Chemokine CCL5 promotes robust optic nerve regeneration and mediates many of the effects of CNTF gene therapy

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Ciliary neurotrophic factor (CNTF) is a leading therapeutic candidate for several ocular diseases and induces optic nerve regeneration in animal models. Paradoxically, however, although CNTF gene therapy promotes extensive regeneration, recombinant CNTF (rCNTF) has little effect. Because intraocular viral vectors induce inflammation, and because CNTF is an immune modulator, we investigated whether CNTF gene therapy acts indirectly through other immune mediators. The beneficial effects of CNTF gene therapy remained unchanged after deleting CNTF receptor alpha (CNTFRα) in retinal ganglion cells (RGCs), the projection neurons of the retina, but were diminished by depleting neutrophils or by genetically suppressing monocyte infiltration. CNTF gene therapy increased expression of C-C motif chemokine ligand 5 (CCL5) in immune cells and retinal glia, and recombinant CCL5 induced extensive axon regeneration. Conversely, CRISPR-mediated knockdown of the cognate receptor (CCR5) in RGCs or treating wild-type mice with a CCR5 antagonist repressed the effects of CNTF gene therapy. Thus, CCL5 is a previously unrecognized, potent activator of optic nerve regeneration and mediates many of the effects of CNTF gene therapy.

ciliary neurotrophic factor | retinal ganglion cells | regeneration | neuroinflammation

Like most pathways in the mature central nervous system (CNS), the optic nerve cannot regenerate once damaged due in part to cell-extrinsic suppressors of axon growth (1, 2) and the low intrinsic growth capacity of adult retinal ganglion cells (RGCs), the projection neurons of the eye (3–5). Consequently, traumatic or ischemic optic nerve injury or degenerative diseases such as glaucoma lead to irreversible visual losses. Experimentally, some degree of regeneration can be induced by intraocular inflammation or growth factors expressed by inflammatory cells (6–10), altering the cell-intrinsic growth potential of RGCs (3–5), enhancing physiological activity (11, 12), chelating free zinc (13, 14), and other manipulations (15–19). However, the extent of regeneration achieved to date remains modest, underlining the need for more effective therapies.

Ciliary neurotrophic factor (CNTF) is a leading therapeutic candidate for glaucoma and other ocular diseases (20–23). Activation of the downstream signal transduction cascade requires CNTF to bind to CNTF receptor-α (CNTFRα) (24), which leads to recruitment of glycoprotein 130 (gp130) and leukemia inhibitory factor receptor-β (LIFRβ) to form a tripartite receptor complex (25). CNTFRα anchors to the plasma membrane through a glycosylphosphatidylinositol linkage (26) and can be released and become soluble through phospholipase C-mediated cleavage (27). CNTF has been reported to activate STAT3 phosphorylation in retinal neurons, including RGCs, and to promote survival, but it is unknown whether these effects are mediated by direct action of CNTF on RGCs via CNTFRα (28). Our previous studies showed that CNTF promotes axon outgrowth from neonate RGCs in culture (29) but fails to do so in cultured mature RGCs (8) or in vivo (6). Although some studies report that recombinant CNTF (rCNTF) can promote optic nerve regeneration (20, 30, 31), others find little or no effect unless SOCS3 (suppressor of cytokine signaling-3), an inhibitor of the Jak-STAT pathway, is deleted in RGCs (5, 6, 32). In contrast, multiple studies show that adeno-associated virus (AAV)-mediated expression of CNTF in RGCs induces strong regeneration (33–40). The basis for the discrepant effects of rCNTF and CNTF gene therapy is unknown but is of considerable interest in view of the many promising clinical and preclinical outcomes obtained with CNTF to date.

