nogo-A is present in oligodendrocytes and activates Nogo-A signaling (Rho-A pathway) on neurons that eventually lead to growth cone collapse and the blockade of axonal regeneration. (B) In intact retinas of adult mice, the bulk of Nogo-A is expressed by Müller glial cells, localized to the end-feet surrounding RGC cell bodies. Even though the function of Nogo-A in Müller cells is not yet understood, this close proximity of the Nogo receptor expressing RGCs suggest close interaction. Nogo-A is up-regulated in about 50% of RGCs following optic nerve crush and these RGCs express both Nogo-A-Δ20 and Nogo-66 receptors, S1PR2 and NgR1.
interaction between the receptor and ligand, namely that the ephrin molecule presented in cis position could block the signaling of the trans ligand through Eph receptors. The cis-ligand may sequester the receptor and block the binding of the exogenous ligand, or inhibit downstream signaling elements activated by the trans-ligand, prevent the receptors cell surface expression or alternatively act as a competitor of the trans ephrin molecule. This hypothesis has been confirmed to help the axonal pathfinding of the lateral motor column neurites in the peripheral targets (Kao and Kania, 2011) and to function as an axon guidance mechanism for the retinotectal projections in the CNS (Hornberger et al., 1999). Additionally, multiple EphA receptors and ephrin-A ligands are expressed in gradients in the developing visual system (Huberman et al., 2005), both in the retina and in different brain targets, raising the possibility that correct patterning of not only retinocollicular but also retinogeniculate projections could depend on cis-interaction. A similar mechanism of ligand-receptor cis-interaction leading to blockade of signal transduction was recently shown for the semaphorins, another family of developmental axonal guidance molecules (Haklai-Topper et al., 2010). The simultaneous presence of Nogo-A and its receptors in RGCs therefore raised the possibility that through cis-interaction Nogo-A could limit signaling via NgR1 and S1PR2 and thereby counteract growth inhibition by myelin inhibitory cues.

In addition to the plausible pro-regenerative function of Nogo-A in neurons, several studies suggested that neuronal Nogo-A might play a cell-autonomous role in improving neuronal survival. One proposed mechanism is the protection by Nogo-A against oxidative insult through interaction with Prdx2 and by scavenging ROS (Mi et al., 2012), by inhibiting NADPH oxidase-mediated oxidative damage (Guo et al., 2013) or by functioning as an anti-stress platform through its central domain NiG, recruiting cytoprotective proteins including Apg-1 and Prdx2 (Kern et al., 2013). Additionally, it has recently been described that the Nogo-A protein levels are increased in cortical and thalamic neurons after stroke (Cheatwood et al., 2008), and that Nogo-A could promote neuronal survival by controlling the Rac1/Rho-A balance (Kilic et al., 2010). Along this line, blocking Rho-A activation after spinal cord injury protected cells from p75NTR-dependent apoptosis (Dubreuil et al., 2003). Additionally, recently a study showed that the loss of RTN4 alter ER morphology and RTN4 depleted cells fail to sustain elevated cytoplasmic Ca\(^{2+}\) levels, therefore are less susceptible to Ca\(^{2+}\) overload induced apoptosis (Jozsef et al., 2014).

4 Conclusions

In the injured CNS, Nogo-A derived from oligodendrocytes appears to be a major inhibitor for axonal regeneration. Many studies have reported that neutralization of Nogo-A or Nogo
receptor complex members improved axonal growth and plasticity and enhanced functional recovery after injury. Clinical studies are currently ongoing with function blocking anti-Nogo-A antibodies in multiple CNS disease areas such as spinal cord injury, stroke, ALS and MS. However, the expression of Nogo-A in physiological conditions and in a large population of neurons during development and even in the adulthood suggests further functions for Nogo-A, such as its influence in cell migration and myelination during development, synaptic plasticity and cell survival. Our recent studies also suggested that not only glial, but also neuronal Nogo-A could influence axonal growth and that this neuronal counterpart might be beneficial for axonal regeneration. The research presented in this thesis therefore aimed to provide, at least in part, answers for the glial versus neuronal cell type-specific functions of Nogo-A, on how they regulate axonal growth and neuronal survival.

References


Chapter 1: Introduction


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Chapter 1: Introduction


Chapter 1: Introduction


Chapter 2

Cell type-specific Nogo-A gene ablation promotes axonal regeneration in the injured adult optic nerve

by Flóra Vajda

Further contributions by:

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Contributions:
F.V., V.P. and M.E.S.: designed the study. F.V. characterized the glial and neuronal conditional Nogo-A knock-out lines (with the help of S.J and F.C.), performed the optic nerve crush surgeries and anatomical tracings (with the help of V.P.), harvested and processed tissues for qRT-PCRs, for Western blotting and immunohistochemical stainings, acquired microscopic images, analyzed axonal regeneration and neuronal survival. N.J. created and analyzed 3D optic nerve reconstructions. D.D prepared the AAV2.Cre virus. B.T designed the Rtn4^lox/lox_ line. V.P. performed AAV2.Cre virus injections. F.V. prepared all the figures and wrote the manuscript (revised by V.P. and M.E.S.).

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1 Abstract

Nogo-A is a well-known myelin-enriched inhibitory protein for axonal growth and regeneration in the central nervous system (CNS). Besides oligodendrocytes, our previous data revealed that Nogo-A is also expressed in subpopulations of neurons including retinal ganglion cells in which it can play a positive role in the neuronal growth response after injury, through an unclear mechanism. In the present study, we analyzed the opposite roles of glial versus neuronal Nogo-A in the injured visual system. To this aim, we created oligodendrocyte (Cnp-Cre+/xRtn4/Nogo-A^flox/flox) and neuron-specific (Thy1-Cre^tg+xRtn4^flox/flox) conditional Nogo-A knock-out (KO) mouse lines. Following complete intraorbital optic nerve crush, both spontaneous and inflammation-mediated axonal outgrowth was increased in the optic nerves of the glia-specific Nogo-A KO mice. In contrast, neuron-specific deletion of Nogo-A in a KO mouse line or acute gene recombination in retinal ganglion cells mediated by adeno-associated virus serotype 2 (AAV2).Cre virus injection in Rtn4^flox/flox animals decreased axon sprouting in the injured optic nerve. These results therefore show that selective ablation of Nogo-A in oligodendrocytes and myelin in the optic nerve is more effective at enhancing regrowth of injured axons than what has previously been observed in conventional, complete Nogo-A KO mice. Our data also suggest that neuronal Nogo-A in retinal ganglion cells could participate in enhancing axonal sprouting, possibly by cis-interaction with Nogo receptors at the cell membrane that may counteract trans Nogo-A signaling. We propose that inactivating Nogo-A in glia while preserving neuronal Nogo-A expression may be a successful strategy to promote axonal regeneration in the CNS.
Keywords:
glial Nogo-A; neuronal Nogo-A; optic nerve crush injury; retinal ganglion cells; axonal regeneration; cis-interaction

Abbreviations:
AAV, adeno-associated virus; AKT, Protein Kinase B; APC, adenomatous polyposis coli; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CRALBP, cellular retinaldehyde binding protein; CREB, cAMP response element-binding protein; CTB-A594, cholera toxin B-Alexa594; DRG, dorsal root ganglion; ERK1/2, extracellular signal-regulated kinases 1/2; Gap-43, growth-associated protein 43; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GS, glutamine synthetase; INL, inner nuclear layer; Jak3, janus kinase 3; KO, knock-out; LIF, leukemia inhibitory factor; LTP, long-term potentiation; MAG, myelin-associated glycoprotein; NgR1, Nogo66 receptor 1; OFL, optic fiber layer; OMgp, oligodendrocyte myelin glycoprotein; ONC, optic nerve crush injury; ONL, outer nuclear layer; Pam3Cys, (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-Npalmitoyl-(R)-Cys-(S)-Ser(S)-Lys(4)-OH.trihydrochloride; PBS, phosphate-buffered saline, PNS, peripheral nervous system; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative real-time polymerase chain reaction; RGCs, retinal ganglion cells; ROCK, Rho-associated protein kinase; RTN, reticulon; SEM, standard error of the mean; SOCS3, suppressor of cytokine signaling 3; S1PR2, sphingosine 1-phosphate receptor 2; Sprr1A, small proline-rich protein 1A; STAT3, signal transducer and activator of transcription 3; TBS, tris-buffered saline.
2 Introduction

In the adult mammalian central nervous system (CNS), axons have a very limited capacity to regenerate after traumatic injury. This lack of axonal regeneration is thought to be mainly due to the presence of growth-inhibiting molecules in the injured CNS environment (Yiu and He, 2006, Schwab, 2010) and to the low intrinsic growth capacity of mature neurons (Goldberg et al., 2002).

Nogo-A is a well-studied inhibitory protein for axonal growth, plasticity and regeneration after CNS injury (Schwab, 2004, Pernet and Schwab, 2012). Nogo-A is predominantly expressed in oligodendrocytes in the adult CNS, where it is thought to stabilize neuronal circuits in healthy conditions and to inhibit neurite growth and plasticity after lesion (Schwab, 2010). Neutralizing Nogo-A by function blocking antibodies or genetic knock-out has been shown to improve axonal sprouting and regeneration in the injured spinal cord and brain (Thallmair et al., 1998, Bareyre et al., 2002, Kim et al., 2003, Simonen et al., 2003, Dimou et al., 2006, Schwab and Strittmatter, 2014).

In addition to oligodendrocytes and myelin, Nogo-A is expressed in growing and immature neurons as well as in some adult neurons (Huber et al., 2002, Wang et al., 2002). Neurons express Nogo-A receptors such as the Nogo-66 receptor 1 (NgR1) (Fournier et al., 2001) and the Nogo-A-A20 specific sphingosine 1-phosphate receptor 2 (S1PR2) (Kempf et al., 2014). They can co-express them along with Nogo-A (Wang et al., 2002), an observation that raises the possibility of cis-interactions between the ligand and its receptors within or at the cell surface of the same cell. This mechanism has previously been described for axonal guidance molecules such as Ephrins and Semaphorins and could play a major role in the neuronal response to extracellular growth inhibitors during development (Egea and Klein, 2007, Haklai-Topper et al., 2010).

In the adult CNS, the expression of neuronal Nogo-A remains elevated mainly in plastic regions such as in the hippocampus, the olfactory bulb or the neocortex and in dorsal root ganglia (Huber et al., 2002). Nogo-A and NgR1 were shown to regulate synaptic plasticity, e.g. long-term potentiation (LTP) in the hippocampus and in the sensory-motor cortex (Raiker et al., 2010, Delekate et al., 2011, Wills et al., 2012, Akbik et al., 2013, Tews et al., 2013), while after injury, the effects of neuronal Nogo-A are not yet well understood. During development, neuronal Nogo-A influences neuronal migration (Mingorance-Le Meur et al., 2007, Mathis et al., 2010), survival (Mi et al., 2012, Guo et al., 2013), cell spreading and neurite growth (Oertle et al., 2003, Petrinovic et al, 2010). In injured adult retinal ganglion cells (RGCs), silencing neuronal Nogo-A resulted in marked reduction of regenerative sprouting and decreased the expression of growth-associated molecules (Pernet et al., 2012).
2011). Furthermore, in the optic nerve, axonal regeneration was not improved in conventional Nogo-A KO animals, in which both glial and neuronal Nogo-A was deleted (Pernet et al., 2011). The present study therefore aimed to investigate whether glial and neuronal Nogo-A differently influence axonal growth in vivo using cell type-specific Nogo-A KO mouse lines and adeno-associated virus-mediated recombination of the Nogo-A gene in neurons. The results show that significantly more axons grew through the lesion site in the oligodendrocyte-specific Nogo-A KO mice. In contrast, neuron-specific ablation of Nogo-A in RGCs reduced the number of regenerating axons after optic nerve crush injury (ONC). In summary, we show that inactivating Nogo-A specifically in oligodendrocytes appears to be the most successful strategy to promote axonal regeneration in the adult optic nerve.

3 Results

3.1 Targeted deletion of Nogo-A in oligodendrocytes

To obtain an oligodendrocyte-specific Nogo-A KO mouse line, mice expressing Cre-recombinase under the control of the 2′,3′-cyclic nucleotide 3′-phosphodiesterase (Cnp-Cre+/-) (Lappe-Siefke et al., 2003) were crossed with mice in which exon 3 of the Rtn4 gene was flanked by loxP sites (Rtn4flox/flox) (Figure 1A). CNPase is a well characterized component and marker for myelin and oligodendrocytes (Sprinkle, 1989). In the optic nerve Olig2-positive oligodendrocytes specifically express CNP (Supplementary Figure S1A). By Western blotting in the highly myelinated optic nerve, the level of Nogo-A protein decreased by ~80% in Cnp-Cre+/- xRtn4flox/flox mice compared to WT, Cnp-Cre+/- and Rtn4flox/flox genotypes (Figure 1B). In the spinal cord, where Nogo-A is expressed in oligodendrocytes and in neurons, the level of Nogo-A was decreased by ~70% (Figure 1C). In the neocortex of Cnp-Cre+/- xRtn4flox/flox mice, Nogo-A levels were decreased by ~50%, reflecting the higher proportion of neuronal Nogo-A (Figure 1D) (Huber et al., 2002). In the hippocampus, we observed a ~30% decrease of Nogo-A (Figure 1E). The level of the Nogo-A splice variant Nogo-B was elevated in the optic nerve, spinal cord, cortex, and hippocampus, suggesting a compensatory up-regulation of this protein in oligodendrocytes (Simonen et al., 2003) (Figures 1B-E). By immunohistochemistry in the optic nerve (Figure 1F) and corpus callosum (Figure 1G), adenomatous polyposis coli (APC)-positive oligodendrocytes contained Nogo-A in control Cnp-Cre+/- animals, but lacked Nogo-A in Cnp-Cre+/- xRtn4flox/flox mice.
Supplementary figure 1. CNP is expressed in oligodendrocytes and in retinal Müller cells. (A) In the optic nerve, Olig2-positive myelin forming oligodendrocytes expressed CNP. (B) Developmental expression pattern of *Cnp* mRNA in the retina shows a peak at birth (P0). Scale bar: A = 10 μm.
Chapter 2: Targeted Nogo-A ablation promotes axonal growth

Figure 1. Generation and characterization of Cnp-Cre+/xRtn4fl/fl Nogo-A knock-out animals. (A) To obtain an oligodendrocyte-specific Nogo-A KO mouse line, mice expressing Cre-recombinase under the control of the 2',3'-Cnp (Lappe-Siefke et al., 2003) were crossed with mice in which exon 3 of the Rtn4 gene was flanked by loxP sequences (Rtn4fl/fl). (B-E) Western blot analysis revealed that Nogo-A protein was down-regulated in the optic nerve (~80%), spinal cord (~70%), cortex (~50%) and hippocampus (~30%) to different extents. In all these four examined CNS regions Nogo-B protein showed compensatory up-regulation. (F-G) By double immunohistochemistry for Nogo-A and adenomatous polyposis coli (APC) on optic nerve and corpus callosum sections, Nogo-A was specifically excised from oligodendrocytes in myelinated regions. APC-positive oligodendrocytes contained Nogo-A only in control animals, and lacked Nogo-A in the Cnp-Cre+/xRtn4fl/fl KO mice. Fluorescent pictures were from merged confocal microscopy stacks. Statistics: one-way ANOVA, Bonferroni's Multiple Comparison Test, *p<0.05, **p<0.01, ***p<0.001. Scale bars: F, G = 25 μm; insets = 5 μm.
3.2 *Cnp-Cre*-driven ablation of Nogo-A increases regenerative axonal sprouting after optic nerve crush injury

The effects of myelin Nogo-A on regenerative growth of CNS axons were assessed in the optic nerve crush model. The retinal ganglion cell axon growth was analysed after anterograde tracing with Alexa594-conjugated cholera toxin B on histological sections two weeks after injury. We observed more axonal sprouting after the lesion site in *Cnp-Cre*+/−*xRtn4*^floxflox^ mice than in control groups (Figure 2A). Quantitatively, compared to control genotypes the *Cnp-Cre*+/−*xRtn4*^floxflox^ optic nerves showed more regenerating axons at 50, 100, 150 and 200μm past the injury site (Figure 2B). The injury-induced growth response of RGCs was further analysed by following gene expression changes for *Sprr1A* and *Gap-43*, two known indicators of neuronal growth in the CNS and the PNS (Aigner et al., 1995, Bonilla et al., 2002, Pernet et al., 2011, Pernet et al., 2013). The mRNA levels of the two growth markers were increased five days after optic nerve crush and were more elevated in the *Cnp-Cre*+/−*xRtn4*^floxflox^ KO retinae than in control samples (Supplementary Figure S2A and B). These data show that the targeted deletion of Nogo-A in myelinating cells of the optic nerve enhances the neuronal growth response after axonal injury, in marked contrast with our previous analysis in conventional, systemic Nogo-A KO animals (Pernet et al., 2011).

3.3 Nogo-A deletion in *Cnp-Cre*+/−*xRtn4*^floxflox^ mice enhances inflammation-induced axonal regeneration after optic nerve injury

The growth state of RGCs can be enhanced by intraocular injection of inflammatory agents such as the Toll-like receptor 2 agonist Zymosan (Yin et al., 2006, Yin et al., 2009). In our study, we observed that the injection of Zymosan at the time of the injury increased the number of regenerating axons by ~3-fold in all the groups compared to untreated mice. The enhancement of growing axons was higher in the optic nerves of *Cnp-Cre*+/−*xRtn4*^floxflox^ mice than in WT, *Cnp-Cre*+/− or *Rtn4*^floxflox^ animals (Figure 2C and D). Statistically more axonal fibers grew across the lesion site in the *Cnp-Cre*+/−*xRtn4*^floxflox^ KO mice at 50, 100, 150, 200 and 300μm past the injury site than in control genotypes (Figure 2D). These results show that the combined growth program stimulation with a pro-inflammatory molecule along with the deletion of Nogo-A myelin potentiates axonal regeneration.
Figure 2. Axonal growth is increased after optic nerve crush injury in Cnp-Cre<sup>-/-</sup>xRtn4<sup>flox/box</sup> mice. (A) In comparison to WT, Cnp-Cre<sup>+/+</sup> and Rtn4<sup>flox/box</sup> control mice, Cnp-Cre<sup>-/-</sup>xRtn4<sup>flox/box</sup> mice showed significantly more growing CTB-594-labelled fibers across the optic nerve lesion site (black stars). (B) The number of growing axonal fibers was statistically higher in Cnp-Cre<sup>-/-</sup>xRtn4<sup>flox/box</sup>, glia-specific Nogo-A KOs (mean±S.E.M., two-way ANOVA, Bonferroni posttest, *p<0.05, **p<0.01, ***p<0.001) at 50, 100, 150 and 200μm past the injury site. (C) The intraocular delivery of pro-inflammatory reagent Zymosan at the time of the lesion placed RGCs in an active growth state resulting in robust axonal growth in the injured optic nerve. (D) In the optic nerves of mice injected with Zymosan, CTB-594-labelled regenerating fibers were dramatically increased in all genotypes, but were the most abundant in Cnp-Cre<sup>-/-</sup>xRtn4<sup>flox/box</sup> animals. Statistically more axons grew across the lesion site in the Cnp-Cre<sup>-/-</sup>xRtn4<sup>flox/box</sup>, glial Nogo-A KO mice (mean±S.E.M., two-way ANOVA,
Bonferroni posttest, *p<0.05, **p<0.01, ***p<0.001) at 50, 100, 150, 200 and 300\(\mu\)m past the injury site than in control genotypes. (E) Tissue clearing allowed to visualize traced axons in three dimensions in transparent whole-mounted optic nerves 2 weeks after injury (lesion site: stars). An example of a 3D optic nerve segment is presented for a control Rtn4\(^{flx/flx}\) mouse after reconstruction of confocal microscopy image stacks with the Imaris software. Axons were analysed at high magnification in the region where Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) mice had more sprouting axons, i.e. between 100 and 300\(\mu\)m past the injury site (two dashed lines). (F) In this range, branched (arrows), unbranched (arrowheads) and U-turn-forming (stars) axons could be identified. (G) The proportion of branching axons did not significantly vary between Rtn4\(^{flx/flx}\) and Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) mice. (H) The percentage of U-turn-forming axons was not different between the two genotypes. Scale bars: A and C insets = 25\(\mu\)m; E = 100\(\mu\)m, F = 50\(\mu\)m.

