Role of CXCL12 and CXCR4 in axonal regeneration of mature retinal ganglion cells

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Summary

Neurons of the adult central nervous system (CNS) are not able to regenerate their axons after lesion, unlike neurons of the peripheral nervous system (PNS). Retinal ganglion cells (RGCs) are CNS neurons that project their axons along the optic nerve connecting them to visual targets in the brain. Damage of the optic nerve results in permanent functional loss of vision with disastrous consequences for the people concerned. Regenerative failure of RGCs has been mainly assigned to axotomy-induced cell death, an insufficient intrinsic ability of mature neurons to regrow injured axons as well as an inhibitory environment for axonal growth cones consisting of myelin-associated molecules and expression of chondroitin sulfate proteoglycans (CSPGs) in the forming glial scar at the lesion site leading to growth cone collapse. By now, it is known that an inflammatory stimulation (IS) caused by an injury of the ocular lens, followed by release of lens proteins into the vitreous chamber, transforms RGCs into a robust regenerative state so that the degeneration process of axotomized RGCs is significantly decelerated and the axonal growth rate is increased. This positive effect can be mimicked by intravitreal injections of purified lens proteins, the yeast wall extract zymosan or the toll-like receptor 2 agonist Pam_3Cys. Also other factors and signaling pathways were already described to be involved in optic nerve regeneration. However, regeneration is still limited after experimental therapeutic treatment and only few regenerating axons reach the optic chiasm after an optic nerve injury.

The chemokine CXCL12, which is reportedly expressed and secreted by reactive astrocytes, monocytes/macrophages and endothelial cells has been shown to be neuroprotective, facilitate elongation of cultured cerebellar granule neurons and regulate axon growth and branching in hippocampal neurons. Furthermore, intrathecal infusion of CXCL12 into the spinal cord injury site resulted in enhanced sprouting of corticospinal tract axons and a pivotal role of CXCL12 and CXCR4 during embryonic development was described guiding RGC axons within the retina to the optic stalk to exit the eye bulb.

Using pharmacological and genetic approaches, this thesis investigated the role of CXCL12 and its cognate receptor CXCR4 and signaling pathways in axonal regeneration in the adult mouse and rat optic nerve.

With experiments in adult rats we could demonstrate that CXCL12 exerted a moderate neurite growth-promoting effect on RGCs in culture and was disinhibitory towards myelin. CXCL12-mediated effects on axon growth were CXCR4 and PI3K/AKT/mTOR-signaling cascade dependent. We furthermore showed that CXCR4 was expressed in cell bodies and predominantely axons of adult RGCs. *In vivo*, intravitreal application of the recombinant protein slightly, but significantly, facilitated axon regeneration into the rat optic nerve, while combinatorial treatment with an IS further enhanced optic nerve regeneration. Interestingly, application of CXCL12 both *in vitro* and *in vivo* did not affect the survival of RGCs.

In addition to rats as model organisms the study was expanded with taking advantage of a transgenic floxed mouse line enabling RGC-specific CXCR4 depletion. Intravitreal injection of an adeno-associated virus of serotype 2 (AAV2) expressing the Cre-recombinase leads to excision of *CXCR4* in transduced RGCs. As seen in rats, CXCL12 moderately promoted neurite growth and exerted disinhibitory features towards myelin in a CXCR4- and PI3K/AKT/mTOR-signaling cascade dependent manner. However, neither application of CXCL12 nor CXCR4 depletion in adult mice affected the survival of RGCs both *in vitro* and *in vivo*. As seen in rats, CXCR4 was expressed in cell bodies and predominantely axons of mouse RGCs. Interestingly, the receptor was significantly upregulated after optic nerve crush (ONC) alone and ONC in combination with an IS. With cell culture experiments we could demonstrate a CXCR4-dependent chemoattractive feature of CXCL12 towards RGC axons. *In vivo*-experiments indicated, that endogenous CXCL12 was presumably produced and secreted by astrocytes at the optic nerve head, while CXCR4 depletion in RGCs reduced axonal U-turns of regenerating axons. This points to the possibility that endogenous CXCL12 chemoattracts CXCR4-positive axonal growth cones to the proximal segment of the crushed optic nerve, thereby limiting axon regeneration.

Intravitreal injection of CXCL12 on the one hand or depletion of CXCR4, by application of antagonists or shRNA at the lesion site, on the other hand, may therefore serve as novel adjuvant therapeutic approaches to improve CNS repair.

Zusammenfassung

Im Gegensatz zu Neuronen des peripheren Nervensystems, besitzen Nervenzellen des adulten zentralen Nervensystems (ZNS) nicht die Fähigkeit nach einer Verletzung zu regenerieren. Retinale Ganglienzellen (RGZ) sind Neurone des ZNS, die ihre Axone entlang des Sehnervs zu ihren visuellen Zielgebieten im Gehirn projezieren. Eine Verletzung des Sehnervs führt zu einem irreversiblen Sehverlust mit verheerenden Folgen für den Betroffenen. Verschiedene Faktoren sind für die eingeschränkte Regenerationsfähigkeit von RGZ verantwortlich: Adulte, verletzte Nervenzellen begehen einen Axotomie-bedingten Zelltod und ihnen fehlt die intrinsische Fähigkeit nach einer Verletzung zu regenerieren. Des Weiteren sind die Wachstumskegel der RGZ einer inhibitorische Umwelt exponiert. Die Expression von Myelin-assoziierten Proteinen und Chondroitinsulfat-Proteoglykanen an der Läsionsstelle führen zum Zusammenbruch der Wachstumskegel. Mittlerweile ist bekannt, dass eine inflammatorische Stimulation (IS) durch eine Verletzung der Linse mit einer Ausschüttung von Linsenproteinen einhergeht. Diese transformieren RGZ in einen aktiven regenerativen Zustand, der zur Folge hat, dass die Degeneration von axotomierten RGZ verlangsamt und das axonale Wachstum gesteigert wird. Der positive Effekt kann durch die intravitreale Injektion von aufgereinigten Linsenproteinen, des Hefeproteins Zymosan oder des Toll-like-Rezeptor 2 Agonist Pam_3Cys imitiert werden. Auch andere Signalwege und Faktoren, die an der axonalen Regeneration im Sehnerv beteiligt sind, konnten identifiziert werden. Dennoch zeigen Experimente mit therapeutischer Behandlung, dass die Regeneration im Sehnerv nach Verletzung eingeschränkt ist und nur wenige Axone das Chiasma erreichen.
Das Chemokin CXCL12 wird nachweislich von reaktiven Astrozyten, Monozyten/Makrophagen und Endothelzellen exprimiert und sezerniert. Es wirkt neuroprotektiv, fördert das Wachstum von kultivierten Körnerzellen des Kleinhirns und reguliert das Wachstum und die Verzweigung von Neuronen des Hippocampus. Weitere Studien zeigten, dass die intrathekale Infusion von CXCL12 in die Läsionsstelle des Rückenmarks das Aussprossen von Axonen des Kortikospinaltrakts verstärkt. Darüberhinaus sind CXCL12 und sein Rezeptor CXCR4 in der embryonalen Entwicklung für die Navigation von Axonen aus dem Auge zum Sehnerv verantwortlich.

Mit Hilfe von pharmakologischen und genetischen Methoden wurde in dieser Arbeit die Rolle von CXCL12, CXCR4 und der beteiligten Signalwege im Hinblick auf die axonale Regeneration im Sehnerv der adulten Maus und Ratte untersucht.

Experimente in der adulten Ratte zeigten, dass CXCL12 das Neuritenwachstum von RGZ in Zellkultur moderat förderte und zudem disinhibitorisch gegenüber Myelin war. Die CXCL12-mediierten Effekte waren dabei abhängig von CXCR4 und dem PI3K/AKT/mTOR-Signalweg. CXCR4 wurde im Zellkörper und insbesondere in den Axonen von RGZ exprimiert. Eine intravitreale Injektion des rekombinanten Proteins CXCL12 resultierte in einer moderat verbesserten axonalen Regeneration im Sehnerv *in vivo*. Durch eine kombinatorische Therapie mit einer IS konnte die axonale Regeneration sogar noch gesteigert werden. Interessanterweise beeinflusste die Applikation von CXCL12 weder *in vitro* noch *in vivo* das Überleben von RGZ.

Neben Ratten als Modellorganismen wurde eine transgene gefloxte Mauslinie verwendet, die eine spezifische Depletion von CXCR4 in RGZ ermöglicht. Eine intravitreale Injektion von Adeno-assoziierten Viren des Seroptyps 2 (AAV2), die die Cre-Rekombinase exprimieren, führt zur Exzision von CXCR4 in tranduzierten RGZ. Ähnlich wie in der Ratte verbesserte die Applikation von CXCL12 das Neuritenwachstum von kultivierten RGZ der Maus und wirkte disinhibitorisch gegenüber Myelin in einer CXCR4 und PI3K/AKT/mTOR-Signalweg-abhängigen Weise. Weder die Applikation von CXCL12, noch die Depletion von CXCR4 beeinflussten das Überleben von RGZ. CXCR4 wurde im Zellkörper und vornehmlich in den Axonen von RGZ exprimiert. Interessanterweise wurde die Expression des Rezeptors nach einer Quetschung des Sehnervs und nach einer Quetschung mit einhergehender IS hochreguliert. Zellkultur-Experimente zeigten, dass CXCL12 CXCR4-abhängig chemoattraktiv auf regenerierende Axone von RGZ wirkte. I*n vivo*-Modelle deuteten darauf hin, dass CXCL12 vermutlich von Astrozyten am Sehnervkopf exprimiert und sezerniert wurde, während eine Depletion von CXCR4 in RGZ die Anzahl der in den proximalen Teil des Sehnervs zurückwachsenden Axone nach Verletzung reduzierte. Dies lässt vermuten, dass endogenes CXCL12 CXCR4-positive Axone anlockt und somit eine erfolgreiche Regeneration verhindert.

Die intravitreale Injektion von CXCL12 oder die Depletion von CXCR4, durch die Applikation von shRNA oder Antagonisten an der Läsionsstelle, sind somit neue und aussichtsreiche, therapeutische Ansätze um Verletzungen im ZNS zu behandeln.

1 Introduction

1.1 Optic nerve regeneration – Limitations and strategies

In contrast to neurons of the peripheral nervous system (PNS), neurons of the adult central nervous system (CNS) are not able to regenerate their axons after lesion. Retinal ganglion cells (RGCs) are CNS neurons located in the innermost layer of the retina that project their axons along the optic nerve connecting them to visual targets in the brain. Damage of the optic nerve results in permanent functional loss of vision with disastrous consequences for the person concerned. The optic nerve can be damaged by different causes including trauma, cancer, ischemia or hematoma and often comes along with neurodegenerative/neuroinflammatory diseases such as multiple sclerosis (Compston, 2004; Plant, 2008). In glaucomatous optic neuropathies, RGCs degenerate because of the appearance of an increased intraocular pressure in the eye (Buckingham et al., 2008; Soto et al., 2008; Vidal-Sanz et al., 2011).

In the early 1900s, Ramon y Cajal demonstrated that after optic nerve injury RGCs are only able to sprout weakly, however, one of his students proved that RGC axons are able to grow into a transplanted peripheral nerve graft that was stitched to the proximal end stump of the cut optic nerve (S.R., 1991). Several years later, Aguayo et al. picked up Cajals pioneer experiments and confirmed that about 5% of RGCs extended their axons over long distances into a transplanted peripheral nerve graft, even forming synapses when the graft ends directly in front of the superior colliculus (Aguayo et al., 1987; Vidal-Sanz et al., 1987; Aguayo et al., 1991). They assumed, that a subset of RGCs still occupies the intrinsic potential to regrow and regenerate after lesion but fail principally because of an inhibitory environment.

Several years and studies later, regenerative failure of RGCs has been mainly assigned to axotomy-induced cell death, an insufficient intrinsic ability of mature neurons to regrow injured axons as well as an inhibitory environment for axonal growth cones consisting of myelin-associated molecules and expression of chondroitin

sulfate proteoglycans (CSPGs) in the forming glial scar at the lesion site (Fig. 1.1) (Fischer and Leibinger, 2012).

So far, no therapeutic treatment was discovered that is able to promote extensive regeneration of RGCs along the optic nerve reconnecting them to their appropriate targets in the brain. However, research of recent years presented a gradual progression in different approaches, leading to enhanced regeneration. Promising proceedings showed improved regeneration by transforming RGCs into an active regenerative state, applying neurotrophic factors intravitreally or by abrogation of inhibitory factors in the surrounding environmental tissue (Fischer and Leibinger, 2012). Furthermore, signaling pathways and factors have been identified that are involved in CNS regeneration and therefore act as therapeutical targets, like the JAK/STAT3- or the PI3K/AKT/mTOR-pathway (Park et al., 2008; Leibinger et al., 2009; Moore et al., 2009; Sun et al., 2011; Leibinger et al., 2013a; Leibinger et al., 2013b). Nevertheless, regeneration in the optic nerve is still limited which illustrates the impact of other so far not well studied parameters impairing axon regeneration. With common approaches using neurotrophic factors, genetic modifications or combinatorial treatments, only a small percentage of growing axons reach the optic chiasm after optic nerve crush. Lately established methods enable the analysis of regenerating axons three-dimensionally throughout the whole optic nerve (Luo et al., 2013; Pernet et al., 2013a; Luo et al., 2014). They clarify that regenerated axonal trajectories are characterized by aberrant growth and axonal U-turns emphazising misguidance as another profound problem that limitates axon regeneration (Fig. 1.1) (Diekmann et al., 2013; Pernet et al., 2013b; Pernet and Schwab, 2014a). In order to overcome restrictions of CNS regeneration, scientific research has to aspire a combinatorial therapy consisting of the administration of neurotrophic factors with simultaneous abrogation of inhibitory molecules and guidance cues.

1.1.1 Axotomy-induced cell death of mature RGCs

Soon after axotomy mature RGCs degenerate and die progressively (Berkelaar et al., 1994). Onset of cell death begins after 5-6 days and slowly continues with less then 10% surviving RGCs in rats 14 days after injury (Berkelaar et al., 1994; Fischer et al.,

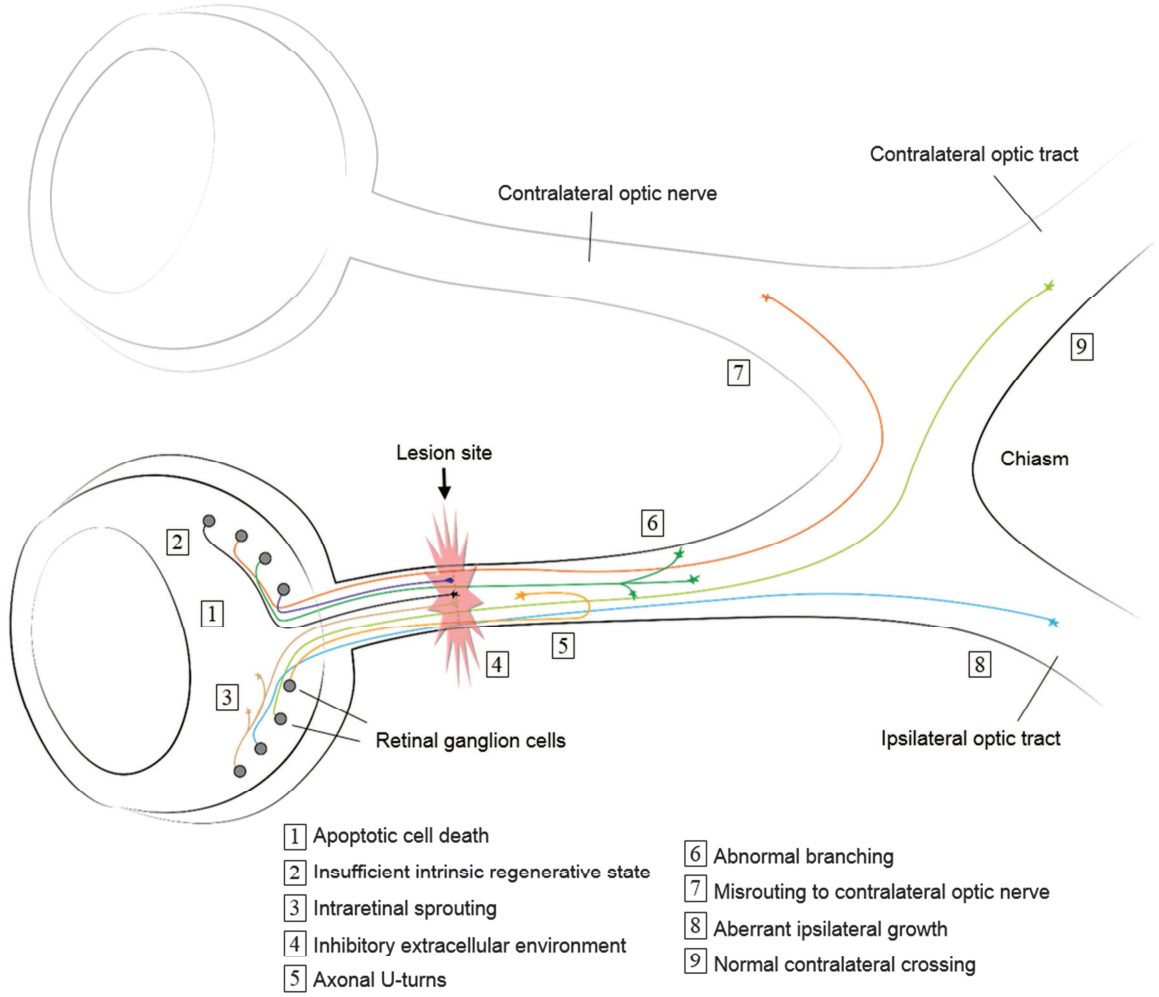

Fig. 1.1: Cellular and molecular mechanisms limiting axon regeneration in the optic nerve of adult mice. Injured RGCs have to deal with several obstacles that prevent successful axon regeneration into the mature optic nerve. Axotomy results in apoptotic cell death (1) and the intrinsic regenerative state remains low (2). Additionally, growth cones of damaged axons have to face an inhibitory environment and the presence of a gliotic scar (4) resulting in growth cone collapse. Axons that cross the lesion site typically exhibit morphological aberrations: they form U-turns toward the eye bulb (5) or form abnormal branches (6). When regenerating axons reach the optic chiasm they are partially misguided into the contralateral optic nerve (7) or grow into the ipsilateral optic tract (8). Only few axons cross contralaterally (9) (modified by Pernet and Schwab, 2014b).

2004c). Starting point and progression of axotomy-induced cell death depends on the distance of the crush site to the eye ball. When the optic nerve is injured 8-9 mm behind the eye, the onset of cell death is delayed until day 8 and about 80% of RGCs survive 4 weeks later (Berkelaar et al., 1994).

Axotomized RGCs are disconnected from their target areas located in the brain that normally ensure supply with neurotrophic factors. These are retrogradely transported to the cell body. Loss of it is presumably jointly responsible for RGC death after optic

nerve injury (Berkelaar et al., 1994; Rabacchi et al., 1994; Quigley et al., 2000; Harrington and Ginty, 2013).

Axotomy-induced death of mature RGCs is mainly due to an apoptotic mechanism, that is also called programmed cell death (Pettmann and Henderson, 1998; Cellerino et al., 2000). This process is dependent on the influx of Ca^{2+} and a Bax translocation on mitochondrial membranes, resulting in the activation of several caspases. Caspases have been termed “executioner” proteins for their role in the cell to cleave proteins responsible for the cellular integrity. They are expressed as precursors (procaspases) that get activated by enzymatic cleavage (Degterev et al., 2003; Inoue et al., 2009). After axotomy of RGCs, especially caspase-3, caspase-8 and caspase-9 occupy crucial roles in the process of apoptosis. (Kermer et al., 1998; Muzio et al., 1998; Kermer et al., 1999; Kermer et al., 2000; Weishaupt et al., 2003; Cheung et al., 2004). Once activated, caspase-3 regulates the proteolytic cleavage of an extensive variety of cellular targets, which leads to subsequent cell death. Moreover, the involvement of caspases-2 and caspase-6 were already described (Ahmed et al., 2011; Monnier et al., 2011). Alongside with caspases, members of the Bcl-2 family are also involved in apoptotic cell death, like Bcl-2, Bcl-XL and Bax (Chaudhary et al., 1999; Chierzi et al., 1999; Inoue et al., 2002; Malik et al., 2005).

1.1.1.1 Application of trophic factors to obtain neuroprotection

An advantage of using the optic nerve in order to study CNS regeneration is that substances can be applied directly into the vitreous body thereby circumventing drug entry limitations occuring because of the blood-brain barrier (blood-retina barrier). In order to prevent cell death of RGCs and enable functional optic nerve repair, trophic factors have been administered intravitreally. The family of neurotrophins are secreted proteins that regulate the development, survival and differentiation of neurons (Ebadi et al., 1997). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and neurotrophin 4/5 (NT4/5) belong to this family and interact with the specific tyrosine kinase receptors (TrK) Trk-A (NGF), Trk-B (BDNF, NT4/5) and Trk-C (NT3). All three Trk-receptors are expressed by mature RGCs, but neuroprotective effects were only described for BDNF and NT4/5 (Mey and Thanos,

1993; Cohen et al., 1994; Mansour-Robaey et al., 1994). Also other molecules are implicated in neuroprotection on RGCs, like some members of the Interleukin-6 (IL-6) family. Leukemia inhibitory factor (LIF) and ciliary neurotrophic factor (CNTF) mediate their effects via the glycoprotein 130 (gp130) and LIF-receptor and are potent neuroprotective factors for RGCs both *in vitro* and *in vivo* (Mey and Thanos, 1993; Leaver et al., 2006; Leibinger et al., 2009). Similarly, IL-6 mediates its effects on RGCs via gp130 complexed with the Interleukin-6 receptor α (IL-6R α) or via a soluble IL-6 receptor (Yasukawa et al., 1990; Heinrich et al., 2003). Previous studies demonstrated, that also IL-6 exerts neuroprotective features on mature RGCs (Leibinger et al., 2013b). Several other trophic factor receptors are expressed by mature RGCs, so that the application of fibroblast growth factor 2 (FGF2), glial cell line-derived neurotrophic facor (GDNF), hepatocyte growth factor (HGF) and granulocyte macrophage colony-stimulating factor (GM-CSF) decelerate RGC cell death after axotomy (Bahr et al., 1989; Koeberle and Ball, 1998, 2002; Schmeer et al., 2002; Schallenberg et al., 2009; Tonges et al., 2011). However, it is unclear whether these factors exert their neuroprotective effects directly on RGCs or whether they are due to other stimulated cell types that secrete neuroprotective factors as a response. Although neurotrophic factors delay axotomy-induced cell death, they do not always promote regeneration. For example the application of BDNF leads to robust neuroprotection of RGCs, but simultaneously blocks axon growth-promoting features (Cui et al., 1999; Pernet and Di Polo, 2006).

1.1.2 Insufficient intrinsic growth state of mature neurons

During development of the embryonic visual system, RGCs have to extend their axons to their appropriate target areas in the brain. At this timepoint, neurons are in an active growth state comprising the expression of several growth associated genes and the activation of specific pathways (Goldberg et al., 2002a; Moore et al., 2009). Expectedly, cultured embryonic neurons of mammals are characterized by strong neurite growth. However, this potent intrinsic growth mode decreases dramatically with ageing of animals (Goldberg et al., 2002b). Mature RGCs fail to enter into an active regenerative state upon injury and only few axons are able to regenerate into a

crushed optic nerve. For optic nerve repair, switching RGCs into a robust regenerative growth state is therefore indispensible.

1.1.2.1 Activation of intrinsic regeneration state by inflammatory stimulation

As pointed out previously, mature RGCs have lost their embryonic ability to regrow axons after injury and fail to switch into an active growth mode. Studies could nicely demonstrate that even mature RGCs can be transformed into an active regenerative state by inducing an inflammatory stimulation (IS) in the eye that allows RGCs to regrow axons into the lesioned optic nerve (Fischer et al., 2000; Leon et al., 2000). A lens injury by puncturing the ocular capsule, intravitreal injections of lens proteins (crystallins) or toll-like receptor 2 agonists like Pam_3Cys/Pam_2Cys or the yeast wall extract zymosan are sufficient to induce an IS in the eye (Fischer et al., 2000; Leon et al., 2000; Fischer et al., 2008; Hauk et al., 2010; Gensel et al., 2015). When combined with an optic nerve crush, regeneration-associated genes (RAGs) like Galanin, SPRR1a and GAP43 are upregulated, indicating a transformation of the cells into an active axonal growth state (Bonilla et al., 2002; Fischer et al., 2004c). Other important features provided by an IS are neuroprotection and disinhibiton. The central mediators of an IS are the cytokines CNTF and LIF, that are produced and released by activated retinal astrocytes and Müller glial cells (Fischer, 2008; Leibinger et al., 2009). Correspondingly, knockout of both CNTF and LIF in transgenic mice results in a complete blockade of neuroprotective and regenerative IS effects (Leibinger et al., 2009). Recently, IL-6 was identified as an additional contributor to IS-mediated optic nerve regeneration, mainly by providing its disinhibitory feature (Leibinger et al., 2013b). Mature RGCs express CNTFR, LIFR, IL-6R and gp130, enabling CNTF, LIF and IL-6 to mediate their beneficial effects directly on RGCs (Sarup et al., 2004; Leibinger et al., 2013b). Application of these cytokines or IS activate several signaling cascades, including the PI3K/AKT/mTOR- and JAK/STAT3-signaling pathways (Park et al., 2004; Lingor et al., 2008; Muller et al., 2009; Leibinger et al., 2013b).

1.1.3 Inhibitory extracellular environment

In addition to the insufficient intrinsic regenerative capability, RGC axons are exposed to an inhibitory environment developing after injury at the lesion site. Growth cones of damaged RGCs have to face inhibitory proteins that are associated with CNS myelin or the glial scar (Silver and Miller, 2004; Yiu and He, 2006; Berry et al., 2008). These inhibitory molecules bind to specific receptors thereby activating the ras homolog gene A/rho-associated protein kinase (RhoA/ROCK)-pathway. Activation of RhoA by the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) by certain guanosine exchange factors (GEFs) leads to ROCK activation. Subsequently, several downstream effectors are activated, which affect the cytoskeletal organization resulting in growth cone collapse and neurite growth inhibition (Lehmann et al., 1999; Mueller, 1999; Wong et al., 2002; Hsieh et al., 2006; Lingor et al., 2007).

1.1.3.1 CNS myelin associated inhibitors

During development, RGC axons in the optic nerve are enwrapped with myelin by brain-derived oligodendrocytes. After optic nerve injury, fragmentation of myelin sheaths occurs leading to an inhibitory environment for RGC growth cones. Myelin-associated proteins interact with specific receptors on the surface of the axon leading to a subsequent destabilization of the actin cytoskeleton (Yiu and He, 2006; Berry et al., 2008). Well-studied inhibitory proteins are Nogo, the transmembrane myelin-associated glycoprotein (MAG) and oligodendrocyte-myelin glycoprotein (OMgp) (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Wang et al., 2002b). Although these three proteins exhibit different structures, they are all able to bind to the glycosylphosphatidylinositol-(GPI-)anchored Nogo receptor (NgR) (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002a). NgR is associated with co-receptors that activate specific downstream signaling pathways. It complexes with either p75NTR or TROY, which are members of the tumor necrosis factor (TNF) receptor family, and additionally with leucine rich repeat (LRR) and Ig domain-containing NgR interacting protein 1 LINGO-1 (Wang et al., 2002a; Wong et al., 2002; Yamashita et al., 2002; Mi et al., 2004). In addition, the

paired immunoglobulin-like receptor B (PirB) was identified as a further functional receptor for the three inhibitory myelin-associated proteins (Atwal et al., 2008; Cai et al., 2012).

1.1.3.2 Formation of a glial scar

Alongside with a myelin-derived inhibitory environment, a glial scar forms at the lesion site (Silver and Miller, 2004). Damage of optic nerve tissue leads to proliferation and activation of resident microglia and recruitment of macrophages. Moreover, astrocytes proliferate that produce and secrete inhibitory CSPGs (Gallo et al., 1987; Gallo and Bertolotto, 1990; McKeon et al., 1991). In addition, several other inhibitory proteins have been identified in the glial scar, including Semaphorin 3A and Tenascin-R (Niederost et al., 1999; Tang, 2003). Secretion of CSPGs is dependent on various cytokines and growth factors (Logan and Berry, 2002). The transmembrane protein tyrosine phosphatase (PTPσ) is expressed by neurons and is a functional receptor for CSPGs (Shen et al., 2009). Recently, it was shown, that inhibition of this receptor promotes recovery after spinal cord injury (Lang et al., 2015). Some years ago, with NgR1 and NgR3 two further CSPG-receptors have been identified (Dickendesher et al., 2012). Along with myelin-associated proteins, binding of glial scar-associated molecules to their specific receptors leads to RGC growth cone collapse.