Because intravitreal virus injections induce inflammation (41), we investigated the possibility that CNTF, a known immune modulator (42–44), might act by elevating expression of other immune-derived factors. We report here that the beneficial effects of CNTF gene therapy in fact require immune system activation and elevation of C-C motif chemokine ligand 5 (CCL5). Depletion of neutrophils, global knockout (KO) or RGC-selective deletion of the CCL5 receptor CCR5, or a CCR5 antagonist all suppress the effects of CNTF gene therapy, whereas recombinant CCL5 (rCCL5) promotes axon regeneration and increases RGC survival. These studies point to CCL5 as a potent monotherapy for optic nerve regeneration and to the possibility that other applications of CNTF and other forms of gene therapy might similarly act indirectly through other factors.

Significance

CNTF is a leading therapeutic candidate for glaucoma and other ocular diseases and is widely used experimentally to promote axon regeneration after optic nerve injury. Paradoxically, whereas CNTF gene therapy is neuroprotective for retinal ganglion cells and promotes considerable regeneration following optic nerve injury, recombinant CNTF has little effect. We show that CNTF gene therapy exacerbates the inflammatory reaction to virally mediated gene therapy, leading to widespread expression of chemokine CCL5. Blocking CCL5 signaling abrogates most neuroprotective and axon-promoting effects of CNTF gene therapy, whereas recombinant CCL5 largely mimics the beneficial effects of CNTF gene therapy. Thus, this study identifies a potent, previously unknown agent for optic nerve regeneration and raises general questions about interpreting results of gene therapy studies.

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The authors declare no competing interest.

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Results

CNTF Gene Therapy Induces Optic Nerve Regeneration. Our initial study tested whether virally mediated CNTF delivery does in fact produce a qualitatively different outcome from high doses of rCNTF protein. We tested a range of rCNTF concentrations corresponding to those reported to be effective (5, 30), adjusting for differences in volume between the rat and mouse eyes as needed. Compared to bovine serum albumin (BSA)-injected controls, rCNTF at concentrations of 0.03, 0.1, 0.3, and 0.5 μg/μL did not improve regeneration (Fig. 1 *A* and *C*) nor RGC survival (Fig. 1 *B* and *D*). To more closely approximate the continuous delivery of CNTF by gene therapy, we also tested three injections of rCNTF (2 d before, the day of, and 3 d after optic nerve injury), which again yielded little optic nerve regeneration (*SI Appendix*, Fig. S1 *A–D*). CNTF gene therapy was carried out using an AAV that targets RGCs (and some amacrine cells). AAV2-CNTF increased CNTF mRNA levels ∼30-fold above background and ∼20-fold above the control virus 2 wk after transfection ($P < 0.05$: AAV2-CNTF vs. AAV2-GFP; $n = 4$ retinas per group; *SI Appendix*, Fig. S1*E*). CNTF gene therapy led to both robust axon regeneration and improved RGC survival ($P < 0.001$ and $P < 0.01$, respectively; Fig. 1 *C* and *D*). rCNTF has been reported to substantially elevate SOCS3 expression (45) and can induce regeneration if SOCS3 is deleted in RGCs (5). Therefore, we investigated whether CNTF gene therapy induces change in SOCS3 expression in RGCs. However, no such changes were found (Fig. 1 *E* and *F*). In sum, the above studies confirm that CNTF gene therapy induces far greater optic nerve regeneration than rCNTF.