### 3.4 Three-dimensional analysis of the growth pattern of regenerating axons in the transparent optic nerve

The higher numbers of retinal fibers in Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) optic nerves elongating after the injury site could either be due to a higher number of individually growing axons or to enhanced branching. In order to assess the pattern of growing axons, a three-dimensional analysis of the regenerating fibers was carried out in whole-mounted optic nerves after tissue clearing using a modified protocol previously described (Dodt et al., 2007, Luo et al., 2013). In optic nerve segments situated between 100 and 300\(\mu\)m past the lesion site, branched and unbranched axons could be distinguished, as well as axons that formed U-turns within this region (Figure 2E and F). We found that the proportion of branching axons did not significantly vary between Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) and Rtn4\(^{flx/flx}\) control mice (Figure 2G). The percentage of U-turn-forming axons was not different between the two genotypes (Figure 2H).

### 3.5 Compensatory up-regulation of EphrinA3 and EphA4 in Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) mice

We then wondered if compensatory mechanisms impede axonal regeneration in Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) mice after crush lesion. Our laboratory has previously found that Nogo-A gene deletion induced the up-regulation of inhibitory molecules such as EphA4 and one of its ligands EphrinA3 in the spinal cord (Kempf et al., 2013). In EphA4 KO mice, axonal growth was increased in the injured spinal cord and optic nerve compared with WT animals (Kempf et al., 2013, Joly et al., 2014). In the present study, the analysis of EphrinA3 and EphA4 expressions by Western blotting revealed a strong up-regulation of EphrinA3 in the optic nerve and an increase of EphA4 in the retina of Cnp-Cre\(^+/-\)xRtn4\(^{flx/flx}\) compared with Cnp-Cre\(^+/-\) mice (Supplementary Figure S3A and E). In contrast, myelin-associated glycoprotein (MAG), a molecule that can block axonal regeneration by activating the same receptor (NgR1) as Nogo-A (Cafferty et al, 2010), did not change in optic nerves (Supplementary...
Supplementary figure 2. Optic nerve crush injury induces gene expression changes. (A-B) After injury, the mRNA levels of growth-associated molecular markers Sprr1A and Gap-43 were increased in the Cnp-Cre<sup>+/−</sup>xRtn<sup>−/−</sup> mice compared to control animals (two-way ANOVA, *P<0.05, **P<0.01).
Supplementary figure 3. Compensatory up-regulation of inhibitory guidance molecules following deletion of Nogo-A from glial cells. The expression of EphrinA3, EphA4, L-MAG and S1PR2 were analysed in 20% of protein lysates from intact retinas and optic nerves. GAPDH was used as internal control. (A) In the optic nerve of Cnp-Cre+/Rtn4flx/flx mice, the expression of EphrinA3 was up-regulated by ~75% compared to control animals. (B) The L-MAG isoform was not significantly (ns.) elevated in Cnp-Cre+/Rtn4flx/flx optic nerves relative to controls. (C) The level of S1PR2 protein was not altered in the optic nerve. (D) Retinal EphrinA3 did not change between groups. (E) EphA4 was significantly increased in the retinae of Cnp-Cre+/Rtn4flx/flx mice. (F) Similarly to the optic nerve, S1PR2 was not modified in Cnp-Cre+/Rtn4flx/flx retina when compared to Rtn4flx/flx controls (mean±S.E.M., student’s T-test, *p<0.05).
Figure S3B). The retinal expressions of NgR1 and S1PR2, a newly identified receptor for Nogo-A (Kempf et al., 2014), were not modified (Supplementary Figure S5A, Supplementary Figure S3C and F). These data suggest that EphA4 and EphrinA3 up-regulation may restrict the distance of axonal regrowth in Cnp-Cre+/xRtn4^{floxflox} optic nerves following injury.

3.6 Nogo-A distribution and neuronal survival in the retinae of Cnp-Cre+/xRtn4^{floxflox} mice

Western blot analysis revealed a down-regulation of Nogo-A by ~85% in glial Nogo-A KO retinae (Figure 3A), although no oligodendrocytes are present in this tissue. As in the intact retina of adult mice the bulk of Nogo-A is expressed by Müller glial cells (Pernet et al., 2011), we hypothesized that Nogo-A down-regulation may derive from expression changes occurring in these cells. In fact, qRT-PCR measurements showed a transitory expression of Cnp mRNA in the postnatal retina (Supplementary Figure S1B). By immunohistochemistry, the CNP protein was localized in Müller cell processes labelled with the specific marker cellular retinaldehyde binding protein (CRALBP) at P8 (Figure 3E). Nogo-B levels were not changed in Cnp-Cre+/xRtn4^{floxflox} retinae (Figure 3A). Compared to intact, control mouse retinal flat-mounds, in which Nogo-A appeared mostly in the end-feet of Müller cells around βIII-Tubulin-labelled retinal ganglion cell bodies, Nogo-A expression was abolished in the Müller cells of Cnp-Cre+/xRtn4^{floxflox} KO retinae (Figure 3B). Strikingly, in the same retinae, Nogo-A was clearly up-regulated in RGCs. Quantitatively, in intact control retinae ~7% of RGCs expressed Nogo-A, whereas in the intact Cnp-Cre+/xRtn4^{floxflox} KOs ~55% of RGCs showed a bright signal for the Nogo-A protein (Figure 3C). Two weeks after optic nerve crush injury, the proportion of cells expressing Nogo-A rose to ~50% in the surviving retinal ganglion cell bodies of Rtn4^{floxflox} control mice, but remained at ~55% in Cnp-Cre+/xRtn4^{floxflox} animals, a percentage that did not differ from the intact condition (Figure 3D). The density of surviving βIII-Tubulin-positive RGCs was examined two weeks after optic nerve crush injury on retinal flat-mounds (Figure 3F). The retinal ganglion cell survival was slightly, but significantly increased in Cnp-Cre+/xRtn4^{floxflox} KO animals compared to the control genotypes, from ~25% in Rtn4^{floxflox} control to ~33% in Cnp-Cre+/xRtn4^{floxflox} KO retinae (Figure 3G).

3.7 Nogo-A expression is required in retinal ganglion cells for optic axon regeneration

We selectively ablated Nogo-A in RGCs of Rtn4^{floxflox} mice by infection with an adeno-associated virus serotype 2 containing the Cre cDNA (AAV2.Cre). After four weeks of
Figure 3. Retinal ganglion cell body analysis reveals Nogo-A up-regulation and higher cell survival in Cnp-Cre\textsuperscript{+/-}\times\textit{Rtn4}floxed/floxed knock-outs. (A) In the retina, Nogo-A expression was down-regulated by \(~85\%\) while Nogo-B was unchanged in the Cnp-Cre\textsuperscript{+/-}\times\textit{Rtn4}floxed/floxed KOs. (B) In intact, Cnp-Cre\textsuperscript{+/-} control retinae, Nogo-A was mostly located in Müller cell end-feet surrounding βIII-Tubulin-labelled retinal ganglion cell bodies. In contrast, in Cnp-Cre\textsuperscript{+/-}\times\textit{Rtn4}floxed/floxed KOs, Nogo-A expression was abolished in Müller cells and was strongly up-regulated in RGCs. (C) Quantitatively, \(~7\%\) of RGCs expressed Nogo-A in intact control retinae, whereas \(~55\%\) of RGCs expressed the Nogo-A protein in the glial Nogo-A KOs (n=4 per group). (D) Two weeks after ONC injury the density of RGCs expressing Nogo-A was not significantly different between \textit{Rtn4}floxed/floxed and Cnp-Cre\textsuperscript{+/-}\times\textit{Rtn4}floxed/floxed mice (n=3 per group). (E) In the retina, the CNP protein was expressed in Müller cells identified by using the cell-type specific marker retinaldehyde binding protein (CRALBP). (F) Two weeks after injury, the density of surviving RGCs was evaluated by immunostaining for βIII-Tubulin on retinal flat-mounts. (G) Retinal ganglion cell survival was slightly, but significantly increased in Cnp-Cre\textsuperscript{+/-}\times\textit{Rtn4}floxed/floxed KO animals compared to the control genotypes (n=3-7). Statistics: one-way ANOVA, Bonferroni’s Multiple Comparison Test, *p<0.05, **p<0.01, ***p<0.001. Scale bars: B, E, F = 50μm; B insets = 10μm.
Figure 4. Neuronal Nogo-A ablation in retinal ganglion neurons reduces axonal sprouting in the injured optic nerve. (A-B) WT and Rtn4\textsuperscript{flx/flx} mice were intraocularly injected with AAV2.Cre virus 4 weeks prior to optic nerve crush injury. Five days after crush operation (5dpo), Nogo-A was detected in ~50% of RGCs in WT animals. In contrast, the proportion of injured RGCs expressing Nogo-A was reduced to ~15% after transduction with AAV2.Cre virus in Rtn4\textsuperscript{flx/flx} animals. (C) Two weeks after injury ~52% of RGCs expressed Nogo-A in WT control mice, whereas only ~15% of surviving RGCs contained Nogo-A in AAV2.Cre-injected Rtn4\textsuperscript{flx/flx} mice, and ~25% of RGCs were positive for Nogo-A in AAV2.Cre-injected Cnp-Cre\textsuperscript{+/−}\times Rtn4\textsuperscript{flx/flx} KOs. (D) For the axonal regeneration study, the AAV2.Cre virus was injected 4 weeks before the optic nerve crush injury and the number of CTB-A594 positive fibers was counted two weeks after injury. Quantitatively, compared to WT mice injected with AAV2.Cre, the number of axons growing after the lesion was statistically lower in the AAV2.Cre-injected Rtn4\textsuperscript{flx/flx} animals. AAV2.Cre injection in the Cnp-Cre\textsuperscript{+/−}\times Rtn4\textsuperscript{flx/flx} prevented axonal regeneration improvement in glial-Nogo-A KOs (mean±S.E.M., two-way ANOVA, Bonferroni posttest, *p<0.05). Scale bar: A = 50μm.
Supplementary figure 4. AAV2.Cre mediated deletion of Nogo-A from the retinal ganglion cells of glial Nogo-A KO mice. (A-B) By double immunostaining for Nogo-A and βIII-Tubulin, we observed that ~55% of retinal ganglion cells expressed Nogo-A in intact Cnp-Cre^{+/+}xRtn4^{fl/fl} mice, while 4 weeks after infection with AAV2.Cre, the number of Nogo-A expressing retinal ganglion cells reduced to ~30%. Scale bar: A = 50μm.
incubation, AAV2.Cre infection reduced the number of Nogo-A-expressing RGCs in intact Cnp-Cre+/xRtn4floxflox mice from ~55% to ~30% (Supplementary Figure S4A and B). The injury-induced up-regulation of Nogo-A at days 5 and 14 was markedly reduced in retinal ganglion cells transduced with AAV2.Cre in Rtn4floxflox animals, from 50% Nogo-A-positive, surviving cells in WT mice to ~15% in the knock-down retinae at the two time points (Figures 4A-C). In Cnp-Cre+/xRtn4floxflox mice, the percentage of Nogo-A-expressing RGCs decreased from ~55% in intact retinae to ~25% at 2 weeks after nerve crush (Figure 4C). The knock-down of Nogo-A in the RGCs resulted in a statistically lower number of sprouting fibers at two weeks post-injury compared with WT mice injected with AAV2.Cre. Moreover, AAV2.Cre injection in Cnp-Cre+/xRtn4floxflox mice completely prevented the enhancement of regenerative sprouting that we observed in these animals without the AAV2.Cre injection (Figure 4D).

To genetically ablate Nogo-A in RGCs, a neuron-specific Nogo-A KO mouse line was generated by crossing mice expressing Cre recombinase under the control of the Thy1 promoter (Moore et al., 2009) with Rtn4floxflox mice (Figure 5A). By Western blotting in the optic nerve, neuronal KOs showed a Nogo-A reduction by ~40% compared to ~80% down-regulation in glial KOs (Figure 5B). In the primary motor cortex, Nogo-A was reduced by ~40% in glial and by ~70% in neuronal KOs (Figure 5C). As shown before, compared with the glial Nogo-A KO retinae where Nogo-A expression was reduced by ~90%, we found ~40% reduction of Nogo-A in the neuron-specific KO retinae (Figure 5D). Importantly, regenerative axonal sprouting was decreased in neuronal Nogo-A KO mice after optic nerve injury with respect to Rtn4floxflox mice. Quantitatively, the number of axons growing through the lesion was statistically diminished in Thy1-Cre+tg×Rtn4floxflox animals at 50 and 100μm past the injury site compared with the control Rtn4floxflox group (Figure 5E).

3.8 The immunohistochemical signal for the Nogo receptor NgR1 is increased in Cnp-Cre+/xRtn4floxflox retinae

NgR1 is a well characterized GPI-linked Nogo-A receptor expressed by intact RGCs in Rtn4floxflox control and Cnp-Cre+/xRtn4floxflox KO mice (Figure 6A). By immunohistochemistry in intact retinal flat-mounts, the level of the NgR1 protein detected in the retinal ganglion cell bodies was found to be higher in the neurons that expressed a higher level of Nogo-A compared with cells that showed weak Nogo-A signal in the Cnp-Cre+/xRtn4floxflox KO
Figure 5. Axonal regeneration is reduced in neuron-specific Nogo-A knock-out animals. (A) To obtain a neuron-specific Nogo-A KO mouse line, mice expressing Cre-recombinase under the control of Thy1 promoter (Moore et al., 2009) were crossed with \( \text{Rtn}^{\text{floxed}} \) mouse line. (B-D) Nogo-A and Nogo-B protein expression levels were studied by Western blotting in control (\( \text{Rtn}^{\text{floxed}} \)), glia- (\( \text{Cnp}^-\text{Cre}^+/-x\text{Rtn}^{\text{floxed}} \)) and neuron-specific Nogo-A KO (\( \text{Thy1-Cre}^+/-x\text{Rtn}^{\text{floxed}} \)) mice. (B) In the optic nerve, the down-regulation of Nogo-A was more pronounced in glial (~80%) than in neuronal (~40%) KOs, while Nogo-B was similarly up-regulated in the two KO mouse lines. (C) In the primary motor cortex, the down-regulation of Nogo-A was less pronounced in glial (~40%) than in neuronal (~70%) KOs. Nogo-B was up-regulated in the two KO mouse lines. (D) In the retina, Nogo-A expression was reduced by ~90% in glial and by ~40% in the neuron-specific KOs. Contrary to other tissues, the level of Nogo-B was not changed in the retina of the two conditional KO mouse lines. Statistics: one-way ANOVA, Bonferroni’s Multiple Comparison Test, *p<0.05, **p<0.01, ***p<0.001. (E) The number of axonal fibers growing spontaneously through the lesion was statistically diminished in neuronal Nogo-A KO, \( \text{Thy1-Cre}^+/-x\text{Rtn}^{\text{floxed}} \) animals at 50, 100 and 200μm past the injury site when compared with \( \text{Rtn}^{\text{floxed}} \) control groups (mean±S.E.M., student’s T-test, *p<0.05).
Figure 6. The correlation of Nogo-A and NgR1 expression levels in retinal ganglion cells suggests possible cis-interaction. (A) NgR1, Nogo-A and βIII-Tubulin were examined by immunohistochemistry on retinal flat-mounts in Rtn4\textsuperscript{floX/floX} and Cnp-Cre\textsuperscript{-}\texttimes Rtn4\textsuperscript{floX/floX} KO mice. (B) Nogo-A-positive RGCs in the Cnp-Cre\textsuperscript{-}\texttimes Rtn4\textsuperscript{floX/floX} mice were divided into high (above the mean level of the control mice) and low expressing cells. (C) The levels of NgR1 correlated with the Nogo-A levels in these two populations of intact RGCs. (D-E) Rho-A pull-down experiments revealed lower activated Rho-A-GTP levels in the injured retinae of the Cnp-Cre\textsuperscript{-}\texttimes Rtn4\textsuperscript{floX/floX} KO mice. (F) Cartoon showing trans-activation of the NgR1-S1PR2/Rho-A signaling pathway by oligodendroglial Nogo-A. (G) Hypothetical cis-interaction between Nogo-A and its receptors in neurons, on the intracellular side, in the endoplasmic reticulum or at the cell membrane, leading to the downstream signaling blockade of Rho-A. Scale bar: A = 20μm.
Chapter 2: Targeted Nogo-A ablation promotes axonal growth

Supplementary figure 5. *NgR1* and *Nogo-A* mRNA expression analysis in intact and injured retinas. (A) qRT-PCR analysis of *NgR1* mRNA levels in intact and injured retinae did not reveal expression differences between the *Rtn4*^fl/fl^ and *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ genotypes. (B) *Rtn4/Nogo-A* transcript levels were strongly decreased in *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ KO mice compared with *Rtn4*^fl/fl^ control retinal lysates with and without optic nerve injury.

