Material and Methods

Gene constructs

For CXCR4 knockdown in wild-type animals, we designed and cloned an shRNA using the following synthesized oligonucleotide sequences: forward: 5'-GATCC-GCCTGCTGGCTGCCATATTAT-CTCGAG-ATAATATGGCAGCCAGCAGGC-TTTTT-A-3', reverse: 5'-AGCTT-AAAAA-GCCTGCTGGCTGCCATATTAT-CTCGAG-ATAATATGGCAGCCAGCAGGC-G-3'. The annealed oligonucleotides were then inserted into pAAV-U6-EGFP (Vector Biolabs, USA) using enzymatic digestion with BamHI and HindIII, and subsequent ligation. The resulting plasmid was then used to generate the AAV2-U6-CXCR4-shRNA (AAV-CXCR4[shRNA]).

In order to confirm the transport and release of CXCL12 in RGC axons, murine CXCL12 was amplified by PCR from mouse retina cDNA using the following primers: forward: 5'-ATAGTCGAATTCactttcactctcggtcca-3', reverse: 5'-aggactttccagtagacccCTCGAGTTACGT-3'. Capital letters indicate the added EcoRI and XhoI restriction sites, including overhangs. The amplified PCR product was inserted into pcDNA3.1 using the TOPO TA kit (ThermoFisher, USA) before cloning into pAAV-IRES-hrGFP (Agilent Technologies, USA) using enzymatic digestion with EcoRI and XhoI, and subsequent ligation. To distinguish exogenous CXCL12 from endogenous an HA-tag was added at the C-terminus of CXCL12 in the pAAV-CXCL12-IRES-hrGFP plasmid using a site-directed mutagenesis approach with the following primers: forward: 5'-ATGTTCCAGATTACGCTtaagcacaacagccc-3', reverse: 5'-GGAACATCGTATGGGTActtgtttaaagctttctcc-3'. Capital letters indicate the sequence of the HA-tag. From this, an AAV2 coding for HA-tagged CXCL12 was produced (AAV-CXCL12-HA). To overexpress CXCL12 in RGCs *in vitro*, CXCL12 was cloned from pAAV-CXCL12-IRES-hrGFP into a modified pENTR2B vector containing an IRES-eGFP sequence [1] by restriction digestion with EcoRI and XhoI, and subsequent ligation. From there on, a CXCL12-expressing baculovirus was generated as described elsewhere [1].

Murine CXCR4 was amplified from mouse retina cDNA using the following primers: forward: 5'-GACGAATTCgttgccatggaaccgatc-3', reverse: 5'-cttgcataagtgttagctggagtg-3'. Capital letters

indicate the added EcoRI restriction site, including the overhang. The amplified PCR product was inserted into pcDNA3.1 using the TOPO TA kit (ThermoFisher, USA) before cloning into pAAV-IRES-hrGFP (Agilent Technologies, USA) using enzymatic digestion with EcoRI and XhoI, and subsequent ligation.

Induction of cell specific knockouts

Specific knockouts of CXCR4 or CXCL12 in RGCs were induced by intravitreal injections of 2 µl of AAV2-Cre-HA (AAV-Cre) of respectively floxed mice. At the same time, controls received the same volume of AAV2-GFP (AAV-GFP) instead, enabling about 80-90% of transduction rates. For CXCR4 knockdown in wild-type mice, 2 µL of an AAV2-CXCR4-shRNA was intravitreally injected, enabling transduction rates of about 75%. All AAV were injected at least 3 weeks before optic nerve injury or tissue isolation to allow for maximum depletion of the respective gene.

To induce genetic knockout of CXCL12 specifically in astrocytes, GFAP-CreER CXCL12-floxed mice received daily intraperitoneal injections of 0.2 mg tamoxifen (Sigma, St Louis, USA) per gram bodyweight for 5 consecutive days [2]. Transduction efficiencies of up to 91% were shown in this mouse line [2,3].

To investigate the transport and release of CXCL12 in RGC axons, we made an AAV2 coding for HA-tagged CXCL12 (AAV-CXCL12-HA) and intravitreally injected it 2 weeks before ONC. This virus was diluted in PBS before injection to reduce the RGC transduction rate to about 30%.