1.1.3.3 Desensitization of RGCs toward inhibitory molecules

1.1.3.3.1 Inhibition of the RhoA/ROCK pathway

Upon binding of inhibitory myelin-associated molecules or proteins of the glial scar to their respective receptors expressed on a RGC axon, the RhoA/ROCK-pathway gets activated. Downstream stimulated LIM kinase and cofilin lead to actin filament degradation resulting in growth cone collapse and regeneration stop (Mueller, 1999; Wong et al., 2002; Hsieh et al., 2006; Lingor et al., 2007). Therapeutical strategies aim for inhibition of RhoA and/or ROCK in order to interrupt the inhibitory signaling cascade. One potent RhoA-inhibitor is the ADP ribosyltransferase C3 of bacterial

origin. First studies demonstrate that treatment with C3 desensitizes neurons toward myelin *in vitro* and enables improved regeneration into the optic nerve (Lehmann et al., 1999; Bertrand et al., 2005). Likewise, the use of the ROCK-inhibitor Y27632 in retinal cultures and also intravitreally applied partially overcomes myelin and CSPG inhibition (Monnier et al., 2003; Lingor et al., 2007; Ahmed et al., 2009; Leibinger et al., 2013b). So far, compounds have been optimized to obtain more potent and selective ROCK inhibitors: Y39983 inhibits ROCK about 30 times more effectively than Y27632 and promotes optic nerve regeneration (Uehata et al., 1997; Sagawa et al., 2007; Tokushige et al., 2007).

1.1.3.3.2 Microtubules stabilization

Microtubules are components of the cytoskeleton that are composed of α- and β-tubulin-heterodimer subunits. A dynamic polymerization and depolymerization of microtubules is a prerequisite for axonal regeneration and therefore serves as a target for optic nerve repair after injury. Paclitaxel (Taxol), which is a clinically established anti-cancer drug, directly affects microtubule dynamics. When used at relatively low concentrations, Taxol promotes microtubule polymerization resulting in desensitization of mature RGCs toward myelin and CSPGs *in vitro* and enhances axon regeneration *in vivo* (Sengottuvel et al., 2011). Recently, the use of the anti-cancer agent epothilone B (epoB) was described to decrease scarring in the injured spinal cord while enhancing axon regeneration resulting in restoration of locomotive function (Ruschel et al., 2015). In contrast to Taxol, epoB is able to cross the blood-brain barrier and can therefore be administered systemically, which makes it a potential treatment approach for CNS regeneration.

1.1.4 Misguidance in the optic nerve

Lately established methods enable the investigation of regenerated axons three-dimensionally throughout the whole optic nerve, demonstrating that regeneration is characterized by the formation of U-turns, branches and misguidance including aberrant growth into the ipsilateral optic nerve or contralateral optic tract (Fig. 1.1) (Ke

et al., 2013; Luo et al., 2013; Pernet et al., 2013a; Luo et al., 2014). This abnormal growth is presumably due to guidance cues expressed by cells along the optic nerve or in the optic chiasm that misguide axons into the wrong direction. During development of the visual system, several guidance cues being expressed along the optic nerve are required for correct axon pathfinding. They direct RGC axons to exit the retina, to cross the optic chiasm and to find their target area in the brain (Tessier-Lavigne and Placzek, 1991; Tessier-Lavigne and Goodman, 1996; Erskine et al., 2000; Oster and Sretavan, 2003; Erskine and Herrera, 2007). Growth cones of neurons are extremely motile, sensory structures that retract and elongate their processes (filopodia and lamellipodia) due to their environmental conditions. Guidance cues can be expressed by other cell types or be existent in the surrounding extracellular matrix. Interaction of guidance molecules with axonal receptors subsequently activates different downstream signaling pathways affecting actin cytoskeleton organisation. Thereby, axons can be chemoattracted or chemorepelled. Well-studied guidance cues are a number of secreted, transmembrane and phospho-inositide (PI)-linked proteins from the ephrin, semaphorin, slit and netrin families. Previous studies identified Ephrins/Ephs (Klein, 2004), netrin/Dcc/UNC5 (Barallobre et al., 2005), Slits/Robos (Brose and Tessier-Lavigne, 2000) and Semaphorins/Neuropilins/Plexins (Fujisawa, 2004) as ligand/receptor signaling systems involved in guidance mechanisms. In contrast to netrins, which exhibit mainly attractant potential, most of the guidance cues act as chemorepellents that repress CNS axon growth by destabilizing the cytoskeleton in a RhoA/ROCK-dependent manner. Beside these well-studied molecules, also chemokines, like CXCL12, are involved in guiding axons during development (Chalasani et al., 2003a; Li et al., 2005). In the adult CNS, so far ephrinB3 (Benson et al., 2005; Duffy et al., 2012), Sema5A and 4D (Goldberg et al., 2004) and netrin-1 (Ellezam et al., 2001) are described to be expressed by oligodendrocytes and upregulated after optic nerve injury. Recently, Joly et al. demonstrated that knockdown of EphA4 receptor in mice enhanced axon regeneration and reduced aberrant axonal branching (Joly et al., 2014) demonstrating the influence of guidance cues on regenerating axons after optic nerve injury.

A combination of growth-promoting and neuroprotective features, while abrogating inhibitory molecules and affecting guidance specific ligand/receptor systems are a

promising therapy in order to induce optic nerve repair and regeneration presumably also in other CNS regions.

1.2 CXCL12 and its cognate receptor CXCR4

1.2.1 The chemokine family and their receptors

The chemokine superfamily comprises 50 small (8-14 kDa) polypeptides, that were initially discovered as chemotactic cytokines attracting a subset of different leukocytes (Rollins, 1997; Jaerve and Muller, 2012). In the last decades it became obvious that chemokines possess more important roles in a variety of physiological processes then expected. Beside their role in cell recruitment, the protein family is additionally involved in wound healing, homeostasis, metastasis, angiogenesis/angiostasis, organogenesis and inflammation (Rossi and Zlotnik, 2000; O'Hayre et al., 2008).
The chemokine family can be divided into four subfamilies based on the arrangement of the two N-terminal cysteine residues that form disulfide bonds to two other cysteine residues within the peptide: CXC, CC, (X)C, and CX3C. CXC chemokines exhibit one amino acid that separates the first two cysteines, whereas in CC chemokines, these two cysteines are directly adjacent to each other. In the CX3C subfamily the conserved cysteine residues are separated by three amino acids, whereas the first cysteine is missing in the (X)C subfamily. Some years ago, another potential type of chemokine (CX) has been identified in zebrafish (Nomiyama et al., 2008), which lacks one of the two N-Terminus cysteines but still exhibits the third and fourth ones.
In addition to the structural classifications mentioned above, chemokines can also be functionally classified in inflammatory and homeostatic chemokines (Moser et al., 2004). Pro-inflammatory chemokines are those upregulated under inflammatory conditions in order to recruit leukocytes to inflamed tissues. Homeostatic chemokines are those expressed constitutively in lymphoid or other organs and usually control physiological migration processes of leukocytes and their precursors (Yoshie et al., 1997). Furthermore, homeostatic chemokines are involved in the development and maintenance of tissues and organs, for example in the brain, where chemokines influence migration and patterning processes as well as differentiation to different lineages (Reiss et al., 2002; Padovani-Claudio et al., 2006; Hattermann et al., 2008).

Chemokines mediate their effects via seven transmembrane domain receptors coupled to the Gαi class of heterotrimeric G proteins (GPCRs). They are also grouped into four subfamilies according to the subfamily of their major chemokine ligands (Zlotnik et al., 2006). However, compared to the ligands, chemokine receptors are well conserved between species (Nomiyama et al., 2011). To date, about 20 chemokine receptors have been identified. Some of the ligands exclusively mediate their effects via one receptor, others are able to interact with several members of the respective receptor family. This redundancy assumingly allows fine tuning of (immune) responses and compensates mutations and deletions of single receptors.

1.2.2 The chemotactic cytokine CXCL12

Originally, CXCL12 (or stromal cell derived factor 1 (SDF-1)) was cloned from a mouse bone marrow stromal cell line (Tashiro et al., 1993) and initially described as a secreted pre-B-cell growth stimulating factor (PBSF) supporting bone marrow B cell progenitor proliferation (Nagasawa et al., 1994). Three isoforms of CXCL12 that are generated by alternative splicing of one common mRNA precursor molecule have been described in mice and rats, called CXCL12α, CXCL12β and CXCL12γ (Gleichmann et al., 2000; De La Luz Sierra et al., 2004). In contrast, humans possess with CXCL12δ, CXCL12ε and CXCL12ϕ three further isoforms (Yu et al., 2006). All splice variants encode for functional 8-14 kDa secreted proteins. An exception is the isoform CXCL12γ which is an intracellular protein localizing to the nucleolus of cells in the adult mouse heart (Torres and Ramirez, 2009). The major protein CXCL12α encodes for a 89-amino acid protein containing a 21-amino acid long signal peptide that is shared among all isoforms. CXCL12 is a homeostatic chemokine being constitutively expressed in a variety of different organs (e.g., bone marrow, heart, liver, lung, lymph nodes, liver, brain, kidney, pituitary). It has a crucial role in developmental processes, including hematopoiesis, cardiogenesis, vascular formation, and neurogenesis, as well as the maintenance of tissue stem cells (Nagasawa et al., 1996a; Aiuti et al., 1997; Tachibana et al., 1998; Zou et al., 1998; Sugiyama et al., 2006) and furthermore, CXCL12 production is correlated with pathological processes,

such as inflammation, heart failure, cell damage after organ irradiation or during chemotherapy.
CXCL12 mediates its effects via two chemokine receptors: CXCR4 and CXCR7.

1.2.3 CXCL12 receptors CXCR4 and CXCR7

CXCR4 (or LESTR/Fusin) is a G-protein coupled seven transmembrane domain receptor that was identified independently of CXCL12 and cloned from a monocyte library (Loetscher et al., 1994). Instantly, CXCR4 was identified as a HIV-entry cofactor (Feng et al., 1996) and a functional interaction of CXCL12 and CXCR4 could be demonstrated (Bleul et al., 1996). The CXCL12-CXCR4 axis is highly conserved between species and by now, a central role in development and physiology could be uncovered (Zlotnik et al., 2006). Accordingly, deletion of CXCL12 or CXCR4 in mice is perinatal lethal. Remarkably, CXCR4 or SDF-1 deficient mice show disturbed hematopoiesis, lymphopoiesis and developmental abnormalities in the hippocampus, cerebellum, retina and spinal cord (Ma et al., 1998; Zou et al., 1998; Nagasawa, 2001; Zhu et al., 2002; Zhu et al., 2009), which underlines the important role of the CXCL12-CXCR4 axis during development.
For many years CXCR4 has been considered the unique receptor for CXCL12 and CXCL12 the sole ligand for CXCR4. However, some years ago, based on structural similarities to other chemokine receptors, CXCR7 (or RDC-1) was identified as an alternative receptor for CXCL12 (Balabanian et al., 2005). It could be demonstrated that the seven-transmembrane receptor CXCR7 is able to bind and internalize CXCL12 on T lymphocytes. CXCR7 does not mediate its effects via classic G-protein pathways. Unlike CXCR4, it is unable to elevate Ca^{2+} levels and it presumably functions as a scavenger receptor to regulate the extracellular availability of CXCL12 (Naumann et al., 2010). Like CXCL12 and CXCR4 knockout mice, deletion of CXCR7 is associated with severe developmental defects affecting the central nervous system, heart and vasculature (Sierro et al., 2007). In addition to CXCL12, CXCR7 can also interact with the chemokine CXCL11 (interferon-inducible T-cell α chemoattractant, ITAC), however, classical GPCR mediated chemokine signaling could not be demonstrated (Burns et al., 2006).

1.2.3.1 MIF and ubiquitin as non-cognate ligands for CXCR4

Originally, the 114 amino acid cytokine macrophage migration inhibitory factor (MIF) was discovered as an inhibitor of random macrophage migration (Gregory et al., 2004). MIF plays a critical role in inflammatory diseases and atherogenesis (Weber et al., 2004; Charo and Ransohoff, 2006; Morand et al., 2006). It binds to CD74 which is a single-pass type II membrane protein, also known as MHC class II invariant chain that is expressed on class II-positive cells including monocytes/macrophages and B lymphocytes (Leng et al., 2003). Furthermore, MIF is a non-cognate ligand for the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al., 2007). Upon binding to CXCR4, MIF promotes T-cell chemotaxis and controls endothelial progenitor cell migration and cancer cell metastasis (Dessein et al., 2010; Simons et al., 2011).
Moreover, CXCR4 is a receptor for extracellular ubiquitin (Saini et al., 2010). Ubiquitin is a small (8.6 kDa) and evolutionary conserved protein which occupies important biological functions as a post-translational protein modifier inside the cell (Hershko and Ciechanover, 1998). However, ubiquitin is also present in normal plasma and various diseases are linked to increased ubiquitin levels in the systemic circulation (Majetschak, 2011). Ubiquitin acts as an immune modulator and administration of exogenous ubiquitin has anti-inflammatory and therapeutically relevant effects in various animal models (Tripathi et al., 2013). The physiological role of CXCR4-ubiquitin interaction still has to be elucidated. In contrast to CXCL12, ubiquitin does not bind to CXCR7 and its interaction with CXCR4 is independent of its N-Terminus (Saini et al., 2011a; Saini et al., 2011b; Tripathi et al., 2013). Nevertheless, both ligands activate a very similar pattern of signaling pathways downstream of CXCR4 such as the MAPK/ERK- and PI3K/AKT/mTOR-cascades (Saini et al., 2011b).

1.2.4 Signaling cascades stimulated upon CXCL12 binding

Binding of CXCL12 to CXCR4 activates a diversity of signaling pathways, that regulate chemotaxis, cell survival, proliferation, Ca^{2+} influx and gene transcription (Fig. 1.2) (Teicher and Fricker, 2010; Duda et al., 2011). Key signaling cascades that are activated upon ligand binding are presumably tissue-dependent and thus may vary

between different cell types. CXCR4 is a typical chemokine receptor that is coupled to an intracellular heterotrimeric G-protein consisting of Gα- and Gβ- and Gγ-subunits that are associated with the inner surface of the plasma membrane. In its basal state, the heterotrimer binds GDP. Upon CXCL12 interaction CXCR4 changes its three-dimensional conformation leading to replacement of GDP to GTP. This results in the dissociation of the G-protein into an α-monomer and a βγ-dimer, that consequently activate multiple transductional pathways (Bajetto et al., 2001). Some studies suggest that mainly G_i proteins are involved in chemokine signaling (Thelen, 2001), while by now it is clear, that also other G-protein subtypes and also non-G-protein-mediated pathways are involved (Bacon et al., 1995; Arai and Charo, 1996; Molon et al., 2005). The α-subunit was long thought to be responsible for major CXCL12 signaling. However, the βγ-subunit is also crucial for the regulation of many chemokine-induced pathways. Amongst others, phospholipase C (PLC) is activated, which hydrolizes phosphatidylinositol 4,5-biphosphate (PIP2) inducing the generation of diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP3) controlling the release of intracellular Ca^{2+}. $G_{\alpha i}$ subunits activate the phosphoinositide-3 kinase PI3K/AKT pathway, regulating cell survival and proliferation. The βγ-subunit is involved in Ras activation of the MAPK/ERK cascade, leading to altered gene expression and cell cycle progression. Furthermore, βγ-dimers interact with ion channels and activate PI3K, modulating CXCL12-dependent chemotaxis. CXCL12 also causes CXCR4 desensitization and uncoupling from G-proteins by GPCR kinase (GRK)-dependent phosphorylation. Subsequent interaction of CXCR4 with β-arrestin mediates internalization of the receptor (Cheng et al., 2000) and targets desensitized CXCR4 to clathrin-coated pits for endocytosis.

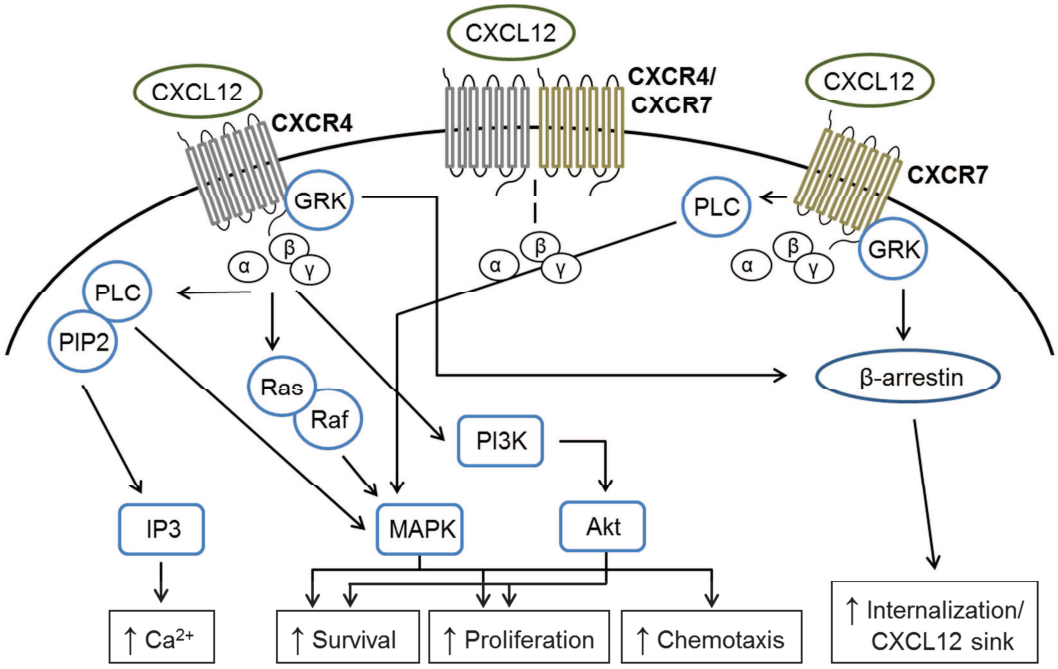

Fig. 1.2: Major CXCL12 signaling pathways. CXCL12 mediates its effects via CXCR4 and CXCR7, which are GPCRs forming homodimers or heterodimers. When the receptors heterodimerize, CXCR7 alters the conformation of the CXCR4/G-protein complexes and prohibits signaling. Besides intracellular Ca^{2+} mobilization, the activation of CXCR4 by CXCL12 leads to G-protein-coupled signaling through PI3K/AKT- and MAPK-pathways, which promote cell survival, proliferation, and chemotaxis. In addition, the β-arrestin pathway can be activated through GRK to internalize CXCR4. When CXCR7 binds CXCL12, the classical GPCR mobilization of Ca^{2+} does not occur, and activation of the β-arrestin pathway may lead to scavenging of CXCL12. In certain cancer cells (e.g., gliomas), CXCR7 can also signal through PLC/MAPK to increase cell survival (modified by Duda et al., 2011).

In contrast to CXCR4, binding of CXCL12 to the monomer CXCR7 does not activate $G_{\alpha i}$ signaling but promotes β-arrestin-mediated signaling (Levoye et al., 2009; Rajagopal et al., 2010; Decaillot et al., 2011). The C-Terminus of CXCR7 affects the ligand-uptake/degradation rate, G-protein coupling, and also receptor stability (Hoffmann et al., 2012). Shimizu et al. indicated that CXCR7 may interact with CXCR4 at the intracellular level in differentiated neurons, possibly influencing CXCR4 trafficking and/or coupling to other proteins (Shimizu et al., 2011).

Upon CXCR4-CXCR7 heterodimerization, the conformation of the receptor complex is altered which impairs chemokine receptor typical G-protein signaling (Levoye et al., 2009).

1.2.5 Role of CXCL12 and CXCR4 in the developmental and adult CNS

Apart from its crucial and well-studied role in developmental and immunological processes, like hematopoiesis, cardiogenesis and vascular formation, the impact of the CXCL12-CXCR4 axis in the adult CNS is not completely uncovered yet. Both proteins are widely and constitutively expressed not only in the developing but also the adult nervous system (Stumm et al., 2002; Stumm et al., 2003; Stumm and Hollt, 2007; Schonemeier et al., 2008). G-protein coupled receptor CXCR4 is expressed on neuronal progenitor cells and CNS neurons, like retinal ganglion cells (RGCs) (Tran et al., 2004; Chalasani et al., 2007; Peng et al., 2007), while its ligand CXCL12 is mainly produced and secreted by reactive astrocytes, monocytes/macrophages and endothelial cells (Hill et al., 2004; Miller et al., 2005; Sanchez-Martin et al., 2011; Tysseling et al., 2011).

1.2.5.1 Migration of neuronal progenitor cells and remyelination

During CNS development, the CXCL12-CXCR4 axis plays a crucial role in directing neuronal migration. Previous studies demonstrated that CXCL12 is a chemoattractant for migratory neurons constituting the cerebellum, cerebral cortex, dentate gyrus, dorsal root ganglia and nuclei in the brainstem in rodents (Ma et al., 1998; Zou et al., 1998; Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Belmadani et al., 2005; Odemis et al., 2005; Borrell and Marin, 2006).
In addition, the chemokine CXCL12 and its cognate receptor are involved in the survival and migration of neuronal and oligodendrocyte precursor cells (OPC) and may thereby contribute to the process of remyelination after CNS injury (Dziembowska et al., 2005; Banisadr et al., 2011). Patel and colleagues described that use of a pharmacological CXCR4-antagonist, as well as the administration of CXCR4-specific shRNA in order to abrogate CXCR4 signaling, reduced OPC maturation resulting in remyelination failure (Patel et al., 2010).

1.2.5.2 CXCL12-CXCR4 signaling regulates axonal pathfinding, sprouting and elongation

Beside the accurate regulation of progenitor cell migration during CNS development, the formation of a complex functioning circuitry by neurons extending neuritic processes (axons or dendrites) and synaptic connections to their targets is just as important and crucial. As delineated before, axonal growth cones sense guidance cues which can influence their navigation as they get chemoattracted or -repelled (Tessier-Lavigne and Goodman, 1996). A variety of molecules contribute to axonal pathfinding, like the chemokine CXCL12. CXCL12-CXCR4 signaling is important for guiding RGC axons within the retina to the optic stalk to exit the retina (Li et al., 2005). Moreover, ectopically expressed CXCL12 in the retina chemoattracts axons and deviates them from their normal pathway (Li et al., 2005) emphasizing its role in influencing RGC neurite growth. *In vitro* assays could demonstrate that CXCL12 decreases the effectiveness of many repellent factors while it does not exhibit chemoattracting or repelling effects itself (Chalasani et al., 2003a; Chalasani et al., 2007). Likewise, CXCR4-knockout mice exhibit hyperfasciculated and aberrantly projecting axons (Chalasani et al., 2003a). Previous studies demonstrated furthermore that CXCR4 is essential for the precision of initial motor axon trajectories of motor neurons in the spinal cord (Lieberam et al., 2005) and that growth cones of rat cerebellar axons in culture are chemorepelled by a CXCL12 gradient (Xiang et al., 2002). Moreover, CXCL12 induces axonal elongation in cultured cerebellar granule neurons in a Rho-dependent manner and differentially regulates axon growth and branching in hippocampal neurons (Arakawa et al., 2003; Pujol et al., 2005). Intrathecal infusion of CXCL12 into the spinal cord injury site results in enhanced sprouting of corticospinal tract axons into the inhibitory white and grey matter of adult rats (Opatz et al., 2009b). In the same study it was shown that CXCL12 reduces the sensitivity of axonal growth cones of DRG neurons towards myelin (Opatz et al., 2009b). However, the molecular mechanism underlying this effect remained elusive.

1.3 The visual system of rodents as a model to study axonal regeneration

The optic nerve is a popular morphological model in order to study CNS regeneration. It represents a defined unidirectional myelinated tract, containing astrocytes and oligodendrocytes, and consists almost entirely of fibres of one origin, the axons from RGCs. Eyes of rats and mice exhibit the same basic structure and function of all mammalian eyes, including the human eye. Thus, they are commonly used to investigate optic nerve diseases. Rats and mice are inexpensive, easy to maintain in the laboratory, and their eyes and optic nerves are easily accessible. In addition, the use of a transgenic mice model confers the unique advantage that genes can be specifically knocked out, overexpressed or altered in their sequence.

Still, the structures of the rodent optic nerve and retina have some differences from those of humans: eyes of rats and mice do not have a macula or fovea and 85 - 90% of their optic nerve axons cross the midline at the optic chiasm and innervate the

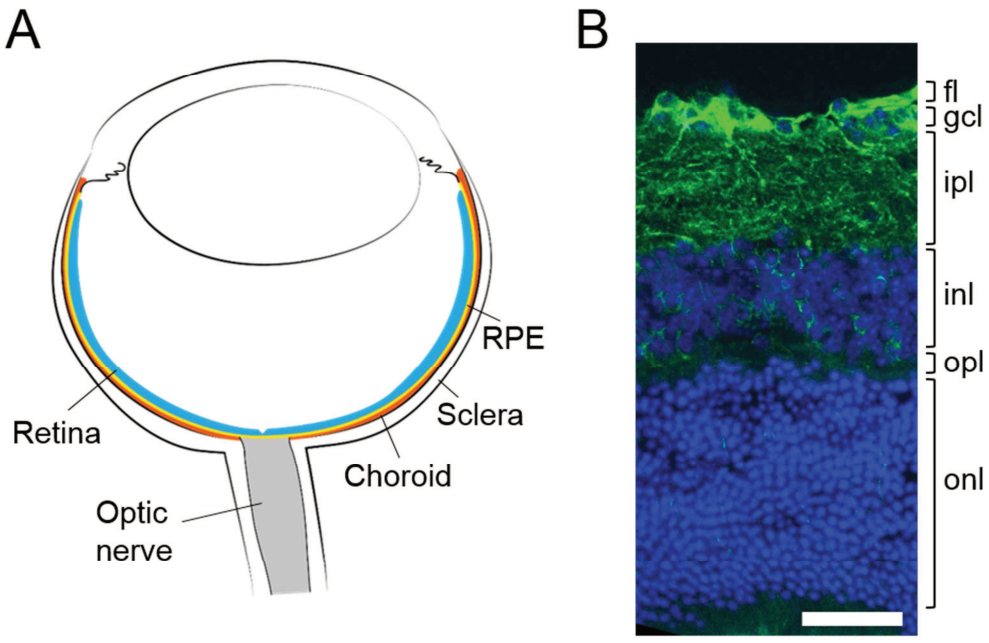

Fig. 1.3: Organization of the mouse eye and retina. (A) The retina is the innermost layer of the eye. The retinal pigment epithelium (RPE) is located between the retina and choroids, a vascularized and pigmented connective tissue. (B) A DAPI (blue) stained cross section of a mouse retina highlights the cell distribution in the retina. βIII-tubulin staining (green) shows RGCs in the innermost ganglion cell layer (gcl) of the retina where also displaced amacrine cells are present. In the inner nuclear layer (inl) amacrine cells, displaced RGCs, horizontal, bipolar and Müller glial cells are located, while in the outer nuclear layer (onl) rods and cones are present. fl: fiber layer, ipl: inner plexiform layer, opl: outer plexiform layer. Scale bar: 50 µm.

contralateral hemisphere, while in humans about 60% of fibers cross in the chiasm (Drager and Olsen, 1980; Jeffery et al., 1981).

The mammalian retina consists of the innermost ganglion cell layer which compromises the cell bodies of RGCs and displaced amacrine cells, the inner plexiform layer containing the processes and terminals of bipolar cell, amacrine cells and ganglion cells, the inner nuclear layer with amacrine cells, displaced RGCs, Müller glial cells, bipolar cells and horizontal cells and the outer plexiform layer containing the processes and synaptic terminals of the photoreceptors, the outer nuclear layer with rods and cones. The processes of Müller glial cells fill all space in the retina that is not occupied by neurons and blood vessels (Fig. 1.3). The layers are bordered by the retinal pigment epithelium, the choroid and the sklera forming the eye bulb (Fig. 1.3).

The RGCs are the output neurons of the retina that project their axons to their target areas in the brain by forming the optic nerve. Both optic nerves decussate in the optic chiasm and divide into the optic tracts leading to the brain. Central projections in the brain of RGC axons are the lateral geniculate nucleus (LGN) of the thalamus (diencephalon) that is connected with the primary visual cortex, the superior colliculus (SC) at the dorsal surface of the midbrain responsible for orienting the movements of head and eyes, the pretectum (between thalamus and midbrain) responsible for reflex control of pupil and lens, and the suprachiasmatic nucleus of the hypothalamus responsible for the regulation of the circadian rhythms. In both humans as well as other mammals, the LGN and the SC are the two most important projection areas.

1.4 Aim of this thesis

Optic nerve regeneration is limited by an insufficient intrinsic regenerative state of RGCs, an inhibitory environment at the lesion site and axotomy-induced cell death. Lately, it became obvious that axonal regeneration is still restricted even when neurotrophic factors are applied or growth cones were made insensitive toward the inhibitory environment. New experimental approaches demonstrated that the majority of regenerating axons are misguided; they form U-turns, branches and grow aberrantly. Presumably, this is due to repellent and attractive guidance cues expressed along the optic nerve. Already during development different molecules are produced within the optic nerve in order to guide growing axons through the optic nerve to their proper target areas. The chemokine CXCL12 is a developmental guidance cue that was already shown to chemoattract RGC axons to the optic stalk enabling them to exit the eye. Within this thesis, the role of the CXCL12-CXCR4 axis in mature rodent RGCs after optic nerve injury and its influence on regenerating axons in the optic nerve was investigated. Thereby, potential treatment approaches for optic nerve repair should be identified.

In detail, the following main objectives were addressed in this thesis:

1) Testing if CXCL12 is a neuroprotective, growth promoting and disinhibitory factor for mature rodent RGCs by using various cell culture and *in vivo* models.
2) If CXCL12 expectedly exerts positive effects on RGCs, it will be addressed whether its cognate receptor CXCR4 is involved in CXCL12-mediated signaling using pharmacological as well as genetic approaches.
3) Identification of potential downstream signaling pathways in mature RGCs that are affected upon CXCL12 treatment by immunocytochemical stainings and cell culture models.
4) Clarifying a potential functional role of the CXCL12-CXCR4 axis in optic nerve regeneration *in vivo* with a threedimensional analysis of regenerated axons.