Supplementary figure 6. Analysis of axonal regeneration and survival related signaling cascades. (A-B) By western blotting, the phospho-Cofilin levels were not different between intact and injured, control and *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ KO mice. (C) On intact retinal cross-sections, phospho-Cofilin appeared in the inner nuclear layer (INL) and ganglion cell layer (GCL) by immunohistochemistry. After injury phospho-Cofilin was also detected in the optic fiber layer (OFL). (D-E) The phospho-CREB levels did not differ between intact and injured, control and *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ KO mice. (F) Phospho-CREB was weakly expressed in the INL of the retina. However, 3 days after optic nerve crush injury, a strong expression was detected in the retina ganglion cell layer. (G-H) The phospho-ERK1/2 levels were similar between intact and injured, control and *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ mice. (I) Phospho-ERK1/2 expression was induced in some Müller cells 3 days after optic nerve injury. (J-K) The phospho-STAT3 level was almost undetectable in intact retinae, but was up-regulated by ~6-fold after ONC injury. The *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ KOs tended to display lower phospho-STAT3 activation levels than in control conditions. (L-M) The phospho-Akt levels did not vary between intact and injured, control and *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ KO mice. No significant differences were found between the analysed phosphorylated protein levels of *Cnp-Cre*^+/−^x*Rtn4*^fl/fl^ and *Rtn4*^fl/fl^ animals by one-way ANOVA, Bonferroni’s Multiple Comparison Test. Scale bars: C, F and I = 50μm.
Chapter 2: Targeted Nogo-A ablation promotes axonal growth

A

B

C

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animals (Figure 6B and C). The overall mRNA expression level of NgR1, however, was not different between the two genotypes in intact and injured conditions (Supplementary Figure 5A). The Nogo-A mRNA level reduction was also confirmed with qRT-PCR experiments (Supplementary Figure 5B). We hypothesized that the stronger NgR1 signal observed by immunohistochemistry could reflect the increased association of Nogo-A with NgR1 in Nogo-A-positive RGCs. This close proximity could lead to a cis-interaction between the ligand and its receptor intracellularly or on the cell membrane and potentially lead to the attenuation of trans Nogo-A signaling (Figure 6F and G). To test this hypothesis, the activation of the downstream Nogo-A/NgR1 signaling mediator Rho-A was studied in Rho-A-GTP pull-down experiments. In the intact conditions where the Rho-A signaling pathway is not activated, no differences could be detected in Rho-A-GTP levels between Rtn4\textsuperscript{fl/fl} control and Cnp-Cre\textsuperscript{+/-}xRtn4\textsuperscript{fl/fl} KO retinae. However, after optic nerve crush injury, significantly less Rho-A-GTP was detected in the retinae of the Cnp-Cre\textsuperscript{+/-}xRtn4\textsuperscript{fl/fl} mice than in samples collected from control animals (Figure 6D and E). Downstream of Rho-A, the level of the inactive, phosphorylated form of the actin filament severing enzyme Cofilin was however not changed in Cnp-Cre\textsuperscript{+/-}xRtn4\textsuperscript{fl/fl} retinae (Supplementary Figure S6A-C). None of the regeneration and survival related signaling pathways such as the cAMP response element-binding protein (CREB), extracellular-signal-regulated kinase 1/2 (ERK1/2), signal transducer and activator of transcription 3 (STAT3) and protein kinase B (AKT) analyzed by Western blotting (Supplementary Figure S6D-M) and immunohistochemistry (Supplementary Figure S6C, F, I) showed significant difference between control and Cnp-Cre\textsuperscript{+/-}xRtn4\textsuperscript{fl/fl} retinae.

In vitro, the role of neuronal Nogo-A was tested by plating DRG-derived F11 cells on substrates coated with increasing concentrations of the Nogo-A inhibitory fragment Nogo-A-\Delta20 (\Delta20); Nogo-A inhibited neurite outgrowth in a concentration dependent way (Supplementary Figure S7B). Overexpression of Nogo-A in the F11 cells by AAV2.Nogo-A attenuated the inhibition of neurite outgrowth on the Nogo-A-\Delta20 substrate (Supplementary Figure S7A and B). This result suggests a cell-autonomous effect of neuronal Nogo-A on neurite outgrowth and supports the possibility that cis-interaction between Nogo-A and its receptors may counteract the inhibitory effect of Nogo-A present in the environment.
Supplementary figure 7. AAV2-mediated over-expression of Nogo-A in F11 cells partially reduces neurite outgrowth inhibition by extracellular Nogo-A. (A) After transduction with AAV2.GFP or AAV2.Nogo-A viruses and 48h after forskolin-induced differentiation, the neurites of F11 cells were labelled with an anti-βIII-Tubulin antibody. (B) The inhibitory effect of coated Nogo-A-Δ20 on F11 cell neurite outgrowth is attenuated at 2.5µg/cm² by AAV2.Nogo-A infection (t-test; **p<0.01). Scale bar: A = 100µm.
4 Discussion

In the present study we aimed to investigate whether glial and neuronal Nogo-A differently influence axonal growth in vivo. We created cell type-specific Nogo-A KO mouse lines and applied adeno-associated virus-mediated recombination of the Nogo-A gene in neurons. Our results show that axonal sprouting is significantly increased in the optic nerves of oligodendrocyte-specific Nogo-A KO mice and accompanied by a decrease in Rho-A activation in the retina.

4.1 Axonal regeneration in conventional versus cell type-specific Nogo-A KO lines

The characterization of Cnp-Cre+/xRtn4floxflox mice (glial Nogo-A KO mice) revealed oligodendrocyte-specific ablation in myelinated CNS regions and deletion of Nogo-A from Müller glial cells in the retina. In these mice, axonal regeneration was increased compared with control animals, in line with the effects obtained by acute pharmacological treatments such as the anti-Nogo-A antibody infusion, the application of the soluble NgR1 decoy receptor, or Rho-A/ROCK blocking agents (Dergham et al., 2002, Li et al., 2004, Liebscher et al., 2005). The improved regeneration that we reported here for the optic nerve is in contrast with what has previously been observed in systemic, conventional KO mice (Pernet et al., 2011). We speculate that the lack of axonal regeneration improvement observed after optic nerve crush in the conventional, full KO mouse line may be due, at least in part, to the lack of neuronal Nogo-A expression (Pernet et al., 2011). Nogo-A levels are up-regulated after injury in many RGCs, and the excision of Nogo-A gene by two different experimental approaches, the AAV2.Cre-mediated recombination and generation of a neuron-specific Nogo-A KO mouse line, decreased the retinal ganglion cell growth potential and prevented regeneration improvement in Cnp-Cre+/xRtn4floxflox KOs. Taken together, our data show that targeted glial Nogo-A deletion appears to be the most effective way to stimulate axonal growth in the injured optic nerve.

4.2 The limitations of axonal regeneration by Nogo-A and guidance molecules in the injured optic nerve

Glial Nogo-A neutralization potentiated inflammation-induced axonal regeneration after optic nerve crush injury. The combined treatment effect emphasizes the importance of making the environment more permissive to boost regeneration upon intrinsic growth mechanism activation. The poor intrinsic neuronal growth can be improved by the deletion
of phosphatase and tensin homolog (PTEN) and suppressor of cytokine signaling 3 (SOCS3), two key players for cell growth inhibition (Sun et al., 2011). Moreover, inflammation-inducing agents such as Zymosan or Pam3Cys have been shown to increase the intrinsic growth potential of adult neurons, thereby leading to enhanced regeneration (Yin et al., 2003, Hauk et al., 2010). Acting at two levels, the neuronal growth program activation with Zymosan and the blockade of Nogo-A signaling potentiated regenerative growth (Fischer et al., 2004, Dickendesher et al., 2012, Wang et al., 2012). However, our data suggested that the range of axonal regeneration may be restricted by compensatory up-regulation of EphA4 and EphrinA3 in glial cells. Further investigation are required to determine 1) how Nogo-A gene ablation causes Ephrin expression increase, 2) if additional Ephrins and Semaphorins are up-regulated as well and 3) if this mechanism can be neutralized to potentiate axonal regeneration in the damaged CNS.

Our previous three-dimensional analyses revealed axonal guidance and patterning defects reflected by increased axonal U-turns and branching after growth induction in the injured optic nerve (Luo et al., 2013, Pernet et al., 2013). In the present study, we hypothesized that the myelin Nogo-A could play a role in axon guidance after optic nerve lesion. However, by examining individual growing axons in 3D, we found no significant difference in the proportion of branching or U-turn-forming axons between mouse genotypes. The compensatory enhancement of EphrinA3 in the optic nerve and EphA4 in the retina that we detected in Cnp-Cre+/xRtn4flx/flox KOs could influence the guidance of regenerating axons in a complex manner. For example, we reported that EphA4 KO mice were less prone to form branches in the injured optic nerve than WT animals (Joly et al., 2014). In Cnp-Cre+/xRtn4flx/flox KOs, the lack of guidance and branching changes could result from the increase of EphA4 or EphrinA3 signaling in regenerating neurons. In future studies, the impact of compensatory myelin protein elevations and possibly of that of other repulsive molecules should be further addressed on axonal guidance in Cnp-Cre+/xRtn4flx/flox KO mice (Giger et al., 2010, Pernet and Schwab, 2014).

4.3 The role of Nogo-A in neuronal survival

We found that retinal ganglion cell survival was slightly, but significantly increased in the glial Nogo-A KO animals compared to control genotypes. Recently, several studies have suggested that neuronal Nogo-A might play a cell-autonomous role in improving neuronal survival under conditions of oxidative stress by scavenging reactive oxygen species (Mi et al., 2012), inhibiting oxidative damage (Guo et al., 2013) or by recruiting cytoprotective proteins (Kern et al., 2013). The moderate retinal ganglion cell survival observed in our
study could be related to the neuronal Nogo-A up-regulation in the glial Nogo-A KOs. Moreover, the activation of Rho-A by myelin Nogo-A could be damaging for neurons after injury; blocking Rho-A activation after spinal cord injury protected cells from p75NTR-dependent apoptosis (Dubreuil et al., 2003). Similarly, the increase of retinal ganglion cell survival in Cnp-Cre+/xRtn4flx/flx mice could be due to the reduction of Rho-A activation that we observed in axotomized retina.

### 4.4 Nogo-A and Nogo receptors in retinal ganglion cells: trans and cis-interaction?

In our study, the increase of the NgR1 immunohistochemical signal in the highly Nogo-A-positive RGCs of Cnp-Cre+/xRtn4flx/flx KOs could reflect cis-interactions between the ligand and receptor. At the cell membrane, the sequestration and inactivation of ligands by cis-binding to adjacent receptors has previously been described to prevent trans-activation in the case of guidance molecules such as Ephrins (Egea and Klein, 2007), Semaphorins (Haklai-Topper et al., 2010) or Slit-Robo (Jaworski and Tessier-Lavigne, 2012), but also for the Notch signaling pathway (Sprinzak et al., 2010). The co-expression of Nogo-A and NgR1 in RGCs of Cnp-Cre+/xRtn4flx/flx KO mice could prevent downstream activation of Rho-A after optic nerve crush injury and Nogo-A over-expression in F11 cells could partially counteract neurite outgrowth inhibition induced by Nogo-A-D20. These results suggest a cell-autonomous effect of neuronal Nogo-A on neurite outgrowth, potentially through cis-interaction with the Nogo receptors NgR1 or S1PR2.

In summary, the generation of cell type-specific KO mice allowed to observe that targeted deletion of Nogo-A from glial cells promotes more neuronal growth in the injured optic nerve than what has previously been reported in conventional KO animals. The inactivation of Nogo-A in glia appears as an optimized strategy to promote axonal regeneration in the CNS.

**Conflict of Interest**
The authors declare no conflict of interest.

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5 Materials and Methods

Animals

\(Rtn4^{\text{flox/flox}}\) mice were generated by TaconicArtemis. Briefly, the conditional \(Rtn4\) exon 3 allele was generated by homologous recombination in C57BL/6N ES cells. Exon 3 was flanked by LoxP sites using a targeting vector with 7.2 kb (long arm) and 3.2 kb (short arm) homologous sequences, respectively. Correct targeting was verified in ES cells using Southern Blotting before performing blastocyst injections. Highly chimeric male offsprings were bred to C57BL/6 females, transgenic for the presence of a recombinase gene (Flp-Deleter line). Germline transmission was identified by the presence of black coat color, strain C57BL/6. Flp mediated removal of the FRT site flanked positive selection marker gene (PuroR) was verified in the next generation. Cre mediated excision of exon 3 resulted in specific loss of function of the \(Rtn4\) isoform A (Nogo-A), whereas the \(Rtn4\) isoforms B and C (Nogo-B and Nogo-C) were still expressed. Glia and neuron-specific Nogo-A knock-out mice were generated to study the role of glial versus neuronal Nogo-A on axonal regeneration. \(Rtn4^{\text{flox/flox}}\) mice were crossed with mice expressing Cre-recombinase under the control of the 2',3'-cyclic nucleotide phosphodiesterase (Cnp) or Thy1 promoter, respectively. The Cnp-Cre line was generated in the laboratory of Klaus-Armin Nave (Lappe-Siefke et al., 2003). The \(Thy1-Cre\) line was obtained from the Jackson Laboratory (FVB/N-Tg(Thy1-cre)1Vln/J) and back-crossed to C57BL/6 background more than eight times before the breeding was started with \(Rtn4^{\text{flox/flox}}\) animals. All procedures and surgeries were performed on 2–4 month old mice of mixed genders and different genotypes. Animal experiments were performed in accordance with the guidelines of the Veterinary Office of the Canton of Zurich.

AAV vector production and application

An AAV2.Cre-mCherry expressing virus was generated to selectively infect RGCs. Adeno-associated viral vectors were produced by standard methods. To selectively excise Nogo-A from retinal ganglion cell neurons in the retina, the AAV2.Cre-mCherry virus was injected to
mice with $Rtn4^{flx/flx}$ genotype. 1.5μl of AAV2.Cre-mCherry virus (10$^{14}$ vg/ml) was administered intraocularly using a 10μl Hamilton syringe adapted with a pulled-glass tip. To allow the diffusion of the virus, the needle was kept in place for 3–4 min and then carefully removed. Attention was paid not to damage the lens during the injections. To allow time for optimal transgene expression in vivo, the injections were performed 4 weeks before the optic nerve crush (ONC) injury.

**Axonal regeneration analysis on longitudinal optic nerve sections**

We performed ONC injuries to study axonal regeneration in the optic nerve and neuronal survival in the retina. The optic nerve was exposed intra-orbitally and then crushed by tying a knot with a 9-0 suture at ~0.5mm from the back of the eye. The suture was carefully removed and the integrity of the ophthalmic artery was examined by fundus examination. To induce intraocular inflammation, 2μl of 1% Zymosan dissolved in PBS was injected intraocularly at the time of the injury. Thirteen days after ONC, the optic axons were anterogradely traced by intraocularly injecting 1.5μl of 0.5% cholera toxin B conjugated to Alexa594 (CTB-A594, Molecular Probes). On the following day, the animals were perfused with 4% paraformaldehyde (PFA) and the optic nerves were processed as described below. CTB-A594-positive axons were observed on longitudinal optic nerve sections (14μm) with a Zeiss Axioskop 2 Plus microscope and images were taken with a CCD video camera at 20X magnification. The number of growing axons was estimated at different distances (50, 100, 150, 200, 300, 400, and 500 to 1’000μm) after the lesion site. Five-six optic nerve sections were analysed per animal. An estimation of the number of axons per optic nerve ($\Sigma$) was calculated with the following formula: $\Sigma_d = \Pi \times R^2 \times$ (average number of axons/mm)/T. The sum ($\Sigma$) of axons at a given distance (d) was obtained using the average optic nerve radius (R) of all optic nerves at 500μm past the lesion site, and a thickness (T) of the tissue slices of 14μm. For statistical analysis with multiple comparisons, ANOVA test was applied followed by a Bonferroni’s post hoc test. Animals with retinal haemorrhages or ischemia were excluded from the analysis.

**Optic nerve preparation and clearing for 3D analysis of regenerating axons**

To visualize CTB-A594-labeled axons in the whole injured optic nerve, the tissue was cleared following the adapted protocol from (Luo et al., 2013). After intracardial perfusion with PBS and 4% PFA, optic nerves were further fixed overnight, then rinsed with PBS and stored at 4°C. The samples were dehydrated in baths of increasing concentrations of tetrahydrofuran (THF, Sigma-Aldrich) (50, 80, and 100%) for 30min each and for additional 30min in 100% THF at room temperature under constant agitation. To remove traces of
water, optic nerves were placed in dichloromethane for 3-4 hours at room temperature. In the BABB clearing solution (mixture of benzyl alcohol and benzylbenzoate (1:2, Sigma-Aldrich)) the white optic nerve turned transparent within 30s. The whole optic nerves were mounted in the clearing medium before imaging. Image stacks were taken using an inverted confocal Leica SP5 microscope equipped with a 63X glycerin immersion objective (NA: 1.3). This setup was used to scan axons throughout the whole thickness of the optic nerve. To obtain 3D reconstruction of the optic nerves, image stacks were stitched using the XuvTools42 software (Emmenlauer et al., 2009) and the resulting macro-stack was exported to the Imaris Software (Bitplane) to create 3D projections. Individual axons were analysed semi-automatically with the Filament Tracers’ advanced manual tracing mode (‘AutoDepth’). The percentage of U-turns and branching was calculated between 100 and 300μm past the lesion site. The best traced 30-60 fibers were selected for growth pattern analysis.

Neuronal survival analysis
Retinal ganglion cell survival was examined two weeks after ONC injury. The animals were intracardially perfused with 4% PFA and the injured and intact retinas were flat-mounted. RGCs were visualized by immunostaining for βIII-Tubulin (1:1’000, Promega), a specific and reliable marker labelling all RGCs. The antibodies were diluted in blocking solution (0.3% Triton-X-100, 5% of normal serum and 0.05% sodium azide in PBS), and the retinas were incubated at 4°C with primary and secondary antibodies, for 5 and 3 days, respectively. The βIII-Tubulin-positive RGCs were imaged in the four quadrants of the retina using a Leica SPE-II confocal microscope equipped with a 40X oil immersion objective (NA 1.25). Image stacks were acquired in the ganglion cell layer with a step size of 0.5μm and a resolution of 1'024×1'024 pixels (0.275μm/pixel). The number of retinal ganglion cell bodies was quantified in grids of 62'500μm² at 1 and 1.5mm distances from the optic disc. The density of surviving RGCs was calculated per mm².