Preparation of dissociated retinal cell cultures

Retinae were rapidly dissected and incubated in digestion solution containing papain (10 U/ml; Worthington) and L-cysteine (0.2 mg/ml, Sigma-Aldrich) in Dulbecco's modified Eagle medium (DMEM, Thermo-Fisher) at 37 °C for 30 min. After rinsing twice with DMEM, retinae were triturated in 1.5 ml DMEM. To remove other factors released from retinal cells of the *in vitro* condition, we immediately washed the retinal cell suspension with 50 ml DMEM and centrifuged it for 7 min at 500 g. The supernatant was discarded, and the pellet resuspended

in DMEM containing B27-supplement (1:50, Thermo-Fisher) and penicillin/streptomycin (200 U/ml, Biochrom). Afterward, dissociated cells were passed through a cell strainer (40 µm, BD Falcon), and the following reagents have been added to test their effect on neurite outgrowth or survival: recombinant CNTF (200 ng/ml, PeproTech), recombinant, human CXCL12 (500 ng/ml, PeproTech).

To test the effect of CXCR4-knockout on spontaneous neurite outgrowth, AAV2-Cre-HA treated animals and controls received ONC/IS 5 days before tissue isolation (*in vivo* primed). The retinae were dissociated according to the *in vitro* condition described above until after the rinsing step. Retinae were triturated in DMEM containing B27-supplement (1:50, Thermo-Fisher) and penicillin/streptomycin (200 U/ml, Biochrom) and passed through a cell strainer (40 µm, BD Falcon). Four well plates (Nunc) were coated with poly-D-lysine (PDL, 0.1 mg/ml, molecular weight between 70,000 and 150,000 Da, Sigma) alone (*in vivo* primed) or additionally with laminin (20 µg/ml, Sigma) and an inhibitory myelin extract (*in vitro* condition). Afterward, a cell suspension of 300 µl was added and incubated for 24 h (*in vivo* primed) or 96 h (*in vitro* condition) and fixed in 4% PFA/PBS. RGCs with regenerated neurites two times the cell body's diameter were photographed with a fluorescent microscope (200x magnification, Axio Observer.D1, Zeiss), and neurite length was determined using ImageJ software. Also, the total number of βIII-tubulin-positive RGCs with an intact nucleus (DAPI stain) per well was quantified to reveal potential neurotoxic effects. The average neurite length per RGC was calculated by dividing the sum of neurite length by the total number of RGCs per well. Due to inter-experimental variations, the neurite length per RGC was normalized to respective CXCR4$^{+/+}$ control (s. also figure legends). Data represent means ± SEM of 3-6 independent experiments, each with at least three replicate wells per treatment.

Immunohistochemical CXCR4-staining (1:400, Abcam, RRID: AB_10975635) of RGC neurites was performed on RGCs that were dissociated according to the *in vitro* condition.

In the survival assay, RGCs were processed as described above for the *in vitro* condition and seeded into poly-D-lysine (PDL, 0.1 mg/ml, molecular weight between 70,000 and 150,000 Da, Sigma) coated wells of a 96 well plate with either recombinant CNTF (200 ng/ml, PeproTech),

recombinant, human CXCL12 (500 ng/ml, PeproTech) or vehicle. To assess the number of initially dissociated RGCs, some vehicle-treated cells were fixed after two hours in culture. The remaining dissociated RGCs have been kept in culture for seven days before fixation with 4% PFA/PBS. Numbers of βIII-tubulin RGCs per well have been counted for each condition. Data represent means ± SEM of 3 independent experiments, each with at least three replicate wells per treatment.

To analyze CXCL12 localization, RGCs were dissociated according to the *in vitro* paradigm described above. For increasing CXCL12-expression in RGCs, cultures were treated with a CXCL12-expressing baculovirus or a respective GFP-expressing control-baculovirus, as described previously [1]. In brief, dissociated cultures were allowed to attach to the well for at least 5 h before adding 10 µL of appropriate baculovirus. Sixteen hours after that, the medium was exchanged, and the cultures fixed 3 days after that. Immunohistochemical staining against CXCL12 (1:250, R&D Systems, AB_2088149), GFP (1:1000; Novus; RRID: AB_10128178), and βIII-tubulin (1:2000; BioLegend, RRID: AB_2313773) were performed as described above to visualize the expression of the chemokine in RGCs. Stained axons were photographed using a confocal microscope (630 x, SP8, Leica).