CNTF Gene Therapy Does Not Require CNTFRα Expression in RGCs. Because the effects of CNTF are mediated through a tripartite receptor complex that includes CNTFRα, we investigated whether the beneficial effects of CNTF gene therapy require CNTFRα to be expressed in RGCs. Immunohistochemistry in retinal whole mounts indicates that CNTFRα colocalizes primarily with glial fibrillary acidic protein (GFAP), a marker for astrocytes and Müller cells (Mander's value [tM] = 65.9 ± 2.7%) but not with βIII tubulin (antibody TUJ1), a marker for RGCs (Fig. 2*A*) (tM = 2.7 ± 0.7%; $P < 0.001$ for the difference in Mander's values for CNTFRα with GFAP vs. CNTFRα with TUJ1; Fig. 2*C*). Thus, in the retina, as elsewhere in the mature CNS, CNTFRα is expressed primarily in nonneuronal cells (46). This localization pattern does not appear to be altered by CNTF gene therapy (Fig. 2*A*). Despite the absence of protein staining in RGCs, in situ hybridization (RNA Scope) detects measurable CNTFRa mRNA in these cells (Fig. 2 *B, Top*). The accuracy of this signal was verified using an AAV2 expressing a small hairpin RNA (shRNA) to knock down CNTFRα expression in RGCs (AAV2-anti-CNTFRa shRNA). Two weeks after AAV2-sh-CNTFR injection, in situ hybridization revealed a near-complete loss of CNTFRα mRNA in RGCs ($P < 0.001$; Fig. 2*B*). CNTFRα knockdown did not diminish the effects of CNTF gene therapy on either axon regeneration ($P = 0.344$; Fig. 2*B*) or RGC survival ($P = 0.538$; Fig. 2 *E–H*). Thus, the effects of CNTF gene therapy do not appear to require the expression of CNTFRα in RGCs. Although it remains possible that RGCs might import biologically relevant levels of CNTFRα from another source, the near absence of CNTFRα protein detected in RGCs and results reported below (in *The Effects of CNTF Gene Therapy Are Mediated Primarily via Chemokine CCL5*) argue against this proposition (Fig. 2*A*).

CNTF Gene Therapy Induces Systemic Immune Changes. Because CNTF is an immune modulator (42, 43), we next investigated whether CNTF gene therapy alters systemic or local immune responses. We collected immune cells from peripheral blood 2 wk after intravitreal injection of AAV2-CNTF or a control viral vector; stained cells with a commercial mixture of fluorescent-conjugated antibodies to CD11b, Ly6G, and Ly6C; and analyzed monocyte-to-neutrophil ratio by flow cytometry (*SI Appendix*, Fig. S2 *A* and *B*). Ly6C is a 14-kDa protein that is commonly used to distinguish different subsets of monocytes that can play either a beneficial or deleterious role depending on their site of activation (47, 48). In naïve mice, the ratio of monocytes to neutrophils, an established measure of inflammation that serves as a potential diagnostic index for multiple diseases (49, 50), was 0.52 ± 0.08. Whereas AAV2-GFP control vector did not significantly alter this ratio (0.45 ± 0.05, $P = 0.474$; Fig. 3 *A* and *B*), CNTF gene therapy increased the monocyte-to-neutrophil ratio