Retina and optic nerve processing and immunostaining
Mice were sacrificed by injecting an overdose of anaesthetics intraperitoneally and perfused intracardially with PBS and 4% PFA. Optic nerves and eyes were dissected, the latter by removing the cornea and the lens from the eyecup. For retinal cross sections and longitudinal optic nerve sections, the eye cups and optic nerves were post-fixed in 4% PFA overnight at 4°C. The tissues were then cryoprotected in 30% sucrose and frozen in OCT compound (Tissue-TEK, Sakura) with a 2-methylbutane bath cooled with liquid nitrogen. Optic nerves and retinal sections were cut (14μm) with a cryostat. Immuno-histochemical
stainings were performed in a blocking solution (5% of normal goat serum or 5% BSA, 0.3% Triton-X-100, and 0.05% sodium azide in PBS). Primary antibodies were applied overnight at 4°C and after PBS washes, sections were incubated with the appropriate secondary antibody for 1h at room temperature. The slides were mounted in Mowiol solution (10% Mowiol 4–88 (Calbiochem) in 100mM Tris, pH 8.5, 25% glycerol and 0.1% DABCO). Primary antibodies were: rabbit anti-Nogo-A (Laura, Rb173A40) serum (1:200), mouse anti-GS (1:300, Chemicon), rabbit anti-CRALBP (1:1’000, kindly provided by Prof. JC Saari, Washington), mouse anti-APC (1:300, Chemicon), mouse anti-CNP (1:300, Chemicon), goat anti-NgR1 (1:50, R&D Systems), rabbit anti-phospho-ERK1/2 (1:100, Cell Signaling), rabbit anti-phospho-CREB (1:100, Cell Signalng), rabbit anti-phospho-Cofilin (1:200, Abcam) and mouse anti-βIII-Tubulin (1:1’000, Promega). Immunofluorescent labelling was analysed with a Zeiss Axioskop 2 Plus microscope or with Leica SPE-II confocal microscope.

Western blot analysis
After cervical dislocation, retinae and optic nerves were quickly dissected out and flash frozen in liquid nitrogen. Tissues were kept at -80°C until extraction in lysis buffer (RIPA buffer: 150mM NaCl, 1% NP40, 0,5% deoxycholate, 0.1% SDS in 50mM Tris buffer at pH8) containing protease inhibitors (Complete mini, Roche Diagnostics). Samples were fully homogenized and let on ice for 60 min. After centrifugation for 15 min at 15'000xg, 4°C, supernatants were collected and processed for protein concentration analysis (Bio-Rad Laboratories, RC DC Protein Assay). Retinal and optic nerve proteins (20μg/lane) were separated by electrophoresis on a 4–12% polyacrylamide gel and transferred to nitrocellulose membranes. Blots were incubated in a blocking solution of either 2% Top Block (Lubio Science) or 5% BSA (bovine serum albumin) in 0.2% TBST (0.2% Tween-20 in Tris-base 0.1 M, pH7.4) for 1 hour at room temperature, then incubated with primary antibodies overnight at 4°C. Following the washing steps, membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10’000-1:25’000; Pierce Biotechnology). Primary antibodies were rabbit anti-Nogo-A/B (Bianca, Rb140) serum (1:20’000), mouse anti-L-MAG serum (1:1’000, polyclonal) (Erb et al., 2003), mouse anti-EphA4 (1:200, Invitrogen), rabbit anti-EphrinA3 (1:200, Invitrogen), mouse anti-S1PR2 (1:500, Santa Cruz Biotechnology), rabbit anti-phospho-STAT3 (1:500, Cell Signaling), rabbit anti-STAT3 (1:500, Cell Signaling), rabbit anti-phospho-ERK1/2 (1:1’000, Cell Signaling), rabbit anti-ERK1/2 (1:1’000, Cell Signaling), rabbit anti-phospho-AKT (1:500, Cell Signaling), rabbit anti-AKT (1:500, Cell Signaling), rabbit anti-phospho-CREB (1:500, Cell Signaling), rabbit anti-CREB (1:500, Cell Signaling), rabbit anti-phospho-Cofilin (1:500, Abcam), rabbit anti-Cofilin (1:500, Cell Signaling), and mouse anti-GAPDH
Chapter 2: Targeted Nogo-A ablation promotes axonal growth

(1:20’000; Abcam). Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) in a Stella detector (Raytest). Band intensities were measured with the Image J software (NIH).

Rho-A pull-down

After rapid dissection of intact and injured (3 days post operation) retinas in TBS, the samples were flash frozen in liquid nitrogen and stored at -80°C until performing the Rho-A pull-down. The pull-down of activated RhoA-GTP was subsequently performed using the RhoA Activation Assay Biochem Kit according to the manufacturer's instructions (Cytoskeleton, Inc.) combined with a previously described protocol (Pellegrin and Mellor, 2008). The Rho-A-GTP pull-down and total lysate samples were then subjected to electrophoresis on a 4–12% polyacrylamide gel and transferred to nitrocellulose membranes. After blocking with 5% BSA in 0.2% TBST for 1 hour, the membranes were incubated overnight at 4°C with a rabbit anti-Rho-A (1:400, Cell Signaling) primary antibody. The secondary antibody incubation and signal detection was performed as described before for the Western blots.

Semi-quantitative RT-PCR (qRT-PCR)

After cervical dislocation, intact and injured whole retinas were rapidly dissected in PBS. The samples were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Retinal RNA was prepared using the RNaseasy RNA isolation kit (Qiagen), including a DNase treatment to digest the residual genomic DNA. For reverse transcription, equal amounts of total RNA were transformed to cDNA by using oligo(dT) primers and M-MLV reverse transcriptase (Promega). 10ng of cDNA was amplified in the Light Cycler 480 thermocycler (Roche Diagnostics AG) with the polymerase ready mix (SYBR Green I Master; Roche Diagnostics). The following specific primers were designed to span intronic sequences or cover exon–intron boundaries: Gapdh (forward, 5’-CAGCAATGCATCCTGCACC-3’; reverse, 5’-TGGACTGTGGTCATGAGCCC-3’), Cnp (forward, 5’-AGGAGAAGCTTGAGCTGGTC-3’; reverse, 5’-CGATCTCTTCACACCCTCCT-3’), Nogo-A/Rtn4 (forward, 5’-CAGTGGATGACCCCTTTTTTG-3’; reverse, 5’-GCTGCTCCTTCAATCCATAA-3’), NgR1/Rtn4R (forward, 5’-CTCGACCCGAGATGAG-3’; reverse: 5’-TGATACACACAAAGCACCAG-3’), Gap-43 (forward, 5’-TGCTGTCATGATGCTGC-3’; reverse, 5’-GGCTTCGTCTACAGCGTCT-3’), small proline-rich protein 1A (Sprr1A, forward, 5’-GAACCTGCTCTTCTCTGAGT-3’; reverse, 5’-AGCTGAGGGAGGTACAGTG-3’). For relative quantification of gene expression, mRNA levels were normalized to GAPDH using the comparative threshold cycle ($\Delta\Delta^{CT}$) method and a
control sample was used to calculate the relative values. Each reaction was done in triplicate and 3-4 mice per condition were analysed.

Cell cultures

The effects of AAV2.GFP and AAV2.Nogo-A on neurite outgrowth were evaluated in vitro by infecting F11 cells. The F11 cell line, kindly provided by Prof. RE van Kesteren (Amsterdam, The Netherlands), was maintained as described before (MacGillavry et al., 2009). F11 cells were plated at a density of 100’000 cells per well in 6-well plates and were treated with AAV2.GFP or AAV2.Nogo-A viruses 24 h later. Four days after infection, F11 cells were transferred to 4-well dishes at a density of 2’000 cells per well. The 4-well dishes were coated by different concentrations of Nogo-A-∆20 substrate (0, 0.5, 1 and 2.5μg/cm²) diluted in PBS at 4°C for 12 hours. Twenty four hours after plating the cells on Nogo-A-∆20 substrate, F11 cells were differentiated by adding 10μM of Forskolin (Sigma-Aldrich) for 48 h. To visualize neurite extension, F11 cells were stained with βIII-Tubulin antibody. Measurement of neurite length was carried out using the Neuron J plugin in the Image J software (NIH). The mean of the total neurite length was calculated for the AAV2.GFP and AAV2.Nogo-A treatments on all four Nogo-A-∆20 substrate concentrations measuring between 80 and 110 cells.

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Chapter 3

Contribution of glial Nogo-A to molecular mechanisms underlying axonal regeneration and cell survival in the visual system

by Flóra Vajda

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Contributions:
F.V., V.P. and M.E.S.: designed the study. F.V. performed the optic nerve crush surgeries, harvested and processed tissues for qRT-PCRs (with the help of S.J.), for Western blotting and immunohistochemical stainings, acquired microscopic images, analyzed mRNA and protein expression levels. F.V. analyzed the data, prepared all the figures and wrote the manuscript (revised by V.P. and M.E.S.).

 manusctipt in preparation
1 Abstract

We previously found that both spontaneous and inflammation-mediated axonal regeneration and neuronal survival was increased in the glia-specific Nogo-A knock-out (KO), Cnp-Cre+/xRtn4^flx/flx mice. In the visual system, Nogo-A is highly expressed in oligodendrocytes of the optic nerve and in Müller cells of the retina. Additionally, Nogo-A is up-regulated in retinal ganglion cells (RGC) after optic nerve injury, where we described its positive influence on axon growth and potentially on cell survival. Based on these previous findings, here we further investigated the role of Nogo-A in gliosis and its possible involvement in the molecular mechanisms of cell death. We found that the deletion of Nogo-A from Müller cells in the glial Nogo-A KOs did not influence the expression regulation of the Müller cell marker glutamine synthetase (GS/GLUL) or the Müller glial activation marker glial fibrillary acidic protein (GFAP). However, in injured conditions, the glial Nogo-A KO retinæ contained slightly increased level of vimentin mRNA. As Nogo-A is highly expressed in the endoplasmic reticulum (ER) and is up-regulated in the RGCs of the glial Nogo-A KO animals, we investigated the expression of ER-stress markers that could contribute to the higher RGC survival observed in these mice. Although the expression of C/EBP homologous protein (CHOP) and binding immunoglobulin protein/78kDa glucose-regulated protein (BiP/GRP78) were increased in the retinae of optic nerve injured mice, the mRNA levels were not significantly different between the observed genotypes. As the role of autophagy was reported in both neuroprotection and in programmed cell death of RGCs, we examined the levels of several autophagy-related genes (Atg) and proteins, but the glial Nogo-A KOs displayed no obvious differences compared to control genotypes. In addition, we screened the mRNA expression of all Nogo-66 receptor complex members and of related receptors, and found an up-regulation of NgR2 and TROY that could render RGC growth cones more sensitive to extrinsic inhibitory myelin cues. To confirm our regenerative and survival results obtained with the Cnp-Cre+/xRtn4^flx/flx mice, we generated and characterized a second oligodendrocyte specific conditional Nogo-A knock-out mouse line by crossing our Rtn4^flx/flx mice to the Mog-Cre^+/− deleter line. To our surprise, we only detected modest Nogo-A down-regulation in the optic nerves of Mog-Cre^+/−xRtn4^flx/flx animals, but as the expression of Nogo-A in Müller cells was not altered, we propose this model to be suitable for certain questions addressing the role of oligodendroglial Nogo-A separately.

In summary, here we tested possible molecular processes that may contribute to the increased axonal regeneration and neuronal survival in the glial Nogo-A KO mice. Despite the potential role that has been attributed to Nogo-A in ER-stress and autophagy in neuronal survival, our results show that Nogo-A up-regulation in neurons does not take part in ER
stress or autophagy-associated apoptosis. However, the changes we detected in NgR2 or TROY receptor expression might influence the growth responses after axonal injury.

**Keywords:**
glial Nogo-A; neuronal Nogo-A; retinal ganglion cells; Müller cells; optic nerve crush injury; gliosis; ER stress; autophagy

**Abbreviations:**
Atf6, activating transcription factor-6; Atg, autophagy-related genes; BiP/Grp78, binding immunoglobulin protein/78 kDa glucose-regulated protein; CHOP/Gadd153, C/EBP homologous protein; adeno-associated virus; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CSPG, chondroitin sulphate proteoglycans; eIF2α, eukaryotic initiation factor 2; ER, endoplasmatic reticulum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GCL, ganglion cell layer; GFAP, glial fibrillary acidic protein; GS/Glut, glutamine synthetase; INL, inner nuclear layer; KO, knock-out; LC3, microtubule-associated protein 1 light chain 3; LIF, leukemia inhibitory factor; LINGO-1, leucine rich repeat - LRR - and IgG domain containing Nogo receptor interacting protein 1; MAG, myelin-associated glycoprotein; MOG, myelin oligodendrocyte glycoprotein; NgR, Nogo66 receptor; OFL, optic fiber layer; OMgp, oligodendrocyte myelin glycoprotein; ONC, optic nerve crush injury; ONL, outer nuclear layer; p75NTR, low affinity neurotrophin receptor; PBS; phosphate-buffered saline, PirB, paired immunoglobulin-like receptor B; PNS, peripheral nervous system; qRT-PCR, quantitative real-time polymerase chain reaction; RGCs, retinal ganglion cells; RTN, reticulin; SEM, standard error of the mean; STAT3, signal transducer and activator of transcription 3; TBS, tris-buffered saline; TROY, tumor necrosis factor - TNFα - receptor superfamily member 19; UPR, unfolded protein response.
2 Introduction

Nogo-A is mostly known as an extracellular inhibitor of axonal regeneration, expressed in the myelin (Schwab, 2010). However, in the visual system in particular, its localization and expression regulation suggests further distinct functions in different cell types.

Firstly, in the intact retina, Nogo-A is expressed in the end-feet of retinal Müller glia surrounding RGC cell bodies. In response to axonal injury and the subsequent inflammation, radial processes of the Müller cells start expressing the gliosis marker GFAP (Chen and Weber, 2002, Lewis and Fisher, 2003, Pernet et al., 2011), a marker that has been widely used to quantify the extent of Müller cell activation. This gliosis of Müller cells following an optic nerve lesion leads to the secretion of cytokines and inflammatory molecules that influence the regenerative state of neurons. For instance, in response to optic nerve crush (ONC), Müller cells express CNTF (ciliary neurotrophic factor) and LIF (leukemia inhibitor factor), two cytokines that had been shown to be pro-regenerative (Leibinger et al., 2009, Pernet et al., 2013) and neuroprotective for RGCs and photoreceptors (Ju et al., 2000, Joly et al., 2008).

The expression of Nogo-A in RGCs in injured WT or in intact and injured glial Nogo-A KO mice suggests that Nogo-A acts as intrinsic growth and survival regulator in neurons (Pernet et al., 2011, Mi et al., 2012). In our previous study presented in Chapter 2, we have shown that possibly by cis-interaction with its receptors, expression of Nogo-A in neurons leads to decreased activation of Rho-A, serving an explanation how glial deletion of Nogo-A could lead to increased regeneration and survival. Additionally, intracellular Nogo-A located in the ER has been proposed to play a neuroprotective function by attenuating ER-stress (Yang et al., 2009). Disturbances such as axonal damage leading to increased Ca^{2+} levels or altered cellular redox regulation cause accumulation of unfolded proteins in the ER. This triggers an unfolded protein response (UPR) that facilitates either the adaptation to stress or leads to apoptosis, depending on the severity of the injury (Rutkowski and Kaufman, 2004). The expression of the ER-stress marker C/EBP homologous protein (CHOP) has been correlated to Nogo-A expression in neurons, and although the expression of both CHOP and Nogo-A was increased in injured RGCs, no causal link was found in their expression (Pernet et al., 2011). About 95% of Nogo-A in neurons is expressed intracellularly in membrane-bound state mostly associated to the ER, and this pattern could suggest that Nogo-A could also be expressed in other organelles such as in autophagosome isolation membranes. A recent study suggested that RTN3, a reticulon family member related to Nogo-A (RTN4) may regulate autophagy under ER stress conditions (Chen et al., 2011), and Nogo-A itself has been implicated in the protection against autophagic cell death (Teng and Tang, 2013).
Autophagy is a highly conserved catabolic intracellular degradation pathway that has been implicated in either pro-survival or pro-death mechanisms in RGCs (Russo et al., 2013). Autophagy is activated in RGCs 3-5 days after optic nerve axotomy and the up-regulation of Atg5, an autophagy regulator, has been described to play a cytoprotective role (Rodriguez-Muela et al., 2012).

In this study we aimed to investigate the possible role of Nogo-A in Müller cells in axonal regeneration and of neuronal Nogo-A in cell survival, possibly through its involvement in ER-stress or autophagic processes. Additionally, to gain further insights into the increased regeneration phenotype we previously observed in glial Nogo-A KO animals, we followed the expression changes of members of the Nogo-A receptor complex. Furthermore we generated and tested an alternative glial conditional Nogo-A KO mouse line (Mog-Cre\(^+/-\)xRtn4\(^{flox/flox}\)) that might allow us to study the separate contribution of oligodendroglial Nogo-A in the blockade of axonal regeneration, independently of the deletion of Nogo-A in Müller cells that occurred in the mouse line we have previously used (Cnp-Cre\(^+/-\)xRtn4\(^{flox/flox}\)).

3 Results

3.1 Müller cells injury response in glial Nogo-A KOs

The effect glial Nogo-A deletion on gliosis in intact and injured retina samples was first evaluated by qRT-PCR. The overall mRNA level of the gliosis marker glial fibrillary acidic protein (Gfap) increased, whereas glutamine synthetase (Glul) decreased 5 days after ONC injury, however no significant difference was found between Cnp-Cre\(^+/-\) control and Cnp-Cre\(^+/-\)xRtn4\(^{flox/flox}\) glial Nogo-A KO mice in either conditions (Figure 1A and B). In injured conditions, the glial Nogo-A KO retinae showed slightly increased levels of vimentin mRNA (Figure 1C), however this increase was modest and not consistent with the Gfap mRNA levels that were not different between the different genotypes. GFAP protein levels and localization was followed by Western blot analysis and immunohistochemistry in intact conditions and 3 days post injury. On the bulk of the protein content, we could not detect a difference between control and glial Nogo-A KO, or intact and injured retina lysates (Figure 1D and E). On retinal cross-sections, compared to intact retinae, where only astrocytes are stained for GFAP in the optic fiber layer (Figure 1F), we found high expression of GFAP in radial processes in injured conditions, reflecting strong Müller cells gliosis (Figure 1G). In our study, GFAP expression levels were not different between injured control and glial Nogo-A KO mice, where Nogo-A is excised from Müller cells. In summary, based on our
Chapter 3: Nogo-A in molecular processes contributing to regeneration and survival

(A) Gfap mRNA
(B) Glul mRNA
(C) Vimentin mRNA

(D) Retina

(E) GFAP protein

(F) Intact

(G) Injured
findings glial Nogo-A does not seem to contribute to the injury-triggered activation of Müller cells.