Preparation of retinal whole-mounts

Flat-mounted retinae were prepared without prior perfusion, fixed in 4% PFA/PBS for 30 min at room temperature, and then incubated in 2% TritonX-100/PBS for one hour to improve antibody penetration. RGCs in flat-mounted retinae and eye sections were visualized using monoclonal antibodies against βIII-tubulin (1:1000; BioLegend, RRID: AB_2313773, RRID: AB_663339) and axons in optic nerve cross-sections by neurofilament (1:1000; Abcam, RRID: AB_1566479). Visualization of CXCR4 and CXCL12 was performed with monoclonal antibodies (1:1000, Abcam, RRID: AB_10975635; 1:250, R&D Systems, AB_2088149). A polyclonal antibody (1:1000, Sigma-Aldrich, RRID: AB_260070) against the HA-tag was used to label RGCs that had been successfully transduced before by AAV2-Cre-HA. Secondary antibodies included donkey-anti-mouse, anti-goat, and anti-rabbit IgG antibodies conjugated

to Alexa Fluor 350, 488, or 594, respectively (all 1:1000, Molecular Probes) or DyLight 405 (all 1:500, Jackson ImmunoResearch).

Quantification of surviving RGCs in wholemounts

Immunostained, flat-mounted retinae were divided into four quadrants, and 4-5 discontinuous pictures were taken in each quadrant using a fluorescent microscope (400x, Axio Observer.D1, Zeiss), proceeding from the retinal center to the periphery. The number of βIII-tubulin-positive RGCs was determined and extrapolated to 1 mm². Values were averaged per retina and across all similarly treated animals to obtain group means ± SEM.

Tissue clearing

Optic nerves were dehydrated in ascending tetrahydrofuran concentrations, incubated in dichloromethane, and eventually transferred to a clearing solution (benzyl alcohol: benzyl benzoate; 1:2).

Western blot analysis

Tissues were homogenized in lysis buffer (10 mM Tris HCl, pH 7.5, 5 mM KCl, 125 mM sucrose, 5 mM NaF, 0.5 mM DTT, 0.05 mM Na3VO4, 0.5% Triton X-100, 5% SDS) with protease inhibitors (Calbiochem) and phosphatase inhibitors (Roche) using five sonication pulses at 40% power (Bandelin Sonoplus). Proteins were isolated by centrifugation in an Eppendorf tabletop centrifuge at 4,150 × g for 10 min at 4°C, then separated by SDS/PAGE using Mini TGX gels (10%, Bio-Rad) according to standard protocols and finally transferred to nitrocellulose membranes (0.2 μm; Bio-Rad). Immediately after that, blots were incubated in a denaturing solution (2% SDS, 100 mM β-Mercaptoethanol in TGS) at 50 °C for 30 min. Blots were blocked in 5% dried milk in phosphate-buffered saline solution with 0.05% Tween-20 (PBS-T; Sigma) and incubated with antibodies against CXCR4 (1:1000, Abcam, RRID: AB_10975635) and βIII-tubulin (1:2000; BioLegend, RRID: AB_2313773), or actin (1:8000, Sigma, RRID: AB_476744). All antibodies were diluted in PBS-T containing 5% BSA (Sigma).

Anti-rabbit IgG conjugated to HRP (1:80,000; Sigma) and anti-mouse conjugated to Alexa Fluor Plus 555 (1:1000, ThermoFisher) were used as secondary antibodies. Antigen-antibody complexes were visualized using an enhanced chemiluminescence substrate (Bio-Rad) on a FluorChem E detection system (ProteinSimple). CXCR4 band intensities were quantified relative to respective loading controls using ImageJ. Experimental groups included three retinae.

Quantification of regenerating axons in the optic nerve

Pictures of at least five longitudinal optic nerve sections per animal were taken under 200x magnification using a fluorescent microscope (Axio Observer.D1, Zeiss) or a confocal laser scanning microscope (LSM510; Zeiss; or SP8; Leica). Numbers of CTB-labeled axons extending 0.5, 1, 1.5, 2, 2.5, and 3 mm from the injury site were quantified and normalized to the width of the optic nerve at the respective measuring point. Experimental groups included 5 - 9 mice.

To quantify the amount of CXCR4-positive axons in naïve optic nerves, cross-sections were immunohistochemically stained against CXCR4 (1:1000, Abcam, RRID: AB_10975635) and neurofilament (1:1000; Abcam, RRID: AB_1566479) and acquired using a confocal laser scanning microscope (SP8; Leica). Three randomly selected areas of 3 sections were quantified for neurofilament total axon count and CXCR4-positive axons from 5 optic nerves.