2 Materials and Methods

2.1 Materials

2.1.1 Media and supplementary reagents

Table 2.1: Media, reagents and kits

Name	Supplier
AG490	Calbiochem (Merck Millipore), Germany
AMD3100 octahydrochloride hydrate	Sigma-Aldrich, Germany
B27	GIBCO (Invitrogen), Germany
BCA assay	Interchim, France
BSA	Sigma-Aldrich, Germany
CNTF, recombinant (rat)	Peprotech, Germany
Collagenase type I	Sigma-Aldrich, Germany
Cryo Compound	Klinipath, Netherlands
CTB conjugated to Alexa Fluor® 594	Invitrogen, Germany
CXCL12, recombinant (human)	R&D Systems or Peprotech, Germany
DMEM high glucose	GIBCO (Invitrogen), Germany
DMEM low glucose	GIBCO (Invitrogen), Germany
Donkey serum	Serotec, Germany
PBS	GIBCO (Invitrogen), Germany
Ethanol	Sigma-Aldrich, Germany

Fetal bovine serum	GIBCO (Invitrogen), Germany
FocusClear™	Cedarlane, Australia
Ketamine	Pfizer, Germany
Laminin	Sigma-Aldrich, Germany
L-Cysteine	Sigma-Aldrich, Germany
LY249002	Sigma-Aldrich, Germany
Methanol	Sigma-Aldrich, Germany
MIF, mouse	Novus Bio, Great Britain
MountClear™	Cedarlane, Australia
Mowiol	Merck Millipore, Germany
Normal goat serum	GIBCO (Invitrogen), Germany
Pam_3Cys ((S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH)	EMC Microcollections, Germany
Papain	Worthington, USA
Paraformaldehyde	Sigma-Aldrich, Germany
Penicillin/streptomycin	Biochrom AG, Germany
Poly-D-lysine hydrobromide (molecular weight 70,000 - 150,000 Da)	Sigma-Aldrich, Germany
Protease inhibitor cocktail	Merck, Germany
Rapamycin	LC Laboratories, USA
RNase OUT	Invitrogen, Germany
RNeasy Mini Kit	Quiagen, Germany

Superscript II Reverse Transcriptase	Invitrogen, Germany
SYBR Green PCR Master Mix	Invitrogen, Germany
Triton X-100	Sigma-Aldrich, Germany
Trypsin/EDTA, 0.5%	GIBCO (Invitrogen), Germany
TSA	Life Technologies, Germany
Tween 20	Sigma-Aldrich, Germany
Ubiquitin, recombinant (human)	R&D Systems, Germany
Xylazine	Bayer, Germany
Y27632	Sigma-Aldrich, Germany

2.1.2 Antibodies

Table 2.2: Antibodies

Name (catalog no.)	**species**	**dilution**	**supplier**
anti-CXCL12 (ab25117)	rabbit	1:250	Abcam, Great Britain
anti-CXCR4 (ab124824)	rabbit	1:400 1:1000 with TSA	Abcam, Great Britain
anti-GAP43	sheep	1:1000	LI Benowitz, USA
anti-GFAP (sc-33673)	mouse	1:50	Santa Cruz Biotechnology, USA
anti-HA (H6908)	rabbit	1:500	Sigma-Aldrich

anti-200 kD Neurofilament (ab7795)	mouse	1:1000	Abcam, Great Britain
anti-phospho-S6 (Ser235/236) (91B2) (#4857)	rabbit	1:500	Cell Signaling Technology, Germany
anti-phospho-STAT3 (Tyr705) (D3A7) (#9145)	rabbit	1:500	Cell Signaling Technology, Germany
anti-βIII-tubulin (TUJ1) (MMS-435P)	mouse	1:2000	Covance, USA
anti-mouse Alexa Fluor® 488/594 (A21202/A21203)	donkey	1:1000	Molecular Probes (Invitrogen), Germany
anti-rabbit Alexa Fluor® 488/594 (A21206/A21207)	donkey	1:1000	Molecular Probes (Invitrogen), Germany
anti-sheep Alexa Fluor® 488 (A11015)	donkey	1:1000	Molecular Probes (Invitrogen), Germany
biotinylated anti-rabbit IgG (BA-1000)	goat	1:150	Vector Laboratories (Alexis), Germany

2.2 Methods

2.2.1 Animals and surgical procedures

2.2.1.1 Animals

Adult (5 - 12 weeks) Sprague-Dawley rats or adult (6 - 10 weeks) homozygous floxed CXCR4$^{f/f}$ mice (B6.129P2-Cxcr4^{tm2Yzo}/J), floxed CXCR4$^{f/f}$/ROSA$^{f/-}$ mice (B6.Cg-Cxcr4^{tm2Yzo}/J Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J) and corresponding wild-type mice (Jackson Laboratory, USA) were maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. All animals were kept under the same conditions for at least 10 days before being used in experiments. All surgical procedures were approved by the local animal care committee in Recklinghausen and conducted in compliance with federal and state guidelines for animal experiments in Germany.

All surgical procedures were kindly performed by Prof. Dr. Dietmar Fischer.

2.2.1.2 Optic nerve crush

Animals were anesthetized by intraperitoneal injections of ketamine (60 - 80 mg/kg) and xylazine (10 - 15 mg/kg) and a 1.0 - 1.5 cm incision was made in the skin above the right orbit. The optic nerve was surgically exposed under an operating microscope and the dural sheath was longitudinally opened. The nerve was completely crushed 1 mm behind the eye for 10 s using jeweler's forceps, avoiding injury to the retinal artery. By fundoscopic examination after each surgery, the vascular integrity of the retina was verified.

2.2.1.3 Inflammatory stimulation via lens injury or injection of Pam_3Cys

In order to induce an inflammatory stimulation (IS), a lens injury was performed using a retrolenticular approach. Therefore the lens capsule was punctured with the tip of a microcapillary tube as described previously (Fischer et al., 2000). Alternatively, 2 µl of the toll-like receptor 2 agonist (S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-

(R)-Cys-(S)-Ser-(S)-Lys4-OH (Pam_3Cys, dissolved in H_2O (1 µg/µl)) was intravitreally injected immediately following optic nerve crush (Hauk et al., 2010; Fischer and Leibinger, 2012; Leibinger et al., 2012).

2.2.1.4 Intravitreal injections and genetic knockdown of CXCR4 in mice

For evaluating the regenerative state of rat RGCs, either bovine serum albumin (BSA), CNTF or CXCL12 (each 1.5 µg dissolved in PBS) was intravitreally injected alongside with and again 3 days after optic nerve injury. RGCs were dissociated as described below 5 days after surgery and cultured for 24 h.

Regeneration in the optic nerve was evaluated in rats that received two intravitreal injections either of BSA or CXCL12 (each 1.5 µg dissolved in PBS) 3 and 7 days after optic nerve surgery. Additional injections were not performed in order to minimize the potential risk of damaging the lens.

In order to induce a genetic knockdown of CXCR4, adult floxed CXCR4 or floxed CXCR4/ROSA mice received an intravitreal injection of either 2.5 µl AAV2-Cre or as a control 2.5 µl AAV2-GFP (adeno-associated virus of serotype 2, preparation see below) 3 weeks before further surgeries. Mainly RGCs are transduced upon intravitreal injection of AAV2 (Harvey et al., 2002; Sapieha et al., 2003; Fischer et al., 2004b; Fischer et al., 2004c) as this virus serotype is highly neurotropic (Rane and Reddy, 2000; Park et al., 2008), and RGCs are the first neurons to be encountered by the virus. By this, AAV2-Cre transduced RGCs express the HA-tagged Cre-recombinase, which leads to excision of the loxP-site flanked exon 2 of *CXCR4* (Nagy, 2000; Nie et al., 2004). Transduced cells can be visualized via immunohistochemical staining against the HA-tag. In CXCR4/ROSA mice, the transduction of RGCs with AAV2-Cre leads to a strong cytoplasmatic expression of the red fluorescent protein tdTomato.

It has to be mentioned that the control injection with AAV2-GFP leads to a very faint green fluorescent signal of transduced cells only, so that additional staining of proteins with the secondary antibody Alexa Fluor® 488 was not impaired or falsified.

2.2.2 Preparation of adeno-associated virus serotype 2 (AAV2)

For AAV2 production, the pAAV-MCS plasmid (Stratagene, USA) carrying the cDNA for Cre-HA (kindly provided by Dr. Zhigang He) (Park et al., 2008) or GFP downstream of the CMV promoter was used. For recombinant virus generation, AAV-293 cells (Stratagene, USA) were co-transfected with pAAV-RC (Stratagene, USA) encoding the AAV genes rep and cap together with the helper plasmid (Stratagene, USA) encoding E24, E4 and VA. Purification of virus particles was performed as described previously (Zolotukhin et al., 1999; Park et al., 2008). The viral solutions had titer of about 1 x 10^9 GC/ml, which lead to a RGC transduction rate of up to 90%.

2.2.3 Molecular biology

2.2.3.1 RNA isolation and quantitative Real-Time PCR

Total RNA was isolated from rat and mouse retinae that were subjected to no treatment, ONC alone or ONC + IS for 5 days using the RNeasy kit (Quiagen, Germany) according to the manufacturer's instructions. Retina-derived RNA (40 ng) was reverse transcribed using the superscript II reverse transcriptase kit (Invitrogen, Germany). The quantitative Real-Time PCR for CXCR4, SPRR1a, Galanin, GAP43 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was performed using SYBR Green PCR Master Mix and QuantiTect primers (Mm_Sprr1a_2_SG, Mm_Gapdh_3_SG, Mm_Gap43_1_SG Mm_Gal_1_SG QuantiTect Primer Assay (200); Qiagen, Germany) and the Real-Time PCR System (Applied Biosystems 7500). For mouse CXCR4 forward primer 3'-CTGGCT GAAAAGGCAGTCTATGT-5' and reverse 3'-TGACTTCATCTTTGCGACG-5' were used. Retina-derived cDNA was amplified during 45 cycles according to the manufacturer's protocol. All reactions were performed in duplicates and at least three independent samples per group were analyzed. The expression levels of CXCR4, GAP43, Galanin and SPRR1a was calculated relative to the endogenous housekeeping gene GAPDH using the Applied Biosystems 7500 software. Relative quantification was calculated using comparative threshold cycle method ($\Delta\Delta C^T$). The specificity of the PCR products from each run

was determined and verified with the dissociation curve analysis feature of the Applied Biosystems 7500 software.

2.2.3.2 Cloning of CXCR4 and CXCL12

Total RNA from adult rat or mouse retina was isolated and reverse transcribed into cDNA using the superscript II reverse transcriptase kit (Invitrogen, Germany). Rat and mouse CXCR4 was amplified by PCR using specific primers (see Table 2.3) and cloned into the pcDNA3.1/V5-His-Topo expression vector (Invitrogen, Germany). Rat CXCL12 was amplified with specific primers and then subcloned into the pAAV-IRES-hrGFP vector (Stratagene, USA). The identity of the amplified genes with the published sequences (rat CXCR4: NM_022205.3; mouse CXCR4: NM_009911.3; rat CXCL12: NM_022177.3) was confirmed by sequencing.

Table 2.3: Primer sequences used for amplification of CXCR4 and CXCL12

gene	host	primer forward 5' → 3'	primer reverse 5' → 3'
CXCR4	rat	GAGCGAGCATTGCCATGGAAATA	GCGTAAGTGTTAGCTGGAGTGAA
CXCR4	mouse	GACGAATTCGTTGCCATGGAACCGATC	CTTTGCATAAGTGTTAGCTGGAGTG
CXCL12	rat	TTTCACTCTCCGTCCACCTCG	ATGACCCCAGTCAGTGCCGT

2.2.4 Preparation and maintenance of cells

2.2.4.1 Dissociation of RGCs

Dissociated retinal cultures were prepared as described previously (Grozdanov et al., 2010). In brief, untreated or *in vivo* primed rats were killed by inhalation of CO_2, while mice were killed by an overdose of intraperitoneal injections of ketamine and xylazine. Immediately thereafter, eyes were removed from surrounding tissue and enucleated. Retinae were rapidly separated from the eyecups and incubated at 37°C for 30 min in a digestion solution containing papain (16.4 U/ml) and L-cysteine (0.3 µg/ml) in high glucose Dulbecco's Modified Eagle medium (DMEM). They were then rinsed and triturated in 1.5 ml DMEM. To remove cell fragments and factors released from the cells, the retinal cell suspension was immediately adjusted to a volume of 50 ml with DMEM and washed once by centrifugation (5 min at 900 x g for rats and 5 min 500 x g for mice). The pellet was carefully resuspended in an appropriate volume of high glucose DMEM containing B27-supplement (1:50) and penicillin/streptomycin (1:50). Dissociated cells were passed through a cell strainer (40 µm, BD, USA) and 300 µl cell suspension were added per well of a 4-well tissue culture plate (Nunc, Germany). To promote the adherence and growth capacity of RGCs, plates were coated first with poly-D-lysine (0.1 mg/ml) and subsequently with laminin (20 µg/ml) before use. Retinal cells of animals that were subjected to surgery were cultured for 24 h, while those of unprimed rats and mice were in culture for 3 and 4 days, respectively. All cultures were incubated at 37°C and 5% CO_2 prior to fixation with 4% PFA. Experimental conditions were pseudo-randomly arranged on the plates, so that the investigator would not be aware of their identity. RGC survival studies were performed in 96-well plates coated with poly-D-lysine (0.1 mg/ml). To this end, 50 µl retinal cell suspension was added per well and cultured for 2 h, 5 or 7 days prior to fixation with 4% PFA.

2.2.4.2 Dissociation of DRGs

DRG neurons of wildtype mice were obtained under sterile conditions as described previously (Scott, 1977; Gobrecht et al., 2014). In brief, DRGs (T8-L6) were cut into sections, digested and mechanically dissociated in a collagenase mixture (0.3% collagenase type IA, 0.25% trypsin/EDTA) dissolved in serum-free DMEM at 37°C for about 45 min, until all ganglia were digested. Trypsin was inactivated by adding serum-containing DMEM. Dissociated ganglia were collected by centrifugation at 1,000 x g for 9 min and the resulting pellet was resuspended in pre-warmed DMEM (low glucose) with FBS (10%) and Penicillin/Streptomycin (2%). Cells were cultured on poly-D-lysine (0.1 mg/ml) and laminin (20 mg/ml) coated 96-well plates (BD, USA) at 37°C and 5% CO_2 for 2 days.

2.2.5 Preparation of CNS myelin

Inhibitory central myelin extract was prepared according to earlier publications (Ahmed et al., 2006; Sengottuvel et al., 2011). In brief, Sprague-Dawley rat brains were mechanically homogenized in 0.32 M sucrose and centrifuged at 800 x g for 10 min (4°C) to collect cell debris. The supernatant was kept, the cell pellet again homogenized in 0.32 M sucrose and centrifuged as described above. Both supernatants were combined and centrifuged at 13,000 x g for 20 min (4°C). The supernatant was discarded and the pellet resuspended in 0.32 M sucrose. 0.9 M sucrose was pipetted in a tube and overlain with the resuspended solution thoroughly. The tube was then centrifuged at 20,000 x g for 60 min (4°C). After the centrifugation step, the white myelin extract can be found between the two phases. It was carefully collected, resuspended in 0.32 M sucrose solution and centrifuged at 13.000 x g for 25 min (4°C). Subsequently, the pellet was resuspended in water and kept on ice for 30 min. After final centrifugation at 20,000 x g for 25 min (4°C), the supernatant was discarded and the CNS myelin extract was resuspended in the desired volume, aliquoted and subsequently stored at -80°C. The concentration of CNS myelin extract used for further experiments was optimized for each lot by testing different concentrations in cell culture for its inhibitory potential.

2.2.6 *In vitro* compound treatments

Human CXCL12 was added to the medium of dissociated rat cell cultures at 0, 100, 250 and 500 ng/ml. In subsequent experiments (for rat and/or mouse), CXCL12 was used at 500 ng/ml, CNTF at 200 ng/ml, MIF at 50 ng/ml and ubiquitin at concentrations ranging from 0.5 to 5000 nM. The CXCR4 antagonist AMD3100 was added at 5 µM, the JAK2 inhibitor AG490 at 2.5 µM, the PI3K inhibitor LY294002 at 1 µM, the mTOR inhibitor rapamycin at 10 nM and the ROCK inhibitor Y27632 at 40 µM. Each pharmacological inhibitor was used either alone or in combination with CXCL12 (500 ng/ml). CNS myelin extract was coated on 4-well plates (see 2.2.5) at a preoptimized concentration. Neurocan was added 3 h after dissociation to the medium of retinal cultures at a preoptimized concentration of 5 µg/ml (Leibinger et al., 2012).

2.2.7 Immunohistochemical techniques

2.2.7.1 Staining of sections and whole mounts

For retinal sections, rats or mice were anesthetized and perfused through the heart with cold saline followed by PBS containing 4% PFA. Eyes with the optic nerve segments attached were separated from connective tissue, post-fixed overnight in 4% PFA at 4°C, transferred to 30% sucrose for at least 6 h at 4°C, and subsequently embedded in Cryo Compound (Klinipath, Netherlands). Frozen sections were cut longitudinally at a thickness of 14 µm on a cryostat (CM 3050S, Leica), thaw-mounted onto charged glass slides (Superfrost Plus, VWR, Germany) and stored at -20°C until further use.

For retinal whole mount stainings, rats or mice were killed and eyes removed. The retina was prepared, attached to blackened nitrocellulose filter and fixed in 4% PFA for 30 min.

Dependent on the antibody, sections and whole mounts were permeabilized with methanol or Triton X-100 (2% or 0.2%) for 10 min or 1 h, respectively.

To prevent unspecific binding of primary or secondary antibodies, retinal sections and wholemounts were incubated with blocking solution (PBS-Tween (0.01%) with 5% donkey serum and 2% BSA) for 30 or 60 min, respectively. Primary and secondary

antibodies diluted in blocking solution were used as designated in Table 2.2. Sections and whole mounts were embedded in Mowiol (Merck Millipore, Germany) and analyzed using a fluorescent microscope (Axio Observer D1, Zeiss) or a Confocal Laser Scanning Microscope (LSM510, Zeiss).
The specificity of the CXCR4 and CXCL12-antibody was confirmed by comparing the staining of HEK293 cells co-transfected with either a CXCR4 or CXCL12- and a GFP-expression vector (Stratagene, USA) or the empty vector with the GFP-expression vector (Fig. 3.3, Fig. 3.17, Fig. 3.19). HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen, Germany) according to the manufacturer's instructions.
An improved CXCR4-staining in mouse whole mounts and sections was obtained with a Tyramide Signal Amplification-Kit that was used according to the manufacturer's instructions (Life Technologies, Germany).

2.2.7.2 Immunocytochemical analysis of CXCL12 signaling pathways

Dissociated rat RGCs were incubated with CXCL12 (500 ng/ml) or CNTF (200 ng/ml) for 15 and 60 min, respectively. Subsequently, cells were fixed in 4% PFA and stained with an antibody specific for phosphorylated STAT3 (pSTAT3). To detect mTOR activity, cells were stained with an antibody specific for phosphorylated S6 ribosomal protein (pS6) after 2 h or 3 days in culture as described previously (Leibinger et al., 2012). Retinal cultures were additionally co-stained for βIII-tubulin in order to identify RGCs. Each experiment was repeated at least twice to verify the data.
After floxed CXCR4/ROSA mice received intravitreal AAV2-Cre or AAV2-GFP injections for 3 weeks, eyes of the animals were removed and retinae dissociated. Cells were incubated with or without CXCL12 (500 ng/ml) for 2 h or 4 days. Additionally some groups were cotreated with AMD3100 (5 µM) or Rapamycin (10 nM). Two hours or 4 days after dissocation, cells were fixed, stained for βIII-tubulin and pS6 ribosomal protein in order to quantify RGCs with high mTOR activity. This experiment was performed once.

2.2.7.3 Determination of neurite growth of RGCs and DRG neurons *in vitro*

For evaluating neurite growth of rat or mouse RGCs that were incubated either with or without *in vitro* compounds (see 2.2.6), cells were fixed after 3 days (rats) or 4 days (mice) in culture. Injury-primed cells from animals subjected to surgery prior to dissociation were fixed after 24 h in culture. Retinal cultures were fixed in 4% PFA solution in PBS for 25 min and then in 100% methanol for 10 min. RGCs were specifically stained with an βIII-tubulin-antibody. RGCs with regenerated neurites were photographed under a fluorescent microscope (x200, Axio Observer D1, Zeiss) and neurite length was determined using ImageJ software. Moreover, the total number of βIII-tubulin-positive RGCs with an intact nucleus (DAPI staining) was quantified per well to evaluate potential neurotoxic treatment effects. Mean neurite length per RGC was calculated by dividing the sum of the neurite length per well by the total number of RGCs per well. Four wells were evaluated per experimental condition, and each experiment was repeated at least twice. Data are given as the mean ± SEM of replicate wells.

Cultures of adult mice DRG neurons, were fixed after 2 days with 4% PFA and immunocytochemically stained with an antibody against βIII-tubulin. Imaging and quantification of total axon length and neuron numbers per well were automatically performed with the Pathway 855 microscope system (BD, USA) and Attovision software, avoiding bias of quantification. Average neurite length per neuron and neuron counts per group were normalized to control groups as indicated. Data are presented as the mean ± SEM of six replicate wells from two separate experiments.

2.2.7.4 Quantification of regenerating axons in the optic nerve and surviving RGCs in retinal cross-sections/whole mounts

Axon regeneration in rats was quantified as described previously (Leon et al., 2000; Müller et al., 2007; Leibinger et al., 2009). In brief, regenerated axons were identified by GAP43 staining and the number of axons extending ≥0.5 mm, ≥0.75 mm and ≥1.0 mm beyond the injury site was counted under 400x magnification in 6 - 8 sections per animal and treatment. Axon numbers were extrapolated to 1 mm cross-sectional width of the optic nerve section.

The numbers of survived βIII-tubulin-positive RGCs were counted in 6 - 8 retinal sections per animal. To obtain a group mean and SEM as described previously (Müller et al., 2007; Hauk et al., 2008), the cell numbers were first averaged per animal and then averaged across all animals per treatment group. Each experimental group included at least 3 rats.

Survival of mouse RGCs was quantified in whole mounted retinae of control animals or animals that were subjected to ONC or ONC + IS 14 days prior to tissue isolation. For quantification of surviving RGCs per mm^2, retinal whole mounts were stained with an antibody against βIII-tubulin and were divided into four quadrants. In each quadrant 4 - 5 independent fields were sampled, proceeding from the center to the periphery. The average number of βIII-tubulin-positive RGCs per field was determined and divided by the area of the field. Values were averaged per retina and across all similarly treated animals to obtain a group mean and SEM. At least 5 - 7 retinae per case were analyzed.

2.2.8 Chemoattraction

For testing the capability of CXCL12 to chemoattract neurites of adult mouse RGCs or DRG neurons, the orientation of regenerating neurites was analzyed toward a CXCL12 source. The CXCL12 gradient was provided by HEK293 cells that were beforehand transfected with pAAV-CXCL12-IRES-GFP (referred to as pAAV-CXCL12) or pAAV-IRES-hrGFP (referred to as pAAV-GFP) as a control. The functionality of the secreted CXCL12 was tested with CXCR4-transfected HEK293 cells as described above (see 3.2.9) demonstrating that the CXCL12-transfected HEK293 cells secrete the biological active protein and can be used for further chemoattraction-experiments. GFP-expressing HEK293 cells are used as respective controls.

2.2.8.1 Adult mouse RGCs

To see whether the chemoattractive effect of CXCL12 is CXCR4 dependent, floxed CXCR4 mice received an intravitreal injection of either AAV2-GFP or AAV2-Cre and were subjected to ONC + IS 3 weeks afterwards to transform cells into an active regenerative state. Five days after surgery, animals were killed, RGCs were dissociated as described previously (see 2.2.4.1) and plated on myelin coated 6-well-plates (Nunc, Germany). Simultaneously, HEK293 cells were Lipofectamine 2000-transfected (Invitrogen, Germany) with a GFP- or CXCL12-expression vector, respectively. One day after dissociation and transfection, approximately 3 x 10^5 of the prepared HEK293 cells were seeded into a cell culture insert containing a membrane with a pore size of 3 µm (Merck Millipore, Germany; Fig. 2.1 A). The HEK293 cells were incubated for at least 6 h at 37°C. Then the insert was placed into the middle of the well with the dissociated RGCs so that a chemokine gradient was established by

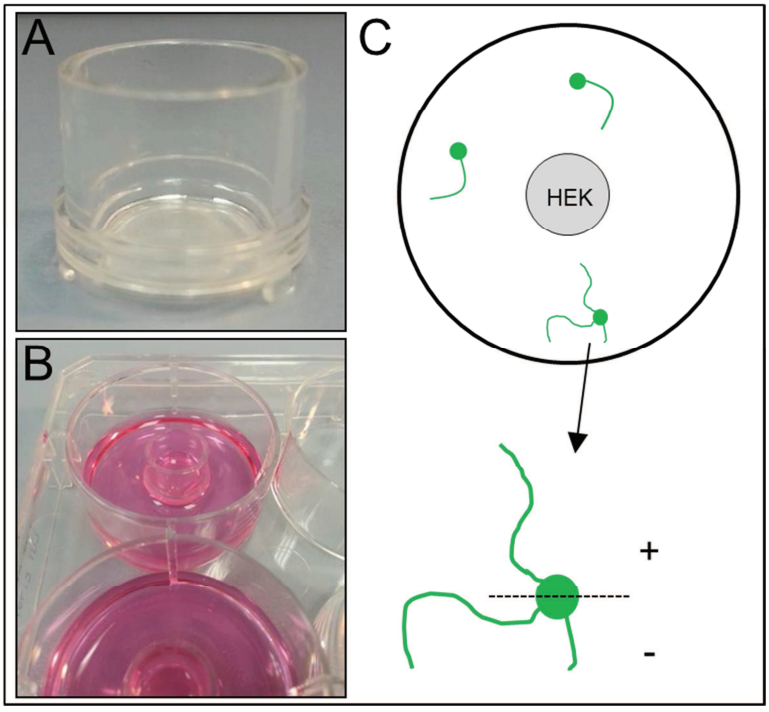

Fig. 2.1: Experimental set-up of the chemoattraction assay with mouse RGCs. (A) A polycarbonate cell culture insert with a pore size of 3 µm was used for cultivation of transfected HEK293 cells (Merck Millipore, Germany). (B) HEK293 cells were transfected with pAAV-GFP or pAAV-CXCL12 and seeded into a cell culture insert. The standing insert was placed in the middle of a 6-well-plate (Nunc, Germany) with dissociated RGCs. (C) After fixation of the retinal cell cultures, RGCs were stained for βIII-tubulin and regenerated neurites were analyzed for their orientation toward the culture insert.

the CXCL12-secreting HEK293 (Fig. 2.1 B). After an additional day, the medium of the wells was carefully replaced in order to prevent supersaturation of the medium with CXCL12. Three days after dissociation, cells were fixed and subsequently stained for βIII-tubulin. Pictures of RGCs with extended neurites were photographed under a fluorescent microscope (x200, Axio Observer D1, Zeiss) parallel to the standing insert and neurites were analyzed for their orientation to the cellular chemokine source (Fig. 2.1 C).

The experiment was repeated twice with a total number of 849 analyzed neurites for AAV2-GFP- and 935 for AAV2-CXCL12-transfected HEK293 cells.

2.2.8.2 Adult mouse DRG neurons

In order to assess whether CXCL12 chemoattracts neurites of peripheral neurons, adult mouse DRGs were dissociated as described previously (2.2.4.2) and plated on myelin-coated 6-well-plates (Nunc, Germany). HEK293 cells were prepared as described in 0. After 2 days in culture, cell culture inserts (Merck Millipore, Germany) containing GFP- or CXCL12-expression vector transfected HEK293 cells were placed in the middle of the well with dissociated DRG neurons. Consequently, CXCL12-transfected HEK293 cells secreted CXCL12 into the medium so that a chemokine gradient was established. After 1 day, the medium of the well was carefully replaced. Four days after dissocation, cells were fixed, stained for βIII-tubulin. Pictures of DRG

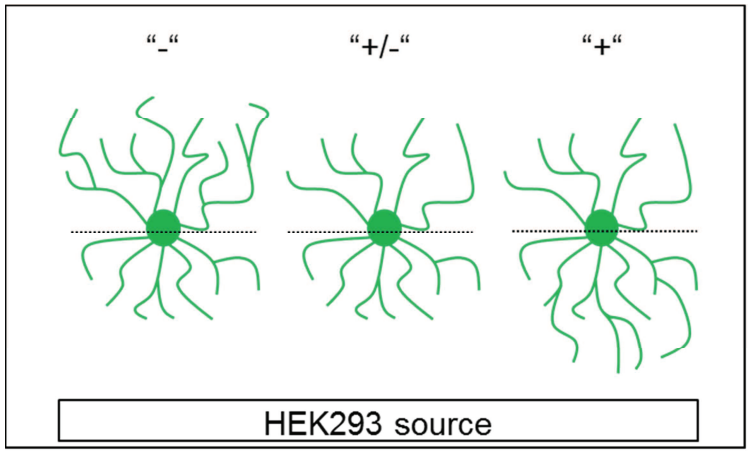

Fig. 2.2: Experimental set-up of the chemoattraction assay with mouse DRGs. DRG neurons were stained for βIII-tubulin and the neurite mass was analyzed in orientation toward the HEK293 source. The percentage of repelled/non-attracted ("-"), positively attracted ("+") and not influenced ("+/") DRG neurons was quantified.

neurons with neurites were photographed under a fluorescent microscope (x200, Axio Observer D1, Zeiss) parallel to the standing insert and neurites were analyzed for their orientation to the HEK293 source A DRG neuron was assessed as not influenced (Fig. 2.2, “+/-“) when 45 - 55 % of axonal mass was formed toward the HEK293 source. A DRG neuron was considered as positively attracted, when more than 55% of axonal mass was produced in orientation to the HEK293 cells, while it was assessed as non-attracted, when less than 45% grew in the direction of the HEK293 source (Fig. 2.2, “+” and “-“). The experiment was performed twice with a total of 28 analyzed DRG neurons for pAAV-GFP- and 33 for pAAV-CXCL12-transfected HEK293 cells.