### 3.2 Regulation of ER stress-related genes in the retina of Cnp-Cre+/xRtn4floxflox mice

Optic nerve injury provoked strong mRNA up-regulation of two important ER stress-related molecules: Chop/Gadd153 (C/EBP homologous protein), a pro-apoptotic transcription factor suppressing the transcription of Bcl-2 (Figure 2A), and Bip/Grp78, an important protein chaperon abundant in the ER lumen possessing anti-apoptotic functions (Figure 2B). The level of elf2α (eukaryotic initiation factor 2) upstream of CHOP was also induced 3 days after ONC (Figure 2C). No changes were detected in the level of Atf6 (activating transcription factor-6), another mediator of the ER-stress cascade (Figure 2D). We have not found different regulation for any of the ER-stress related genes in intact or injured glial Nogo-A KO retinae. The anti-apoptotic marker Bcl-2 also showed increased mRNA expression after injury (Figure 2E), but its expression was comparable between different genotypes. On the protein level, CHOP was strongly increased in the nucleus of injured RGCs, but not in other retinal layers, showing the specificity of the signal detection. The deletion of Nogo-A from Müller cells and the initial up-regulation of Nogo-A in RGCs did not influence this CHOP activation pattern in Cnp-Cre+/xRtn4floxflox mice (Figure 3A).

### 3.3 The expression of autophagy related genes is not modified by glial deletion of Nogo-A

To study whether autophagy serves as an anti-apoptotic strategy in the glial Nogo-A knock-out mice, we measured the mRNA levels of several autophagy-related genes (Atg) (Figure 4). The microtubule-associated protein 1 light chain 3 (LC3, in mammals three homologous LC3 proteins: α, β and γ), a homolog of yeast Atg8, is either a cytosolic protein (LC3I) or localizes to autophagosomal membranes after post-translational modifications (LC3II). The
Figure 2. Regulation of the ER stress related genes and in the glial Nogo-A KO mice. The effect of Nogo-A deletion from glia on ER stress mechanisms in intact and injured retina samples was measured by qRT-PCR 3 days after ONC injury. (A) Chop/Gadd153 and (B) Bip/Grp78 were strongly up-regulated in response to the injury, but the magnitude if increase was comparable between the different genotypes. The mRNA levels of two upstream regulators (C) eIF2α and (D) Atf6, were slightly increased after injury to similar extent in all groups. (E) Bcl-2 also showed a small increase in mRNA expression after injury.

Figure 3. Protein expression and localization of CHOP after ONC injury. The elevation of CHOP was confirmed on protein levels. (A) CHOP was strongly increased in the nucleus of only injured RGCs, but not in other retinal layers. The activation pattern was not altered in Cnp-Cre+/xRtn4lox/lox mice. Scale bar: A = 50μm.
Figure 4. Regulation of the autophagy related genes and in the glial Nogo-A KO mice. The levels of (A) Atg3, (B) Atg5, (D) Atg12, (E) LC3α and (F) LC3β mRNA were increased in whole retinal lysates 3 days after optic nerve crush injury and no difference was detected between the glial Nogo-A KO and control groups. (C) The Atg7 mRNA level was unchanged after injury.
proteolytic cleavage and lipidation that turns LC3 into its active form that translocates to the autophagosomal membrane involves a cascade of events requiring the action of Atg3 and 7. In the macroautophagic process, the elongation and closure of the isolation membrane requires the covalent attachment of the protein Atg5 to Atg12 through an ubiquitin-like conjugation system and this association then also promotes the activation of LC3. Except Atg7, all analyzed autophagy mRNA products such as Atg3, Atg5, Atg12, LC3α and LC3β were increased to different extents in whole retinal lysates 3 days after optic nerve crush injury (Figure 4A-F), consistently with previous studies (Russo et al., 2013). However the levels of these mRNAs were not significantly different in the Cnp-Cre+/xRtn4flx/flx mice compared to control genotypes. The LC3 (I and II) protein was localized in the ganglion cell layer, but the ONC injury did not change the distribution of LC3 in the retina in either genotypes (Figure 5A). By Western blotting, the level of the Atg5/Atg12 protein complex was not changed in the glial Nogo-A KOs in intact or injured retinae (Figure 5B and C).

3.4 Regulation of Nogo-A receptors in the retina of Cnp-Cre+/xRtn4flx/flx mice

We observed interesting mRNA level alterations of the Nogo-66 receptor complex 5 days after ONC injury. As reported before, NgR1 mRNA decreased by ~40% in response to the injury (Pernet et al., 2011), but the overall level was not changed between the genotypes (Figure 6A). We also analyzed the expression level of a related NgR family member, NgR2, a receptor for MAG but not for Nogo-A (Venkatesh et al., 2005). As we previously found that MAG increased in conventional Nogo-A KO mice (Pernet et al., 2008), the interplay between the regulation of Nogo-A, MAG and NgR2 was especially interesting. Indeed, the NgR2 mRNA expression level was increased in the Cnp-Cre+/xRtn4flx/flx mice in both intact and injured retinae (Figure 6B). The amount of NgR3, one of the receptors for CSPGs, was not changed between conditions and genotypes (Figure 6C). We found a significant up-regulation of Troy in injured glial Nogo-A KO retinae (Figure 6D), but the levels of a further NgR1 receptor complex partner, Lingo-1 were not altered between the genotypes (Figure 6D). The expression level of PirB, an additional Nogo-66 receptor, was not statistically different in the glial Nogo-A KOs (Figure 6E). In the retina, p75 is mostly expressed in Müller cells (Hu et al., 1998), but its expression levels were not changed in the glial Nogo-A KOs where Nogo-A is ablated in this cell type in neither intact or injured conditions (Figure 6G).
Figure 5. Protein expression and localization of LC3 and Atg5/12 after ONC injury. (A) LC3 was localized in the ganglion cell layer, but the ONC injury have not changed the distribution of LC3 in the retina in neither of the genotypes. (B-C) The level of the Atg5/Atg12 protein complex was not changed in the glial Nogo-A KOs in intact or injured retinas. Scale bar: A = 50 μm.
Figure 6. Nogo-A receptor expression changes in the retina of Cnp-Cre\(^+/−\)/Rtn4\(^{flx/flx}\) mice. (A) The NgR1 mRNA decreased by ~40% in response to the injury, but the overall level was not changed between the genotypes. (B) The NgR2 receptor mRNA expression was significantly increased in both intact and injured Cnp-Cre\(^+/−\)/Rtn4\(^{flx/flx}\) retinae. (C) NgR3 was not changed between conditions and genotypes. (D) The NgR1-Lingo-1 co-receptor Troy was significant up-regulation in injured glial Nogo-A KO retinae. (E) PirB increased after injury, but not to a different extent in the glial Nogo-A KOs. (F) Lingo-1 was not altered between genotypes. (G) p75 expression levels were not changed in any conditions. All receptor mRNA levels were measured in intact retinae and 5 days after ONC injury (two-way ANOVA, *P<0.05, **P<0.01, ***p<0.001).
3.5 Model systems: *Mog-Cre+/xRtn4flox/flox* vs. *Cnp-Cre+/xRtn4flox/flox*

In the *Cnp-Cre+/xRtn4flox/flox* mice we found complete excision of Nogo-A from Müller cells that might have influenced regeneration in these glial Nogo-A KO animals, indirectly through influencing gliosis or by possible changes in the direct interaction with retinal ganglion cell bodies. To create a more specific oligodendroglial Nogo-A KO mouse line where Nogo-A is solely excised from oligodendrocytes but not from other glial cells, we crossed *Rtn4flox/flox* mice with the *Mog-Cre+/−* deleter mouse line. Surprisingly, in this different system where Cre expression is driven by the *Mog* gene expressed in oligodendrocytes, we only found ~25% Nogo-A down-regulation in the optic nerve at age P60 compared to ~80% down-regulation in the *Cnp-Cre+/−xRtn4flox/flox* mice (Figure 7A). The double immunostaining for Olig2 and Cre-recombinase confirmed that compared to the *Cnp-Cre+/−xRtn4flox/flox* optic nerves, where all Olig2 positive oligodendrocytes expressed Cre recombinase, Cre expression was very sparse and almost undetectable in the *Mog-Cre+/−xRtn4flox/flox* mice (Figure 7B). In the retina, we found no Nogo-A expression changes in *Mog-Cre+/−xRtn4flox/flox* animals (Figure 8A) and no alterations in Nogo-A localization in RGCs or Müller cells (Figure 8B).
Figure 7. Nogo-A expression in the optic nerves of Mog-Cre<sup>+/−</sup>xRtn4<sup>flox/flox</sup> mice. (A) Western blot analysis revealed that Nogo-A protein was only down-regulated by ~25% in P60 optic nerves of Mog-Cre<sup>+/−</sup>xRtn4<sup>flox/flox</sup> in contrast to the previously observed ~80% decrease in Cnp-Cre<sup>+/−</sup>xRtn4<sup>flox/flox</sup> mice (one-way ANOVA, **P<0.01, ***p<0.001). Nogo-B protein showed compensatory up-regulation in both glial Nogo-A KO lines. (B) Olig2 and Cre-recombinase immunostainings on sagittal optic nerve sections revealed very weak Cre expression in Mog-Cre<sup>+/−</sup>xRtn4<sup>flox/flox</sup> oligodendrocytes compared to highly Cre-expressing Olig2-positive cells in the Cnp-Cre<sup>+/−</sup>xRtn4<sup>flox/flox</sup> animals. Scale bar: B = 100μm.
Figure 8. Nogo-A expression in the optic nerves of Mog-Cre\(^{+/}\)/Rtn4\(^{lox/lox}\) mice. (A) In the retina, we found no Nogo-A or -B expression changes in Mog-Cre\(^{+/}\)/Rtn4\(^{lox/lox}\) animals. (B) Nogo-A was mostly located in Müller cell end-feet surrounding βIII-Tubulin-labelled retinal ganglion cell bodies in both Mog-Cre\(^{+/}\) and Mog-Cre\(^{+/}\)/Rtn4\(^{lox/lox}\) intact retinae. Scale bar: B = 50μm.
4 Discussion

In Chapter 2 of the present thesis, we found that deletion of Nogo-A from glial cells leads to significantly increased axonal regeneration in the optic nerve and elevated retinal ganglion cell survival in the retina. In the present study, we aimed to further investigate the possible underlying molecular mechanisms that drive cell survival and axonal regeneration in the glial Nogo-A knock-out animals. Our results showed no obvious alterations in gliosis, ER stress or autophagy that may contribute to RGC survival. Some expression changes were detected in NgR related receptors upon deletion of Nogo-A from glial cells. Additionally in this study we introduced an alternative mouse model to study further functions of glial Nogo-A in the CNS, however this new line displayed only modest Nogo-A down-regulation from oligodendrocytes.

4.1 Nogo-A and Müller cell reaction

In the retina, Nogo-A is expressed in Müller cells and after ONC injury about 50% of RGCs respond with Nogo-A up-regulation. The polarized distribution of Nogo-A in the end-feet of Müller cells resembles the localization of RTN3 and MAG (Stefansson et al., 1984, Kumamaru et al., 2004), but the physiological role of these proteins in this cell type is not yet understood. In response to the optic nerve or retinal injury and inflammation, radial processes of the Müller cells start expressing increased amount of GFAP, a widely used gliosis marker. In our study, we tested the possible contribution of Nogo-A to Müller cell activation in the glial Nogo-A KO mice. Our results showed no significant alteration in the up-regulation of GFAP, and only a slight increase in retinal vimentin levels following ONC injury. These findings are consistent with previous data showing that in conventional Nogo-A KO mice the loss of Nogo-A in Müller cells did not alter axotomy-induced gliosis or that following injury Nogo-A levels remain unchanged in Müller glia (Pernet et al., 2011). Interestingly, the glia Nogo-A KO mice showed a tendency towards lower STAT3 activation that could be attributed to the deletion of Nogo-A from Müller glia, but this alteration was not significant and did not lead to changes in the downstream signaling pathways.

Nogo-B is expressed solely in Müller cells of the retina, but in contrast to myelinated CNS regions, its expression was not altered in Cnp-Cre+/xRtn4\textsuperscript{floxed/floxed} animals, where Nogo-A is deleted from glial cells. However, as the amount of Nogo-B exceed Nogo-A expression levels in retina, it is not unlikely that Nogo-B would replace functions of Nogo-A in Müller cells and possibly participate in the gliotic reaction.

Additionally, in our previous study we observed EphA4 up-regulation in the retinae of glial Nogo-A KO mice. EphA4 is mostly expressed in Müller cells in the retina, and genetic
deletion of EphA4 led to increase regeneration that was associated with significantly higher *Lif* and *Bdnf* mRNA expression in the retina (Joly et al., 2014). These expression changes were probably not triggered by altered gliosis, as the levels of the two gliosis markers *Gfap* and *vimentin* were similar between control and EphA4 KO genotypes. The compensatory up-regulation of EphA4 in Müller glia of the *Cnp-Cre*/*Rtn4*^fl/fl^ mice, however, could still be of functional importance by acting as a break on the regenerative response, a possibility that should be further investigated.

4.2 Possible neuro-protective mechanisms of Nogo-A in the retina

Nogo-A belongs to the reticulon protein family composed of four members (RTN1, RTN2, RTN3 and RTN4), the functions of which have been closely related to the maintenance of ER structure (Voeltz et al., 2006, Jozsef et al., 2014). RTN proteins have been also implicated in cell death regulation by Bcl-2 and Bcl-XL, two intracellular anti-apoptotic proteins (Tagami et al., 2000, Oertle et al., 2003, Wan et al., 2007).

In contrast to cell surface Nogo-A's role as a myelin-associated inhibitor of axon outgrowth, the function of the high levels of intracellular Nogo-A is largely unknown. Neuronal Nogo-A has been indicated to act as a neuroprotective molecule after oxidative stress: Nogo-A^Δ20^ has been shown to scavenge reactive oxygen species (Mi et al., 2012), whereas M9, a region of amino-Nogo-A, attenuated cerebral ischemic injury by inhibiting NADPH oxidase derived superoxide production (Guo et al., 2013). Additionally, NiG/Nogo-A recruited cytoprotective proteins such as Apg-1, a member of the stress-induced Hsp110 (heat-shock protein of 110 kDa) family (Kern et al., 2013).

Injured neurons up-regulate Nogo-A (Cheatwood et al., 2008, Pernet et al., 2011) suggesting that neuronal Nogo-A might play a cell-autonomous role in neuronal survival and growth related mechanisms. Due to its localization, RTN members were proposed to influence ER stress activation (Munoz and Zorzano, 2011, Sutendra et al., 2011). Nogo-A contributed to the proper function of the ER resident chaperone PDI, and was found to be protective against ALS-like neurodegeneration (Yang et al., 2009). Optic nerve injury provokes sustained CHOP up-regulation, and deletion of CHOP promotes RGC survival (Hu et al., 2012), however, no expression changes of CHOP were detected in Nogo-A depleted neurons (Pernet et al., 2011). Additionally, overexpression of Nogo-A did not alter the level of CHOP or that of the chaperon protein Bip (Yang et al., 2009). In our study, where we deleted Nogo-A in Müller glia, we could not detect any differences in the regulation of ER stress related genes such as *Chop/Gadd153, Bip/Grp78, elf2a, Atf6*, and *Bcl-2*, or the localization of the CHOP protein, neither in Müller cells nor in RGCs. A recent study...
suggested that although Nogo-A/B deletion altered ER morphology and compromised the Ca\textsuperscript{2+} homeostasis, no indication of ER stress was detected in the Nogo-A/B KO cells, suggesting proper functioning of the protein folding machinery (Jozsef et al., 2014). Our results are therefore consistent with the previously discussed data on ER stress regulation, showing that Nogo-A is not involved in the injury-induced ER stress processes.

Interestingly, the role of autophagy has been controversial in the retina; several studies reported either positive (Kim et al., 2008, Shen et al., 2011, Rodriguez-Muela et al., 2012) or negative (Piras et al., 2011, Park et al., 2012) contribution of autophagy to retinal ganglion cell survival. In the glial Nogo-A KOs, where Nogo-A was up-regulated in about 50% of RGCs before and after injury, we found increased RGC survival after ONC injury. In one study, Nogo-A overexpression suppressed autophagy induction after H\textsubscript{2}O\textsubscript{2} treatment as assessed by the LC3\textbeta{}II/LC3\textbeta{}I ratio, suggesting that Nogo-A would protect against autophagic cell death (Teng and Tang, 2013). In the retinæ of the glial Nogo-A KOs, however, we found no changes in the regulation of autophagy related genes (Atg3, Atg5, Atg7, Atg12, LC3\textalpha{} and LC3\textbeta{}) or proteins (LC3 and Atg5/12).

In light of these data, an influence of Nogo-A on the oxidative stress-induced necrotic neuronal cell death or apoptosis could be an explanation for the increased neuroprotection in glial Nogo-A KOs. However, physiological, in vivo experiments are necessary to confirm this hypothesis.

4.3 NgR receptor complex changes upon glial deletion of Nogo-A

In our previous study with the glial Nogo-A KO mice we proposed that neuronal Nogo-A might interact with its receptor NgR1 in cis, thereby blocking the downstream Rho-A signaling pathway and leading to increased axonal regeneration after ONC injury. The overall level of NgR1 was not changed in the retina, but the mRNA of a related receptor NgR2 was significantly up-regulated in both intact and injured glial Nogo-A KO retinæ. NgR1 is a common receptor for the myelin associated inhibitors (Nogo-A, MAG, OMgp) (Fournier et al., 2001, Domeniconi et al., 2002, Wang et al., 2002), however, NgR2, a receptor for MAG, acts selectively to mediate MAG derived inhibitory responses (Venkatesh et al., 2005). The increase in NgR2 expression might render RGC axons in the glial conditional KOs more sensitive to MAG and block axonal regrowth. On the ligand level, we have previously shown that MAG was not significantly up-regulated in the optic nerves of glial Nogo-A KOs. Additionally, a recent study demonstrated Versican as a novel interaction partner of NgR2 (Baumer et al., 2014), therefore up-regulation of NgR2 might further sensitize growing axons to this subclass of CSPGs.
Another NgR receptor complex member that showed significant mRNA up-regulation in injured glial Nogo-A KOs is TROY, a co-receptor that can replace p75 in the p75/NgR1/LINGO-1 complex (Shao et al., 2005). This up-regulation, however, was not associated with an NgR1/LINGO-1 increase, its two partners in the receptor complex; therefore, the expression increase might derive from distinct cell types from RGCs, such as reactive astrocytes, macrophages, microglia or immune cells (Satoh et al., 2007). A possible contribution of glial and neuronal Nogo-A to the regulation of the retinal immune responses triggered by optic nerve injury needs to be further investigated.

4.4 Lack of significant Nogo-A down-regulation in Mog-Cre+/xRtn4floxflox mice

Contrary to our expectations, only a fraction of Nogo-A was excised from oligodendrocytes of Mog-Cre+/xRtn4floxflox optic nerves, a finding that was also supported by the low expression of Cre recombinase in these cells. Relative to other, well-characterized myelin proteins such as MBP and CNP, the expression of MOG appeared relatively late during CNS development (Scolding et al., 1989, Matthieu and Amiguet, 1990). Cre expression therefore might only rise at the moment of oligodendrocyte development, when Nogo-A is already strongly expressed. Although the Cre staining we performed on optic nerve sections did not label well defined oligodendrocyte nuclei in Mog-Cre+/xRtn4floxflox mice, it remains possible that only a subpopulation of oligodendrocytes express Cre in the Mog-Cre+/xRtn4floxflox animals, thereby driving only a localized effect on Nogo-A expression. This could be due to insufficient amount of Cre present in the genome, as the Mog-Cre mice were kept in a heterozygous (+/−) state to avoid complete deletion of the Mog gene from these Cre knock-in mice. The difference in Nogo-A down-regulation levels in Mog-Cre+/xRtn4floxflox vs. Cnp-Cre+/xRtn4floxflox mice could therefore be due to different amount of Cre recombinase expressed in oligodendrocytes of the two lines.

