

# CXCR4 signaling in central nervous system regeneration: friend or foe?

Daniel Terheyden-Keighley, Alexander M. Hilla, Dietmar Fischer\*

The knockout of the chemokine C-X-C motif chemokine receptor 4 (CXCR4) in growth-stimulated retinal ganglion cells (RGCs) has a multiplicative effect on optic nerve regeneration. C-X-C motif chemokine ligand 12 (CXCL12), the exclusive ligand of CXCR4, is expressed and axonally transported by an RGC subpopulation, releasing the chemokine at the lesion site. CXCL12 attracts injured axons of a CXCR4-positive RGC subpopulation, mostly  $\alpha$ RGC, thereby preventing extension into the distal nerve. Knockout of either CXCR4 or CXCL12 in RGCs overcomes the axonal entrapment at the lesion site and enables long-distance regeneration. Thus, CXCL12/CXCR4-dependent attraction of axons contributes to the failure of optic nerve regeneration. Here we briefly cover CXCR4-based neural motility, current mechanistic background, and future perspectives in central nervous system (CNS) regeneration.

**Regeneration insufficiency in the CNS:** Axons in the CNS usually fail to regrow after injury, leading to the permanent sensory, motor, or cognitive deficits in affected patients. Efforts to remedy regenerative failure can generally be put into two main categories: strategies for overcoming the inhibitory environment of the CNS or activating the neuron-intrinsic regenerative state. Over the past decades, the mouse optic nerve has become one of the more accessible CNS models in the mammalian CNS regeneration field. Many strategies developed in it also show promise in more complex areas such as the spinal cord (Leibinger et al., 2021). RGCs are the output layer of the retina and project their axons along the optic nerve towards the brain. A complete optic nerve crush severs all axons at the lesion site, with practically no regeneration occurring in untreated animals. The regenerative state induced by inflammatory stimulation (lens injury) via cytokine signaling facilitates a moderate amount of axonal extension past the lesion site. However, much of the axonal regrowth is aberrant, with around a quarter of them forming U-turns and heading back towards the lesion site (Hilla et al., 2021).

As for strategies that deal with the CNS's inhibitory environment, the seven-transmembrane G-protein-coupled

receptor CXCR4 and its exclusive ligand, the chemokine CXCL12, have been shown to confer disinhibition towards CNS myelin (Heskamp et al., 2013). Next to its described disinhibitory role, CXCR4/CXCL12 signaling has been the focus of intense research due to its role in embryogenesis, immune cell migration, HIV, and cancer metastasis (Pozzobon et al., 2016). CXCL12 also has essential functions during CNS development, including directing neural precursor cell migration during cerebellum formation (Reiss et al., 2005). Besides cellular migration, RGCs are dependent upon CXCL12 signaling for axon guidance, growing towards the higher CXCL12 concentration in the optic stalk of the embryo (Li et al., 2005). Previously, we demonstrated that CXCR4 is also expressed in adult RGCs (Heskamp et al., 2013), encouraging us to examine the effect of its knockout on optic nerve regeneration (Hilla et al., 2021).

**CXCR4/CXCL12 signaling affects CNS axon regeneration:** To this end, we specifically knocked out CXCR4 in RGCs and found a significant increase in the number and length of regenerating axons in the crushed optic nerve. Mice with floxed CXCR4 received intravitreal injections of AAV2-Cre, resulting in the homozygous knockout of CXCR4 in around 85% of their RGCs. These mice were subjected to optic nerve crush (ONC) 3 weeks later with or without additional inflammatory stimulation to induce a regenerative stimulus for the RGCs. After a further 3 weeks, the optic nerves were examined for anatomical regeneration. Surprisingly, the knockout resulted in slight improvement of regeneration (< 1.5 mm), but even more peculiar, the combination with lens injury resulted in a tenfold increase in the number of axons regenerating over 1.5 mm compared to lens injury alone. Many of them reached over 3 mm in length. Moreover, the regenerative effect of RGC-specific CXCR4-knockout combined with inflammatory stimulation could be replicated when using an shRNA against CXCR4 to knock down its expression in non-transgenic animals. When looking 7 days after ONC and inflammatory stimulation in knockout animals, the axons grew primarily straight with some aberrant growth. Still, in the control animals, almost twice as many axons

could be seen to undergo U-turns back towards the lesion site (Hilla et al., 2021).