2.2.9 Tissue clearing of mouse optic nerves, quantification of U-turns and distance of regenerating axons to the lesion site

The growing behavior of regenerating axons was analyzed in whole-mounted optic nerves in three dimensions (3D) after tissue clearing. Three weeks after intravitreal AAV2-Cre- or AAV2-GFP-injection, floxed CXCR4 mice were subjected to ONC + IS. The anterograde tracer cholera toxin subunit B (CTB) conjugated to Alexa Fluor® 594 (Invitrogen, Germany) was injected intravitreally after 5 days. Animals were killed after 2 more days for visualization of regenerating axons in the optic nerve. Anesthetized animals were perfused intracardially with ice cold 4% PFA, optic nerves were removed carefully and post-fixed overnight in 4% PFA. The fixed optic nerves were washed twice in PBS and treated according to the SeeDB method (Ke et al., 2013) in order to maximize the transparancy. SeeDB is a saturated solution of fructose (80.2% wt/wt) in water that is supplemented with 0.5% α-thioglycerol in order to prevent the Maillard reaction (Dills, 1993; Ke et al., 2013). Fixed optic nerves were incubated in increasing fructose-solutions ranging from 10% wt/vol to 80.2% wt/wt and subsequently transferred into the clearing agent FocusClear™ (Cedarlane, Australia) and incubated for at least 2 days at room temperature. The transparent whole optic nerves were mounted in MountClear™ (Cedarlane, Australia) and image stacks were generated using a Confocal Laser Scanning Microscope (LSM510, Zeiss), equipped with a 20x objective. The specimen holder was turned once in order to scan the optic nerve from both sides. With this setup axons throughout the whole thickness of the optic nerve

could be investigated. The image stacks were 3D reconstructed with the Vaa3D software (Peng et al., 2010; Peng et al., 2014a; Peng et al., 2014b), before the 20 longest axons were semi-automatically traced and analyzed for formation of U-Turns and their distance to the lesion site. At least 9 animals per group were investigated thoroughly.

2.2.10 Statistical analysis

For all experiments, the significance of intergroup difference was evaluated using one- or two-way analysis of variance (ANOVA), followed by corrections for multiple post hoc tests (Bonferroni-Holm, Tukey, Holm-Sidak) using SigmaStat3.1.

3 Results

3.1 Role of the CXCL12-CXCR4 axis in rats regarding optic nerve regeneration

3.1.1 Neurite growth of adult rat RGCs is moderately improved by CXCL12

Recent studies demonstrate the potential of the chemokine CXCL12 to enhance neurite growth of cultured neurons like primary postnatal DRG neurons, cerebellar granule neurons and hippocampal neurons (Arakawa et al., 2003; Pujol et al., 2005; Opatz et al., 2009a). In order to address the question whether CXCL12 exerts positive effects on adult rat RGCs, dissociated retinal cell cultures were incubated with human CXCL12. Although CXCL12 is highly conserved among different species, we initially verified that human CXCL12 is able to bind to rat CXCR4. To this end, rat CXCR4 was overexpressed in HEK293 cells and transfected cells were incubated with recombinant human CXCL12 (100 ng/ml) for 45 min (Fig. 3.3 E). Upon ligand binding, CXCR4 was phosphorylated C-terminally so that the respective CXCR4-antibody lost its ability to detect its appropriate epitope (Mueller et al., 2013). While CXCR4-transfected HEK293 cells exhibited a clear membrane-bound signal when stained with the CXCR4-antibody, the staining altered to a much weaker and more cytoplasmatic signal after treatment with recombinant CXCL12 (Fig. 3.3 E) demonstrating the interaction between human CXCL12 and rat CXCR4.

In order to examine the neurite growth promoting effect of the chemokine on rat RGCs, dissociated retinal cultures were exposed to increasing concentrations of CXCL12 (0 - 500 ng/ml) and the neurite length of βIII-tubulin positive RGCs was determined after 3 days in culture (Fig. 3.1 A, B). As a positive control, one cell culture group was exposed to CNTF, that was previously reported to potently increase neurite growth of mature RGCs (Leibinger et al., 2009; Muller et al., 2009; Sengottuvel et al., 2011). CXCL12 promoted neurite growth of rat RGCs concentration dependently (Fig. 3.1 B). Incubation of cells with 250 ng/ml lead to a moderate, but significant increase

of neurite growth, while 500 ng/ml showed even stronger effects. Nevertheless, the neurite growth promoting effect of CNTF was with a 2.8-fold upregulation in comparison to the untreated control markedly stronger (Fig. 3.1 A, B). The survival of RGCs was expectedly not affected by any treatment after 3 days in culture as neuronal cell death in these cultures can be observed earliest after 5 days (Fig. 3.1 C) (Grozdanov et al., 2010).

To test for potential neuroprotective effects of CXCL12 in culture, we evaluated the survival of RGCs after two later timepoints (Fig. 3.1 D). For this purpose, dissociated RGCs were incubated with CXCL12 (500 ng/ml) or CNTF (200 ng/ml) and the number of surviving βIII-tubulin positive RGCs was quantified after 2 h, 5 and 7 days. Consistent with previous studies, the RGC number of untreated cultures was reduced about 23% after 5 days and 51% after 7 days compared to the cell number of untreated controls determined after 2 h, due to axotomy-induced cell death (Manitt et al., 2001; Sengottuvel et al., 2011). In contrast to CNTF, which elicited a robust neuroprotective effect, no neuroprotection could be observed in CXCL12-treated RGCs (Fig. 3.1 D).

These data suggest that CXCL12 moderately facilitates neurite growth of adult rat RGCs, without affecting neuronal survival.

3.1.2 CXCL12 desensitizes RGCs toward inhibitory myelin but not neurocan

The administration of recombinant CXCL12 to postnatal dorsal root ganglion (DRG) neuron cultures is reportedly sufficient to overcome CNS myelin induced neurite growth inhibition (Opatz et al., 2009a). After axotomy of the optic nerve, growth cones of regenerating RGCs have to face an inhibitory environment containing myelin-associated proteins and CSPGs limiting axon regeneration. In order to investigate the disinhibitory effect of CXCL12 on RGCs, dissociated rat RGCs were incubated with the ROCK inhibitor Y27632, CXCL12 and/or CNTF and plated on laminin, CNS myelin extract or in the presence of CSPGs (neurocan). After 3 days in culture, cells were fixed, stained for βIII-tubulin and neurite growth was quantified thereafter. As published previously, both myelin and neurocan significantly impaired neurite growth in comparison to the untreated control plated on laminin-coated dishes (Fig. 3.2 A - C) (Ahmed et al., 2009; Sengottuvel et al., 2011). Y27632 blocked the ROCK-signaling

cascade and thereby overcame the inhibitory effects on neurocan (Dergham et al., 2002; Monnier et al., 2003), while CXCL12 was not able to overcome the inhibition of CSPGs (Fig. 3.2 A, B).

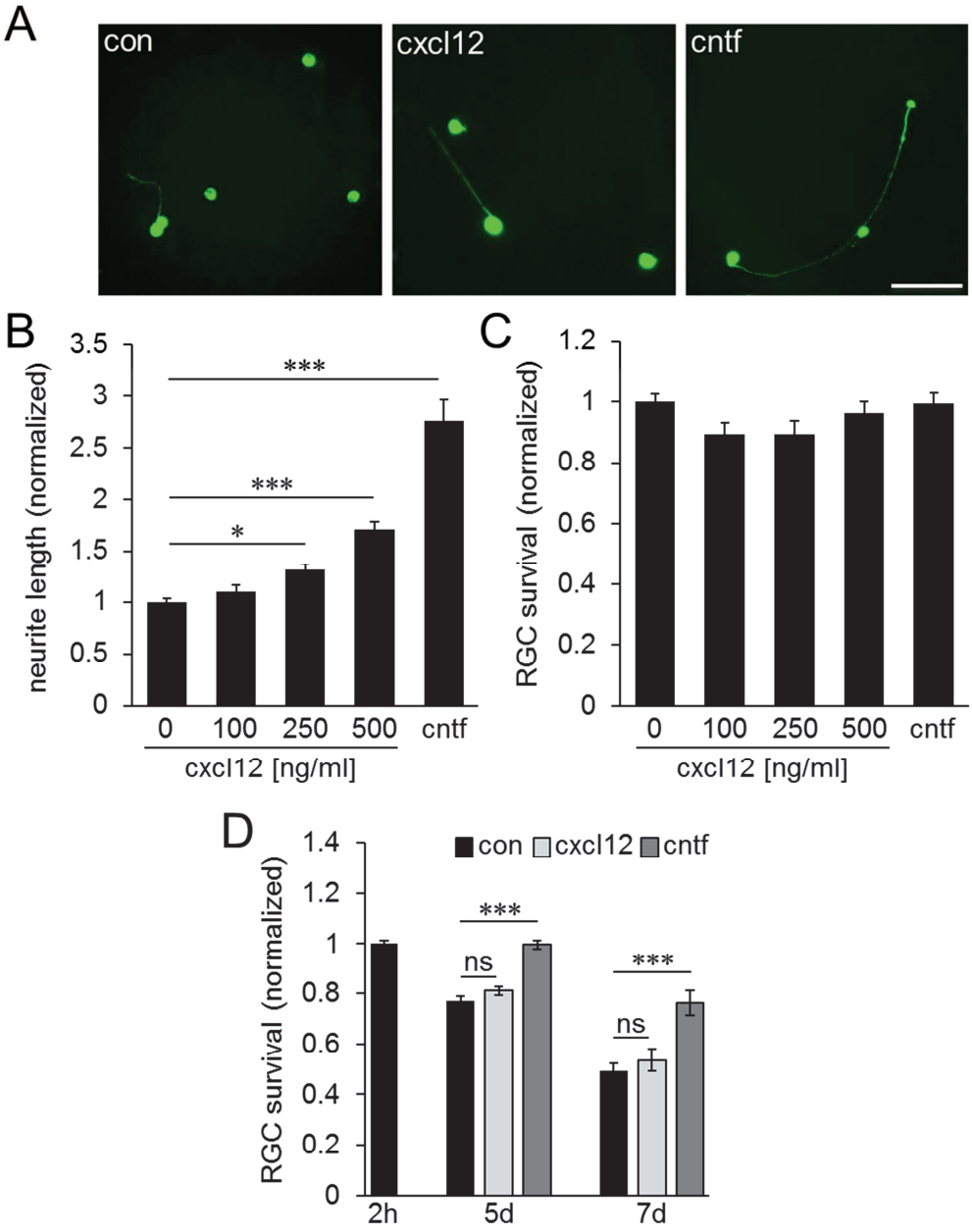

Fig. 3.1: CXCL12 moderately stimulates neurite growth of rat RGCs. (A) Dissociated RGCs were stained for βIII-tubulin after exposure to either vehicle (con), CXCL12 (500 ng/ml) or CNTF (200 ng/ml) for 3 days. Scale bar: 50 µm. (B) RGC neurite growth was quantified in the presence of increasing concentrations of CXCL12 (0 - 500 ng/ml) or CNTF (200 ng/ml). Neurite length was normalized to the control group with an average neurite length of 5.4 µm/RGC. Treatment effects: *p<0.05, ***p<0.001. (C) Survival of RGCs per well of experimental groups described in B was not affected by any treatment. Numbers were normalized to the untreated control with an average number of 1283 RGCs/well (4-well plate). (D) Dissociated RGCs were exposed to either vehicle (con), CXCL12 (500 ng/ml) or CNTF (200 ng/ml), as indicated and incubated for 5 and 7 days, respectively. The number of surviving, βIII-tubulin-positive RGCs was quantified and values normalized to the initial number of RGCs determined after 2 h with an average number of 257 RGCs/well (96-well plate). Treatment effects: ***p<0.001, ns = non-significant. (Heskamp et al., 2013)

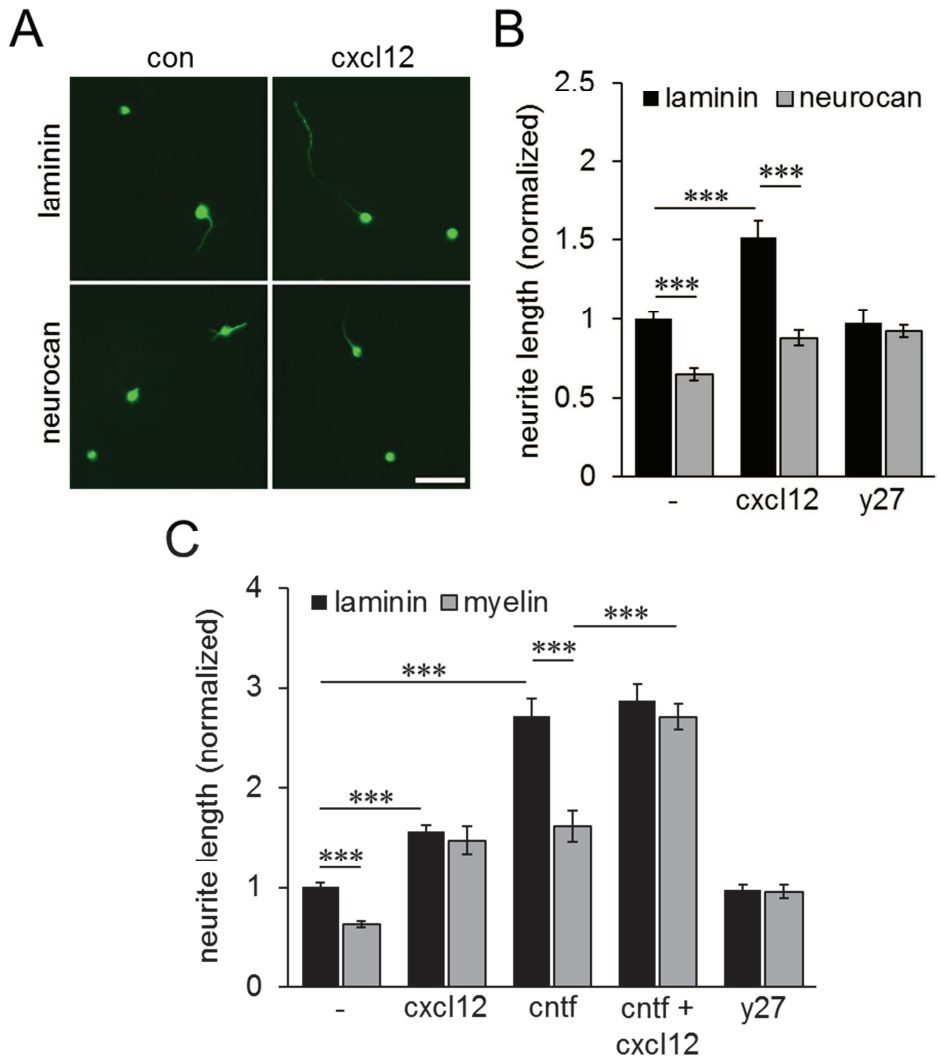

Fig. 3.2: CXCL12 desensitizes RGCs toward inhibitory myelin. (A) Retinal cell cultures were dissociated and plated on laminin or in the presence of neurocan. Cells were exposed to vehicle (con) or CXCL12 (500 ng/ml) for 3 days. Scale bar: 50 µm. (B) RGC neurite growth either on laminin (black bars) or laminin + neurocan (grey bars) and treatment with either vehicle (-), CXCL12 (500 ng/ml) or the ROCK inhibitor Y27632 (40 µM; y27) was quantified after 3 days in culture. Neurite growth was normalized to the untreated control on laminin with an average neurite length of 10.8 µm/RGC. Treatment effects: ***p<0.001. (C) Quantification of neurite growth of dissociated RGCs plated either on laminin (black bars) or laminin + central myelin extract (myelin, grey bars). Cultures were incubated with either vehicle (-), CXCL12 (500 ng/ml), CNTF (200 ng/ml), CNTF + CXCL12 or Y27632 (40 µM; y27) for 3 days. Neurite growth was normalized to the untreated control on laminin with an average neurite length of 2.1 µm/RGC. Treatment effects: ***p<0.001. (Heskamp et al., 2013)

However, both Y27632 and CXCL12 were able to desensitize RGCs toward myelin inhibition, leading to similar growth on laminin, respectively (Fig. 3.2 C). In accordance with previous studies CNTF was not able to overcome myelin inhibition (Ahmed et al., 2009; Sengottuvel and Fischer, 2011) and the combination of CNTF and CXCL12 did not enhance outgrowth on laminin further compared to CNTF alone (Fig. 3.2 C).

Interestingly, on inhibitory myelin the combination of both factors lead to similar growth compared to the group plated on laminin, indicating the potential of CXCL12 to desensitize CNTF-treated RGCs toward inhibitory substrate. The survival of RGCs was not affected by either treatment after 3 days (data not shown).
This demonstrates that CXCL12 itself exhibits disinhibitory features on CNS myelin and furthermore desensitizes CNTF-stimulated RGCs specifically toward the inhibitory environment of myelin.

3.1.3 Adult rat RGCs express CXCR4

CXCL12 interacts with the seven-transmembrane receptors CXCR4 and CXCR7 which are expressed in a variety of different tissues and cell types such as astrocytes, macrophages and neurons (Bleul et al., 1996; Nagasawa et al., 1996b; Chalasani et al., 2003a; Jaerve et al., 2012). To test whether CXCL12 mediates its effects on mature RGCs directly via CXCR4, we initially verified CXCR4 expression in the rat retina. Using specific primers, CXCR4 was detected in the adult rat retina by RT-PCR as a single, definite band of about 1.1 kb. The identity of the amplified cDNA was confirmed by subsequent gel extraction and sequencing (Fig. 3.3 A). In order to examine the protein expression of CXCR4 in the rat retina, cultured βIII-tubulin-positive RGCs, cross-sections and retinal whole mounts were stained immunochemically with an anti-CXCR4 antibody (Fig. 3.3 B, D). This antibody was tested for its ability to detect CXCR4 with HEK293 cells that were transfected with a rat CXCR4-expression vector and an empty expression vector, respectively. All cells were co-transfected with a GFP-expression vector. The CXCR4-antibody precisely recognized exogenously expressed rat CXCR4 in HEK293 (Fig. 3.3 E). CXCR4 was mainly detected in RGC axon bundles and to a lesser extent in RGC bodies of naïve retinae (Fig. 3.3 C, D). Fourteen days after ONC and ONC + IS a slight upregulation of CXCR4 expression in some RGC somas in comparison to untreated controls got visible in whole mounted retinae (Fig. 3.3 D). In dissociated cell cultures which were incubated for 3 days, CXCR4 was detected in the cell bodies and on neurites of RGCs (Fig. 3.3 B).
In conclusion, the CXCR4 receptor is expressed by adult rat RGCs and might therefore be involved in CXCL12 mediated signaling.

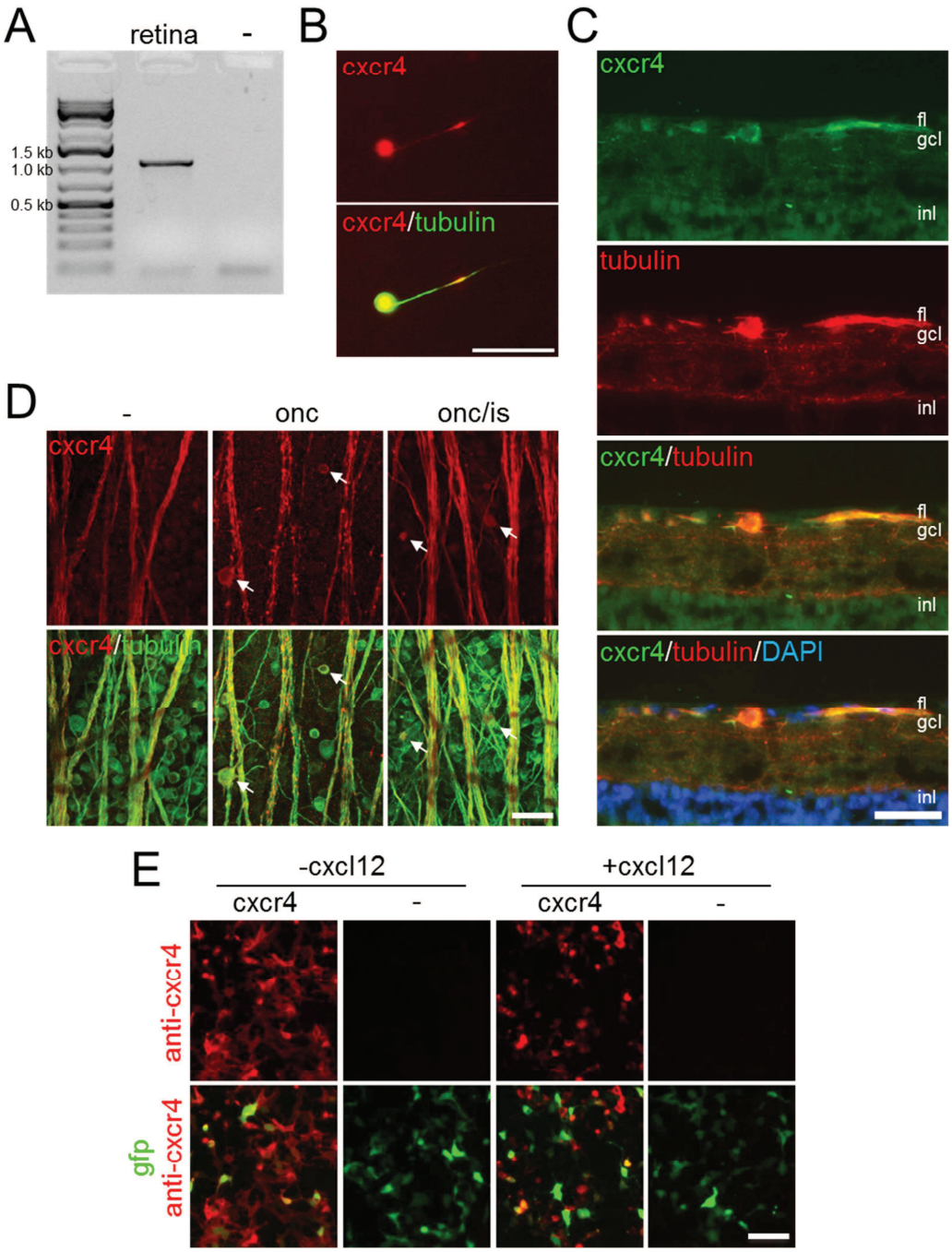

Fig. 3.3: CXCR4 expression in mature rat RGCs. (A) Retinal RNA was isolated and transcribed into cDNA. Using CXCR4-specific primers and retinal cDNA (retina) in a PCR resulted in a single band of ~1.1 kb while no product was obtained in the no template control (-). (B) CXCR4 (red) localized to the cell body and neurite of dissociated βIII-tubulin-positive RGCs (green) that were cultivated for 3 days. Scale bar: 50 µm. (C) Staining of untreated adult retinal sections with CXCR4 (green) and βIII-tubulin (red) antibodies demonstrated that the receptor localized to RGC bodies and in axon bundles. fl: fiber layer, gcl: ganglion cell layer, inl: inner nuclear layer. Scale bar: 30 µm. (D) Staining of adult retinal wholemounts that were subjected to no treatment (-), ONC and ONC + IS for 14 days with CXCR4 (red) and tubulin (green) antibodies. Scale bar: 50 µm. (E) Immunocytochemical staining of rat CXCR4-transfected HEK293 cells (cxcr4, red) in comparison to controls (-) transfected with the empty vector. All cells were co-transfected with a GFP expression vector (green). CXCR4-staining of CXCR4-transfected cells that where incubated with recombinant human CXCL12 (100 ng/ml) for 45 min demonstrated that human CXCL12 is able to interact with rat CXCR4. Scale bar: 50 µm. (Heskamp et al., 2013)

3.1.4 CXCL12 mediates growth promoting and disinhibitory effects PI3K/AKT/mTOR-dependently

Previous experiments demonstrated that CXCL12 enhanced neurite growth of rat RGCs both on growth-permissive and also inhibitory substrates like CNS myelin. In order to define whether CXCL12 modulates signaling pathways that have previously been associated with axon regeneration of mature RGCs, such as the JAK/STAT3- and PI3K/AKT/mTOR-pathway (Muller et al., 2009; Smith et al., 2009; Park et al.,

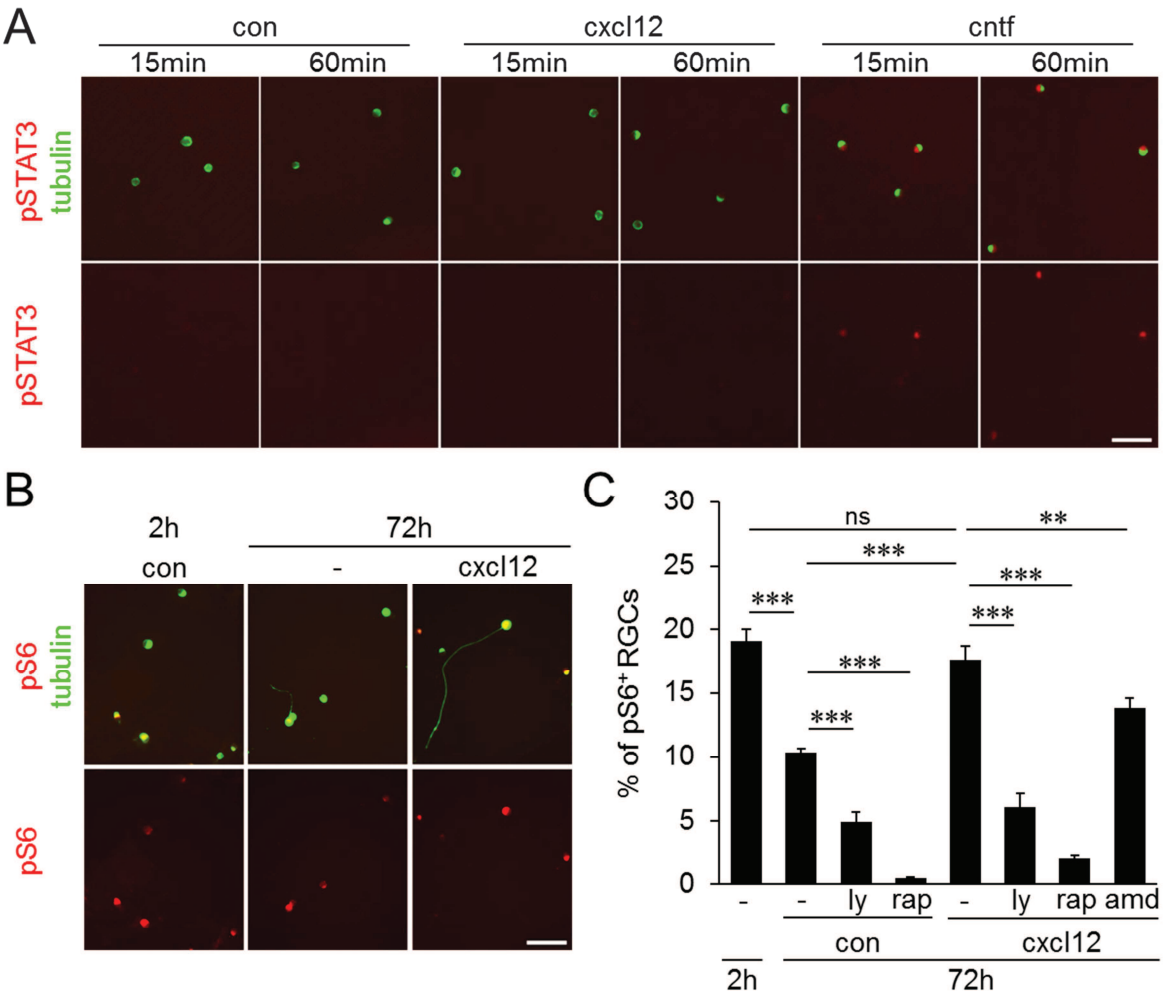

Fig. 3.4: CXCL12 maintains mTOR activity after axotomy. (A) Dissociated rat RGCs were incubated with either vehicle (con), CXCL12 (500 ng/ml) or CNTF (200 ng/ml) for 15 and 60 min. Cells were fixed and stained with antibodies against phosphorylated STAT3 (pSTAT3) (red) and βIII-tubulin (green). Scale bar: 50 µm. (B) Rat RGCs were exposed to vehicle (con) or CXCL12 (500 ng/ml) for 2 or 72 h. After fixation, cell cultures were stained for phosphorylated S6 (pS6, red) and βIII-tubulin (green). Scale bar: 50 µm. (C) Cultures as described in B were additionally exposed to vehicle (-), the PI3K inhibitor LY294002 (ly), the mTOR inhibitor rapamycin (rap) or the CXCR4 antagonist AMD3100 (amd). Strongly stained pS6-positive RGCs were quantified after 2 or 72 h in culture. Treatment effects: **p<0.01, ***p<0.001, ns = non-significant. (Heskamp et al., 2013)

2010; Leibinger et al., 2012), the phosphorylation levels of STAT3 (JAK/STAT3-pathway) and ribosomal protein S6 (PI3K/AKT/mTOR-pathway) were analyzed by immunocytochemistry as described previously (Manitt et al., 2001; Park et al., 2008; Leibinger et al., 2012). Dissociated rat RGCs were incubated with a vehicle control, CXCL12 or CNTF for 15 and 60 min, respectively. After fixation, cells were stained for phospho-STAT3 (pSTAT3; Fig. 3.4 A). As shown before, incubation with CNTF induced STAT3 phosphorylation in RGCs within 15 min, indicating a direct response of RGCs toward CNTF (Peterson et al., 2000; Muller et al., 2009). In contrast to CNTF exposed cultures, no pSTAT3-staining was detected in controls and CXCL12-treated cultures, suggesting that the JAK/STAT3-pathway is not affected upon CXCL12 treatment (Fig. 3.4 A).