Due to the modest Nogo-A down-regulation in the Mog-Cre+/xRtn4floxflox animals, these mice would not serve as an ideal model to study axonal regeneration in the optic nerve. However, this lower efficiency of Nogo-A deletion from oligodendrocytes might allow us to study local, cell specific processes, such as the myelination pattern around subpopulations of oligodendrocytes that express or lack Cre recombinase. The newly generated Mog-Cre driven glial Nogo-A KO line therefore holds the potential to serve as a model to study localized Nogo-A functions in the optic nerve, however the suitability and Nogo-A down-regulation efficiency in other CNS regions need to be investigated.
In summary, in this study we aimed to identify further molecular regulators that drive the increased axonal regeneration and neuronal survival in the glial Nogo-A KO mice. No obvious changes were found in the Müller glial activation, ER-stress or autophagy responses. Small alterations were detected in the expression of some Nogo-A receptor complex members such as TROY and NgR2 that could influence the regenerative response of our glial Nogo-A KOs. Additionally, although we observed some Nogo-A deletion in our newly generated Mog-Cre+/xRtn4flx/flx, due to the relatively low down-regulation efficiency we propose that our Cnp-Cre+/xRtn4flx/flx is more suitable to study the role of oligodendroglial Nogo-A in axonal regeneration.

Conflict of Interest
The authors declare no conflict of interest.

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5 Materials and methods

Animals
The previously described Rtn4flx/flx, in which the exon 3 of the Rtn4 allele was flanked by LoxP sites were crossed with mice expressing Cre-recombinase under the control of the 2',3'-cyclic nucleotide phosphodiesterase (Cnp) or myelin oligodendrocyte glycoprotein (Mog), respectively. The Cnp-Cre line was generated in the laboratory of Prof. Klaus-Armin Nave (Lappe-Siefke et al., 2003). The Mog-Cre line was obtained from Prof. Ari Waisman (Buch et al., 2005, Hovelmeyer et al., 2005). All procedures and surgeries were performed on 2–4 month old mice of mixed genders and different genotypes. Animal experiments were performed in accordance with the guidelines of the Veterinary Office of the Canton of Zurich.

Optic nerve crush (ONC) injury
The left optic nerve was exposed intra-orbitally and then crushed by tying a knot with a 9-0 suture at ~0.5mm from the back of the eye. The suture was carefully removed and the
integrity of the ophthalmic artery was examined by fundus examination. Three to five days after ONC, intact and injured retinas were collected for Western blot and qRT-PCR analysis.

*Retina and optic nerve processing and immunostaining*

Mice were sacrificed by injecting an overdose of anaesthetics intraperitoneally and perfused intracardially with PBS and 4% PFA. Optic nerves and eyes were dissected, the latter by removing the cornea and the lens from the eyecup. For retinal cross sections and longitudinal optic nerve sections, the eye cups and optic nerves were post-fixed in 4% PFA overnight at 4°C. The tissues were then cryoprotected in 30% sucrose and frozen in OCT compound (Tissue-TEK, Sakura) with a 2-methylbutane bath cooled with liquid nitrogen. Optic nerves and retinal sections were cut (14μm) with a cryostat. Immuno-histochemical stainings were performed in a blocking solution (5% of normal goat serum or 5% BSA, 0.3% Triton-X-100, and 0.05% sodium azide in PBS). Primary antibodies were applied overnight at 4°C and after PBS washes, sections were incubated with the appropriate secondary antibody for 1h at room temperature. The slides were mounted in Mowiol solution (10% Mowiol 4–88 (Calbiochem) in 100mM Tris, pH 8.5, 25% glycerol and 0.1% DABCO). Primary antibodies were: rabbit anti-Nogo-A (Laura, Rb173A40) serum (1:200), rabbit anti-GFAP (1:500, Dako), mouse anti-GS (1:300, Chemicon), rabbit anti-CHOP/GADD153 (1:50, Santa Cruz), rabbit anti-LC3 (1:250, MBL), mouse anti-APC (1:300, Chemicon), rabbit anti-Olig2 (1:300, Millipore), mouse anti-Cre recombinase (1:400, Millipore) and mouse anti-βIII-Tubulin (1:1'000, Promega). Immunofluorescent labelling was analysed with a Zeiss Axioskop 2 Plus microscope or with Leica SPE-II confocal microscope.

*Western blot analysis*

After cervical dislocation, retinas and optic nerves were quickly dissected out and flash frozen in liquid nitrogen. Tissues were kept at -80°C until extraction in lysis buffer (RIPA buffer: 150mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS in 50mM Tris buffer at pH8) containing protease inhibitors (Complete mini, Roche Diagnostics). Samples were fully homogenized and let on ice for 60 min. After centrifugation for 15 min at 15'000xg, 4°C, supernatants were collected and processed for protein concentration analysis (Bio-Rad Laboratories, RC DC Protein Assay). Retinal and optic nerve proteins (20μg/lane) were separated by electrophoresis on a 4–12% polyacrylamide gel and transferred to nitrocellulose membranes. Blots were incubated in a blocking solution of either 2% Top Block (Lubio Science) or 5% BSA (bovine serum albumin) in 0.2% TBST (0.2% Tween-20 in Tris-base 0.1 M, pH7.4) for 1 hour at room temperature, then incubated with primary antibodies overnight at 4°C. Following the washing steps, membranes were incubated with
horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10’000-1:25’000; Pierce Biotechnology). Primary antibodies were rabbit anti-Nogo-A/B (Bianca, Rb140) serum (1:20’000), rabbit anti-GFAP (1:500, Dako), mouse anti-Atg5/Atg5-12 (1:400, NanoTools), and mouse anti-GAPDH (1:20’000; Abcam). Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) in a Stella detector (Raytest). Band intensities were measured with the Image J software (NIH).

Semi-quantitative RT-PCR (qRT-PCR)

After cervical dislocation, intact and injured whole retinae were rapidly dissected in PBS. The samples were flash frozen in liquid nitrogen and stored at -80°C until RNA extraction. Retinal RNA was prepared using the RNeasy RNA isolation kit (Qiagen), including a DNase treatment to digest the residual genomic DNA. For reverse transcription, equal amounts of total RNA were transformed to cDNA by using oligo(dT) primers and M-MLV reverse transcriptase (Promega). 10ng of cDNA was amplified in the Light Cycler 480 thermocycler (Roche Diagnostics AG) with the polymerase ready mix (SYBR Green I Master; Roche Diagnostics). The following specific primers were designed to span intronic sequences or cover exon–intron boundaries: *Gapdh* (forward, 5’-CAGCAATGCATCCTGCACC-3’; reverse, 5’-TGGACTGTGGTCATGAGCCC-3’), *Nogo-A/Rtn4* (forward, 5’-CAGTGGATGAGACCCTTTTTG-3’; reverse, 5’-GCTGCTCCTTCAATCCATAA-3’), *NgR1/Rtn4R* (forward, 5’-CTCGACCCCGAAGATGAAGG-3’; reverse: 5’-TGGACTGTGGTCATGAGCCC-3’), *NgR2* (forward, 5’-GAGGCTTGGTCAGCCTACAGT-3’; reverse: 5’-CGCGAACAAGTCATCCTGT-3’), *NgR3* (forward, 5’-ATGCTTCGCAAAGGGTGCTGTG-3’; reverse: 5’-ATGCTTCGCAAAGGGTGCTGTG-3’), *Troy* (forward, 5’-ATGCTTCGCAAAGGGTGCTGTG-3’; reverse: 5’-ATGCTTCGCAAAGGGTGCTGTG-3’), *PirB* (forward, 5’-TGTGGCCTTCATCCTGTTCCT-3’; reverse: 5’-CCTGGTTATGGGCTCTTCAGC-3’), *Lingo-1* (forward, 5’-AAGTGGCCAGTTCATCAGGT-3’; reverse: 5’-TGTAGCAGACGCTGACAGA-3’), *p75* (forward, 5’-CTGCTGCTGCTGCTTCTAGG-3’; reverse: 5’-ACACACGGGTTGGTGCTGCT-3’), *Gfap* (forward, 5’-ATGCTTCGCAAAGGGTGCTGTG-3’; reverse: 5’-GGAAGAGATGGCAGCGA-3’), *Glul* (forward, 5’-TGTGGCCTTCATCCTGTTCCT-3’; reverse: 5’-CCTGGTTATGGGCTCTTCAGC-3’), *Vimentin* (forward, 5’-TACAGGAAGCTGCTGAGG-3’; reverse: 5’-TGCGTGCAACCAGAGGAA-3’), *Chop/Gadd153* (forward, 5’-ACACCACCACCTGAAAGCAG-3’; reverse: 5’-TGACTGAAATCTTGAGCACAG-3’), *Bip/Grp78* (forward, 5’-TGCAGCAGAGATGAGCCC-3’; reverse: 5’-TGGACTGTGGTCATGAGCCC-3’), *eIF2α* (forward, 5’-CCAGGAAATGCAAGCCATT-3’; reverse: 5’-CAGGATCACCAGAAGCAGA-3’), *Atf6* (forward, 5’-TGCTTGGGAGTCCAGACCTA-3’; reverse: 5’-AGGAAGCCGGAGAAAGG-3’), *Bcl-2* (forward, 5’-TGACCTGAGCAGCCTTCAC-3’; reverse: 5’-AGACAGCCAGGAATACCAACAG-3’),
Chapter 3: Nogo-A in molecular processes contributing to regeneration and survival

Atg3 (forward, 5'-CAGCACTGTGTGATGAAGAAGACG-3'; reverse: 5'-CATAGCCAAACACCATAGCGC-3'), Atg5 (forward, 5'-GACAGATTTGACCACTTTTGGG-3'; reverse: 5'-GGGTTTACAGGTCCTATC-3'), Atg7 (forward, 5'-CGATGACGACACTGTGCTTC-3'; reverse: 5'-GACTTCAACGGACTGGAAACTGC-3'), Atg12 (forward, 5'-ATTCAGGCACCAGGAAATGTCTC-3'), Lc3α (forward, 5'-CGACCAGCACCCCAGTAAGA-3'; reverse: 5'-TGACCAACTCGCTCATGTAAACA-3'), Lc3β (forward, 5'-AGTGGAAGATGTCCGGCTCAT-3'; reverse: 5'-TCAGGCACCAGGAACTGGT-3'). For relative quantification of gene expression, mRNA levels were normalized to GAPDH using the comparative threshold cycle (ΔΔCT) method and a control sample was used to calculate the relative values. Each reaction was done in triplicate and 3-4 mice per condition were analysed.

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Chapter 4

In vitro investigation of the potential hindrance of Nogo-A receptor signaling by Nogo-A expressed in cis position

by Flóra Vajda

Further contributions by:

Jody L. Martin, Martin E. Schwab and Vincent Pernet

Contributions:
F.V., V.P. and M.E.S.: designed the study. F.V. performed all the in vitro studies: handled and transduced the cells, harvested the cell cultures for Western blotting and immunocytochemical stainings, acquired microscopic images, analyzed cell spreading and neurite outgrowth. J.L.M produced the Adenovirus (sh.Nogo-A and sh.Control) constructs. F.V. prepared the figures and wrote the manuscript (revised by V.P. and M.E.S.).

(manuscript in preparation)
Abstract

Besides being known as a myelin inhibitory protein for axonal growth, Nogo-A can also be expressed in neurons, where it can exert distinct and beneficial functions in axonal repair and neuronal survival. In Chapter 2 of the present thesis, we reported that overexpression of Nogo-A in F11 cells by AAV2.Nogo-A attenuated the inhibition of neurite outgrowth on Nogo-A-Δ20 substrate and proposed that in Nogo-A expressing neurons, cis interaction between Nogo-A and its receptors may counteract trans Nogo-A signaling. To further test this hypothesis we performed a series of in vitro studies with Nogo-A manipulations in neuronal and fibroblast cell lines and tested their neurite outgrowth and cell spreading capacity, respectively. We characterized the expression of Nogo-A and its receptors in a neuronal cell line, in F11 cells, and found stable expression of the ligand and receptors both in basal conditions and after differentiation, in whole cell lysates and in membrane preparations. F11 cells responded to the inhibitory Nogo-A-Δ20, however silencing Nogo-A with a short hairpin construct (sh.Nogo) did not change the neurite outgrowth extent in these cells. Similarly, sh.Nogo-A mediated down-regulation did not alter spreading capacity of NIH 3T3 fibroblasts, however, full genetic deletion of Nogo-A decreased cell spreading of mouse embryonic fibroblast cells plated on Nogo-A-Δ20. On the molecular level, the up-regulation of Nogo-A in F11 cells increased phospho-ERK1/2 expression, which may contribute to the increased neurite growth or neuronal survival in neurons.

In summary, in this study we further tested whether cell autonomous expression of Nogo-A is beneficial for axonal growth and cell spreading. Our in vitro results support the hypothesis of a positive role of Nogo-A expressed in cis position.
Keywords:
cis Nogo-A; trans Nogo-A; F11 cell neurite outgrowth; fibroblast cell spreading; adenoviruses; Nogo-A up and down-regulation

Abbreviations:
ATF3, activating transcription factor-3; CNS, central nervous system; DRG, dorsal root ganglia; ERK1/2, extracellular signal-regulated kinases 1/2; EtOH, ethanol; Fsk, Forskolin; GAP-43, growth associated protein-43; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescent protein; KO, knock-out; LTP, long-term potentiation; MOI, multiple of infection; NgR1, Nogo66 receptor-1; PBS, phosphate-buffered saline; qRT-PCR, quantitative real-time polymerase chain reaction; RTN, reticulon; S1PR2, sphingosine-1-phosphate receptor 2; SEM, standard error of the mean; shRNA, short hairpin RNA; TBS, tris-buffered saline.
2 Introduction

During development Nogo-A is highly expressed in growing and immature neurons and is down-regulated in many types of mature neurons. In adulthood, the expression of neuronal Nogo-A remains elevated in plastic regions of the nervous system, such as in the hippocampus, cortex and dorsal root ganglia (DRGs) (Huber et al., 2002). Nogo-A was shown to control long-term potentiation (LTP) in the hippocampus and in the cortex (Delekate et al., 2011, Tews et al., 2013), to influence cell migration (Mathis et al., 2010), survival (Mi et al., 2012, Guo et al., 2013), cell spreading and growth (Oertle et al., 2003) of multiple cell types. In this context the possible effects of cell autonomous, neuronal Nogo-A are less well understood. We have previously shown that in contrast to myelin/glial Nogo-A, neuronal Nogo-A appeared beneficial for regeneration in the injured optic nerve, and that Nogo-A expression was increased in retinal ganglion cells (RGC) after optic nerve crush injury (Pernet et al., 2011). In Chapter 2 of the present thesis, we demonstrated that neuronal expression of Nogo-A is necessary for improved regeneration in a more permissive environment, where Nogo-A is deleted from glial cells.

Interestingly, Nogo-A receptors such as Nogo-66 receptor 1 (NgR1) (Fournier et al., 2001) and sphingosine-1-phosphate receptor 2 (S1PR2) (Kempf et al., 2014) can be co-expressed with Nogo-A in retinal ganglion neurons. The proximity of the ligand and its receptors raised the possibility of cis-interaction within the same cell, a phenomenon that has previously been described for several guidance molecules (Yaron and Sprinzak, 2012), such as Ephrins, Semaphorins and Slit2 (Egea and Klein, 2007, Haklai-Topper et al., 2010, Jaworski and Tessier-Lavigne, 2012), but also for the Notch/Delta signaling pathway (Sprinzak et al., 2010) (Figure 1). A possible cis-interaction between Nogo-A and PirB, an additional receptor member, has been raised before, however, in this case Nogo-A on the neuron was proposed to self-limit axon outgrowth in a cell autonomous manner, by activating downstream receptor components (Dickson et al., 2010). Additionally, Olfactomedin 1 (Olfm1), a highly conserved secreted glycoprotein, has been shown to promote axon growth through interaction with the Nogo A receptor (NgR1) complex. This interaction reduced binding of NgR1 to its co-receptors p75NTR and LINGO-1 and therefore attenuated the activation of underlying signaling mechanisms (Nakaya et al., 2012). In vitro, we found that overexpression of Nogo-A in F11 cells by AAV2.Nogo-A attenuated the inhibition of neurite outgrowth on Nogo-A∆20-coated substrate, suggesting a cell-autonomous effect of neuronal Nogo-A on neurite outgrowth and supporting the possibility that cis-interaction between Nogo-A and its receptors may counteract the inhibitory effect of Nogo-A present in the environment (Figure 1).
Chapter 4: In vitro investigation of cis-interaction

Figure 1. Illustration of trans-signaling and cis-interaction. In different classes of ligand/receptor pairs, such as Ephrins/Ephs (Egea and Klein, 2007), Semaphorins/Plexins (Haklai-Topper et al., 2010), Delta/Notch (Sprinzak et al., 2010), trans binding of the ligand to the receptor leads to repulsive signaling activation. However, ligand binding in cis position, referred to as cis-interaction, in some cases blocked inhibitory signaling in various cell types, including neurons. We propose similar action mechanism for Nogo-A, whereby trans Nogo-A would activate Nogo-66 and Nogo-Δ20 mediated growth inhibition, but Nogo-A expressed in cis position would lead to the blockade of Rho-A activation down-stream NgR1 and S1PR2.
In the present study, due to technical reasons our experiments with a Nogo-A knock-down technique could not confirm a cell-autonomous role of Nogo-A in neurite outgrowth or cells spreading. However, genetic deletion of Nogo-A led to decreased spreading of mouse embryonic fibroblast cells at the highest concentration of the tested Nogo-A-Δ20 substrates, supporting the hypothesis that cell autonomous Nogo-A expression is able to counteract the inhibitory Nogo-A-Δ20 presented in trans.