Investigating the origin of these regenerating fibers revealed an almost complete overlap between osteopontin and a robust CXCR4 immunoreactivity in RGCs, indicating that the regenerating neurons mainly belong to the  $\alpha$ RGC subpopulation (Hilla et al., 2021). This group comprises around 30% of surviving RGCs after optic nerve injury and is known to have a higher-than-average intrinsic regenerative capacity and be resistant to axotomy-induced apoptosis (Duan et al., 2015). To see if the improved regeneration could be attributed to increased survival or induction of a regenerative state, the RGCs were analyzed in the retina. In both environments, survival was not affected, and neither was any increase in spontaneous neurite outgrowth observed after CXCR4 knockout, even in combination with neurotrophic cytokine stimulation. The only noticeable effect of CXCR4 knockout was that RGCs could no longer be disinhibited by CXCL12 when plated upon myelin, as expected (Heskamp et al., 2013). With the only visible result of CXCL12 signaling being a disinhibitory one, it was puzzling why its knockout would aid regeneration within the inhibitory CNS.

**Axon attraction and entrapment by disinhibitory CXCL12:** A disinhibitory molecule secreted from a point source results in a gradient, giving it directionality. This directional disinhibition can then act in a pseudo-chemoattractive way in an inhibitory environment such as the CNS. Put another way, when all directions are inhibitory, a disinhibitory signal provides a path of least resistance, channeling the axon in that direction. This effect was demonstrated *in vitro* using culture inserts containing CXCL12-secreting HEK293 cells surrounded by RGCs. When the RGCs were grown on laminin, no measurable growth bias could be seen. However, a significantly higher percentage of neurites oriented themselves towards the central HEK293 culture insert when grown on CNS myelin (Hilla et al., 2021). Thus, directional disinhibition is a possible explanation for the phenomenon seen *in vivo*, where CXCL12 secretion at the lesion site causes growth cone retention.

The next question was which cells release sufficient amounts of CXCL12 to entrap axons at the injury site. CXCL12 mRNA levels didn't change before or after injury in the optic nerve. This left the axons themselves as the next most likely source of CXCL12. Due to its rapid secretion and

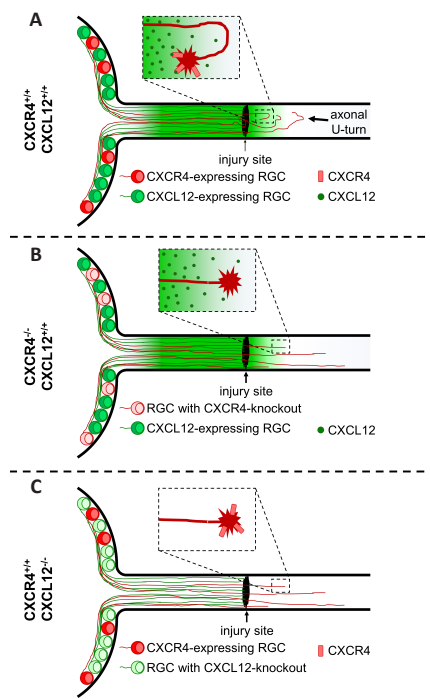
internalization, the chemokine is challenging to detect immunohistologically. However, a leap forward in CXCL12 protein detection came when a protein-secretion inhibitor was applied intravitreally, causing a clearly detectable immunohistological signal in both the cell bodies and axons of approximately 8% of the RGCs. Interestingly, the CXCL12 signal did not colocalize with either the CXCR4- or osteopontin-positive  $\alpha$ RGCs (Hilla et al., 2021).

To confirm the CXCL12 secretion from axons' tips, *in vitro* experiments were done with virally transduced sensory neurons to overexpress CXCL12, which were then seeded into special chambers that physically separated the cell bodies from their axonal tips. Using ELISA, it was possible to detect secreted CXCL12, but not when applying the protein-secretion inhibitor, proving that axons can release CXCL12. Moreover, an HA-tagged version of CXCL12 was virally expressed in RGCs. Interestingly, after ONC, the HA signal accumulated in fibers at the lesion site and diffusely around the growth cones (Hilla et al., 2021), providing more evidence of its secretion into the injury site (Figure 1).