References

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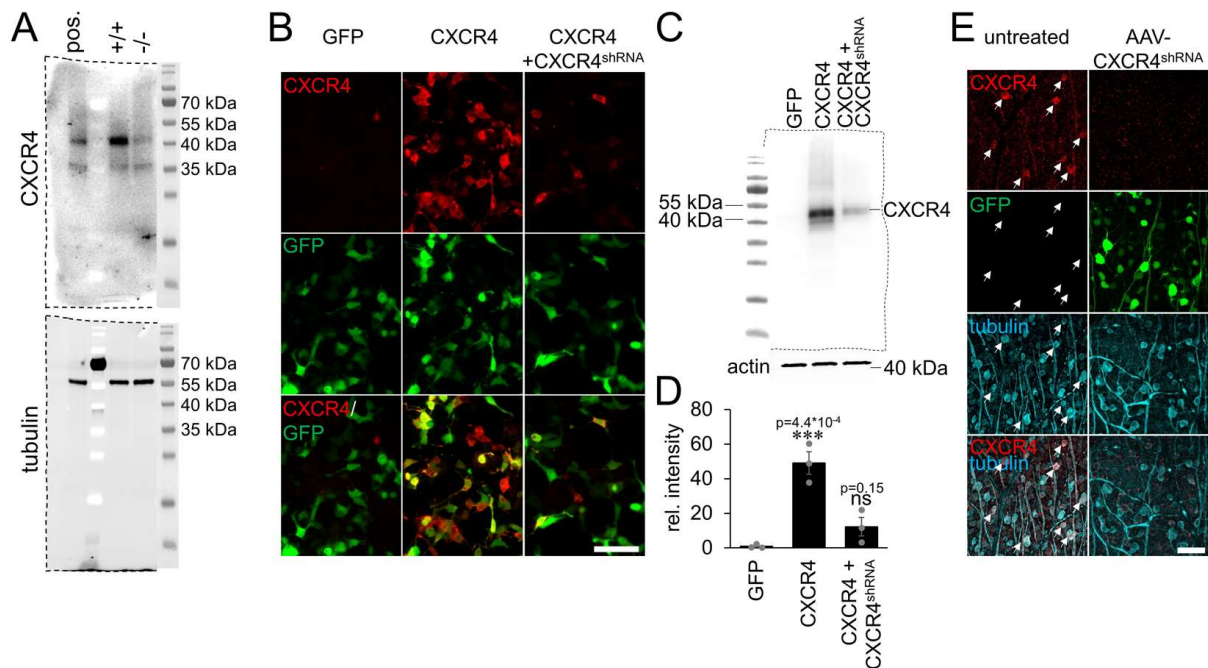
Supp. Fig. 1: CXCR4-knockout and -knockdown A: Complete Western blots displays specific CXCR4- and tubulin-positive bands for groups, as shown in Fig. 1 F. **B:** Immunohistochemical staining of HEK293 cells transfected with plasmids coding either for GFP-, CXCR4/GFP, or a combination of CXCR4/GFP together with a plasmid carrying an shRNA-construct to suppress CXCR4 expression. CXCR4/GFP-transfected cells show a co-localization of CXCR4 (red) and GFP (green), whereas co-transfection with the CXCR4shRNA-plasmid reduces CXCR4 signal. Scale bar: 100 µm. **C:** Western blot of HEK293 cell lysates transfected with plasmids as described in B. The specific CXCR4-band is diminished by co-expression with the CXCR4shRNA-plasmid. Actin served as a loading control. **D:** Densitometric quantification of CXCR4 signals relative to actin and normalized to GFP on Western blots as depicted in C. Bars represent means ± SEM; dots represent single values for n=3 independent