3 Results

3.1 Expression of Nogo-A and Nogo-A receptors in the F11 neuronal cell-type

First, we characterized the expression of Nogo-A and its receptors NgR1 and S1PR2 in F11 cells by Western blotting with whole cell lysates or membrane preparations. The endogenous levels of both Nogo-A and Nogo-B were high in undifferentiated F11 cells (EtOH), and neither of protein's expression was altered upon Forskolin (Fsk)-induced differentiation (Figure 2A). We also tested the expression of the receptors for the two identified inhibitory regions of Nogo-A, the Nogo-A-Δ20 receptor S1PR2 and the Nogo-66 receptor NgR1. Both receptors were present in whole protein lysates of F11 cells and their expression did not change after neurite outgrowth stimulation (Figure 2B-C). Next, we compared the amount of these proteins in total cell lysates and membrane preparations (Figure 2D). The purity of these proteins samples was controlled by GAPDH and Na⁺/K⁺-ATPase detections, which revealed that the membrane preparations almost lacked the cytoplasmic protein GAPDH, but were highly enriched in Na⁺/K⁺-ATPase, a transmembrane ion-pump. Nogo-A, Nogo-B and S1PR2 were all expressed in both the total cell and membrane fractions (Figure 2D), allowing us to formulate the hypothesis of cis-interactions with the membrane-bound/cell surface pool of these molecules. Plating F11 cells on increasing concentrations of Nogo-A-Δ20 did not alter Nogo-A or Nogo-B protein expression (Figure 2E). We have not observed any changes in the expression of the growth-associated protein-43 (GAP-43) after plating on inhibitory Nogo-A-Δ20 substrate and following Fsk treatment (Figure 2E). Compared to the EtOH-treated F11 cells, cells differentiated with Fsk grew fairly long neurites as early as 24 hours after treatment that were visualized by staining with the βIII-Tubulin antibody labelling neuronal processes (Figure 2F). Characterization of the F11 cells confirmed that this cell line is suitable for testing the cis-interaction hypothesis in vitro.
Figure 2. Nogo-A and its receptors are expressed in F11 cells. (A) In total cell lysates of F11 cells, Nogo-A and Nogo-B were both expressed and their protein level did not vary between EtOH (control) treated and Forskolin-induced, differentiated conditions. The Nogo-A receptors (B) S1PR2 and (C) NgR1 were both expressed in F11 cells and their expression did not change upon differentiation. (D) Nogo-A, Nogo-B and S1PR2 were present in the cell membrane preparations from F11 cell cultures. The membrane bound faction of these proteins was not altered upon Forskolin treatment. (E) Plating F11 cells on increasing amounts of Nogo-AΔ20 did not alter Nogo-A or Nogo-B protein expression levels, and did not lead to the decrease of GAP-43 protein. (F) Treatment with Forskolin leading to intracellular cAMP up-regulation induced neurite outgrowth of F11 cells, as demonstrated on the pictures taken 24 hours after the differentiation induction. Neurites were labelled with βIII-Tubulin antibody. Scale bar: F = 50μm.
3.2 Down-regulation or deletion of Nogo-A: effect on neurite outgrowth and cell spreading

With the aid of Adenoviruses containing short hairpin RNA (shRNA) specific to Nogo-A (Adeno.sh.Nogo-A), we aimed to investigate the contribution of cell autonomous Nogo-A to neurite outgrowth and cell spreading on an inhibitory Nogo-A-∆20 substrate. The neurite outgrowth experiments with F11 cells (MacGillavry et al., 2009) showed a Nogo-A-∆20 dose-dependent outgrowth inhibition, with and EC50 of ~12.5pM (Figure 3A). The Nogo-A down-regulation efficiency was controlled after each experiments; transduction with the Adeno.sh.Nogo-A virus led to ~75% decrease of Nogo-A from F11 cells 4 days after viral transduction (Figure 3B). However, silencing Nogo-A did not alter the length of neurites in any conditions (Figure 3A). Cell spreading experiments with NIH 3T3 fibroblasts also failed to show different spreading phenotypes between control and Adeno.sh.Nogo-A transduced cells (Figure 3C) in which Nogo-A was also down-regulated by ~75% (Figure 3D). However, when mouse embryonic fibroblast cells (MEFC) from WT and conventional Nogo-A knock-out (KO) mice were compared for their spreading efficiency on Nogo-A-∆20, the Nogo-A KO MEFC cells showed modest, but significantly decreased cell spreading at the highest (9pM) substrate concentration (Figure 3E).

3.3 Protein expression changes following Nogo-A up and down-regulation

We followed the expression of Nogo-A, its receptor and growth related protein and signaling cascade molecules by Western blotting after Nogo-A overexpression by Adeno.Nogo-A-GFP (Figure 4A) and Nogo-A down-regulation by Adeno.sh.Nogo-A (Figure 4B), respectively. Nogo-A up-regulation was confirmed after transduction of F11 cells with Adeno.Nogo-A-GFP viruses by detection of Nogo-A or GFP proteins. The increased Nogo-A expression lead to strong up-regulation of phospho-ERK1/2, while the total-ERK level remained constant (Figure 4A). The expression level of ATF3, the growth associated activating transcription factor 3, did not increase upon adeno-mediated Nogo-A up-regulation. Additionally, no changes were observed in the protein expression of S1PR2, Nogo-B and βIII-Tubulin (Figure 4A). No protein changes were observed in the control virus (Adeno.LacZ) treated cells (Figure 4A). Inversely, the strong, almost complete down-regulation of Nogo-A in F11 cells (50-100MOI Adeno.sh.Nogo-A) led to the decrease in phospho-ERK1/2 protein levels, although higher MOIs of the Adeno.sh.Control viruses also resulted in the same phospho-ERK1/2 expression changes (Figure 4B). We did not detect a decrease in the growth marker GAP-43 and ATF3 protein levels. The level of Nogo-B also remained constant (Figure 4B).
Chapter 4: *In vitro* investigation of *cis*-interaction

**Neurite outgrowth assay (F11 cells)**

(A) Silencing Nogo-A by Adeno.sh.Nogo-A viruses in F11 cell did not alter Nogo-A-Δ20 mediated outgrowth inhibition. (B) The Adeno.sh.Nogo-A virus transduction led to ~75% deletion of Nogo-A in F11 cells (4 days sh.Nogo-A expression). (C) NIH 3T3 fibroblast spreading was also not effected by Nogo-A silencing. (D) ~75% of Nogo-A was deleted 4 days after the transduction of NIH 3T3 fibroblasts with Adeno.sh.Nogo-A virus. (E) Mouse embryonic fibroblast cells (MEFC) were isolated from WT and Nogo-A KO embryos. Nogo-A KO MEFC cells showed significantly decreased cell spreading on the highest (9pM) Nogo-A-Δ20 concentration (t-test; ***p<0.001).

**Figure 3. Effect of Nogo-A ablation on neurite outgrowth and cell spreading.** (A) Silencing Nogo-A by Adeno.sh.Nogo-A viruses in F11 cell did not alter Nogo-A-Δ20 mediated outgrowth inhibition. (B) The Adeno.sh.Nogo-A virus transduction led to ~75% deletion of Nogo-A in F11 cells (4 days sh.Nogo-A expression). (C) NIH 3T3 fibroblast spreading was also not effected by Nogo-A silencing. (D) ~75% of Nogo-A was deleted 4 days after the transduction of NIH 3T3 fibroblasts with Adeno.sh.Nogo-A virus. (E) Mouse embryonic fibroblast cells (MEFC) were isolated from WT and Nogo-A KO embryos. Nogo-A KO MEFC cells showed significantly decreased cell spreading on the highest (9pM) Nogo-A-Δ20 concentration (t-test; ***p<0.001).
**Figure 4.** Protein expression changes after Nogo-A up and down-regulation in F11 cells. (A) Nogo-A up-regulation by Adeno.Nogo-A-GFP led to the strong increase of phospho-ERK1/2, while the total-ERK level remained constant, and the level of ATF3 did not increase. Furthermore, no changes were observed in the protein expression level of the Nogo-A-Δ20 receptor S1PR2, or Nogo-B and βIII-Tubulin. (B) Nogo-A downregulation by Adeno.sh.Nogo-A viruses led to the decrease in phospho-ERK1/2 protein levels. However, high MOI infection rates (50-100MOI) with Adeno.sh.Control viruses similarly led to a decrease in phospho-ERK1/2. No changes were detected for the growth marker GAP-43 and ATF3 protein levels. The level of Nogo-B also remained constant.
4 Discussion

At the cell membrane, the sequestration of the receptor by adjacent ligand binding, the so-called *cis*-interaction, has previously been described to prevent *trans* receptor activation in the case of guidance molecules such as Ephrins (Egea and Klein, 2007), Semaphorins (Haklai-Topper et al., 2010) or Slit-Robo (Jaworski and Tessier-Lavigne, 2012), but also for the Notch signaling pathway (Sprinzak et al., 2010) (Figure 1). In the visual system, the Ephrin-Eph *cis* and *trans* signaling is of particular importance, as these ligand-receptor interactions have been shown to be major determinants of the topographic retino-tectal projection during development (Hornberger et al., 1999).

It is intriguing that in retinal ganglion neurons both Nogo-A and its receptors NgR1 and S1PR2 are present, and this co-expression raised the possibility of *cis*-interaction between the ligand and receptor (Figure 1). The Nogo-A expressed in neurons could potentially bind to NgR1 and prevent downstream signaling to Rho-A through the NgR1 receptor complex. In our previously described *in vitro* experiments, Nogo-A overexpressing F11 cells could counteract the outgrowth-blocking effect of Nogo-A-Δ20. However, Nogo-A knock-down experiments have not confirmed a crucial cell-autonomous role of Nogo-A in neurite outgrowth or cells spreading. It is possible that these experiments employing RNA interference have led to insufficient down-regulation levels. As confirmed by Western blotting, ~25% of Nogo-A was still expressed in these cells at the time of the functional experiments, and this could mask the real effect of Nogo-A deletion.

Cell spreading studies with embryonic fibroblast cells derived from Nogo-A KO mice indicated that these cells lacking 100% of Nogo-A spread less on the substrate containing high concentration of Nogo-A-Δ20. These results are in line with our previous observation, where Nogo-A overexpression increased neurite outgrowth of F11 cells only on the highest inhibitory Nogo-A-Δ20 conditions. Cell spreading with the MEFC cells therefore confirmed that full deletion of Nogo-A indeed leads to impaired spreading capacity. However, as these Nogo-A KO cells up-regulate Nogo-B, the contribution of compensatory expression changes remains to be investigated.

Interestingly, in F11 cells, the overexpression of Nogo-A led to the up-regulation of phosphorylated extracellular signal-regulated protein kinase1/2 (*phospho-ERK1/2*). Activation of ERK, a member of the mitogen-activated protein kinase family, has been shown to mediate neurite outgrowth-promoting effects (Agthong et al., 2009). However, although in the visual system ERK1/2 activation promoted robust neuroprotection, it did not induce increased regeneration (Pernet et al., 2005). It would be therefore tempting to hypothesize that cell autonomous Nogo-A leads to the activation of pro-regenerative and pro-survival
processes. However, as we introduced Nogo-A or sh.Nogo-A to the cells by Adenoviruses, it cannot be excluded that the virus transduction by itself led to the phospho-ERK1/2 increase (Tibbles et al., 2002). Phospho-ERK1/2 levels decreased not only in Adeno.sh.Nogo-A treated, but also after higher MOI application of Adeno.sh.Control viruses. This might serve as a confirmation for the viral transduction induced phospho-ERK1/2 expression changes. On the other hand, Nogo-A up and down-regulation lead to phospho-ERK1/2 up and down-regulation respectively, which is a contradictory result considering that both cell populations were transduced with Adenoviruses. An additional concern for the results with Nogo-A up-regulation is that the GFP levels controlling MOIs of the control LacZ and Nogo-A overexpressing viruses were unequal, and the higher MOI of the Adeno.Nogo-A-GFP viruses could have induced changes in the ERK1/2 signaling.

In summary, the experiments in this study partially confirmed the beneficial role of Nogo-A in cis position for cell spreading on Nogo-A-Δ20. However, further experiments such as an encounter assay analyzing effects of Nogo-A-Δ20 on the single cell level are required to address this hypothesis in details.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements
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5 Materials and methods

Cell Cultures
F11 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen GIBCO, #21885) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (PenStrep) (Invitrogen, #15140) in tissue culture dishes (Techno Plastic Products). For passaging and before outgrowth experiments 0.05% Trypsin (Invitrogen) was used to dissociate the F11 cells from the culture dish. NIH 3T3 cells were maintained in DMEM (Invitrogen GIBCO, #61965) containing 10% neonatal bovine calf serum (NBCS, Invitrogen).
For passaging and before spreading experiments 0.25% Trypsin was used to dissociate the NIH 3T3 cells from the culture dish. Primary mouse embryonic fibroblast cells (MEFC) were isolated and immortalized as described previously (Todaro and Green, 1963). Each primary fibroblast culture was isolated from the embryos (E15) from a single pregnant C57BL/6 Nogo-A heterozygous knock-out mouse. To identify the genotype of each embryo, genotyping protocol was carried out on tail samples as described before (Simonen et al., 2003). MEFC cultures of each genotypes (WT, heterozygous and Nogo-A KO) were maintained separately in DMEM (Invitrogen GIBCO, #61965) containing 10% FBS and 1% PenStrep. For passaging, 0.25% Trypsin was used to dissociate the MEFCs and before spreading experiments MEFCs were detached with 2mM EDTA-PBS from the culture dish. All cells were grown in the incubator at 37°C with 5% CO₂ concentration.

**AAV and Adenovirus vector production and application**

Adeno-associated viral vectors were produced by standard methods. An AAV2.Nogo-A (5.1x10^{11}vp/ml) and AAV2.GFP (2.1x10^{11}vp/ml) (adeno-associated virus serotype 2) expressing virus was generated to selectively infect neuronal cell populations and were used in 230 and 200 MOIs (multiple of infection), respectively. The Adeno.Nogo-A-GFP (2.1x10^{10}vp/ml) and Adeno.LacZ-GFP (6.5x10^{10}vp/ml) viruses were produced by Dr. Dana A Dodd and were applied in 10 and 20 MOIs, respectively. The Adeno.sh.Nogo-A (3.25x10^{10}vp/ml) and Adeno.sh.Control (3.25x10^{10}vp/ml) adenoviruses were generously provided by Dr. Jody L Martin and used in 1-100 MOIs for Western blotting and in 25 and 50 MOIs for F11 outgrowth and 3T3 NIH cell spreading assays.

**F11 neurite outgrowth assay**

The effects of AAV2.GFP, AAV2.Nogo-A, Adeno.sh.Control or Adeno.sh.Nogo-A on neurite outgrowth were evaluated in vitro by infecting F11 cells. The F11 cell line, kindly provided by Prof. RE van Kesteren (Amsterdam, The Netherlands), was maintained as described before (MacGillavry et al., 2009). F11 cells were plated at a density of 100 000 cells per well in 6-well plates and were treated with the viruses 24 h later. Four days or 24 hours after infection with AAVs or adenoviruses respectively, F11 cells were transferred to 4-well dishes at a density of 2000 cells per well. The 4-well dishes were coated by different concentrations of Nogo-A-Δ20 substrate (0, 0.5, 1 and 2.5μg/cm²) diluted in PBS at 4 degrees for 12 hours. Twenty four hours after plating the cells on Nogo-A-Δ20 substrate, F11 cells were differentiated by adding 10μM of Forskolin (Sigma-Aldrich) for 48 h. To visualize neurite extension, F11 cells were stained with βIII-Tubulin antibody. Measurement of neurite length was carried out using the Neuron J plugin in the Image J software (NIH).
The mean of the total neurite length was calculated for each treatment on all Nogo-A-Δ20 substrate concentrations measuring between 80 and 110 cells.

**NIH 3T3 and MEFC spreading assay**

The NIH 3T3 and MEFC fibroblast spreading assays neurons were performed as described previously (Oertle et al., 2003). Briefly, four-well plates (Greiner) were coated with different concentrations of Nogo-A-Δ20 at 4°C overnight. NIH 3T3 and MEFC cells were plated at 7,000 cells per cm² for 1 h at 37°C and 5% CO2, fixed with 4% paraformaldehyde (PFA) and stained with Phalloidin-Alexa-488. Each experiment was performed at least three times in four replicate wells. Spreading was quantified manually in a blinded manner. NIH 3T3 and MEFC cells were classified as spread cells if they bear at least two lamellipodial processes longer than one cell body diameter. Round cells were classified as non-spread. Data were normalized to baseline and plotted as average with error of the mean (SEM).

**Western-blot analysis**

F11 cells were plated in DMEM containing 10% FBS and 1% PenStrep in 6-well plates by at. 200’000 cells/well density. 48 hours later, the cells were treated with either 10μM forskolin (Sigma-Aldrich, diluted in ethanol) or ethanol diluted in DMEM containing 0.5% FBS and 1% PenStrep. 24 or 48 hours after treatment the cells were scraped and lysed in 100μl CHAPS lysis buffer (20mM Tris-HCl pH8, 0.5% CHAPS) containing protease inhibitors (Complete mini, Roche diagnostics). The samples were fully homogenized and let on ice for 60 min. After centrifugation for 15min at 15,000xg, 4°C, supernatants were collected and processed for protein concentration analysis (Bio-Rad Laboratories, RC DC Protein Assay). 20 μg of proteins from each cell lysates were mixed with 4x SDS-loading buffer (Invitrogen, NuPAGE LDS sample buffer), were boiled at 95°C for 10 min and were separated by electrophoresis on a 4–12% polyacrylamide gel and transferred to nitrocellulose membranes (Whatman, reinforced NC). Blots were incubated in a blocking solution of either 2% Top Block (Lubio Science) or 5% BSA (bovine serum albumin) in 0.2% TBST (0.2% Tween-20 in Tris-base 0.1 M, pH7.4) for 1 hour at room temperature, then incubated with primary antibodies overnight at 4°C. Following the washing steps, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (1:10’000-1:25’000, Pierce Biotechnology). Primary antibodies were rabbit anti-Nogo-A/B (Bianca, Rb140) serum (1:20’000), rabbit anti-phosphoERK1/2 (1:1’000, Cell Signaling), rabbit anti-ERK1/2 (1:1’000, Cell Signaling), rabbit anti-ATF3 (1:200, Santa Cruz Biotechnology), rabbit anti-GAP43 (1:500, Millipore), goat anti-NgR1 (1:300, R&D), human anti-S1PR2 (1:500, AbD Serotec custom made HuCAL antibody AbD14533.1), chicken anti-
GFP (1:5’000, Abcam), mouse anti-βIII-Tubulin (1:500, Promega), mouse anti-Na+/K+ ATPase (1:5’000; Abcam) and mouse anti-GAPDH (1:20’000, Abcam). Protein bands were detected with SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) in a Stella detector (Raytest). Band intensities were measured with the Image J software (NIH).