However, incubation with CXCL12 maintained mTOR activity in cultured RGCs. After axotomy of RGCs, mTOR-dependent phosphorylation of S6 continuously decreased with extended time after axotomy (Park et al., 2008; Leibinger et al., 2012). Accordingly, ~19% of untreated rat RGCs were stained for pS6 after 2 h and this proportion was reduced to ~10% after 3 days in culture (Fig. 3.4 B, C). Importantly, CXCL12 treated RGCs maintained the initial, higher pS6 level (~16%) even after 3 days in culture, indicating sustained mTOR activity upon CXCL12 treatment. This effect was abolished in the presence of LY294002, a PI3K inhibitor (~5-6% pS6-positive RGCs), suggesting that CXCL12 modulates mTOR activity via PI3K/AKT-signaling. As expected, incubation of retinal cultures with the mTOR inhibitor rapamycin strongly reduced pS6-levels as well (Fig. 3.4 C). Treatment of dissociated rat RGCs with the specific CXCR4 antagonist AMD3100 (Hatse et al., 2002) decreased the CXCL12 induced maintenance of mTOR activity significantly (Fig. 3.4 C). These data suggest that CXCL12 maintains mTOR activity in axotomized rat RGCs CXCR4-dependently.

In order to define the role of the JAK/STAT3- and PI3K/AKT/mTOR-signaling pathway in CXCL12 mediated neurite growth promotion and myelin disinhibition, we exposed cultured RGCs plated either on growth permissive substrate or on inhibitory myelin to CXCL12 in the presence of AMD3100 (CXCR4 antagonist), LY294002 (PI3K inhibitor), rapamycin (mTOR inhibitor) and AG490 (JAK/STAT3 inhibitor), respectively (Fig. 3.5 A, B). Treatment with AMD3100, LY294002 and rapamycin significantly abolished the growth promoting effect of CXCL12 on laminin as well as its disinhibitory feature on

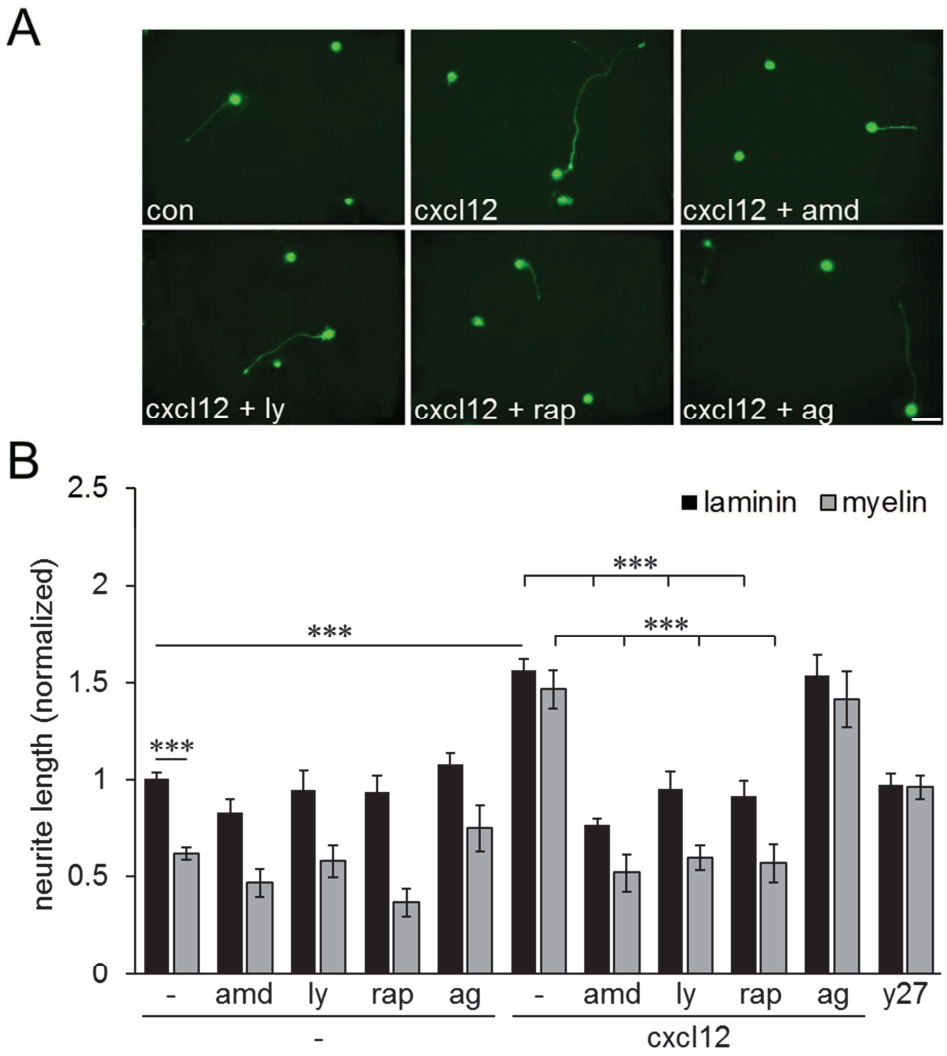

Fig. 3.5: CXCL12 mediates its effects via the PI3K/AKT/mTOR-pathway. (A) Dissociated retinal cell cultures were plated on laminin and incubated with vehicle (con), CXCL12 (500 ng/ml), CXCL12 + AMD3100 (cxcl12 + amd), CXCL12 + LY294002 (cxcl12 + ly), CXCL12 + rapamycin (cxcl12 + rap) and CXCL12 + AG490 (cxcl12 + ag) for 3 days. RGCs were stained for βIII-tubulin after fixation. Scale bar: 25 µm. (B) Dissociated RGCs were treated either with vehicle (-) or CXCL12 (500 ng/ml). Additionally, some groups were treated with amd, ly, rap, ag, or Y27632 (y27) respectively. Some of the cultures were plated on laminin (black bars) or myelin (grey bars) as indicated. After 4 days in culture neurite growth was quantified and normalized to the untreated control group with an average neurite length of 6.9 µm/RGC. Treatment effects: ***p<0.001. (Heskamp et al., 2013)

myelin (Fig. 3.5 B). Exposure to AG490 did not significantly affect CXCL12 mediated neurite growth or myelin disinhibition indicating that CXCL12-binding does not affect JAK/STAT3-signaling (Fig. 3.5) thereby confirming previous results (Fig. 3.4). The survival of RGCs was not influenced by any treatment after 3 days in culture (data not shown).

In conclusion, CXCL12 seems to mediate its growth promoting and disinhibitory effects on mature rat RGCs via the CXCR4/PI3K/AKT/mTOR-pathway.

3.1.5 Intravitreal injection of CXCL12 transforms RGCs into an active axonal growth mode

As delineated before, mature RGCs have lost their ability to switch into an active growth mode which is a prerequisite for successful axon regeneration. In order to see whether intravitreally applied CXCL12 is sufficient to switch RGCs into an active regenerative state, we prepared retinal cultures 5 days after ONC and simultaneous CXCL12 or BSA injection. A positive control group was subjected to ONC + CNTF injection, which has previously been shown to transform RGCs into a potent regenerative state (Cui et al., 2003; Park et al., 2004; Muller et al., 2007; Muller et al., 2009). In contrast to intravitreal control injections with BSA that did result in weak neurite growth, CNTF treatment *in vivo* shifted RGCs into a robust regenerative state indicated by spontaneous neurite growth after 24h in culture (Fig. 3.6 A, B). Intravitreal application of CXCL12 also significantly enhanced spontaneous neurite outgrowth of RGCs which was about 2.5-fold higher compared to the BSA-treated control group, but less pronounced (~1/3) than seen after CNTF treatment (Fig. 3.6 A, B). Nevertheless, CXCL12 lead to sustained mTOR-activity indicated by the quantification

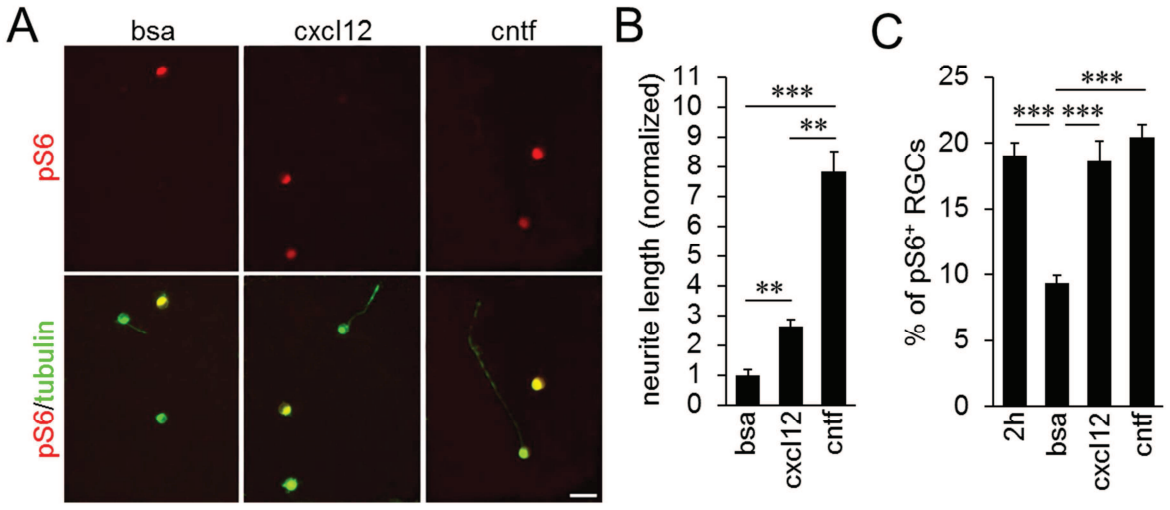

Fig. 3.6: CXCL12 moderately transforms RGCs into an active regenerative state. (A) BSA, CXCL12 or CNTF (each 1.5 µg) was intravitreally injected into rat eyes concomitantly with and again 3 days post ONC. Five days after surgery, dissociated rat RGCs were plated on poly-D-lysine and incubated for 1 day. Retinal cell cultures were then immunocytochemically stained for βIII-tubulin and phospo-S6. Scale bar: 25 µm. (B) Neurite growth of cultures as described in A was quantified. Values were normalized to the BSA-treated control group (bsa) with an average neurite length of 0.6 µm/RGC. Treatment effects: **p<0.01, ***p<0.001. (C) Strongly pS6-positive RGCs of cultures as described in A and an untreated control after 2 h were quantified. Treatment effects: ***p<0.001. (Heskamp et al., 2013)

of phosphorylated S6-positive RGCs which were comparable to the numbers after CNTF treatment (Fig. 3.6 C).
Consequently, CXCL12 seems to be sufficient to transform RGCs into a moderate regenerative state via the PI3K/AKT/mTOR-pathway.

3.1.6 Axon regeneration into the optic nerve is enhanced by CXCL12 injection

Opatz and colleagues demonstrated that a local intrathecal infusion of CXCL12 into the spinal cord lesion site promotes axonal sprouting of corticospinal tract (CST) axons (Opatz et al., 2009a). Our previous experiment showed, that CXCL12-

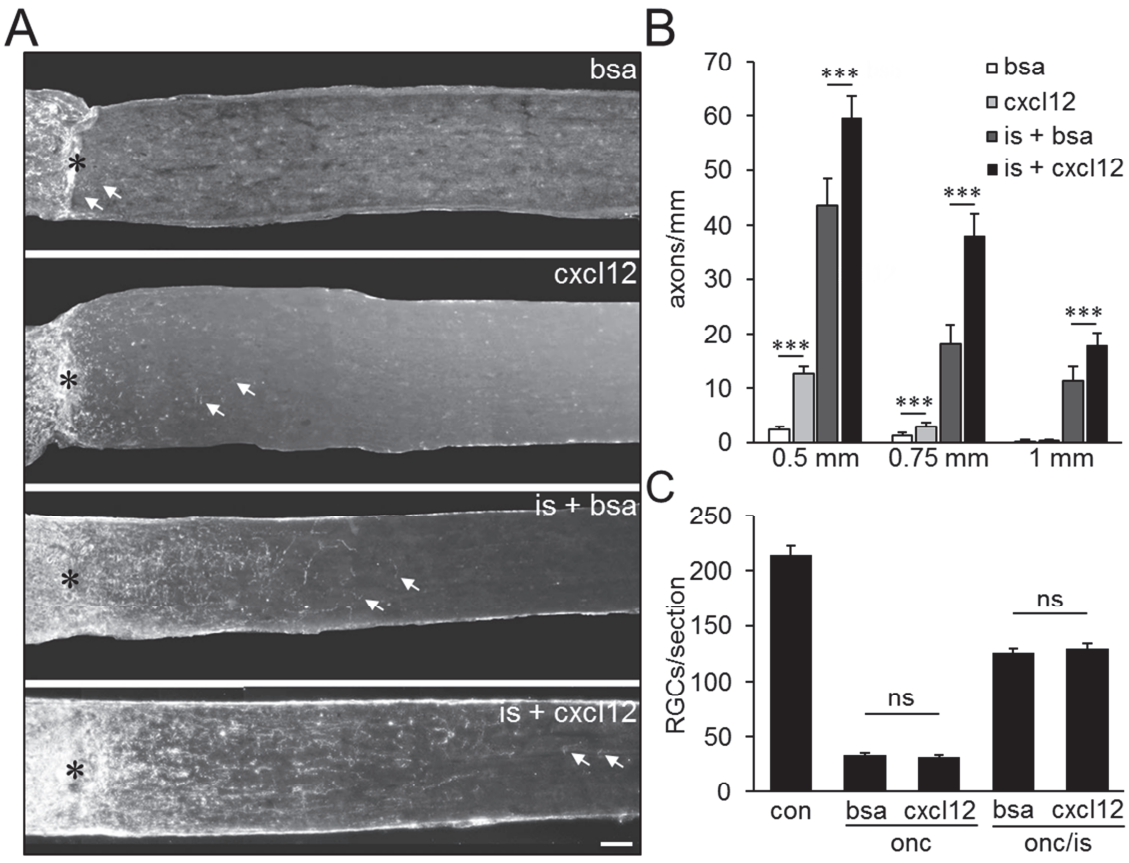

Fig. 3.7: CXCL12 moderately stimulates axon regeneration into the optic nerve. (A) Longitudinal sections through the optic nerve were stained for GAP43 in order to visualize regenerating axons proximal and distal to the injury site (asterisk) in rats 2 weeks after optic nerve crush alone or in combination with an inflammatory stimulation (is). Furthermore, the rats had repeatedly received intravitreal injections of 1.5 µg BSA (bsa) or 1.5 µg CXCL12 (cxcl12) 3 and 7 days after surgery. Scale bar: 100 µm. (B) Regenerating axons growing 0.5, 0.75 and 1.0 mm beyond the injury site of the optic nerve after treatment as described in A were quantified. Data are presented as average numbers of axons per width of optic nerve sections. Treatment effects: ***p<0.001. (C) The number of surviving RGCs (βIII-tubulin-positive RGCs per retinal section) of animals as described in A and of an untreated control animal was quantified. Treatment effects: ns = non-significant. (Heskamp et al., 2013)

application is able to transform adult rat RGCs into an active regenerative state. To investigate the ability of CXCL12 to stimulate axon regeneration into the injured optic nerve per se or in combination with an IS, an ONC with or without additional IS and either injected recombinant CXCL12 or BSA was performed. The number of GAP43-positive regenerating axons was quantified 2 weeks after surgery for 3 distances behind the lesion site (Fig. 3.7 A, B). In comparison to BSA, intravitreal application of CXCL12 resulted in a moderate, but significantly enhanced axon regeneration into the optic nerve, which was, nevertheless, notably less pronounced than regeneration after IS (Fig. 3.7 A, B). However, combinatorial treatment of CXCL12 injection and IS further increased the number of regrowing axons compared to IS alone (Fig. 3.7 A, B).
Moreover, RGC survival of the same animals as described before was analyzed in retinal cross sections. The number of surviving RGCs, while significantly increased upon IS, was not altered upon CXCL12 application either alone or in combination with IS (Fig. 3.7 C).
To sum up, CXCL12 seems to be able to moderately enhance the axon growth promoting, but not the neuroprotective effect of IS *in vivo*.

3.2 Role of the CXCL12-CXCR4 axis in mice regarding optic nerve regeneration

Pharmacological approaches as used in previous experiments in rats to inhibit CXCR4-signaling in RGCs involve several limitations: pharmacological compounds affect all cell types in retinal cultures and tissues. For this reason the observed effects on RGCs could be rather indirect. Moreover, potential off-target effects of inhibitors could falsify the experimental results. To circumvent these potential issues and to investigate the role of CXCR4 in axon regeneration in a more conclusive way we expanded our study to a genetic approach. As previously shown by others, intravitreal injection of an adeno-associated virus of serotype 2 (AAV2) expressing the Cre-recombinase in floxed mice allows specific depletion of genes in RGCs (Park et al., 2008; Smith et al., 2009; Sun et al., 2011; Leibinger et al., 2013a). We therefore used floxed CXCR4 mice to specifically deplete CXCR4 in RGCs and to determine the impact on survival and axonal regeneration upon injury.

3.2.1 CXCR4 expression in mouse RGCs after injury

In a first step we needed to verify receptor expression in naïve and injured mouse RGCs. To this end, we performed both qRT-PCR and immunohistochemical analysis (Fig. 3.8). Mice were subjected to either ONC, ONC + IS or received no treatment. To avoid axotomy-induced RGC loss that would affect the results of qRT-PCR analysis, retinae were isolated after 5 days before the onset of cell death. ONC slightly increased CXCR4-expression, while mRNA levels were even 2.5-fold upregulated after ONC + IS compared to untreated controls. To verify upregulation of CXCR4 protein in axotomized RGCs, we performed immunohistochemical stainings of eyes that were isolated 5 and 14 days after surgeries (Fig. 3.8 B, C). Compared to rats, stainings with the CXCR4-antibody revealed a much weaker signal in naïve mouse retina. In contrast, a clear CXCR4 signal was visible in axon bundles and RGC somas 5 days after surgeries. The signal intensity in surviving RGCs was even more pronounced when retinae were evaluated 14 days after ONC or ONC + IS (Fig. 3.8 B, C). However, at this timepoint signals detected in ONC and ONC + IS treated retinae were similar. Amplification of the primary antibody signal with Tyramide Signal Amplification (TSA) in naïve retina allowed detection of CXCR4 predominantly in axon

bundles, too (Fig. 3.8 D). Thus, similar to rats, CXCR4 is expressed in naïve mouse RGCs. Moreover the sensitivity of CXCR4 towards potential ligands may even be increased after ONC + IS because of its observed upregulation over time.

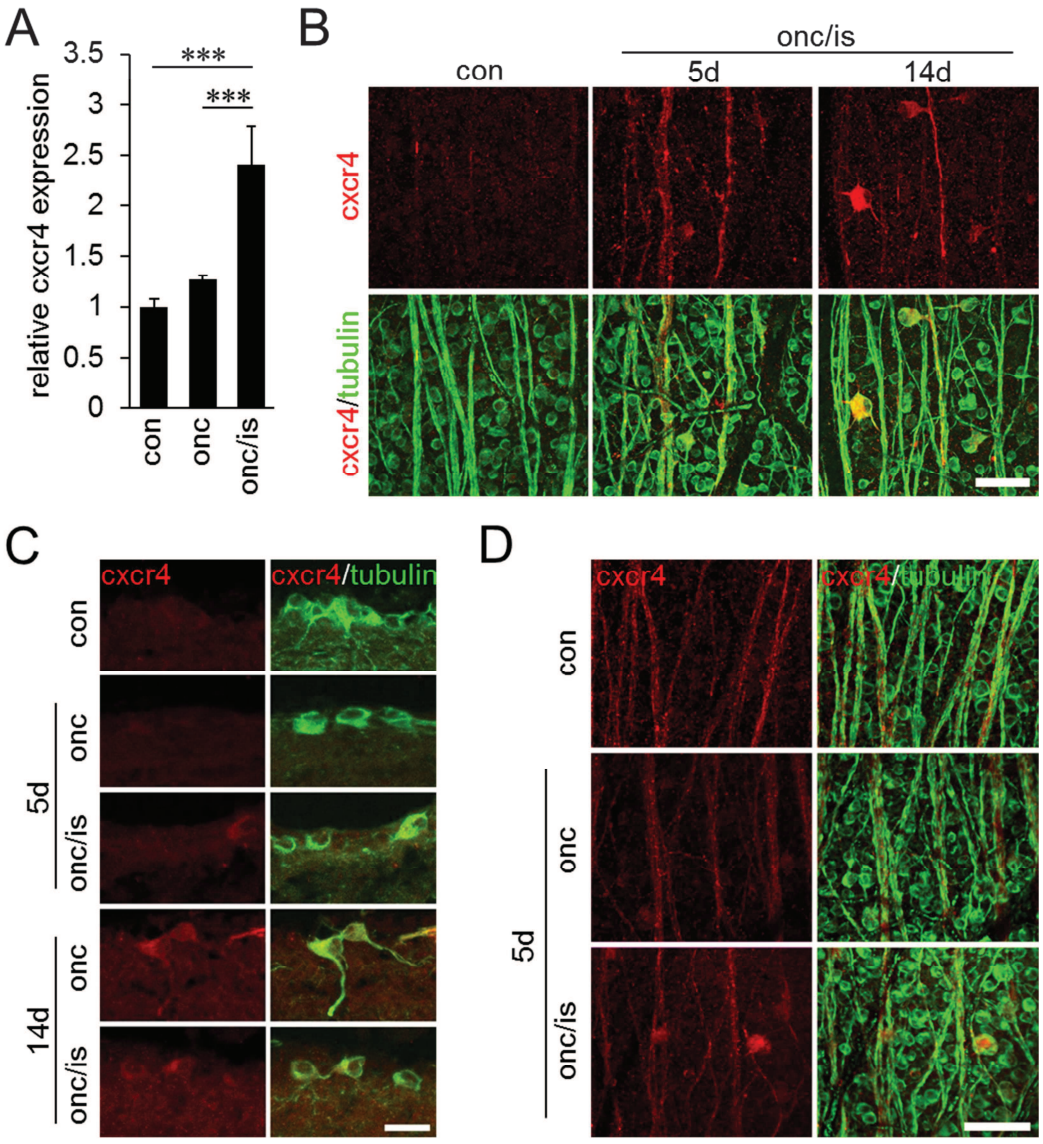

Fig. 3.8: Upregulation of CXCR4 expression in mouse retinae upon injury and IS. (A) Retinal RNA was extracted 5 days after optic nerve crush (ONC) alone, ONC + inflammatory stimulation (IS) or no treatment (con). Quantitative RT-PCR demonstrated a significant upregulation of CXCR4 expression after ONC + IS compared with other groups. Treatment effects: ***p<0.001. (B) Retinal whole mount stainings confirmed the upregulation of CXCR4 expression after ONC + IS in RGC somas and partially in axons. Scale bar: 30 µm. (C) Staining of retinal sections of animals that were subjected to either no treatment (con), ONC alone or ONC + IS 5 and 14 days earlier, with CXCR4 (red) and βIII-tubulin (green) antibodies. Images demonstrated an injury-induced upregulation of receptor expression in RGC bodies. Scale bar: 20 µm. (D) Tyramide signal amplification (TSA) staining of CXCR4 (red) in retinal whole mounts taken from untreated mice and mice that received either ONC or ONC + IS 5 days prior to isolation revealed that CXCR4 is predominantely expressed in axons of βIII-tubulin-positive (green) RGCs. Additionally, CXCR4 protein was upregulated in RGC somas after ONC + IS-treatment. Scale bar: 50 µm.

3.2.2 Depletion of CXCR4 in mouse RGCs by AAV2-Cre injection

To functionally assess, whether CXCR4 expression affects survival or axon regeneration of RGCs and additionally, to confirm the specificity of the CXCR4 antibody used in our experiments, we intravitreally injected HA-tagged AAV2-Cre or AAV2-GFP in eyes of floxed CXCR4 mice. After 3 weeks, eyes were isolated and retinal sections prepared. As indicated by HA-positive nuclei in retinal wholemounts, about 90% of RGCs were successfully transduced by the viruses (Fig. 3.9 A). AAV2-GFP treated control sections showed a clear CXCR4 staining predominantly in axon bundles and RGC bodies. In contrast, the signal for CXCR4 was almost completely absent in AAV2-Cre-treated retinae (Fig. 3.9 A). Likewise, the efficient knockdown of CXCR4 was also confirmed via immunohistochemical stainings of whole mounted retinae of floxed CXCR4/ROSA mice, which express RFP upon Cre-recombinase transduction and therefore indicate the transduction rate (Fig. 3.9 B).
These data clearly demonstrate the effective knockout of CXCR4 3 weeks after AAV2-Cre application and verify the specificity of the respective antibody.

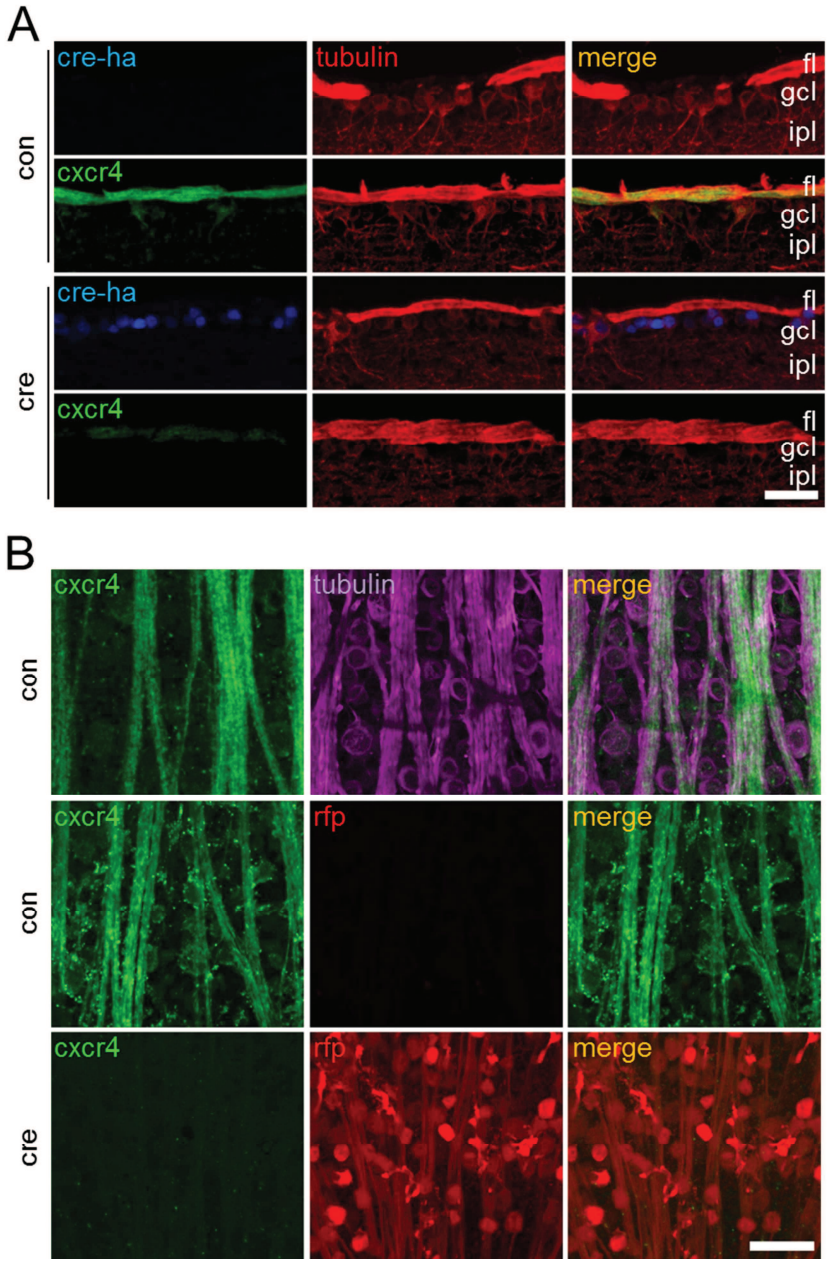

Fig. 3.9: Knockdown of CXCR4 in RGCs after intravitreal AAV2-Cre-injection. (A) Retinal sections of floxed CXCR4 mice prepared 3 weeks after intravitreal injection of either AAV2-GFP (con) or AAV2-Cre (cre) as indicated. Sections were immunohistochemically stained for CXCR4 (green; with TSA-kit), βIII-tubulin (red) and Cre-recombinase (HA-tag, blue). CXCR4 expression was almost absent in Cre-HA-transduced RGCs, verifying the specificity of the staining. Scale bar: 40 µm. fl = fiber layer, gcl = ganglion cell layer, ipl = inner plexiform layer. (B) Retinal whole mounts of floxed CXCR4/ROSA mice isolated 3 weeks after intravitreal injection of either AAV2-GFP (con) or AAV2-Cre (cre) were immunohistochemically stained for CXCR4 (green; with TSA-kit) and βIII-tubulin (magenta). CXCR4 was predominantly expressed in axons and to some extent in somas of RGCs. AAV2-mediated Cre-expression in RGCs induced red fluorescent protein (rfp) expression and knockout of CXCR4 expression. Scale bar: 40 µm.