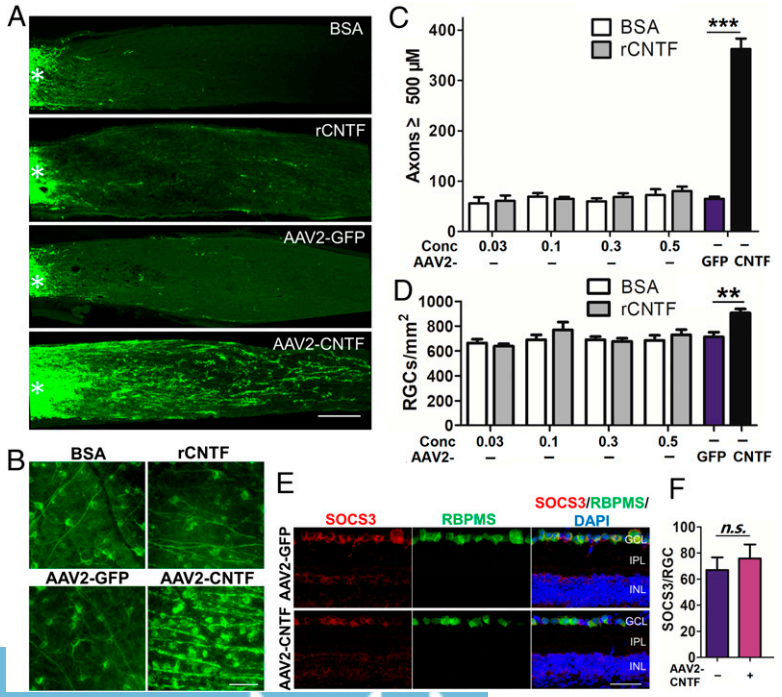

Fig. 1. CNTF gene therapy, but not rCNTF, induces optic nerve regeneration. (*A*) Longitudinal sections through mouse optic nerve immunostained for the anterograde tracer CTB (green) to visualize regenerating axons 2 wk after NC with the indicated treatments. The asterisk indicates the injury site. (Scale bar, 150 μm.) (*B*) Whole-mounted retinas immunostained with antibody TUJ1$^+$ (green) to visualize βIII tubulin, a marker for RGCs (treatments as in *A*). (Scale bar, 60 μm.) (*C*) Quantitation of regenerating axons 0.5 mm distal to the injury site. Whereas recombinant (rCNTF) did not promote regeneration at any concentration (Conc), CNTF gene therapy was highly effective. ***$P < 0.001$ (AAV2-CNTF vs. AAV2-GFP; $n = 10$ nerves per group). (*D*) Quantitation of cell survival. Whereas rCNTF did not protect RGCs, CNTF gene therapy increased cell survival by 39%. **$P < 0.01$ (AAV2-CNTF vs. AAV2-GFP; $n = 7$ retinas per group). (*E* and *F*) Expression of SOCS3. (*E*) Retinal cross-sections immunostained for SOCS3 (red) in TUJ1-positive RGCs (green). (Scale bar, 30 μm.) (*F*) CNTF gene therapy did not alter levels of SOCS3 in RGCs ($P = 0.561$; $n = 6$ to 7 retinas per group). Bars show means ± SEM. n.s., not significant.