Finally, to confirm that neuronal CXCR4/CXCL12 signaling causes the observed differences in regeneration, the experiments were repeated by inducing a specific CXCL12 knockout in RGCs. As expected, the resulting increase in regeneration was similar to CXCR4 depletion. These results also indicated that RGCs are the relevant signal sources and receivers due to the knockout of each protein being dependent upon viral transduction in the retina and not cells at the lesion site (Hilla et al., 2021).

#### Axonal attraction on a molecular level:

The processes involved in axon pathfinding and cell migration are similar. These include mechanisms for gradient detection, cell/growth cone polarization, and output via Rho GTPase cytoskeletal regulation (von Philipsborn and Bastmeyer, 2007). Essentially, a growth cone is a tiny, polarized cytoskeletal structure with similar movement mechanisms to migrating cells. This is a boon for neuroscience, as the extensive research undertaken in the field of cancer metastasis has indirectly delineated many of the molecular mechanisms governing growth cone motility. In the context of CXCR4/CXCL12 signaling, familiar situations are described for cell migration during neural development. For example, during cerebellar development, the external granular layer, which resides directly below the meninges,



**Figure 1 | Schematic drawing visualizing the improved axonal regeneration caused by CXCR4- or CXCL12-knockout.** (A) In wildtype mice, different subpopulations of RGCs express either the chemokine CXCL12 (all greenish objects) or its cognate receptor CXCR4 (all reddish objects). CXCL12-expressing RGCs secrete the chemokine at the injury site resulting in a CXCL12 gradient in the proximal part of the optic nerve. Regenerating axons of RGCs, which express the cognate receptor CXCR4 on their growth cones (magnification in dashed box), are entrapped within the lesion site or deflected back towards it, resulting in axonal U-turns. (B) Upon CXCR4-knockout (CXCR4<sup>-/-</sup>, pale red RGCs), regenerating axons do not express CXCR4 on their growth cones (magnification in dashed box), making them insensitive towards CXCL12. (C) Upon CXCL12-knockout (CXCL12<sup>-/-</sup>, pale green RGCs) RGCs do not secrete CXCL12 at the lesion site, abolishing the chemokine gradient. CXCR4-positive axon tips (magnification in dashed box) can regenerate beyond the injury site.

acts as a germinal zone for cerebellar granular neurons (Reiss et al., 2005). CXCL12 signaling from the meninges anchors the cells in place, allowing them to be exposed to mitogens such as sonic hedgehog before migrating inwards to populate the inner granule cell layer. This signaling takes the form of a step gradient, where heparin sulfate proteoglycans in the extracellular matrix concentrate the CXCL12 by fixing it in place (Reiss et al., 2005). Contrasting these cellular retention mechanisms to that of growth cones observed in the crushed optic nerve reveals profound similarities, providing evidence towards an axon-retention hypothesis (Hilla et al., 2021). Indeed, the glial scar that forms at the optic nerve injury site contains glycosaminoglycans known to protect CXCL12 from degradation,

likely causing prolonged exposure of CXCR4-positive growth cones towards the chemokine.

How CXCL12-mediated chemoattraction of axonal growth cones is realized on a molecular level remains to be answered. A range of different signaling pathways acts downstream of CXCL12/CXCR4, including RhoA/ROCK. By regulating the actin cytoskeleton, this pathway is reportedly involved in growth cone motility. In addition, overcoming RhoA/ROCK signaling renders outgrowing neurites insensitive towards inhibitory CNS substrates (Heskamp et al., 2013). Therefore, RhoA/ROCK is a potential pathway that confers chemoattraction by disinhibition on an inhibitory substrate. Another plausible mechanism involved in directional growth is PI3K/Akt/mTOR signaling that reportedly affects microtubule organization and plays a substantial role in RGC axon regeneration. Particularly the  $\alpha$ RGC subtype, characterized by a high mTOR activity, is described as the main RGC population that regenerates injured axons upon PI3K/Akt activation by PTEN depletion (Duan et al., 2015). In addition, pharmacologic inhibition of PI3K/Akt signaling blocked the growth-promoting effect of CXCL12 in RGC cultures on growth-permissive and -inhibitory substrate (Heskamp et al., 2013), rendering this pathway as a potential mediator of the CXCL12/CXCR4 effects. Future studies will unravel the molecular mechanisms downstream of CXCL12/CXCR4 and the extent to which the signaling pathways mentioned above are involved in CXCL12-mediated chemoattraction.