3.2.3 CXCL12 effect depends on CXCR4-expression in RGCs

In order to elucidate whether CXCL12 promotes neurite growth and exerts disinhibitory features on mouse RGCs, as seen in rats, and furthermore, to determine CXCR4-dependency, floxed CXCR4 mice received intravitreal injections of either

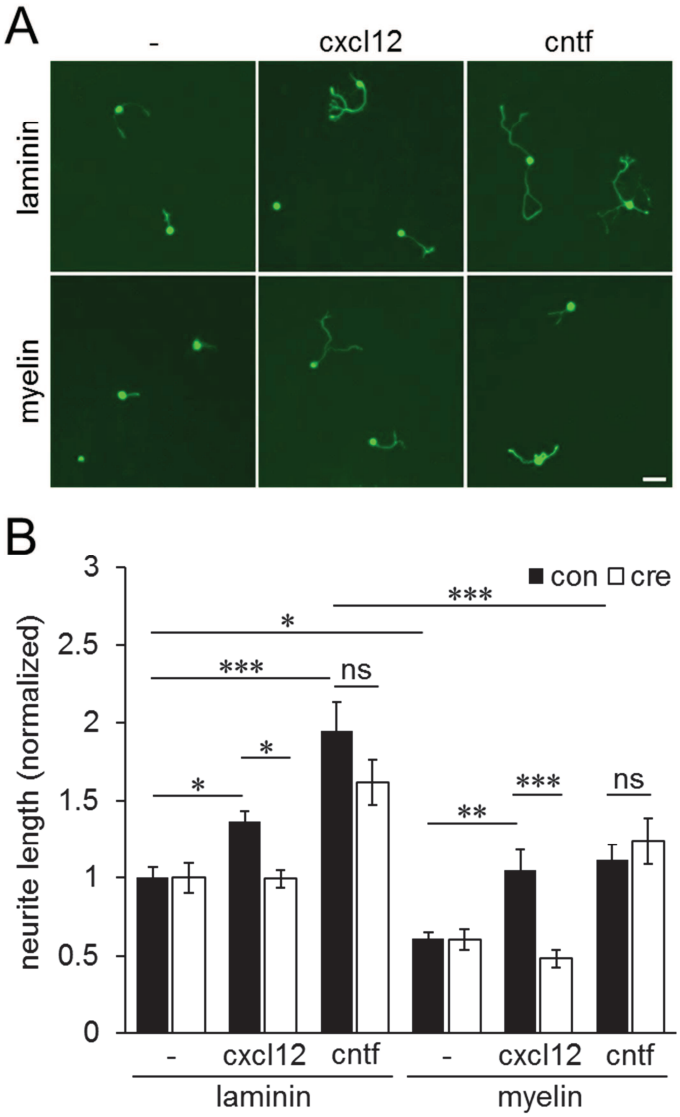

Fig. 3.10: Conditional CXCR4-knockdown obliterates neurite growth promoting and disinhibitory effects of CXCL12 on mature RGCs. (A) Adult mouse RGC cultures were prepared 3 weeks after intravitreal injection of AAV2-GFP in floxed CXCR4 mice. Retinal cultures were exposed to either vehicle (con), CXCL12 (500 ng/ml) or CNTF (200 ng/ml) and plated either on laminin or inhibitory myelin for 4 days. After fixation, cultures were stained with a βIII-tubulin-antibody and subsequently analyzed. Scale bar: 25 µm. (B) Neurite growth of cell cultures as described in A (con, black bars) and additionally cultures prepared 3 weeks after intravitreal injection of AAV2-Cre (cre, white bars) was quantified. Data were normalized to the untreated control on laminin with an average neurite length of 10.7 µm/RGC. Treatment effects: *p<0.05, **p<0.01, ***p<0.001. ns = non-significant.

AAV2-GFP or AAV2-Cre. Retinae were dissociated 3 weeks afterwards to prepare retinal cultures. RGCs were exposed to either vehicle, CXCL12 or CNTF, respectively, for 4 days (Fig. 3.10 A, B). As previously demonstrated in rat cultures, CXCL12 administration enhanced neurite growth of adult mouse RGCs moderately and additionally overcame CNS myelin inhibition (Fig. 3.10 B). Moreover, in contrast to CXCL12, CNTF promoted stronger neurite growth on laminin while it lacked a disinhibitory feature (Fig. 3.10 B). Importantly, in AAV2-Cre transduced RGCs the positive effect of CXCL12 on both laminin and myelin was completely absent (Fig. 3.10 B), strongly suggesting a CXCR4-dependency. At the same time, CNTF-mediated neurite growth was not affected by CXCR4 depletion (Fig. 3.10 B).
Thus, similar to rats, CXCL12 promotes neurite growth of adult mouse RGCs and furthermore desensitizes neurite growth towards inhibitory CNS myelin. Both effects were CXCR4-dependent, indicating the involvement of CXCR4 in CXCL12 mediated signaling in mature RGCs. Importantly, these data also demonstrate the functionality of the CXCR4 depletion.

3.2.4 Non-cognate ligands exert no CXCR4-dependent effect on RGCs

Recently, MIF (Macrophage Migration Inhibitory Factor) and ubiquitin have been identified as two alternative non-cognate ligands for CXCR4 (Bernhagen et al., 2007; Saini et al., 2010). In order to examine their potential to promote neurite growth and disinhibition of mature mouse RGCs, both ligands were also tested in retinal cell cultures. To this end, dissociated retinal cultures were incubated with increasing concentrations of MIF (10 - 500 ng/ml) and plated on laminin- and CNS myelin coated-plates for 4 days. On growth-permissive substrate, none of the tested concentrations was sufficient to enhance neurite growth of mature RGCs. Surprisingly however, all MIF concentrations overcame CNS myelin inhibition (data not shown). To address the question whether this disinhibitory effect was dependent on CXCR4, RGCs of floxed CXCR4 mice were dissociated after intravitreal AAV2-GFP or AAV2-Cre injections. Cells were plated on laminin- and myelin-coated dishes and incubated with vehicle or MIF (50 ng/ml) (Fig. 3.11 A - C). Quantification of neurite growth revealed that knockdown of CXCR4 did not abolish MIF-mediated desensitization of RGCs towards

the inhibitory substrate (Fig. 3.11 A, B). In addition, RGCs survival was not affected by this treatment (Fig. 3.11 C).

Therefore, the disinhibitory effect of MIF seems to be dependent on either indirect effects or other mechanisms than CXCR4 mediated signaling.

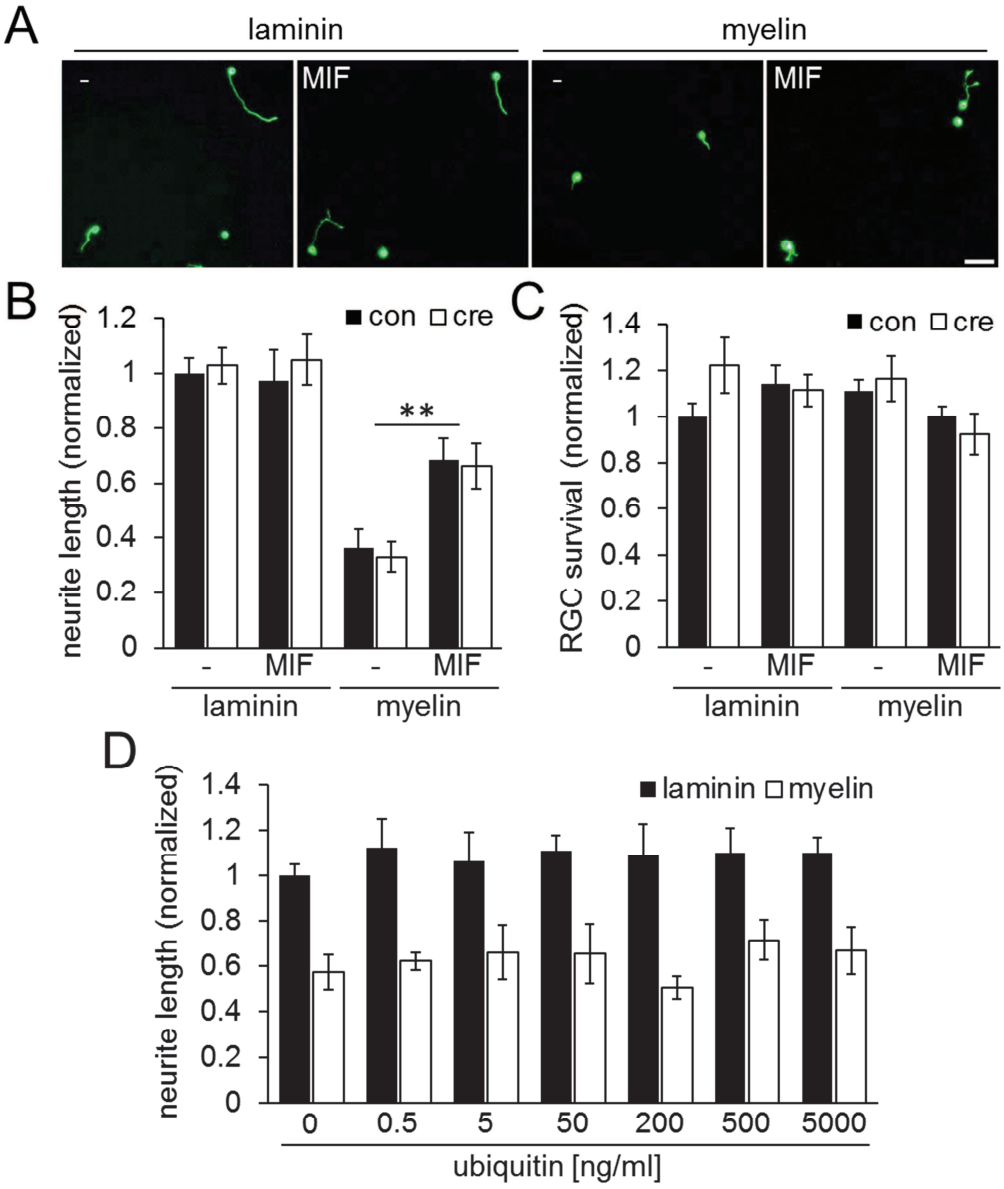

Fig. 3.11: Non-cognate CXCR4 ligands MIF and ubiquitin exert no effect on mouse RGCs via the CXCR4 receptor. (A) Dissociated retinal cultures were incubated without (-) or with MIF (50 ng/ml) on laminin and myelin-coated dishes for 4 days. Cells were stained for βIII-tubulin and subsequently analyzed. Scale bar: 25 µm. (B) Cell cultures as described in A of animals that beforehand received an intravitreal injection of AAV2-GFP (con, black bars) or AAV2-Cre (cre, white bars). Neurite growth was quantified and normalized to the untreated control on laminin with an average neurite length of 6.8 µm/RGC. Treatment effects: **p<0.01. (C) Quantification of RGC numbers of cultures as described in B. (D) Quantification of neurite growth of dissociated retinal cell cultures that were incubated with increasing concentrations of ubiquitin, as indicated (0 – 5000 nM). Cells were plated on laminin- (black bars) or myelin-coated (white bars) dishes for 4 days, subsequently stained for βIII-tubulin and analyzed. Neurite length was normalized to the untreated control on laminin with an average neurite length of 8.4 µm/RGC.

Additionally, the non-cognate CXCR4-ligand ubiquitin was tested with increasing concentrations (0.5 - 5000 ng/ml) on dissociated retinal cultures. Retinal cultures were incubated on laminin- and myelin-coated dishes for 4 days and neurite growth of βIII-tubulin-positive RGCs was subsequently measured. RGC neurite growth was neither affected on laminin-coated dishes, nor in the presence of inhibitory central myelin extract (Fig. 3.11 D).
In conclusion, both MIF and ubiquitin showed no CXCR4-dependent effect on mature RGCs emphasizing the specificity of the effects seen after CXCL12 application.

3.2.5 CXCL12 sustains mTOR-activity CXCR4-dependently

Previous experiments demonstrated that CXCL12 maintains mTOR-activity in rat RGCs upon axotomy (Fig. 3.4). In order to investigate whether this finding is also conserved in mice and furthermore to investigate CXCR4-dependency of CXCL12-mediated mTOR-maintenance, floxed CXCR4/ROSA mice received intravitreal injections of AAV2-GFP or AAV2-Cre 3 weeks before dissociation. Retinal cultures were exposed to a vehicle, CXCL12 alone or in combination with AMD3100 or rapamycin for 4 days. Additionally, an unstimulated group was fixed after 2h to determine the initial value of phospho-S6-positive RGCs as described before (Fig. 3.4). As in rat RGCs, application of CXCL12 was able to maintain initial higher pS6-levels in axotomized mouse RGCs (Fig. 3.12 B, C). This effect was partially blocked with the CXCR4-antagonist AMD3100, while almost totally abolished by rapamycin. Phospho-S6 levels of CXCR4-depleted RGCs in untreated groups that were fixed after 2h and 4 days were not significantly affected. However, CXCL12 was not able to maintain mTOR activity in CXCR4-depleted RGCs in comparison to AAV2-GFP transduced cells after 4 days, confirming the involvement of the receptor in this signaling pathway (Fig. 3.12 B, C).
Thus, similar to rats, CXCL12 maintains CXCR4-dependently mTOR-activity after axotomy in mouse RGCs.

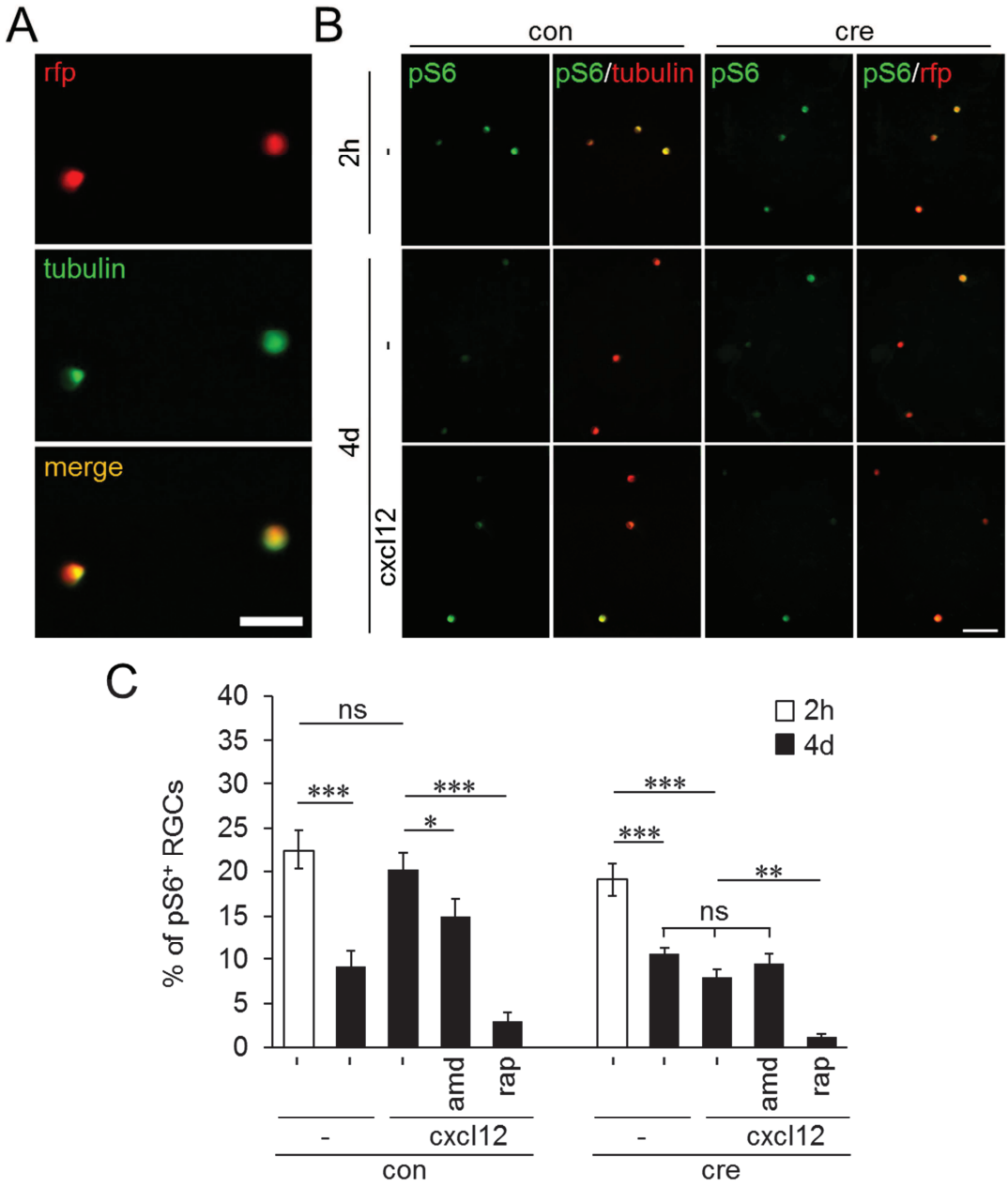

Fig. 3.12: CXCL12 maintains mTOR-activity via the CXCR4 receptor. (A) After intravitreal AAV2-Cre-injection, transduced dissociated RGCs of floxed CXCR4/ROSA mice expressed red fluorescent protein (rfp). Scale bar: 25 µm. (B) RGCs of floxed CXCR4/ROSA mice were dissociated 3 weeks after intravitreal injection of AAV2-GFP (con) or AAV2-Cre (cre). They were exposed to vehicle (-) or CXCL12 (500 ng/ml) and pS6-levels were analyzed after 2 h and 4 days immunocytochemically. Scale bar: 50 µm. (C) Quanification of pS6-positive RGCs of cultures as described B. Cells incubated with CXCL12 were additionally treated with the CXCR4 antagonist AMD3100 (amd) or the mTOR inhibitor rapamycin (rap), respectively. Strongly stained pS6-positive RGCs were quantified after 2 h (white bars) or 4 days (black bars) in culture. Treatment effects: *p<0.05, **p<0.01, ***p<0.001; ns = non-significant.

3.2.6 CXCL12 mediated neurite growth promotion depends on the CXCR4/PI3K/AKT/mTOR-pathway

Previous experiments identified the PI3K/AKT/mTOR-pathway to be responsible for CXCL12-mediated signaling in mature rat RGCs (Fig. 3.5). To assess the contribution of this pathway to CXCL12-mediated neurite growth promotion of adult mouse RGCs,

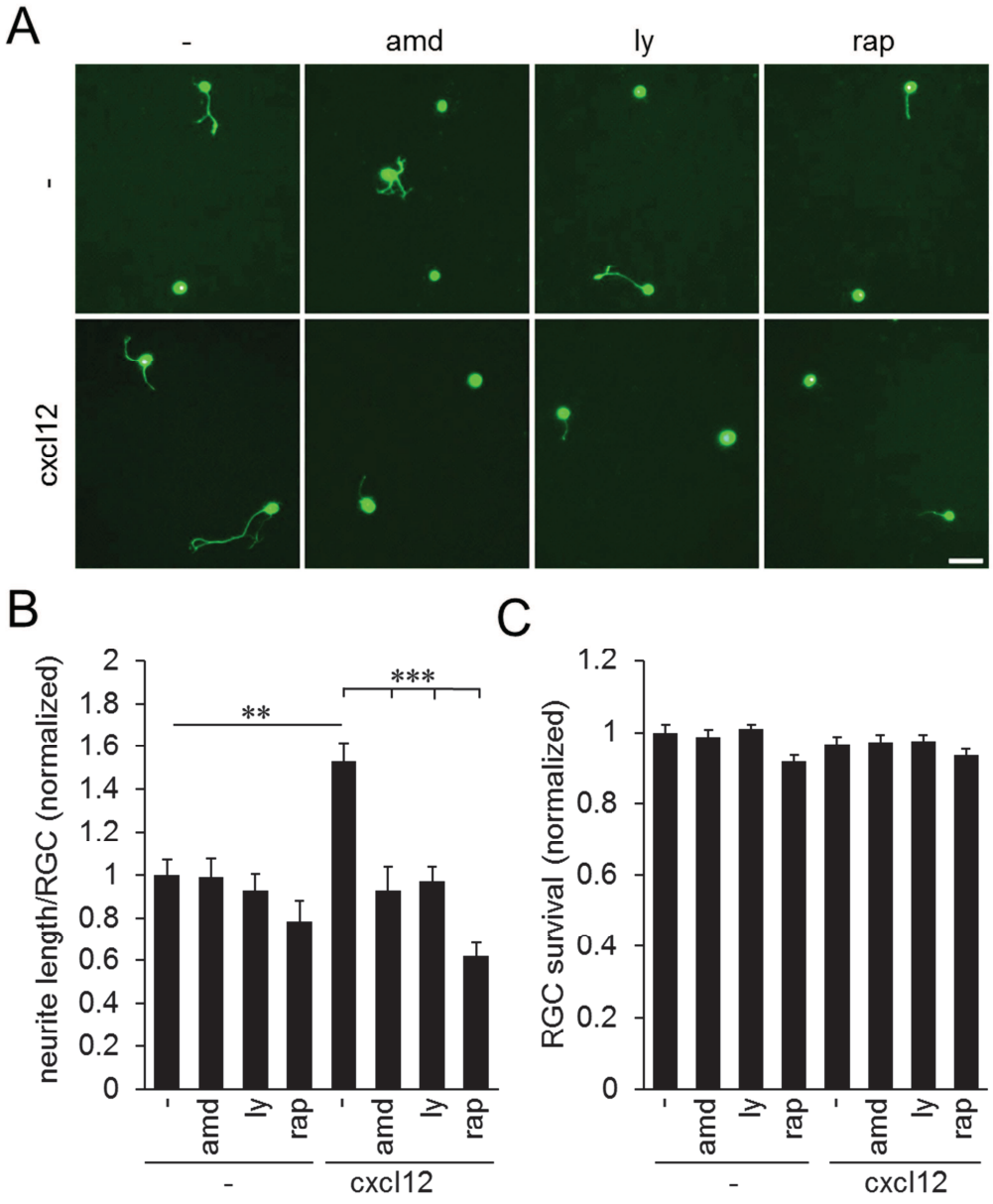

Fig. 3.13: CXCL12 mediates positive effects on RGCs via the CXCR4/PI3K/AKT/mTOR-pathway. (A) Dissociated mouse RGCs were exposed to either vehicle (-) or CXCL12 (500 ng/ml). Each group was additionally incubated with either vehicle (-), the CXCR4 antagonist AMD3100 (amd; 5 µM), the PI3K inhibitor LY294002 (ly; 1 µM) or the mTOR inhibitor rapamycin (rap; 10 nM). RGCs were fixed after 4 days in culture, stained for βIII-tubulin and subsequently analyzed. Scale bar: 30 µm. (B) Neurite growth of cultures as described in A was determined and normalized to the untreated control. Treatment effects: **p<0.01, ***p<0.001. (C) Survival of RGCs as described in A and B after 4 days in culture.

the CXCR4 antagonist AMD3100 (5 µM), the PI3K-inhibitor LY294002 (1 µM) and rapamycin (10 nM) were applied to cell cultures treated with either vehicle or CXCL12 (500 ng/ml), respectively, for 4 days (Fig. 3.13 A-C). As expected, no inhibitor influenced neurite growth per se, while all of them significantly reduced CXCL12-mediated neurite growth to control levels (Fig. 3.13 A, B). The survival of RGCs was not affected by any treatment (Fig. 3.13 C).
As already seen in rats, CXCL12 promotes neurite growth of mature mouse RGCs via the CXCR4/PI3K/AKTmTOR-pathway.

3.2.7 Survival of mouse RGCs is neither affected by CXCL12-treatment nor CXCR4-depletion

Earlier experiments demonstrated that incubation of retinal cultures with CXCL12 and also intravitreal injection of the chemokine did not affect survival of mature rat RGCs both *in vitro* and *in vivo* (Fig. 3.1; Fig. 3.7). In order to see whether genetic knockdown of CXCR4 in RGCs or incubation with recombinant CXCL12 has an influence on RGC neuroprotection *in vitro*, adult floxed CXCR4 mice obtained intravitreal injections of AAV2-GFP or AAV2-Cre. Three weeks afterwards, retinae were dissociated and cultured for 2 h or 7 days (Fig. 3.14 A, B). Some groups were additionally incubated

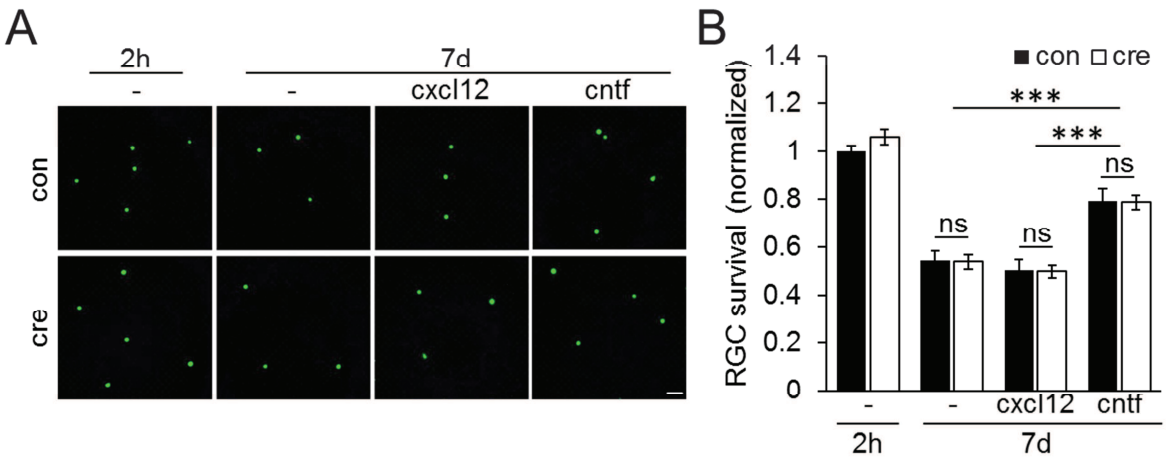

Fig. 3.14: CXCR4 knockdown and CXCL12 treatment do not affect the survival of mature RGCs in culture. (A) Mature RGC cultures were prepared 3 weeks after intravitreal injection of either AAV2-GFP (con) or AAV2-Cre (cre) in floxed CXCR4 mice. Neurons were plated on laminin and cultured for 2 h or 5 days in the absence or presence of either CXCL12 (500 ng/ml) or CNTF (200 ng/ml), respectively. RGCs were stained for βIII-tubulin and subsequently analyzed. Scale bar: 25 µm. (B) Quantification of βIII-tubulin-positive RGCs as described in A. Values were normalized to the initial number of RGCs determined after 2 h with an average number of 175 RGCs/well (96-well plate). Treatment effects: **p<0.01, ***p<0.001, ns = non-significant.

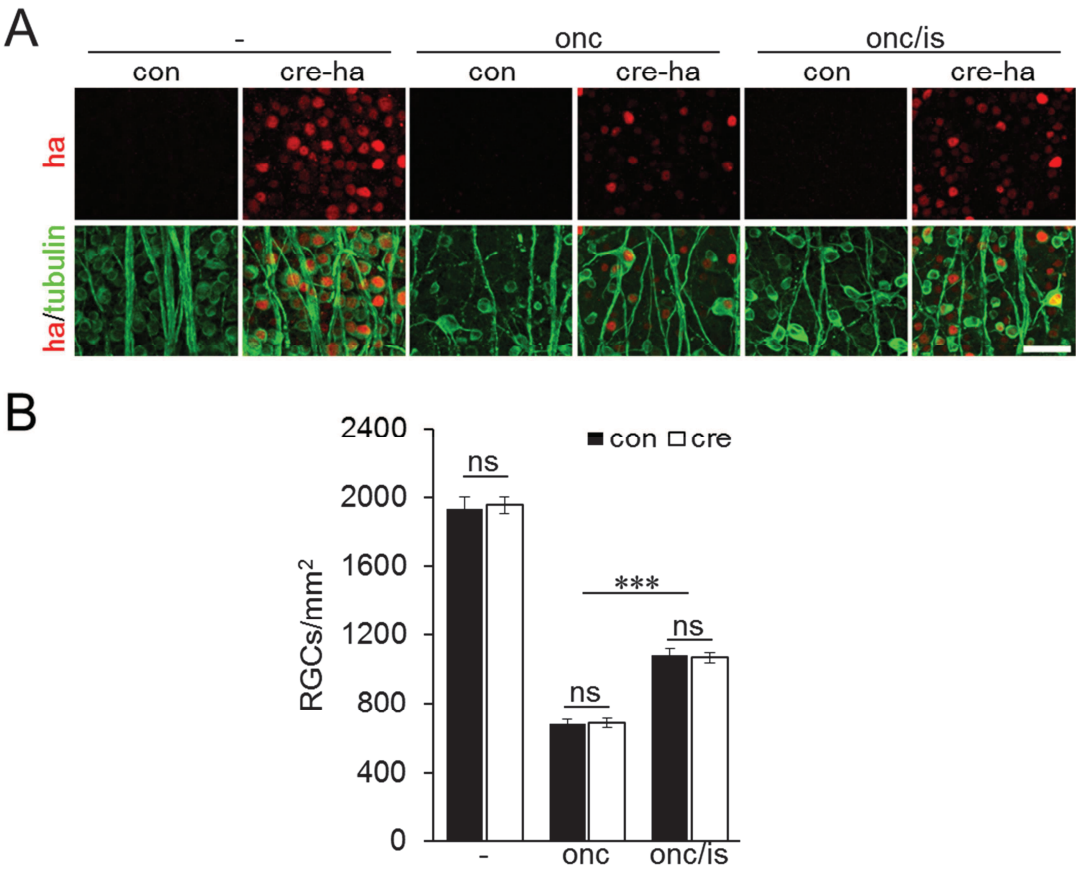

Fig. 3.15: Knockdown of CXCR4 does not affect survival of mature RGCs *in vivo*. (A) Adult floxed CXCR4 mice were subjected to optic nerve crush (ONC) alone, ONC + inflammatory stimulation (IS) or no treatment (-) 3 weeks after intravitreal injection of AAV2-Cre (cre-ha) or AAV2-GFP (con). Fourteen days after surgery, retinae were dissected, whole mounted, PFA-fixed and subsequently stained for the Cre-recombinase (ha) and βIII-tubulin. Scale bar: 50 µm. (B) Quantification of surviving βIII-tubulin-positive RGCs/mm^2 of treatments as described in A. Treatment effects: ***p<0.001, ns = non-significant.

with CXCL12 or CNTF, respectively. CXCL12 exerted no neuroprotective effect on mouse RGCs *in vitro*, while CNTF was a neuroprotective factor as reported previously (Fig. 3.1). Likewise, the knockdown of CXCR4 in RGCs did not affect the survival (Fig. 3.14 A, B).