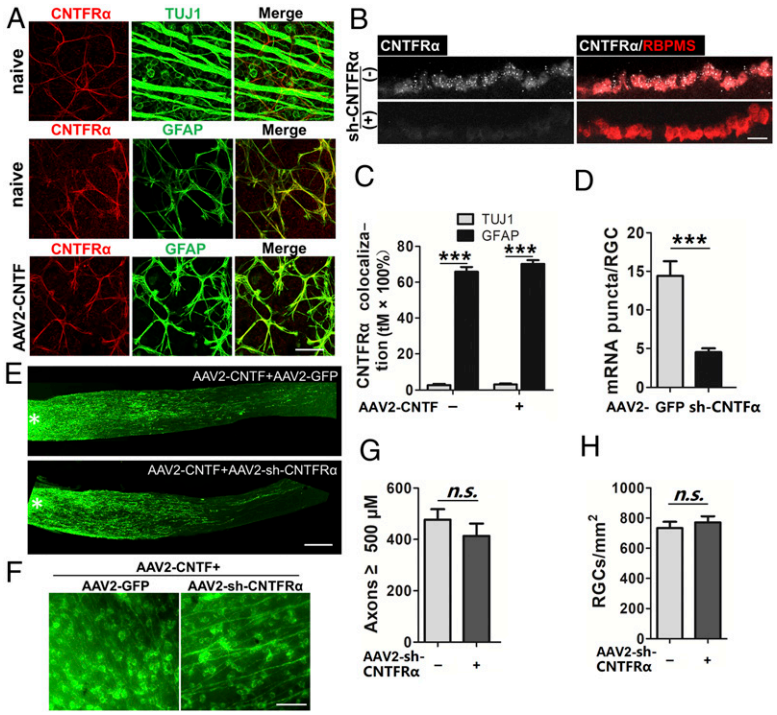

Fig. 2. CNTF gene therapy does not require CNTFRα expression in RGCs. (*A–D*) Localization and knockdown of CNTFRα. (*A*) Whole-mounted retinas immunostained for CNTFRα (red), TUJ1-positive RGCs and fiber bundles (green) (top row), and GFAP-positive astrocytes (green) (lower two rows). (Scale bar, 40 μm.) (*B*) In situ hybridization detected low levels of CNTFRα mRNA (white puncta) in the RGCs (stained with antibody to RBPMS [red] to delineate RGC cell bodies but not axon bundles). (Scale bar, 10 μm.) (*C*) Quantitation of colocalization frequency. CNTFRα colocalizes with astrocytes but not with RGCs or axon bundles. ***$P < 0.001$ (Mander's value; CNTFRα with GFAP vs. CNTFRα with TUJ1; $n = 4$ retinas per group). CNTF gene therapy did not alter CNTFRα intensity or localization. (*D*) Quantitation of CNTFRα mRNA in RGCs. CNTFRα mRNA was knocked down in RGCs 2 wk after intraocular injection of AAV2 expressing an shRNA (AAV2-sh-CNTFRα). ***$P < 0.001$ (AAV2-sh-CNTFRα vs. AAV2-GFP; $n = 4$ retinas per group). (*E–H*) RGC-selective knockdown of CNTFRα does not diminish the effects of CNTF gene therapy. (*E*) Regenerating axons visualized by CTB immunostaining (green). The asterisk indicates the injury site. (Scale bar, 150 μm.) (*F*) Whole-mounted retinas immunostained with antibody TUJ1$^+$ (green) to visualize βIII tubulin-positive RGCs. (Scale bar, 60 μm.) (*G*) Quantitation of axon regeneration 0.5 mm distal to the injury site 2 wk after nerve injury. RGC-selective CNTFRα knockdown did not alter the effects of CNTF gene therapy on axon regeneration ($P = 0.344$; $n = 7$ to 8 nerves per group). (*H*) Quantitation of RGC survival. CNTFRα knockdown in RGCs did not alter the neuroprotective effects of CNTF gene therapy ($P = 0.538$; $n = 9$ retinas per group). Bars show means ± SEM. n.s., not significant.

sixfold ($P < 0.001$, AAV2-CNTF vs. AAV2-GFP; Fig. 3 *A* and *B*). In complementary experiments, we isolated cells from 100 μL of whole blood after lysing red blood cells (RBCs), diluted these six times in Dulbecco's modified Eagle medium (DMEM), seeded cells from 100 μL onto coverslips, and counted these after staining for Gr1 and F4/80. Compared to the control group, we found that, in conformity with our findings using flow cytometry, CNTF gene therapy increased the number of macrophages, the major type of circulating monocytes ($Gr1^{low}F4/80^{high}$ cells), in peripheral blood ($P < 0.01$; Fig. 3 *C* and *D*) without significantly altering the number of neutrophils ($Gr1^{high}F4/80^{negative}$ cells: $P = 0.622$; Fig. 3 *C* and *E*). CNTF gene therapy also enhanced the infiltration of both GR1-positive neutrophils and F4/80-positive macrophages into the retina, particularly in the optic nerve head (Fig. 3*F*). Thus, CNTF gene therapy induces both systemic and local immune responses.

The Effects of CNTF Gene Therapy Require Neutrophil Activation. We next investigated whether inflammation plays a role in the beneficial effects of CNTF gene therapy using mice lacking CCR2, a chemokine receptor that mediates monocyte recruitment and migration (51, 52). Following intraocular injection of AAV2-CNTF and nerve crush (NC), mice lacking CCR2 showed a 48% reduction in optic nerve regeneration ($P < 0.001$; Fig. 3 *G* and *I*) and an 18% decrease in RGC survival ($P < 0.01$; Fig. 3 *H* and *J*) back to baseline levels compared to heterozygous KO controls.

Neutrophils are the first responders of the inflammatory cascade and modify the chemokine network while providing granule proteins to create a milieu favoring the subsequent monocyte influx (53–55). We therefore examined whether neutrophils contribute to the effects of CNTF gene therapy. Neutrophils were immune-depleted by multiple systemic injections of an antibody against Ly6G, a neutrophil-specific surface protein (56) (Fig. 4*A*). Whereas neutrophils normally comprised 15.1 ± 1.3% of all blood cells in the control group, the percentage dropped 20-fold following immune depletion (to 0.3 ± 0.2%, $P < 0.01$; Fig. 4*B*). Neutrophil depletion strongly suppressed the effects of CNTF gene therapy, reducing axon regeneration by 74% ($P < 0.001$; Fig. 4 *C* and *D*) and RGC survival by 21% (i.e., to baseline levels: $P < 0.001$; Fig. 4 *E* and *F*). Thus, the effects of CNTF gene therapy require monocyte migration and neutrophil activation.