Membrane preparation

F11 cells were plated in DMEM containing 10% FBS and 1% PenStrep in 10cm tissue culture dishes. When the plates were 75% confluent, the cells were treated with either 10μM forskolin (Sigma-Aldrich, diluted in ethanol) or ethanol diluted in DMEM containing 0.5% FBS and 1% PenStrep. 24 hours after treatment the cells were collected in 6ml PBS containing protease inhibitors (Complete mini, Roche diagnostics) and centrifuged for 10 min at 2,500xg at 4°C. Pellets from eight dishes per conditions (EtOH and forskolin) were pooled together and were homogenized in 5ml of H buffer (50mM mannitol, 5mM HEPES, pH 8) with Ultra Turrax T25 for 10 min on ice. After addition of CaCl2 (end concentration of 10mM), the samples were homogenized for additional 10min. The cell lysates were centrifuged at 3,000xg for 15min at 4°C. The supernatants were further ultra-centrifuged at 48,000xg for 30min at 4°C (27,000rpm for Type 80 Ti rotor) in special 10 ml thick-wall tubes (Beckmann). The pellet was carefully re-suspended in 200 μl PBS and the membrane preparations were processed for Western blotting as described before.

References


Chapter 5

Concluding remarks
1 Conclusions and Outlook

With the work presented in this thesis, we have made substantial progress towards understanding the role of glial versus neuronal Nogo-A in axonal regeneration in the mouse visual system. So far besides function blocking anti-Nogo-A antibodies or receptor blocking agents, only conventional knock-out (KO) mouse lines have been used to study the the effects of Nogo-A neutralization and gene ablation on axonal growth inhibition. Previous studies from our laboratory have suggested that in contrast to the inhibitory myelin Nogo-A, neuronal Nogo-A could positively influence axonal growth. Therefore, these molecular manipulations and genetic deletions affecting both glial and neuronal cell populations might not yield the most optimal growth conditions for promoting regeneration in the CNS.

In the present studies, we employed the Cre-lox recombination system and generated cell-type specific conditional Nogo-A knock-out animals, or acutely excised the Nogo-A gene by injecting adeno-associated virus serotype 2 (AAV2).Cre viruses in Nogo-A/Rtn4 floxed mice. In Chapter 2, we showed that axonal regeneration is increased in the optic nerves of glia-specific Nogo-A KO mice and this increase is associated with the up-regulation of growth related genes. However, deletion of neuronal Nogo-A decreased the retinal ganglion cell (RGC) growth potential and prevented the regeneration improvement seen in the glial Nogo-A KOs. Therefore, we concluded that targeted glial Nogo-A deletion appears to be the most effective way to stimulate axonal growth in the injured optic nerve. We detected decreased Rho-A activation in the retinae of glial Nogo-A KOs, where Nogo-A is highly expressed in RGCs. We proposed that neuronal Nogo-A in RGCs could possibly interact with Nogo receptors at the cell membrane in cis, thereby preventing inhibitory trans Nogo-A signaling to the neurons. In Chapter 3, we aimed to investigate the molecular mechanisms underlying increased axonal growth and neuronal survival in the glia-specific Nogo-A knock-outs. Chapter 4 provides further evidences on how cell autonomous, neuronal Nogo-A could be beneficial for cell growth and survival.

The results presented in this thesis strongly suggest that oligodendroglial and neuronal Nogo-A play distinct roles in axonal regeneration in the retina and optic nerve, and perhaps also in the central nervous system in general. While myelin Nogo-A presented in trans leads to growth inhibition, Nogo-A expressed extra or intracellularly in neurons may exert a positive function possibly by interaction with and blockade of its receptors in cis position. Neuronal Nogo-A could influence the neuronal growth in the intracellular space, possibly through sequestering Nogo receptors, or at the cell membrane by preventing trans Nogo-A signaling converging to Rho-A activation. Further in vitro experiments, such as encounter assays dissecting the contributions of the two Nogo-A pools to neuronal growth.
and migration on single cell level would be of great importance for finding the exact underlying molecular mechanisms.

Based on these findings we propose that inactivating Nogo-A in glia while preserving neuronal Nogo-A expression may be the most successful strategy to promote axonal regeneration in the CNS.

1.1 Nogo-A as an extrinsic and intrinsic growth, synapse and survival regulator

Nogo-A is mostly seen as an extracellular inhibitor of axonal regeneration. Apart from Rho-A activation leading to growth cone collapse, trans Nogo-A also suppresses the neuronal growth program (Schwab, 2010). Several transcriptional changes have been detected between spinal cord tissue samples from conventional Nogo-A knock-out and wild-type mice (Montani et al., 2009), pointing to regulation of the actin cytoskeleton, the neuronal growth machinery, and in particular the Rho-GTPase/LIMK1/cofilin pathway. Additionally, Nogo-A-∆20 containing signalosomes have been shown not only to activate Rho-A, but also down-regulate cAMP response element binding protein (CREB) phosphorylation and repress the neuronal growth program (Joset et al., 2010). This pathway is a main regulatory mechanism used by neurotrophic factors to elicit growth and neuronal plasticity (Shao et al., 2002) and strikingly, to help neurons to overcome myelin-mediated growth inhibition (Cai et al., 2002, Gao et al., 2004). Interestingly, signaling from negative and positive growth regulators converge on the same transcription factor and this observation might be generalized to regulation of other growth controllers, such as the mammalian target of rapamycin (mTOR), whose synaptic expression was observed to be decreased by Nogo-A (Baldwin et al., 2011, Peng et al., 2011). Additionally, recently, Nogo-A-∆20 mediated growth cone collapse has been shown to be dependent on the mTOR pathway-activated protein translation (Manns et al., 2014). All these findings illustrate how a negative, extrinsic regulator of growth, such as Nogo-A, can interfere with intrinsic growth mechanisms.

Similarly to previous studies, here we report that neuronal Nogo-A could also modify intrinsic neuronal growth properties through a cell autonomous mechanism. We found increased regeneration in the optic nerve of glial Nogo-A KOs, where expression of the neuronal Nogo-A pool is preserved and in vitro neurons over-expressing Nogo-A grew longer neurites on high concentrations of inhibitory Nogo-A-∆20 substrate. In injured retinas of the glial Nogo-A KOs, increased growth associated Sprr1a and Gap43 mRNA levels were detected confirming an elevated neuronal growth state in these animals. Importantly, we detected lower Rho-A activation in these retinas, but the phospho-CREB levels were not
altered. We therefore aimed to find an explanation for how neuronal Nogo-A could act as a positive intrinsic growth regulator. We proposed cis-interaction with neuronal Nogo receptors as a new mechanism for the cell autonomous, positive role of Nogo-A. There is a growing body of evidence in support of this novel type of interaction, in which ligands inhibit their receptor in the same cell. The cis-interaction we suggest within the cell membrane between Nogo-A and Nogo receptors may be analogous to the mechanism previously described for the Ephrin/Eph, Semaphorin/Plexin or Delta/Notch that blocked downstream signaling (Yaron and Sprinzak, 2012). Cis-interaction has so far been shown to control axonal guidance and cell fate specification, however other possible roles in the central nervous system, such as in axonal growth inhibition or synaptogenesis can be postulated. The simultaneous presence of Nogo-A and its receptors led us to the hypothesis that through cis-interaction Nogo-A could limit signaling via NgR1 and S1PR2 and thereby counteract growth inhibition by myelin inhibitory cues. Additionally, in view of the importance of ephrins, some semaphorins and Nogo-A in synapse formation and maintenance, the contribution of cis-interaction to synaptogenesis is plausible.

After CNS injury, Nogo-A has been observed to be increased in neurons (Cheatwood et al., 2008, Pernet et al., 2011), suggesting that it may contribute to growth and cell death related mechanisms. Our and other studies have not detected changes in the regulation of ER-stress associated markers upon Nogo-A expression manipulation. However, in ischemia and after oxidative stress, Nogo-A has been shown to act as a neuroprotective molecule. Nogo-A protein levels were increased in cortical and thalamic neurons after stroke (Cheatwood et al., 2008), and Nogo-A could promote neuronal survival (Kilic et al., 2010). Multiple in vitro studies suggested that Nogo-A might protect neurons against oxidative insult through scavenging ROSs or by interacting with a network of anti-stress molecules (Mi et al., 2012, Guo et al., 2013, Kern et al., 2013). Stroke affects hundreds of thousands of people worldwide; in light of the possible positive role of Nogo-A in neuronal survival, the influence of immunotherapy against Nogo-A on neuronal cells should be further investigated.

As a consequence, discovery of these newly emerging functions for Nogo-A raise important points for designing new treatments for CNS diseases, as blocking Nogo-A may influence regeneration, plasticity and cell survival in a complex manner.
1.2 Contribution of guidance molecules and myelin inhibitory proteins to the blockade of CNS axonal regeneration

In several studies from our laboratory, three-dimensional analysis of regenerating axons revealed axonal guidance and patterning defects reflected by increased axonal U-turns and branching after growth induction in the injured optic nerve (Pernet et al., 2013a, Pernet et al., 2013b, Joly et al., 2014). These findings point to the complexity of molecular players contributing to the blockade and guidance of regeneration.

After fulfilling their role in nervous system development, many axon guidance molecules are down-regulated, while others retain their expression in the adult CNS and this implies additional roles for guidance cues beyond developmental contribution to outgrowth, growth cone navigation and target innervation (Giger et al., 2010). Axon guidance molecules in the mature CNS have been proposed to play a role in synaptic stabilization and limitation of neuronal plasticity in adulthood, and their expression is regulated following injury. As CNS neurons continue to express guidance receptors, the inability of severed axons to undergo spontaneous repair in the adult CNS has been proposed to be partially attributed to the presence of the same guidance molecules that were important during development, a hypothesis that needs further investigation.

A previous study from our laboratory revealed up-regulation of developmental axon guidance cues such as the EphrinA3/EphA4 ligand/receptor pair following constitutive Nogo-A deletion, and demonstrated increased axonal sprouting and regeneration after spinal cord injury in Nogo-A/EphA4 double KO mice compared to EphA4 single KO animals (Kempf et al., 2013). Our data in the glial Nogo-A KOs also suggested that the range of axonal regeneration may be restricted by the compensatory up-regulation of EphA4 and EphrinA3 in glial cells. Although by examining individual growing axons in 3D we found no significant difference in the proportion of branching or U-turn-forming axons between mouse genotypes, it remains possible that compensatory enhancement of EphrinA3 in the optic nerve and EphA4 in the retina could influence the guidance of regenerating axons in a complex manner in the glial Nogo-A KOs. We also reported that EphA4 KO mice were less prone to form branches in the injured optic nerve than WT animals (Joly et al., 2014). Due to the EphA4 up-regulation in the glial Nogo-A KOs, we expected to find increased branching of regenerating neurons in the injured optic nerve. However, possibly due to the simultaneous higher expression of EphrinA3 that may have led to increased signaling through other receptors, we did not detect changes in branching or any other guidance deficits. As additional Ephrins and Semaphorins might be up-regulated in the glial Nogo-A KOs, future
studies should detail the complex regulation system of myelin inhibitory and guidance and other repulsive molecules in the injured optic nerve.

Additionally, further components of the CNS myelin, such as MAG and OMgp have been shown to have inhibitory properties on axon growth. Although we have not detected changes in MAG levels in intact optic nerves of the glial Nogo-A KOs, expression of further myelin components such as PLP or MBP should also be followed up to exclude alterations in the myelin composition. As Nogo-A was proposed to be a key factor for precise myelination in the developing CNS (Pernet et al., 2008, Chong et al., 2012), it is possible that due to the deletion of Nogo-A in oligodendrocytes the myelin ultrastructure is slightly altered and this could lead to altered regeneration phenotypes.

It would be of great interest to understand how and why these repulsive guidance cues and potentially other myelin inhibitors get up-regulated upon Nogo-A deletion, and to find out how these compensatory mechanisms could be neutralized to potentiate axonal regeneration in the damaged CNS.

1.3 Model systems to study axonal regeneration in the CNS

The ultimate goal of the research on CNS regeneration is to improve the recovery from diseases such as spinal cord, brain or optic nerve injuries, where regrowth and protection of injured axons and formation of new functional neuronal connections is of utmost importance. Regenerating axons in the adult spinal cord have low intrinsic growth potential, and additionally face a hostile inhibitory environment: myelin inhibitory and repulsive guidance molecules and the CSPG expressing glial scar.

The same set of inhibitory factors represents a major hurdle for regenerating retinal ganglion cells in the optic nerve. The visual system has been proven to be an excellent and powerful research tool and many important milestones in understanding the mechanisms of axonal regeneration in the CNS have been reached by using this model. The optic nerve crush model allows researchers to investigate mechanisms of both axonal regeneration and cell survival of retinal ganglion cells. Furthermore, as injections in the vitreous liquid are relatively simple, retinal ganglion cells are readily accessible for pharmacological treatments and gene therapy. Combinatorial treatments acting on both intrinsic limitations and extrinsic hurdles affecting regeneration led to long-distance regrowth of a few RGC axons toward the brain targets such as the hypothalamus, lateral geniculate nucleus and in some reports even superior colliculus (de Lima et al., 2012, Luo et al., 2013). However, correct path-finding and guidance of these re-growing axons was questioned by newly developed 3 dimensional tracing techniques of individual axons in the transparent optic
nerve. Several studies reported that although a number of regrowing axons can be induced, these fibers very often follow irregular trajectories, show abnormal morphologies and misrouting at decision points, such as the optic chiasm, possibly due to the presence of myelin inhibitory proteins and repulsive guidance cues (Pernet and Schwab, 2014). However, target finding and circuit formation is rather different in the optic nerve from other parts of the CNS, therefore regenerative outcomes should also be tested in other systems.

Once the efficiency of a treatment has been proven to lead to increased regeneration in the optic nerve, this regenerative improvement and underlying mechanisms are likely to be successful when transferred to the spinal cord (Liu et al., 2010). Regeneration studies after spinal cord injury could have even stronger outcomes, as the gray matter composed of neuronal cell bodies in immediate vicinity of regenerating axons provides a more growth permissive environment and can be the substrate of compensatory sprouting and formation of detour connections (Bareyre et al., 2004). In the spinal cord, studies with clinically relevant lesion models such as compression and contusion are valuable for direct comparison with clinical cases.

With careful selection of the injury model and species, animal experimentations in the laboratory bring us closer to future therapies that could be applied in the clinics.

1.4 Implications for clinical therapies targeting Nogo-A signaling

Multiple ways have been developed to suppress Nogo-A/Nogo receptor interactions and the underlying signaling pathways, such as the application of Nogo-A function blocking antibodies, or NgR1 or RhoA/ROCK blockers. These treatments lead to enhanced regeneration with improved functional recovery and hold high promises for promoting axonal regeneration and plasticity after CNS injury (Schwab and Strittmatter, 2014). Clinical trials with the anti-human Nogo-A antibodies ATI355 (Novartis) or GSK1223249 (GlaxoSmithKline) are currently being carried out with excellent safety and tolerance for multiple CNS disease areas such as spinal cord injury, stroke, multiple sclerosis and amyotrophic lateral sclerosis. Based on our findings, however, such treatments would results in the blockade of both inhibitory myelin and positive neuronal Nogo-A pools, thereby hampering the possible full extent of axonal regeneration. Indeed, the treatment with anti-Nogo-A antibodies has been shown to down-regulate endogenously expressed Nogo-A in both cultured oligodendrocytes and neurons in vitro, as well as in vivo in the spinal cord (Weinmann et al., 2006). To overcome such treatment limitations, this phenomenon should be kept in mind when designing new treatments for spinal cord injured
patients. The ideal therapy targeting Nogo-A signaling should aim to block glial Nogo-A (in trans), but at the same time preserve neuronal Nogo-A functions (in cis). However, reaching this treatment regimen is challenging, as targeted blockers for Nogo-A in oligodendrocytes are not yet available and reinforcing neuronal Nogo-A expression would only be possible by gene therapy applications.

Furthermore, as we gain more knowledge on the factors limiting axonal regeneration in the central nervous systems, new generations of pharmacological treatments may be developed. One potential way is targeting Nogo-A signaling pathways with small molecules instead of antibodies, such as the S1PR2-blocker JTE-013 and the Rho-A/ROCK blocker Cethrin. Our recent data also suggest that targeting the cAMP/CREB pathway might be beneficial to overcome Nogo-A and MAG-mediated growth inhibition. Various degrees of growth and sprouting of injured CNS neurons have been achieved by blocking extrinsic inhibitory cues (myelin, ECM, glial scar), increasing extrinsic growth promoting cues (neurotrophic factors), or by activation of intrinsic growth programs (PTEN deletion, Stat3, mTOR up-regulation). Recently, combinations of these different treatment regimens have yielded even more success in promoting neural growth, sprouting and improving behavioral outcomes (Schnell et al., 2011, Wang et al., 2012). Whether the most promising treatment strategies in animal models are also beneficial for human patients suffering from spinal cord, brain or optic nerve injury is a challenge ahead for testing in clinical settings.
1.5 Final conclusions

The research presented in this thesis provides, at least in part, answers for the glial versus neuronal cell type-specific functions of Nogo-A after optic nerve lesion, in particular for axonal regeneration, retinal ganglion cell survival and Müller cell reactivity. Conditional deletion of Nogo-A from glial cells shed light on the specific growth inhibitory function of Nogo-A in oligodendrocytes and provided further evidence for the growth supporting, positive role of neuronal Nogo-A. We proposed that while Nogo-A leads to the activation of Nogo-A receptors in trans and subsequent neuronal growth inhibition, Nogo-A expressed in cis in neurons could counteract this receptor activation by sequestering, binding, blocking or internalizing NgR1 and/or S1PR2. This possible mode of action for Nogo-A raises many new questions, regarding the exact mechanisms and whether promoting cis-interaction between Nogo-A and its receptors could be exploited for a future potential therapy promoting axonal regeneration. Taken together, our results suggest that in order to promote strong regeneration and plasticity after CNS injury, the next generation of Nogo-A signaling blockers should selectively target Nogo-A in oligodendrocytes, and simultaneously prevent loss or even promote Nogo-A expression and functions in neurons.
References


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# Curriculum vitae

## Personal data

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## Education

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| September 2010 - present      | **PhD candidate in Neuroscience**  
ETH (Swiss Federal Institute of Technology Zurich)  
and UZH (University of Zürich), Brain Research Institute |
| November 2008 - May 2010       | **Master thesis and Research internship**  
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| September 2005 - June 2010     | **Master's Degree in Neuroscience, Bachelor's Degree in Biology**  
Eötvös Loránd University (Budapest), Faculty of Science, Institute of Biology |

## Scholarships and awards

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| March 2013                    | **First Prize for Poster presentation**  
Cambridge Brain Repair Spring School, Cambridge, UK                                             |
| November 2009                 | **First Prize for Presentation**  
Undergraduate Researchers Conference in Animal Physiology and Neurobiology section, Eötvös Loránd University, Budapest |
| June 2008 - August 2008       | **Summer Research Program Scholar**  
École Polytechnique Fédérale de Lausanne (EPFL), Brain Mind Institute |
Publications


Poster presentations


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