experiments. Treatment effects: ***p<0.001, ns: non-significant by one-way ANOVA with Holm-Sidak *post hoc* test. **E:** Confocal images with CXCR4-positive (red) RGCs (tubulin, cyan, white arrows). CXCR4-shRNA AAV transduced RGCs, indicated by GFP (green) expression, show no CXCR4 signal in axotomized retina. Scale bar: 50 µm. Refers to Fig. 2 C, D.
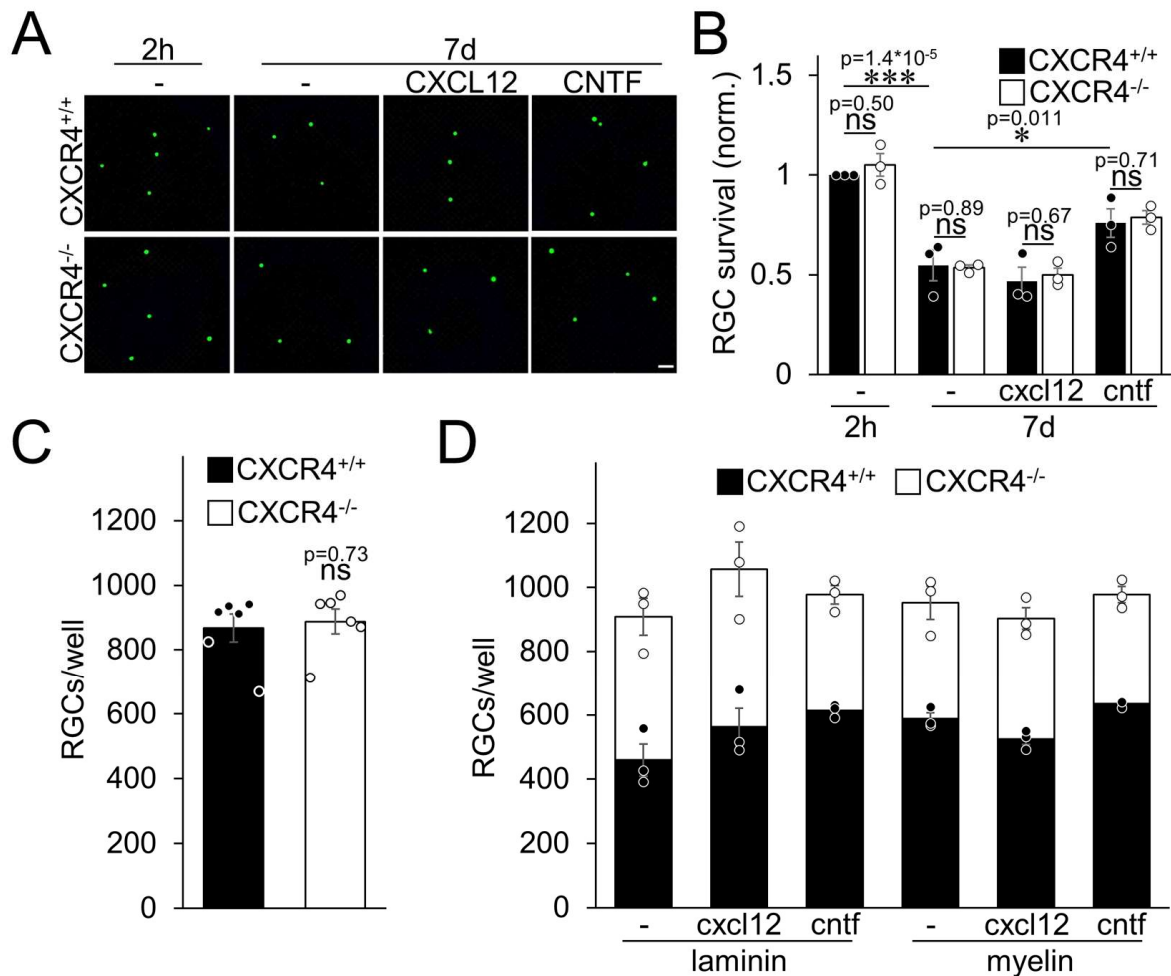
Supp. Fig. 2: Survival of RGCs *in vitro* and cell numbers of *in vitro* experiments.
A: Representative images of dissociated RGCs with a CXCR4-knockout (CXCR4$^{-/-}$) and controls (CXCR4$^{+/+}$) cultivated for 2 h to assess initial RGC numbers and 7 d, a time point at which half of the original RGCs had already degenerated. The presence of CXCL12 in the culture medium did not affect RGC numbers, whereas CNTF treatment elevated RGC survival. Scale bar: 25 µm. **B:** Quantification of surviving RGCs from cultures, as described in A. CXCR4$^{-/-}$ does not show significantly different RGC numbers than controls (CXCR4$^{+/+}$) after either treatment. RGC survival has been normalized to RGC