Regeneration Induced by CNTF Gene Therapy Involves Factors Other than Those Involved in Zymosan-Induced Regeneration. Because CNTF gene therapy depends upon inflammation, we tested whether its effects involve the same proteins that mediate the effects of intraocular zymosan on optic nerve regeneration and RGC survival (8, 10, 17). One of these, oncomodulin (Ocm), is an 11-kDa Ca^{2+}-binding protein that is highly expressed in neutrophils and that mediates most of the axon-promoting effects of zymosan treatment, although not its neuroprotective effects (8, 10, 57). The second protein, SDF-1, is highly expressed in macrophages and complements the effects of Ocm by enhancing RGC survival and augmenting regeneration (58). Two weeks after intraocular injection of AAV2-CNTF, mRNA levels for SDF1 and Ocm increased 2.5- and 2.2-fold, respectively, in whole eye ($P < 0.05$ for both; *SI Appendix*, Fig. S3 *A* and *B*). At the protein level, we observed SDF1 expression in both Gr1-positive neutrophils (*SI Appendix*, Fig. S3*C*) and von Willebrand factor-positive vascular cells (*SI Appendix*, Fig. S3*D*), whereas Ocm was detected in Gr1-positive neutrophils (*SI Appendix*, Fig. S3*E*).

We next examined whether SDF1 and Ocm contribute to the effects of CNTF gene therapy. As a positive control, we tested whether AMD3100, a selective antagonist to the primary receptor for SDF1, CXCR4 (59), combined with P1 peptide, an Ocm antagonist (10), would diminish the proregenerative effects of zymosan. As expected, AMD3100 and P1 combined reduced zymosan-induced axon regeneration and RGC survival to near-baseline levels ($P < 0.001$ and $P < 0.01$ respectively; Fig. 5). In contrast, intraocular injection of AMD3100 and P1 reduced the effects of CNTF gene therapy on axon regeneration by only 19% ($P < 0.05$) and did not diminish RGC survival ($P = 0.441$; Fig. 5). Therefore, the effects of CNTF gene therapy primarily involve factors other than Ocm and SDF-1.

The Effects of CNTF Gene Therapy Are Mediated Primarily via Chemokine CCL5. Chemokine CCL5 (regulated upon activation, normal T cell expressed and secreted [RANTES]) is an important chemotactic agent that promotes immune cell recruitment through binding to