In addition, the survival of mature RGCs was also investigated *in vivo*. To address this, floxed CXCR4 mice were treated with AAV2-GFP or AAV2-Cre and 3 weeks afterwards subjected to no treatment, ONC or ONC + IS. Quantification of RGCs in whole mounted retinae revealed about 2,000 RGCs/mm^2 in the untreated adult mouse retina with about 80-90% of AAV2-Cre transduced RGCs (Fig. 3.15 A). Crush of the optic nerve lead to drastic cell death resulting in about 600 RGCs/mm^2 after 14 days, while an IS exerted neuroprotective effects, leading to enhanced RGC numbers of

1,000 RGCs/mm^2 (Fig. 3.15 A). A CXCR4 depletion did not affect cell numbers after any of these treatments (Fig. 3.15 A, B).

In conclusion, neither treatment with CXCL12, as seen in rats, nor knockdown of CXCR4 affected survival of mouse RGCs both *in vitro* and *in vivo,* suggesting that CXCR4 signaling is not involved.

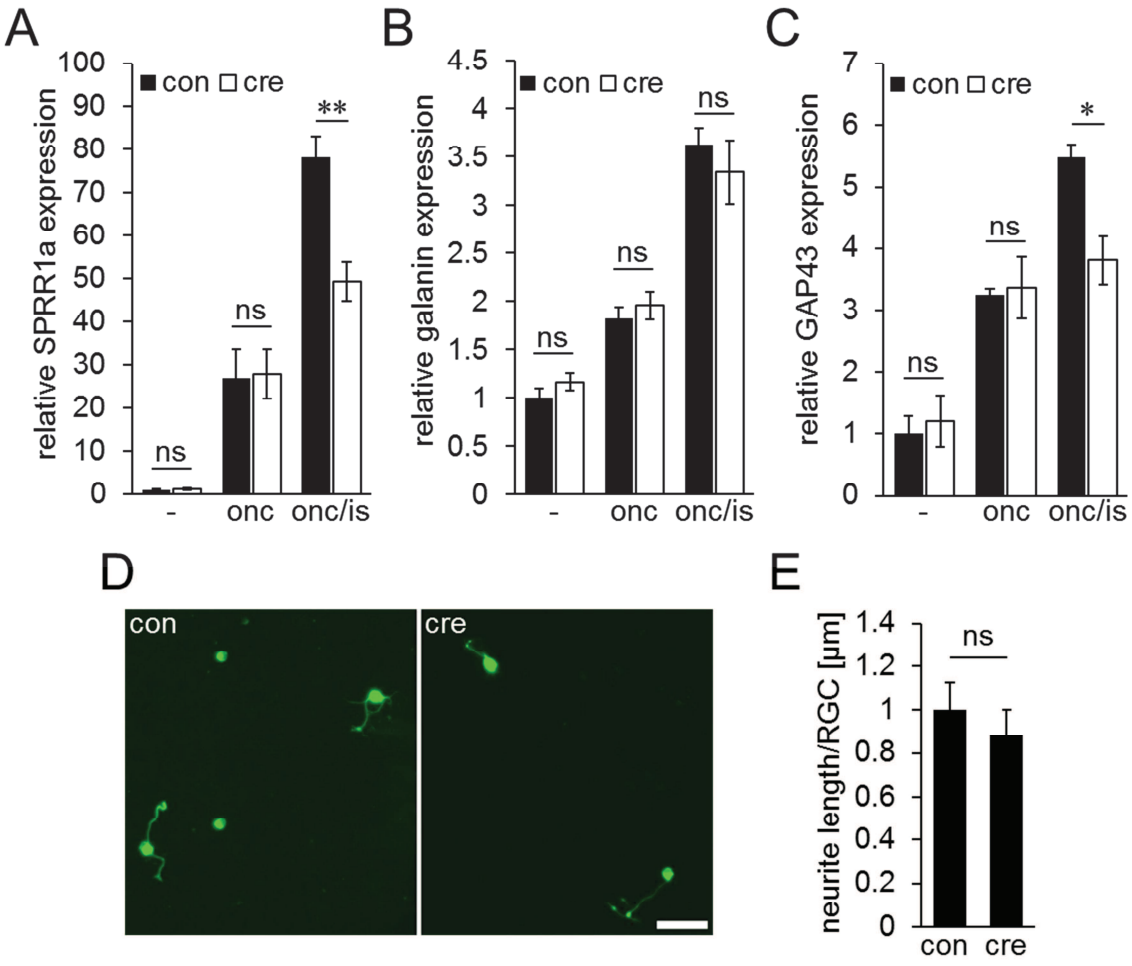

Fig. 3.16: CXCR4 knockdown does not deteriorate the intrinsic regenerative state of RGCs. (A - C) With quantitative Real-time PCR the amount of retinal GAP43 (A), SPRR1a (B) and Galanin (C) was quantified relative to GAPDH expression in floxed CXCR4 mice objected to ONC or ONC + IS 5 days prior to preparation. Animals obtained an intravitreal injection of AAV2-GFP (con) or AAV2-Cre (cre) 3 weeks beforehand. Treatment effects: *p<0.05, **p<0.01, ns = non-significant. (D) Floxed CXCR4 mice received intravitreal injections of AAV2-GFP (con) or AAV2-Cre (cre) and were afterwards subjected to ONC + IS for 5 days. Cells were dissociated, plated on poly-D-lysine-coated dishes for 1 day and subsequently stained for βIII-tubulin. Scale bar: 50 µm. (E) Spontanous neurite growth of groups as described in D was quantified and normalized to the AAV2-GFP control with an average neurite length of 0.7 µm/RGC. Treatment effects: ns = non-significant.

3.2.8 CXCR4 depletion does not affect the transformation of RGCs into an intrinsic regenerative state upon IS

Regeneration associated genes (RAGs) are upregulated after optic nerve injury and expression is further increased after combination with IS in the mouse retina (Bonilla et al., 2002; Fischer et al., 2004c). This upregulation of genes reflects an altered transcriptional programm that boosts biosynthesis enabling elongation and therefore regeneration after injury. In order to see whether CXCR4 depletion influences the intrinsic regenerative state of RGCs, the expression of the three RAGs SPRR1a, galanin and GAP43 in retinae of floxed CXCR4 mice was analyzed by qRT-PCR. Animals obtained intravitreal injections of AAV2-GFP or AAV2-Cre and 3 weeks afterwards they were subjected to ONC or ONC + IS. Five days after surgeries, retinal mRNA was isolated and the mRNA levels of the RAGs was examined. The knockdown of CXCR4 did not influence the expression of the tested RAGs in untreated retinae or after ONC alone (Fig. 3.16 A - C). Galanin expression was not influenced by CXCR4 knockdown at all. However, upregulation of SPRR1a and GAP43 after ONC + IS was significantly impaired by CXCR4 depletion (Fig. 3.16 A - C) suggesting that the absence of CXCR4 negatively affected the regenerative state of mouse RGCs after ONC + IS.

To further investigate the intrinsic growth mode of injured adult mouse RGCs after CXCR4 depletion, spontanous neurite growth was investigated that reflects the active regenerative state of cells. Floxed CXCR4 mice that beforehand received an intravitreal AAV2-GFP or AAV2-Cre injection, were subjected to ONC + IS. Five days afterwards retinae were dissociated and neurite growth of dissociated RGCs was determined after 1 day in culture (Fig. 3.16 D, E). CXCR4-depleted RGCs exhibited the same spontanous neurite growth as the control group where the receptor was still expressed (Fig. 3.16 D, E).

In summary, although some RAGs failed to be IS-dependently upregulated after CXCR4 depletion, spontaneous neurite growth was not significantly affected.

3.2.9 CXCR4 and CXCL12 are expressed in the optic nerve

In order to investigate the *in vivo* localization of both CXCL12 and CXCR4 in naïve optic nerves and cultured dissociated RGCs, immunochemical stainings were

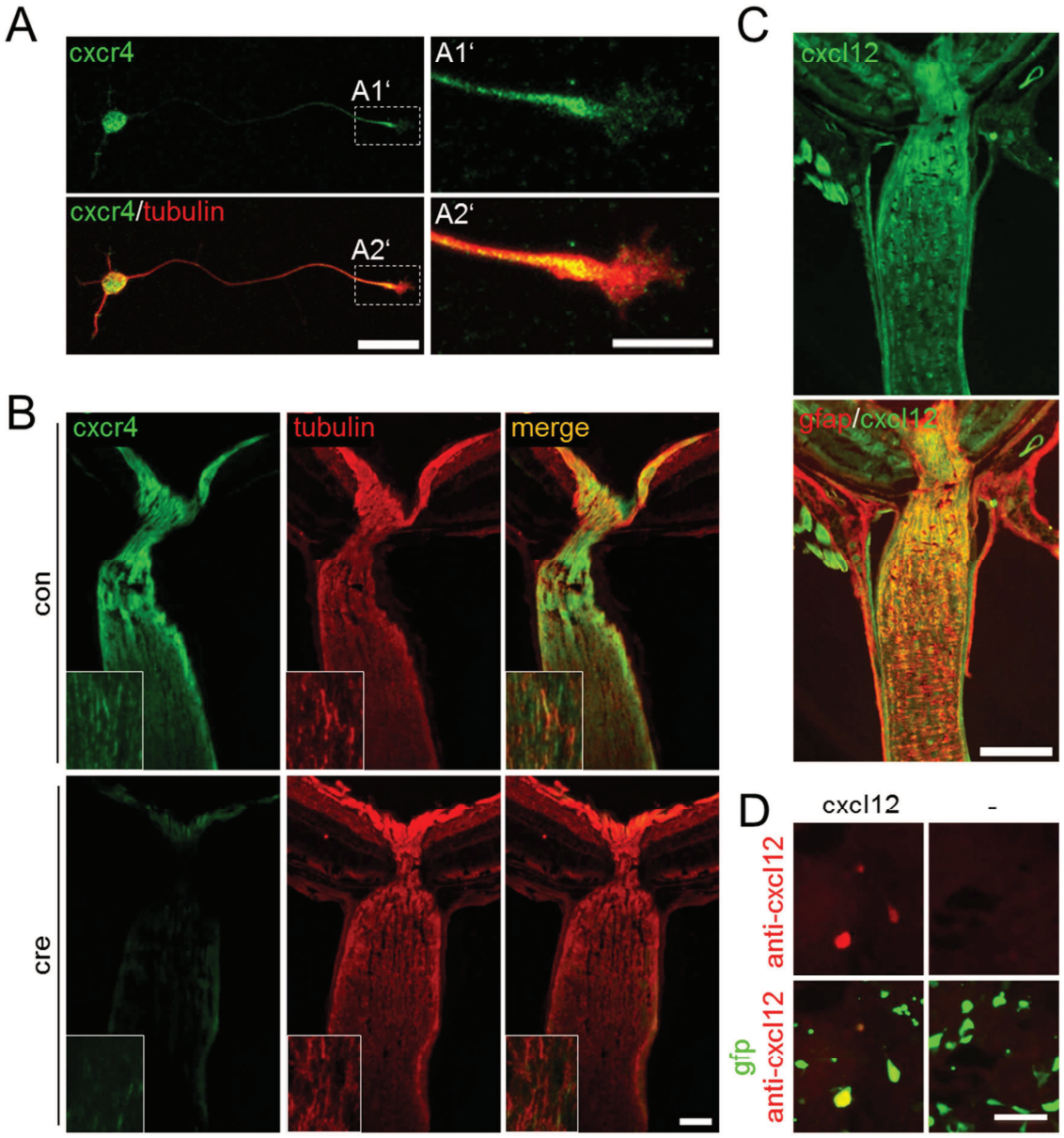

Fig. 3.17: CXCR4 and CXCL12 are expressed in the optic nerve head and optic nerve. (A) Immunocytochemical staining of CXCR4 (green) and βIII-tubulin (red) of a dissociated mouse RGC after 4 days in culture. Scale bar: 25 µm. (A1' + A2') Magnification of boxes as indicated in A. Scale bar: 10 µm. (B) Optic nerve sections of animals that obtained intravitreal injections of AAV2-GFP (con) or AAV2-Cre (cre) 3 weeks before perfusion. CXCR4 (green, with TSA-kit) and tubulin (red) staining were predominantly detected in the optic nerve head. Scale bar: 50 µm. (C) Optic nerve sections of untreated mice were stained for CXCL12 (green) and the astrocyte-marker GFAP (red). Scale bar: 200 µm. (D) Immunocytochemical staining of CXCL12- or empty vector-transfected HEK293 cells with a CXCL12-antibody used in C verifying the ability of the antibody to detect exogenous CXCL12. Scale bar: 50 µm.

performed. Similar to rat RGCs, CXCR4 was expressed on axonal tips and in somas of dissociated mouse RGCs (Fig. 3.17 A). CXCR4-staining of optic nerve sections demonstrated that the receptor was predominantly expressed in RGC axons. As shown before in whole-mounted retinae, this signal was almost entirely abolished when floxed CXCR4 animals received an intravitreal AAV2-Cre-injection beforehand, while the βIII-tubulin signal was not affected at all (Fig. 3.17 B). Interestingly, the staining intensity of CXCR4 was strongest at the optic nerve head area. Similarly, staining of untreated mouse sections with an antibody that specificially detects exogenously expressed CXCL12 in HEK293 cells, exhibited a diffuse signal that colocalized partially with GFAP-positive astrocytes at the optic nerve head (Fig. 3.17 C, D). The antibody signal was strongest at the optic nerve head, while it dramatically declined in the optic nerve, representing a chemokine gradient.
These findings indicate that both receptor and ligand are strongly expressed in the optic nerve predominantly in close proximity to the eye ball.

3.2.10 CXCL12 is a chemoattractant for neurites of mouse RGCs and DRG neurons on inhibitory substrate

After optic nerve crush the presence of both damaged CXCR4-positive axons and CXCL12-secreting astrocytes may lead to a local event that influences optic nerve regeneration. Growth cones of damaged axons are on the one hand exposed to an inhibitory environment but also to a CXCL12 gradient that is produced by astrocytes. Previously, the pivotal role of the CXCL12-CXCR4 axis in axon guidance during retinal development was described (Chalasani et al., 2003a; Li et al., 2005; Lieberam et al., 2005). In order to investigate whether CXCL12 is able to chemoattract neurites of mature RGCs, dissociated cells were exposed to a local CXCL12 gradient and the direction of neurite growth was analyzed.

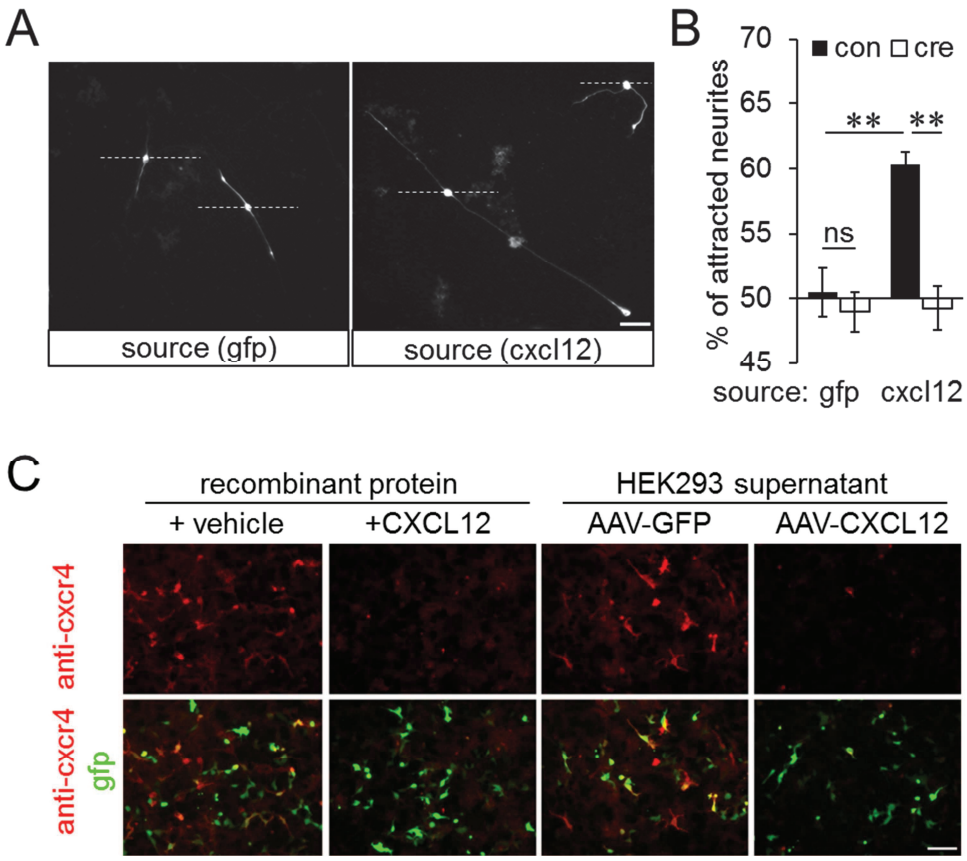

Fig. 3.18: CXCL12 attracts RGC neurites on a non-growth permissive substrate *in vitro*. (A) Floxed CXCR4 mice received an intravitreal AAV2-GFP injection and were subjected to ONC + IS. Five days thereafter, cells were plated on myelin-coated dishes for 1 day until a standing insert containing GFP- or CXCL12-transfected HEK293 cells was placed in the middle of the well for additional 2 days. RGCs were then fixed, stained for βIII-tubulin and the orientation of the regenerated neurites toward the HEK293 source was analyzed. Scale bar: 50 µm. (B) Percentage of positively attracted neurites of retinal cell cultures as described in A (con, black bars) with an additional group that obtained intravitreal AAV2-Cre injection (cre, white bars). Treatment effects: **p<0.01, ns = non-significant. (C) HEK293 cells were transfected with a mouse CXCR4-expression vector und co-transfected with pAAV-GFP. As a positive control recombinant human CXCL12 (250 ng/ml) or a vehicle were added to the medium for 1 h, respectively. Additionally, supernatants of GFP (pAAV-GFP)- or rat CXCL12 (pAAV-CXCL12)-transfected HEK293 cells were applied to the mouse CXCR4-transfected cells and incubated for 1 h. Cells were then fixed and CXCR4-staining (red) analyzed. Scale bar: 50 µm.

AAV2-GFP or AAV2-Cre were intravitreally injected in floxed CXCR4 mice. Three weeks afterwards, RGCs were transformed into an active regenerative state by performing ONC + IS 5 days prior to dissociation. Retinal cells were plated on myelin-coated dishes in order to mimick native conditions present after optic nerve injury. After 1 day, cell culture inserts containing either GFP-expressing or CXCL12-secreting HEK293 cells were positioned in the middle of the well for additional 2 days. During this time, CXCL12 was produced and secreted. To ensure the functionality of secreted

CXCL12, the supernatant of CXCL12-transfected HEK293 cells was beforehand applied to CXCR4-transfected HEK293 cells (Fig. 3.18). Consistent with previous reports, treatment of CXCR4-transfected HEK293 cells with recombinant CXCL12 lead to receptor phosphorylation and therefore loss of CXCR4-antibody recognition (Fig. 3.18 C) (Mueller et al., 2013). Importantly, CXCR4-staining was also diminished when HEK293 cells were incubated with the supernatant of CXCL12-transfected HEK293 cells (Fig. 3.18 C), whereas GFP-expressing HEK293 cells did not affect CXCR4-staining. Thus, CXCL12-transfected HEK293 cells expectedly secreted a biological active chemokine making them suitable for chemoattraction-experiments. After fixation and staining of the retinal cultures, the orientation of regenerating neurites toward the HEK293 source was analyzed (Fig. 3.18 A, B).

Notably, significantly more neurites directed toward the HEK293 source when CXCL12 was secreted (~60%) in comparison to the GFP-transfected control (~50%). Noticeably, this effect was totally absent in CXCR4-depleted RGCs (Fig. 3.18 B) indicating the potential of the CXCL12-CXCR4 axis to chemoattract neurites of adult mouse RGCs on inhibitory myelin

To address the question whether also other cell types react to a CXCL12 gradient, DRG neurons of adult mice were tested in a chemoattraction-assay. To assess whether DRG neurons respond to CXCL12 at all, dissociated neurons were incubated with two concentrations of the chemokine for 2 days on either laminin- or myelin-coated dishes (Fig. 3.19 A). Application of CXCL12 had no growth-promoting effect on DRG neurons plated on growth-permissive substrate, while incubation with CXCL12 (500 ng/ml) on CNS myelin exhibited a robust disinhibitory effect (Fig. 3.19 A). Immunohistochemical stainings for CXCR4 showed an axonal localization with accumulation in tips, branches and also in the soma of adult mouse DRG neurons (Fig. 3.19 B) pointing to the possibility that CXCL12 mediated its disinhibitory effects on DRG neurons via CXCR4.

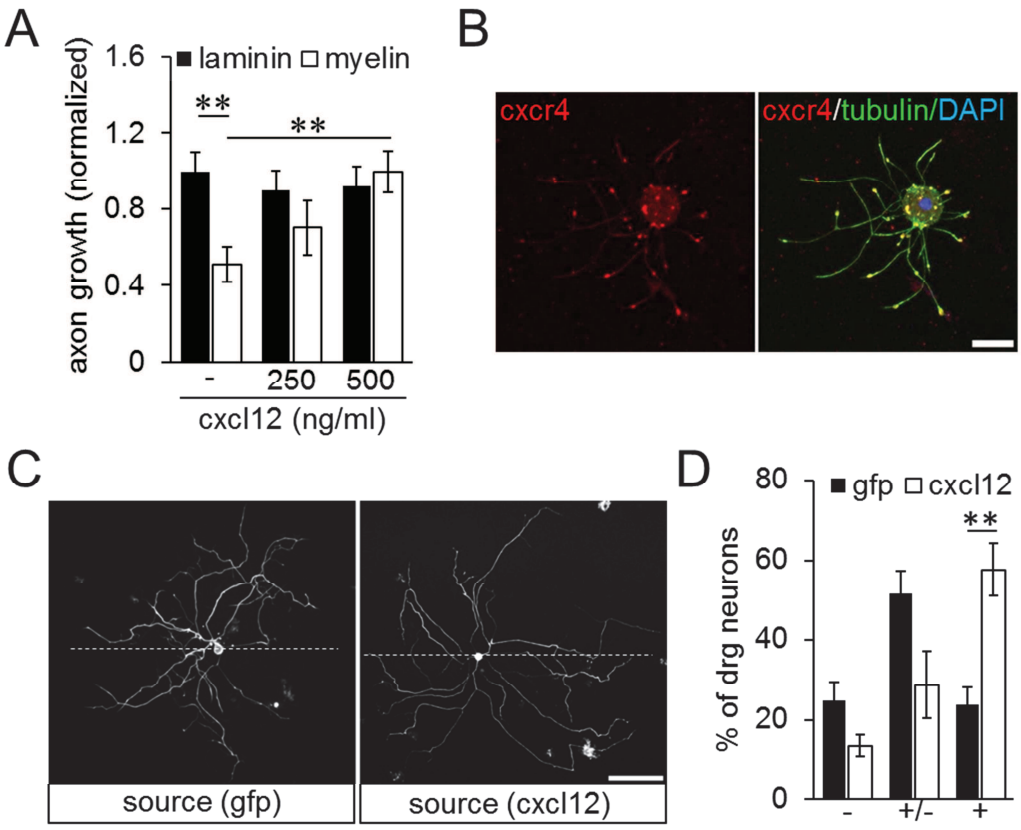

Fig. 3.19: CXCL12 attracts neurites of DRG neurons. (A) DRGs of wildtype mice were dissociated and neurons were exposed to no treatment (-) or CXCL12 (250 ng/ml or 500 ng/ml, as indicated). Cells were plated on laminin (black bars) or myelin-(white bars) coated dishes for 2 days, fixed, stained for βIII-tubulin. Axon growth was then quantified. Treatment effects: **p<0.01. (B) Immunocytochemical staining for CXCR4 (red) and βIII-tubulin (green) of a dissociated DRG neuron that was cultivated for 2 days on a growth-permissive substrate. Scale bar: 50 µm. (C) Dissociated DRG neuron cultures were incubated on myelin-coated dishes for 2 days until a standing insert containing GFP- or CXCL12-transfected HEK293 cells was placed in the middle of the well for additional 2 days. DRG neurons were fixed after 4 days in culture, stained for βIII-tubulin and the neurite mass in orientation toward the HEK293 cells was analyzed. Scale bar: 200 µm. (D) Quantification of positively attracted (+), repelled (-) or not influenced (+/-) DRG neurons toward the HEK293 source expressing gfp (black bars) or CXCL12 (white bars). Treatment effects: **p<0.01.

For chemoattraction assays, dissociated DRG neurons were cultivated for 2 days until cell culture inserts containing pAAV-GFP or pAAV-CXCL12 transfected HEK293 cells were placed in the middle of the well for additional 2 days. Analysis of outgrown DRG neurons revealed that about 50% of all neurons that were exposed to the GFP-transfected HEK293 cells developed an evenly distributed neurite mass ("+/-"), while those that were exposed to a stable CXCL12-gradient developed more neurite mass directed to the chemokine gradient ("+") (Fig. 3.19 C, D). Just like adult RGC, adult mouse DRG neuron axons were able to sense and react to a CXCL12 gradient.

In conclusion, in the presence of inhibitory CNS myelin, axons of CNS and PNS neurons were able to sense and react to a CXCL12 gradient and were attracted to the source of the chemokine.