**Future directions:** Regarding future perspectives of CXCR4/CXCL12 signaling in CNS regeneration, a range of concepts from materials bioengineering to combinatorial clinical strategies are feasible. The first questions are transferability and which other neural populations have their regeneration impeded by CXCR4-mediated axon retention. In the peripheral nervous system where CXCL12 is expressed by dedifferentiated Schwann cells forming structures called bands of Büngner (Negro et al., 2017; Zanetti et al., 2019), it promotes axonal growth by guiding axons back to their initial targets. Hence, when CXCL12 is expressed along the nerve, it aids regeneration. However, when secreted only at or proximal to the lesion site as in the optic nerve, chemoattractive CXCL12 entraps axons and prevents extension into the distal nerve. It is unknown whether CXCR4/CXCL12-mediated attraction

also contributes to regenerative failure in other CNS areas such as the spinal cord. In fact, delivery of CXCL12 by mini pumps into the lesion site of the spinal cord has been shown to improve axonal spouting, suggesting that axons in the spinal cord can respond to the chemokine (Opatz et al., 2009). Whether endogenous CXCL12 is released by axons or other cells close to the lesion site in the spinal cord needs to be investigated in the future. Whatever the outcome, a viral-induced knockout of CXCR4 in respective neurons in the cortex or brain stem could be easily performed (Leibinger et al., 2021). Another approach could be the use of AMD3100, a specific CXCR4 inhibitor. An application of this drug into the lesion site in the CNS in combination with intrinsic regeneration-activating compounds such as hyper-interleukin-6 could increase the number of axons regenerating over longer distances and reduce aberrant growth (Leibinger et al., 2021). On the other hand, CXCL12's directed disinhibition in the CNS could be exploited for guiding regenerating axons to a specific location. The addition of CXCL12 could enhance artificial axon conduits filled with extracellular matrix proteins. Through ligand clearance, advancing growth cones would remove the CXCL12, thus creating a gradient and giving directionality to the conduit, preventing U-turns. Such bioengineered tubes could be used for bridging gaps in damaged spinal cord tracts. Alternatively, targeted expression of CXCR7, a non-signal-transducing receptor of CXCL12, could be used to shape the gradient of CXCL12 by removing it from the extracellular space. It is currently unknown what distances the directionality of secreted CXCL12 can accurately be determined by receptive cells. With CXCR4's role in neural migration/axon guidance mainly seen on tiny embryonic scales, experiments are needed to determine whether CXCL12-based directional disinhibition can attract CXCR4<sup>+</sup> axons across clinically relevant distances, such as after spinal cord injury. Currently, despite a multitude of strategies being able to stimulate RGCs to project long distances along the optic nerve, without correct retinotopic guidance and synapsing, shape-discerning vision is unlikely to be restored. That said, any future strategies

that solve the retinotopic guidance problem will likely benefit from incorporating some form of signal regulation for CXCR4 or its downstream effectors to prevent axon retention at the lesion, thus supporting strategies aiming at restoring vision.

Taken together, CXCL12/CXCR4 signaling and its axon entrapment effects provide an additional mechanism hindering axon regeneration and provide the possibility for the development of new therapeutic strategies to overcome regenerative failure.

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**Daniel Terheyden-Keighley, Alexander M. Hilla, Dietmar Fischer\***

Department of Cell Physiology, Ruhr University of Bochum, Universitätsstraße 150, 44780 Bochum, Germany

\*Correspondence to: Dietmar Fischer, PhD, dietmar.fischer@rub.de.

<https://orcid.org/0000-0002-1816-3014> (Dietmar Fischer)

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