numbers 2 h after dissociation of CXCR4$^{+/+}$ RGCs with an average of 175 RGCs/well. Bars represent means $\pm$ SEM for n=3 experiments, each with at least 3 wells analyzed per condition; dots represent single values. Treatment effect: ns: non-significant by two-way ANOVA with Holm-Sidak *post hoc* test. **C:** RGC numbers per well from cultured RGCs after *in vivo* pre-treatment showed no significant difference between both groups. Bars represent means $\pm$ SEM for n=6 experiments, each with at least 3 wells analyzed; dots represent single values. Treatment effects: ns: non-significant by Student's t-test. Refers to Fig. 3 C, D. **D:** Numbers of cultured RGCs per well are presented as stacked bar graphs showing no significant differences in total RGC numbers between groups. Bars represent means $\pm$ SEM for n=3 independent experiments, each with at least 3 wells analyzed; dots represent single values. Refers to Fig. 3 E, F.
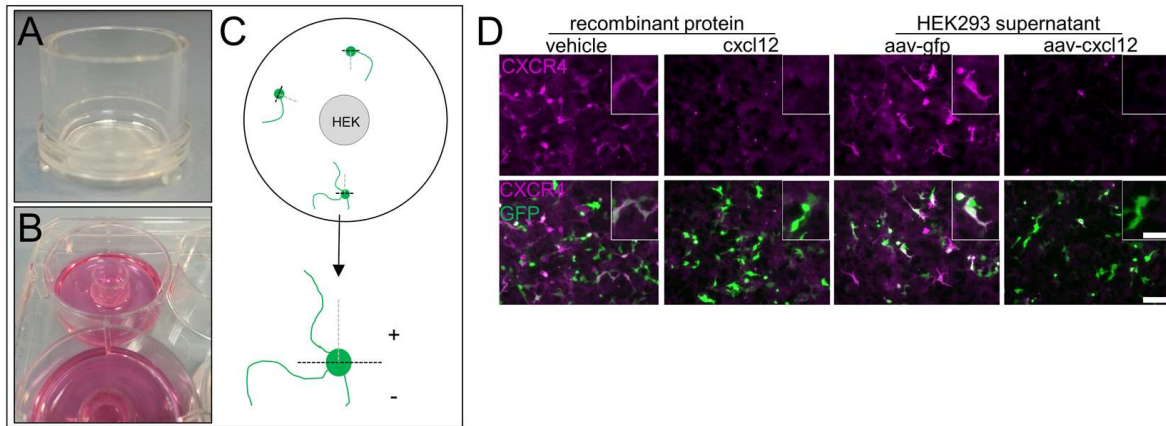
Supp. Fig. 3: Scheme of chemoattraction assay and CXCR4-internalization after CXCL12 treatment.