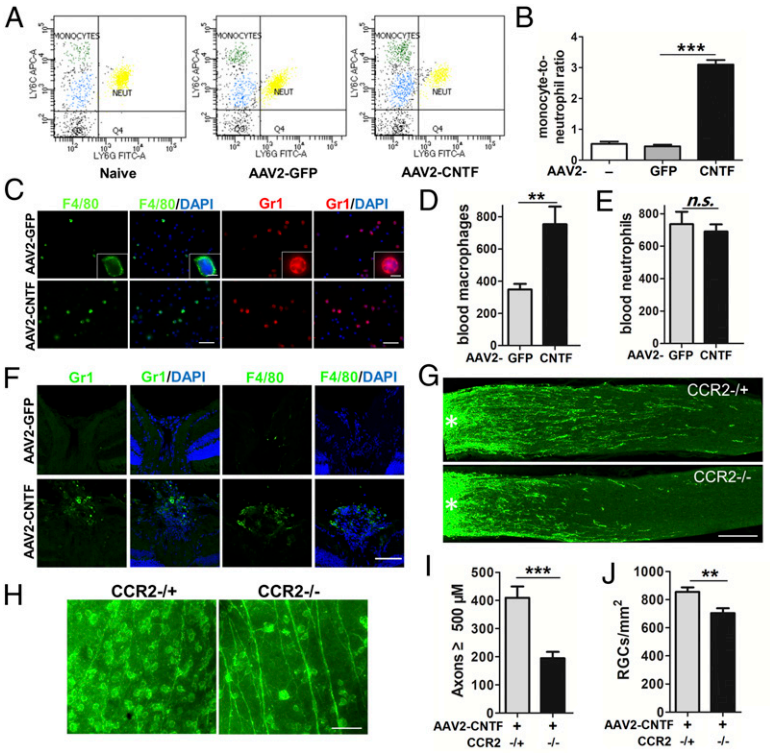

Fig. 3. CNTF gene therapy alters systemic and local inflammation. (*A*) Blood-derived immune cells stained with fluorescent antibodies to CD11b, Ly6G, and Ly6C and analyzed by flow cytometry 2 wk after intraocular injection of AAV2-CNTF or control vector. Monocytes ($CD11b^{+}Ly6G^{low}Ly6C^{high+intermediate}$) and neutrophils (NEUT) ($CD11b^{+}$ $Ly6G^{high}Ly6C^{intermediate}$) were quantified as shown. (*B*) Quantitation of changes. CNTF gene therapy increased the ratio of monocytes to neutrophils approximately sevenfold. ***$P < 0.001$ (AAV2-CNTF vs. AAV2-GFP; n= 3 mice per group). (*C–E*) Immune cells stained with F4/80 (green) (macrophages), Gr1 (red), and the nuclear marker DAPI (blue). Macrophages ($Gr1^{low}F4/80^{high}$) (*D*) and neutrophils ($Gr1^{high}F4/80^{negative}$) (*E*) were counted manually. CNTF gene therapy enhanced macrophage numbers in peripheral blood (**$P < 0.01$; AAV2-CNTF vs. AAV2-GFP; $n = 5$ mice/group) but not neutrophil numbers. (Scale bars: *C*, 5 μm; *C*, *Insets*, 50 μm.) (*F*) CNTF gene therapy enhances neutrophil and macrophage infiltration into the retina (both green), particularly in the optic nerve head. (Scale bar, 40 μm.) (*G–J*) CCR2 KO reduces the effects of CNTF gene therapy. (*G*) Regenerating axons visualized by CTB immunostaining (green). The asterisk indicates the injury site. (Scale bar, 150 μm.) (*H*) Whole-mounted retinas immunostained with antibody $TUJ1^{+}$ (green) to visualize surviving RGCs 2 wk after NC. (Scale bar, 60 μm.) (*I*) Quantitation of regenerating axons 0.5 mm distal to the injury site. CCR2 KO reduced the effects of CNTF gene therapy on axon regeneration by 48%. ***$P < 0.001$ (KO vs. heterozygous KO controls; $n = 9$ to 10 nerves per group). (*J*) Quantitation. CCR2 KO diminished RGC survival by 26%. **$P < 0.01$ (KO vs. heterozygous KO controls; $n = 11$ to 14 retinas per group). Bars show means ± SEM.

one or more G protein-coupled receptors that include CCR1, CCR3, CCR5, and/or GPR75 (60–63). Intraocular CNTF has been reported to up-regulate multiple inflammation-associated genes in the retina including CCL5 (64). In conformity with this result, qRT-PCR revealed that AAV2-CNTF induced a 9.5-fold increase in CCL5 mRNA in the retina ($P < 0.05$; Fig. 6*A*) and elevated immunostaining for CCL5 protein in the innermost retina. Double-immunostaining revealed that CCL5 colocalizes primarily with GFAP ($P < 0.001$; Fig. 6 *B* and *C*). CNTF gene therapy increased CCL5 and GFAP colocalization 32-fold ($P < 0.001$; Fig. 6 *B* and *C*), pointing to expression in astrocytes and/or Müller cell endfeet. Because GFAP is expressed in both types of glia, we further investigated whether CCL5 colocalizes with retinaldehyde-binding protein (CRALBP), a marker for Müller cells. CCL5 expression overlapped extensively with CRALBP, and this overlap increased 8.4-fold with CNTF gene therapy ($P < 0.001$; *SI Appendix*, Fig. S4 *A* and *B*).