3.2.11 CXCR4 depletion reduces aberrant axon growth

Previous *in vitro* experiments indicated that CXCL12 chemoattracts CNS neurites CXCR4-dependently on inhibitory myelin (Fig. 3.18 A, B). Additional immunohistochemical stainings revealed that CXCR4 is expressed in axons along the

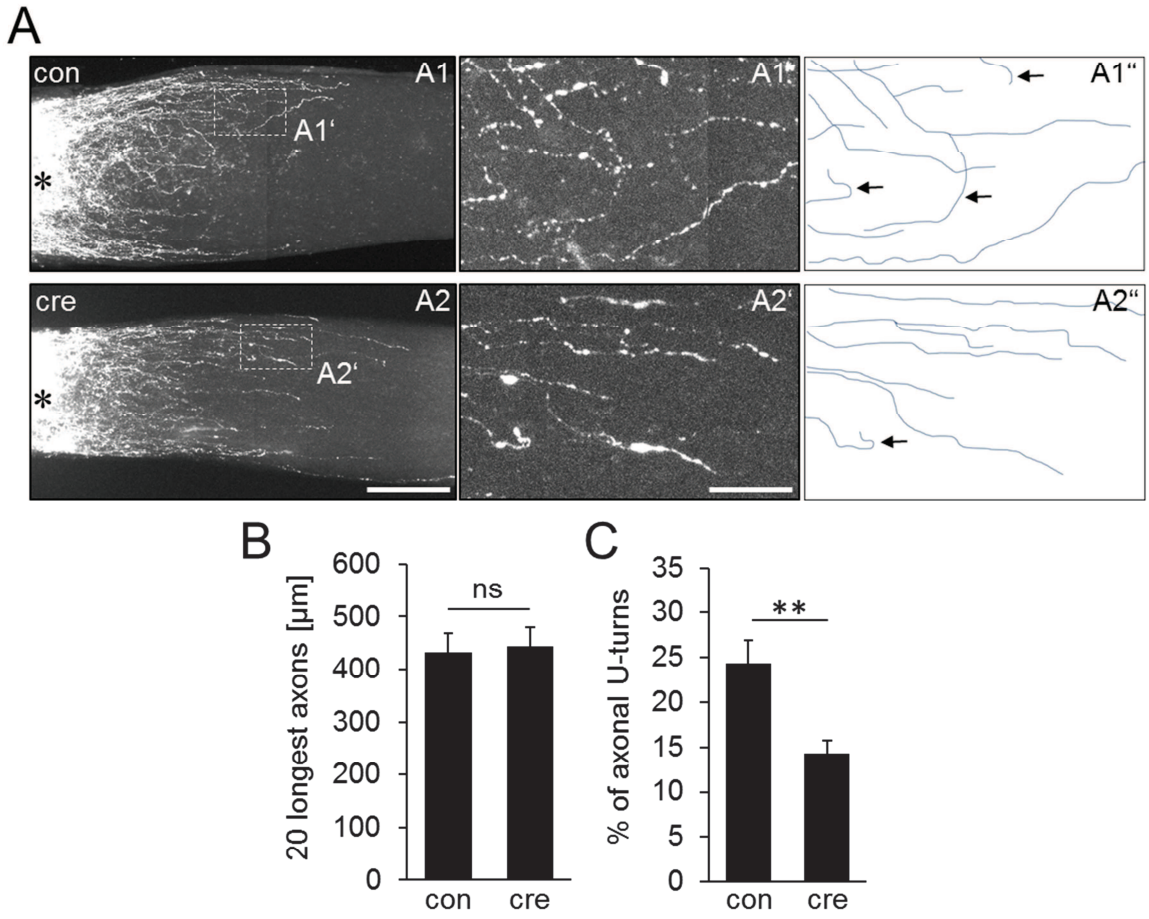

Fig. 3.20: Knockdown of CXCR4 decreases aberrant axonal growth distal to the lesion site. (A) Maximum intensity projections of stacks scanned 50 µm into the cleared optic nerves of floxed CXCR4 mice treated with AAV2-GFP (A1; con) or AAV2-Cre (A2; cre) that were subjected to ONC + IS for 7 days. The lesion site is marked with an asterisk. Scale bar: 75 µm. (A1'+A1'') Magnification of boxes as indicated in A1 + A2. Analysis of semi-automatically traced axons indicated that after ONC + IS, axons tend to form U-turns and grew back toward the lesion site, while after CXCR4-knockdown regenerating axons grew more straight and precicsely toward the chiasm (A2'+A2''). Scale bar: 25 µm. (B) The distance of the 20 longest regenerating axons to the lesion site was determined for groups as described in A. Treatment effects: ns = non-significant. (C) Quantification of U-turn forming axons of the 20 longest axons per optic nerve. Treatment effects: **p<0.01.

optic nerve, while CXCL12 is apparently secreted by astrocytes predominantely at the optic nerve head (Fig. 3.17 B, C). Thus, these findings open the possibility that CXCR4-positive RGC axons are exposed to a chemoattractive CXCL12 gradient upon ONC *in vivo*, which could affect the orientation of axon growth. In order to address this possibility and to test whether this happens CXCR4-dependently, AAV2-GFP and AAV2-Cre were intravitreally injected into floxed CXCR4 mice. Animals were subjected to ONC + IS 3 weeks afterwards in order to transform RGCs into an active regenerative state. Animals were perfused 7 days later. To analyze the regenerating axons distal to the lesion site three-dimensionally (3D), complete unsectioned optic nerves were subjected to tissue clearing after anterograde tracing with cholera toxin subunit B (CTB) conjugated to Alexa Fluor 594®. Whole-mount optic nerves were subsequently scanned with a confocal laser scanning microscope and stacks were reconstructed in 3D in order to characterize the behavior of regenerating axons after injury. Analysis of the 20 longest axonal trajectories in each transparent nerve revealed that the distance of the regenerating axons to the lesion site was similar between those animals transduced with AAV2-Cre and respective controls (Fig. 3.20 A, B). Interestingly, in control animals around 24% of regenerating axons performed U-turns directed backwards toward the lesion site. Importantly, the number was significantly reduced to 14% when CXCR4 was knocked out in RGCs (Fig. 3.20 C). Thus, axons of CXCR4 depleted animals exhibited less irregular trajectories and U-turns in comparison to the GFP-AAV2 treated controls.

The whole-mount 3D analysis method used here suggests that normally ocurring aberrant growth and U-turns in control nerves might be partially mediated by axonal CXCR4 signaling.

4 Discussion

This thesis investigated the role of CXCL12 and its cognate receptor CXCR4 in neuroprotection of mature RGCs and axonal regeneration in the adult mouse and rat optic nerve using pharmacological and genetic approaches. Key novel findings identified in this context were:

1) CXCL12 exerts a moderate neurite growth-promoting effect on mature RGCs in culture and is disinhibitory towards myelin. *In vivo*, intravitreal application of the chemokine slightly, but significantly, facilitates axon regeneration into the rat optic nerve. Combinatorial treatment with an inflammatory stimulation further enhances axon regeneration into the rat optic nerve.
2) Neither application of CXCL12 nor CXCR4 depletion in adult animals affects the survival of mature RGCs both *in vitro* and *in vivo*.
3) CXCR4 is expressed in cell bodies and neurites of adult RGCs. Expression is significantly upregulated after ONC alone and ONC + IS. Its ligand CXCL12 is presumably produced and secreted by astrocytes at the optic nerve head.
4) CXCL12-mediated effects on axon growth are CXCR4 and PI3K/AKT/mTOR-signaling cascade dependent.
5) CXCL12 is a chemoattractant for regenerating axons of RGCs, which is dependent on CXCR4. Consistently, *in vivo* CXCR4 depletion in RGCs reduces axonal U-turns of regenerating axons after ONC + IS *in vivo*.

4.1 CXCR4 is expressed and differentially regulated after optic nerve injury in mature rodent RGCs

Previous studies described the expression of the G-protein coupled receptor CXCR4 in a variety of different cell types including neurons, like chick and zebrafish embryonic RGCs (Chalasani et al., 2003b; Chalasani et al., 2003a; Li et al., 2005; Chalasani et al., 2007; Opatz et al., 2009b; Buckley et al., 2013). Using *in situ hybridization*, a strong expression of CXCR4 was demonstrated in the RGC layer of E6 chick retinae and zebrafish embryos 48 hours post-fertilization (Chalasani et al., 2003b; Li et al., 2005). In analogy with these data, we show that CXCR4 was expressed by mature rodent RGCs. Immunohistochemical stainings indicated, that CXCR4 was expressed at basal levels in the RGC soma, while it predominantly localized to their axons. This suggests, that mature RGCs are directly responsive to the receptors cognate ligand CXCL12. Interestingly, we could see that CXCR4 expression was increased in RGC bodies 5 days after ONC + IS in comparison to untreated controls. A stronger upregulation of the receptor became visible 14 days after ONC alone or ONC + IS, implicating a greater sensitivity of RGCs towards CXCL12 at later time points after axotomy. The molecular mechanisms underlying the CXCR4 upregulation need to be addressed in subsequent studies. Whether CXCL12 preferentially binds *in vitro* and *in vivo* to CXCR4 at the RGC soma or the axon/growth cone still has to be elucidated.

4.2 CXCL12 treatment and CXCR4 knockdown do not affect RGC survival

During embryonic development, RGCs are initially overproduced and about 50% undergo programmed cell death during the first postnatal week due to limited trophic support (Hamburger and Levi-Montalcini, 1949; Cellerino et al., 2000). It was recently shown, that the chemokine CXCL12 occupies a neurotrophic role, preventing cell death of RGCs during development of the chick and mouse retina (Chalasani et al., 2003b). Previous *in vitro* studies demonstrated a neuroprotective feature of CXCL12 on embryonic E6 chick RGCs preventing them from cell death, while likewise in embryonic E13.5 CXCR4 knockout mice RGC numbers were reduced to 65% in comparison to wild-type animals (Chalasani et al., 2003b). However, also a negative

effect of CXCL12 on neurons was described. Previous reports demonstrated, that CXCL12 exerts neurotoxic effects on embryonic rat cerebrocortical cultures and on 13-16 week human fetal neurons (Kaul and Lipton, 1999; Zheng et al., 1999). In contrast, the current study demonstrates that CXCL12 had no effect on mature rodent RGCs. Neither the application of CXCL12 to dissociated mature retinal cultures *in vitro* nor its intravitreal injection *in vivo* affected cell death of axotomized RGCs. This indicates, that in contrast to CNTF and other neurotrophic factors such as BDNF (Mey and Thanos, 1993; Cohen et al., 1994; Mansour-Robaey et al., 1994), the neuroprotective effects of CXCL12 seem to be restricted to the early development of RGCs or non-injury induced cell death.

We furthermore tested the neuroprotective feature of CXCR4 in adult mice. Absence of CXCR4 in RGCs did not affect survival of RGCs both *in vitro* and *in vivo*. Additionally, the IS-mediated neuroprotective effect seen after ONC + IS was also independent of CXCR4 expression as its depletion did not influence it *in vivo*. These data suggest that CXCL12-CXCR4 signaling is neither involved in degenerative processes of mature axotomized RGCs nor in IS-mediated neuroprotection.

4.3 Mature RGCs are desensitized by CXCL12 towards inhibitory CNS myelin

Previous studies demonstrated that incubation of postnatal DRG neurons with CXCL12 reduced the sensitivity af axonal growth cones towards inhibitory CNS myelin (Opatz et al., 2009a). Furthermore, the chemokine decreased the effectiveness of many axonal repellents and is prerequisite for normal axonal pathfinding in zebrafish, implying that the chemokine regulates and modulates the sensitivity towards inhibitory signals (Chalasani et al., 2003a; Chalasani et al., 2007). Consistently, we could demonstrate that CXCL12 application desensitizes mature RGCs towards inhibitory myelin and that these effects depend on the CXCR4/PI3K/AKT/mTOR-signaling cascade. Nevertheless, incubation with the chemokine did not overcome neurocan-mediated inhibition. Therefore the chemokine acts similar to IL-6 which was recently shown to activate the same signaling cascades in mature RGCs, plus the JAK/STAT3-pathway, and to overcome myelin mediated but not neurocan dependent disinhibition

(Leibinger et al., 2013b). However, in contrast to CXCL12 IL-6 facilitated stronger RGC neurite growth which was probably due to its ability to transform mature RGCs into an active regenerative state (Leibinger et al., 2013b). These findings imply that both IL-6 and CXCL12 affect molecular targets upstream of the RhoA/ROCK-signaling cascade. In contrast to that, incubation of retinal cultures with the ROCK-inhibitor Y27632 or Taxol overcame both myelin and neurocan mediated neurite growth inhibition (Ahmed et al., 2009; Sengottuvel and Fischer, 2011; Sengottuvel et al., 2011).

Therefore, despite lacking neuroprotective features, CXCL12 can be used as a co-treatment to desensitize growth cones toward inhibitory myelin environment.

4.4 CXCL12 moderately enhances axon growth

Previous studies already demonstrated that CXCL12 enhances axonal elongation of cerebellar granule neurons *in vitro* in a Rho-dependent manner and furthermore controls axon growth and branching in hippocampal neurons (Arakawa et al., 2003; Pujol et al., 2005). Moreover, intrathecal infusion of CXCL12 into the spinal cord injury site reportedly improved sprouting of corticospinal tract axons (Opatz et al., 2009b).

Here we show for the first time, that incubation of dissociated mature rodent RGCs with recombinant CXCL12 is sufficient to moderately enhance neurite growth on the growth-permissive substrate laminin. These effects were however less pronounced than seen after CNTF application. This could be due to different reasons. In dissociated retinal cultures, relatively high concentrations of CXCL12 have to be applied to promote neurite growth of RGCs. It may be possible that there are not enough receptor molecules present at the surface due to merely basal expression levels of CXCR4 in untreated animals. One could assume that application of CXCL12 to retinal cell cultures at later time points after axotomy may result in stronger effects on neurite growth. Other explanations for the rather moderate effect could be a short half-life of recombinant CXCL12 in culture, the limited stability or the fact that CXCR4 gets internalised upon ligand binding and is removed from the cell surface.

We could furthermore demonstrate, that CXCL12 completely overcame myelin inhibition in cell culture, emphasizing its potent disinhibitory feature. In contrast to

CXCL12, CNTF is a more potent neurite growth promoting factor, but lacks the disinhibitory feature. Importantly, we could demonstrate that co-application of CXCL12 and CNTF leads to similar neurite growth on myelin as observed on laminin. Apparently, CXCL12 is able to desensitize RGCs toward the inhibitory substrate, while CNTF transforms RGCs into an active regenerative state (Muller et al., 2009). Thus, in combination with factors transforming RGCs into an robust growth state, CXCL12 may be useful as a potent adjuvant factor for optic nerve repair.

Further experiments show enhanced optic nerve regeneration *in vivo* after intravitreal injection of CXCL12. However, regeneration was not as pronounced as seen after an IS. This is presumably due to the lack of neuroprotective features and/or the only moderate growth stimulatory effects compared to other cytokines, such as CNTF, which is released by IS (Leibinger et al., 2012).

Nevertheless, the effects seen after IS still can be further enhanced, when the inhibitory environment is abrogated by local Taxol application or inhibition of the Nogo-receptor or RhoA-signaling (Fischer et al., 2004a; Fischer et al., 2004d; Chen et al., 2009; Sengottuvel and Fischer, 2011; Sengottuvel et al., 2011). A combinatorial approach that transforms RGCs into an active regenerative state and simultaneously desensitizes RGCs towards inhibitory environment is therefore advantagous. Thereby, we performed an IS in combination with an intravitreal CXCL12 application and found enhanced axon regeneration in the optic nerve compared to IS alone. The increased regeneration *in vivo* could be due to the disinhibitory effect of intravitreally applied CXCL12 towards myelin.

We can not exclude the possibility that some CXCL12 also diffused into the optic nerve after injections of high concentrations into the vitreous chamber. As demonstrated in this thesis, CXCL12 is a chemoattractant for neurites of mature RGCs *in vitro* and CXCR4 depletion reduces the number of naturally occuring axonal U-turns after ONC + IS. The artificial chemokine gradient present after the intravitral injection could therefore also direct CXCR4-positive regenerating axons towards the optic nerve head. In this case the beneficial effect of CXCL12-stimulated regeneration would be partially compromised. In rat RGCs, we quantified numbers of regenerated axons in thin sections (14 µm). By this, it was not possible to analyze axonal U-turns or branches. Therefore, a threedimensional analysis of rat optic nerves that have been

treated with CXCL12 could exhibit more axonal U-turns and aberrant growth compared to a control and thereby reflect higher quantified axon numbers in sections.

4.5 CXCL12 mediates its effects CXCR4/PI3K/AKT/mTOR-dependently

Several studies identified the PI3K/AKT/mTOR-pathway as one of the major signaling pathways regulated by CXCR4 activation (Teicher and Fricker, 2010; Chen et al., 2012). Additionally, it has been described that CXCL12 activates the JAK/STAT3-pathway (Gao et al., 2009). Both pathways are associated with RGC axon regeneration (Park et al., 2008; Smith et al., 2009; Leibinger et al., 2012; Leibinger et al., 2013a). However, the activation of JAK/STAT3-cascade upon CXCL12-binding to CXCR4 is still under debate. Moriguchi and colleagues suggested that this pathway is not regulated via CXCR4 signaling (Moriguchi et al., 2005). Consistently, we did not find JAK/STAT3 activation in mature RGCs after CXCL12 exposure, while CNTF application induced a strong activation. Likewise, the use of the JAK2-inhibitor AG490 did not impair CXCL12-mediated neurite growth.

Importantly, we found that CXCL12 mediates its effects via the PI3K/AKT/mTOR-pathway as indicated by mTOR maintenance in axotomized RGCs when exposed to CXCL12 (Park et al., 2008; Leibinger et al., 2012) and by the observation that pharmacological inhibitors of this pathway abrogated the positive neurite growth promoting effects of CXCL12.

With the use of the pharmacological CXCR4-antagonist AMD3100 and floxed CXCR4 mice, we demonstrated the involvement of CXCR4 in CXCL12-mediated signaling in mature rodent RGCs. Likewise, the CXCR4 antagonist AMD3100 completely abolished CXCL12 mediated neurite growth while significantly reducing mTOR activity. Previous studies indicated that both CXCR4 and CXCR7 are able to homo- and heterodimerize (Levoye et al., 2009; Luker et al., 2009). Whether CXCR7 is also involved in CXCL12-mediated signaling in mature RGCs still has to be addressed in subsequent studies.

4.6 CXCR4 depletion does not affect the regenerative state of RGCs

Several combinatorial treatments were described, like co-deletion of PTEN and SOCS3 or the deletion of PTEN alone combined with an IS or cAMP, promoting robust and massive axon regeneration in the optic nerve (Park et al., 2008; Smith et al., 2009; Park et al., 2010; Sun et al., 2011). Nevertheless, only few axons reach the optic chiasm, indicating that the transformation of RGCs into a robust growth mode is not decisive enough to restore visual function. Moreover, transduction of RGCs with AAV2-CNTF induced massive axonal sprouting in the retina presumably by U-turns originating from the optic nerve or from collaterals of retinal axon bundles, demonstrating that transforming RGCs into a robust regenerative state can lead to aberrant growth (Pernet et al., 2013b). We show in this study an impaired upregulation of the RAGs GAP43 and SPRR1a after ONC + IS after depletion of CXCR4 in RGCs. Assumedly, CXCR4 depletion should therefore impair optic nerve regeneration compared to respective controls. With another experiment, we could however reveal that CXCR4-depleted RGCs that were subjected to ONC + IS for 5 days, did show same spontanous neurite growth in cell culture like CXCR4-producing RGCs. Similarly, 7 days after ONC + IS we could observe that floxed CXCR4 mice, receiving either a AAV2-GFP or AAV2-Cre injection, exhibited the same distance of regenerated axons to the lesion site. This implicates, that the transformation of RGCs into an active regenerative state is important but not decisive for successful optic nerve regeneration.

4.7 CXCL12-CXCR4 axis is involved in aberrant growth after optic nerve injury

Recent studies indicated that axon regeneration into the lesioned optic nerve is characterized by the formation of U-turns, axonal branching and aberrant growth (Luo et al., 2013; Pernet et al., 2013b; Pernet et al., 2013a; Pernet and Schwab, 2014a) highlighting axon guidance as another profound and so far underestimated problem that limits optic nerve regeneration.

Pernet and colleagues showed that expression of constitutively active STAT3 in RGCs or of the CNTFRα super-agonist DH-CNTF in retinal Müller glial cells promotes massive axon regeneration in the injured optic nerve, that however comes along with immense sprouting in the retina, aberrant growth and the formation of U-turns distal to the lesion site (Pernet et al., 2013b; Pernet et al., 2013a). They hypothesize that inhibitory molecules like Nogo-A, MAG and OMgp (Schwab, 2010) or ephrinB3 (Benson et al., 2005; Duffy et al., 2012) and Sema5A (Goldberg et al., 2004) chemorepell RGC growth cones and are responsible for guidance mechanisms in the adult optic nerve after injury. They support this note by performing an intravitreal administration of the ROCK-inhibitor Y27632 resulting in reduced axonal U-turns and thereby improved axonal regeneration (Pernet et al., 2013a). Additionally and likewise, enhanced axon regeneration and reduced aberrant axonal branching by ablation of the inhibitory molecule EphrinA3-specific receptor EphA4 was described, while the appearance of U-turns was not affected (Joly et al., 2014).

During development, many ligand/receptor systems, including Ephrins/Ephs (Klein, 2004), netrin/Dcc/UNC5 (Barallobre et al., 2005), Slits/Robos (Brose and Tessier-Lavigne, 2000) and Semaphorins/Neuropilins/Plexins (Fujisawa, 2004) are reportedly involved in guiding axons to their appropriate targets in the brain. As delineated before, the CXCL12-CXCR4 axis also inhabits a pivotal role in axonal pathfinding during retinal development (Chalasani et al., 2003a; Li et al., 2005; Chalasani et al., 2007). This suggests the possibility of guidance molecules like CXCL12 to impair successful axonal regeneration into the injured optic nerve.

Here, we could demonstrate that generally about 24% of regenerating axons perform U-turns distal to the lesion site 7 days after ONC + IS. However, after CXCR4 depletion the percentage was reduced to 14%. Using immunohistochemistry we observed a diffuse CXCL12 staining predominantly at the optic nerve head. This staining colocalized with GFAP-positive astrocytes, likewise to previous studies that already described CXCL12-production by astrocytes (Hill et al., 2004; Miller et al., 2005; Knerlich-Lukoschus et al., 2010). This points to the possibility, that the chemotactic cytokine CXCL12 is produced and secreted by astrocytes at the optic nerve head. Slow diffusion of CXCL12 into the distal optic nerve would result in the presence of a constant chemokine gradient (Fig. 4.1). With *in vitro* experiments we showed a CXCR4-dependent chemoattractive feature of CXCL12 on the growth non-

permissive substrate myelin. We therefore postulate an attraction of CXCR4-positive axons by a CXCL12 gradient into the wrong direction after optic nerve injury (Fig. 4.1). Both axons of CXCR4-depleted RGCs and respective controls exhibited the same regeneration distance to the lesion site 7 days after optic nerve injury. We hypothesize that CXCR4-depletion will be in the long term beneficial for optic nerve regeneration and will result in longer regeneration distances than seen in respective controls. In comparison to respective controls, where aberrant growth and the formation of U-turns take place more frequently, CXCR4-depleted axons are “blinded” resulting in more axons growing towards the optic chiasm. Whether depletion of CXCR4 improves optic nerve regeneration in comparison to respective controls in the long term has to be addressed in follow-up studies.

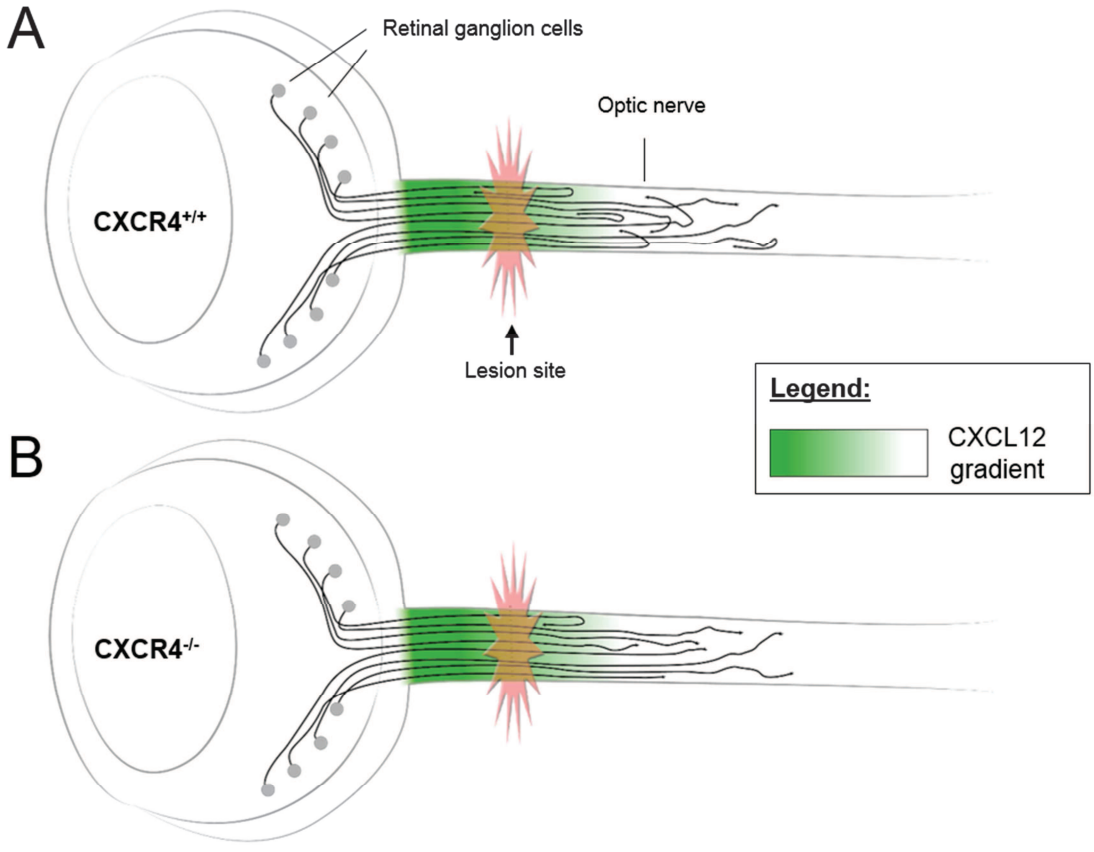

Figure 4.1: Schematic morphology of CXCR4 depleted and control RGC axons after ONC. (A) In control animals ($CXCR4^{+/+}$) many axons grow aberrantly and perform U-turns growing to the proximal part of the optic nerve after ONC. They are able to “sense” the CXCL12 gradient that is strongest in close proximity to the optic nerve head. (B) CXCR4 depleted RGC axons ($CXCR4^{-/-}$) are “blinded” to the chemokine gradient and perform less U-turns, growing straight to the optic chiasm after ONC.

4.8 Conclusion and further directions

The current study provides new therapeutic mechanisms to improve optic nerve regeneration. Potentially, these findings may also be transferred to other damaged areas of the CNS.

We could show that intravitreal application of CXCL12 moderately facilitated axon regeneration in the injured optic nerve, most likely by stimulating signaling pathways relevant for axon growth and exerting disinhibitory effects towards myelin. Additionally, intravitreal injection of CXCL12 in combination with IS significantly enhanced the regeneration in the optic nerve. Consequently, CXCL12 may be a useful adjuvant factor for the development of combinatorial strategies for optic nerve repair, particularly when combined with factors activating the intrinsic regenerative state of RGCs.

On the other hand, we showed that CXCL12 chemoattracted axons of cultured RGCs and DRG neurons and these effects were CXCR4 dependently. Depletion of CXCR4 in mature RGCs significantly reduced axonal U-turns compared to respective controls. Presumably, CXCL12 is produced and secreted by astrocytes at the optic nerve head, thereby guiding CXCR4-positive axons after optic nerve injury into the wrong direction (Fig. 4.1). As aberrant growth represents a major, so far underestimated obstacle for optic nerve regeneration, CXCR4 depletion might be beneficial for axon regeneration in the long term. This hypothesis still needs to be tested in the future. In that case knockdown of the receptor, for example by intravitreal application of CXCR4-specific siRNA or inhibition of the receptor by the CXCR4-antagonist AMD3100 or other commercially available antagonists at the lesion site could provide beneficial effects for optic nerve regeneration. Both approaches would decrease the sensitivity of growth cones toward the chemoattractive CXCL12 gradient and provide novel therapeutic strategies to improve CNS repair.

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6 Abbreviations

AAV2	Adeno-associated virus of serotype 2
Bax	Bcl-2-associated X protein
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell lymphoma-extra large
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CSPG	Chondroitin sulfate proteoglycan
CTB	Cholera toxin subunit B
CXCL12	C-X-C chemokine ligand 12
CXCR4	C-X-C chemokine receptor type 4
DAG	Diacylclycerol
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco’s modified eagle medium
DRG	Dorsal root ganglion
ERK	Extracellular-signal regulated kinases
FGF-2	Fibroblast growth factor 2
FL	Fiber layer
GAP43	Growth associated protein 43
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCL	Ganglion cell layer
GDNF	Glial cell line-derived neurotrophic factor

GDP	Guanosindiphosphat
GEF	Guanine nucleotide exchange factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage colon-stimulating factor
gp130	Glycoprotein 130
GPCR	G protein coupled receptor
GPI	Glycosylphosphatidylinositol
GRK	GPCR kinase
GTP	Guanosintriphosphat
HEK293	Human embryonic kidney
HGF	Hepatocyte growth factor
IL-6	Interleukin-6
INL	Inner nuclear layer
IP3	Inositol 1, 4, 5 triphosphate
IPL	Inner plexiform layer
IS	Inflammatory stimulation
JAK	Janus kinase
LGN	Lateral geniculate nucleus
LIF	Leukemia inhibitory factor
LINGO-1	Leucine rich repeat and Immunoglobin-like domain-containing protein 1
LRR	Leucine rich repeat
MAG	Myelin-associated glycoprotein
MAPK	Mitogen-activated protein kinases
MHC	Major Histocompatibility Complex
MIF	Macrophage migration inhibitory factor

mTOR	Mammalian target of rapamycin
NGF	Nerve growth factor
NgR	Nogo-receptor
NT3, 4, 5	Neurotrophin 3, 4, 5
OMgp	Oligodendrocyte-myelin glycoprotein
ONC	Optic nerve crush
ONL	Outer nuclear layer
OPC	Oligodendrocyte precursor cell
OPL	Outer plexiform layer
Pam3Cys	(S)-[2,3-Bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys4-OH
PBS	Phosphate buffered saline
PBSF	Pre-B cell growth stimulating factor
PCR	Polymerase chain reaction
PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP2	Phosphatidylinositol 4, 5-biphosphate
PirB	Paired immunoglobulin-like receptor B
PLC	Phospholipase C
PNS	Peripheral nervous system
RAG	Regeneration associated gene
RGC	Retinal ganglion cell
RhoA	Ras homolog gene family, member A
ROCK	Rho-associated protein kinase
RPE	Retinal pigment epithelium
SC	Superior colliculus

SEM	Standard error of the mean
SPRR1a	Small proline-rich protein 1A
STAT3	Signal transducer and activator of transcription 3
TNF	Tumor necrosis factor
Trk	Tyrosine receptor kinase
TSA	Tyramide signal amplification

Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Diese Dissertation hat in gleicher oder ähnlicher Form noch keiner anderen Prüfungsbehörde vorgelegen. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 18. Dezember 2015