A-B: Photographs of the cell culture inserts (A) in which the GFP- or CXCL12-expressing HEK cells had been seeded before placing them into the middle of a dissociated RGC culture (B). **C:** Schematic drawing of RGCs and their outgrowing neurites highlight how the chemoattraction assay analysis has been performed. A line perpendicular towards the cell culture insert divides the RGC into two virtual spaces. All neurite tips present in the space toward the insert (+) are counted as attracted neurites. **D:** Lipofection with a CXCR4-carrying vector caused the expression of CXCR4 (magenta) in HEK293 cells. Transfected cells were identified by co-expression of GFP (green). Treatment with recombinant CXCL12 or CXCL12-containing HEK293 supernatant led to CXCR4 internalization resulting in the absence of CXCR4-signal, verifying the biological activity of CXCL12. In contrast, CXCR4 signals persisted upon respective control treatments (vehicle and aav-gfp). Insets in the top right of each panel show magnifications of a representative region. Scale bar: overview: 50 μm, magnification 25 μm. Refers to Fig. 4.
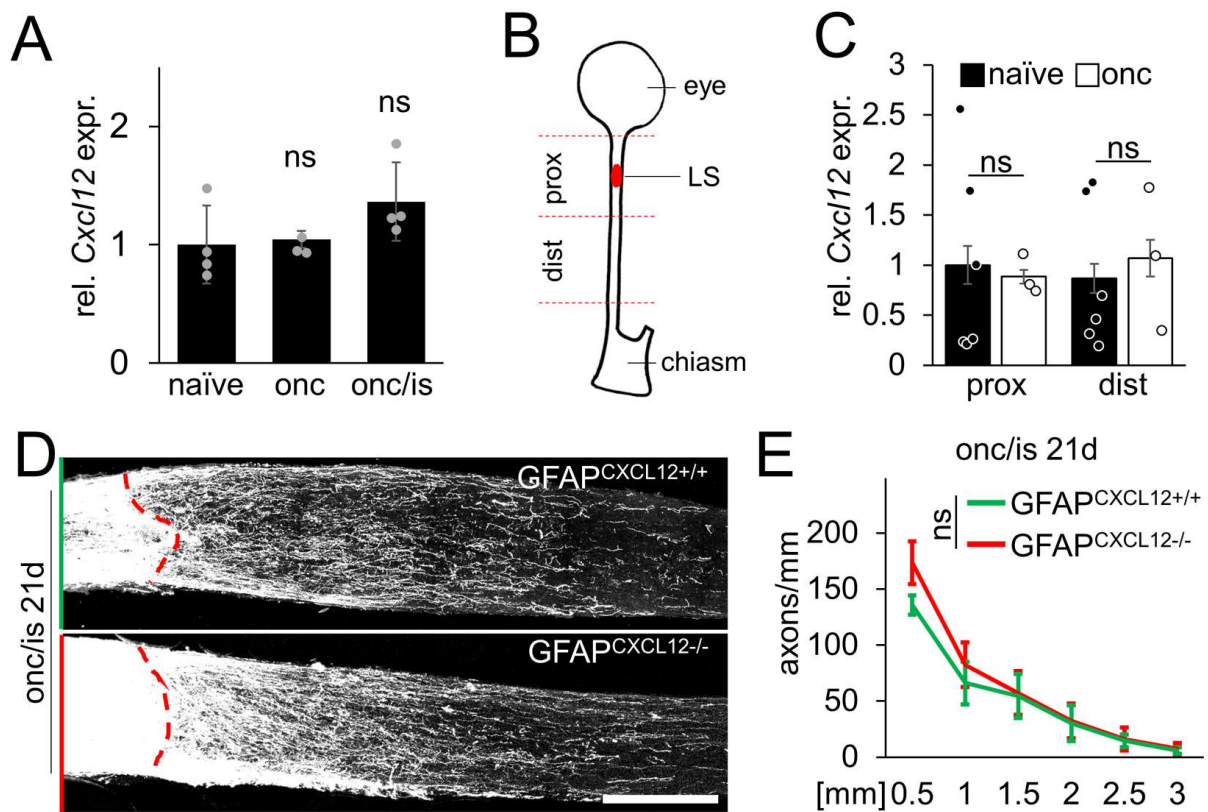
Supp. Fig. 4: CXCL12 knockdown in astrocytes does not affect regeneration
A: Quantitative real-time PCR using mRNA isolated from retinae 5 d after optic nerve crush (onc) or onc + inflammatory stimulation (onc/is) compared to untreated controls (naïve). All samples show similar expression levels of *Cxcl12*. Bars represent means ± SEM for n = 3-4 retinae per condition; dots represent single values. Treatment effect: ns: non-significant by one-way ANOVA. **B:** Schematic drawing of an optic nerve divided into proximal (prox) and distal (dist) segments that have been used for quantitative mRNA expression analysis (C). The red cloud indicates the lesion site (LS) in the optic nerve 5 d after optic nerve crush. **C:** Quantitative real-time PCR using mRNA isolated from proximal or distal segments of naïve or axotomized (onc) optic nerve as described in B. All samples show similar expression levels of *Cxcl12*. Bars represent means ± SEM; dots represent single values. Treatment effect: ns: non-significant by one-way ANOVA. **D:** Longitudinal optic nerve sections with CTB-labeled regenerating axons 21 d after optic nerve crush (onc) and additional inflammatory stimulation (onc/is). Nerves were isolated from mice with a conditional CXCL12-knockout in astrocytes (GFAP$^{CXCL12-/-}$) or respective controls (GFAP$^{CXCL12+/+}$). The dashed red line indicates the injury site. Scale bar: 250 µm. **E:** Quantification of regenerating axons extending 0.5, 1, 1.5, 2, 2.5, and 3 mm from the injury site on sections as shown in D. Values represent means ± SEM for three to four mice per experimental group (GFAP$^{CXCL12+/+}$: n=3; GFAP$^{CXCL12-/-}$: n=4). Treatment effect: ns: non-significant by one-way ANOVA with Holm-Sidak *post hoc* test. Refers to Fig. 5.