Based on our finding that neutrophil depletion diminishes the effect of CNTF gene therapy on axon regeneration and RGC survival, we examined whether the effect of CNTF gene therapy on retinal CCL5 expression depends upon neutrophil activation. Neutrophil depletion eliminated the effects of CNTF gene therapy on CCL5 mRNA expression in both the retina and blood immune cells, returning both to baseline ($P < 0.01$ and $P < 0.05$, respectively; Fig. 6 *D* and *E*) and reducing CCL5 protein levels in the inner retina ($P < 0.05$; Fig. 6 *F* and *G*).

Although CCL5 can act through multiple receptors, adult RGCs only express CCR5 and GPR75 in multiple subtypes (16), and we therefore focused on these two. Immunostaining revealed that GPR75 was expressed mainly on RGC somata (*SI Appendix*, Fig. S5*B*), whereas CCR5 was present on what appear to be cilia extending from RGC cell bodies (Fig. 6*H*). The latter localization was confirmed by double-immunostaining and confocal microscopy, revealing a strong overlap between CCR5 and adenylyl cyclase type 3 (ACIII), a marker for primary cilia (65, 66) (Fig. 6*I*). CCR5 did not appear to be expressed on the primary cilia of every RGC, however, and was also expressed in the inner plexiform layer. Based on data from single-cell sequencing, CCR5 is expressed in 18 RGC subtypes in normal intact mice and becomes expressed in 16 additional subtypes after optic nerve injury (16).

Loss-of-function studies. To determine whether CCL5 contributes to the proregenerative and neuroprotective effects of CNTF gene therapy, we first used CCR5 KO mice to eliminate the cognate receptor globally. Following intraocular AAV2-CNTF and NC, CCR5 deficiency reduced optic nerve regeneration by 72% and decreased RGC survival to baseline (24% decrease) compared to similarly treated littermate controls ($P < 0.001$ and $P < 0.01$, respectively; Fig. 7 *A–D*). The roles of CCL5 and CCR5 were further tested pharmacologically using the highly selective antagonist D-Ala-peptide T-amide (DAPTA). Daily intraperitoneal injection of DAPTA reduced the effects of CNTF gene therapy on axon regeneration by 75% compared with vehicle-injected controls and reduced RGC survival to baseline (20% decrease: $P < 0.001$ and $P < 0.05$, respectively; Fig. 7 *A–D*). Finally, we carried out RGC-selective deletion of CCR5 using CRISPR-mediated gene editing. We injected an AAV (AAV2) expressing Cas9 driven by the RGC-selective γ-synuclein (Sncg) promoter (AAV2-Sncg-Cas9) together with an AAV2 expressing a small guide RNA targeting either CCR5 or GPR75 (AAV2-sgCCR5 or AAV2-sgGPR75) into the eye 2 wk prior to CNTF vector injection. GFP and RNA-binding protein with multiple splicing (RBPMS) immunostaining in retinal cross-sections showed ≥95% of RGCs to be GFP-positive 2 wk after either AAV2-Sncg-Cas9 or AAV2-sgRNA injection (*SI Appendix*, Fig. S1*F*). Following intraocular AAV2-Sncg-Cas9 combined with AAV2-sgCCR5, CCR5 immunostaining decreased dramatically compared to mice injected with the control vector (*SI Appendix*, Fig. S5*A*). RGC-selective CCR5 deletion decreased the effects of CNTF gene therapy on axon regeneration by 64% and diminished RGC survival by 17% compared to mice injected with AAV2-Sncg-Cas9 combined with the control virus ($P < 0.001$ and $P < 0.05$, respectively; Fig. 7 *A–D*). In contrast, deletion of GPR75, an alternative receptor for CCL5,

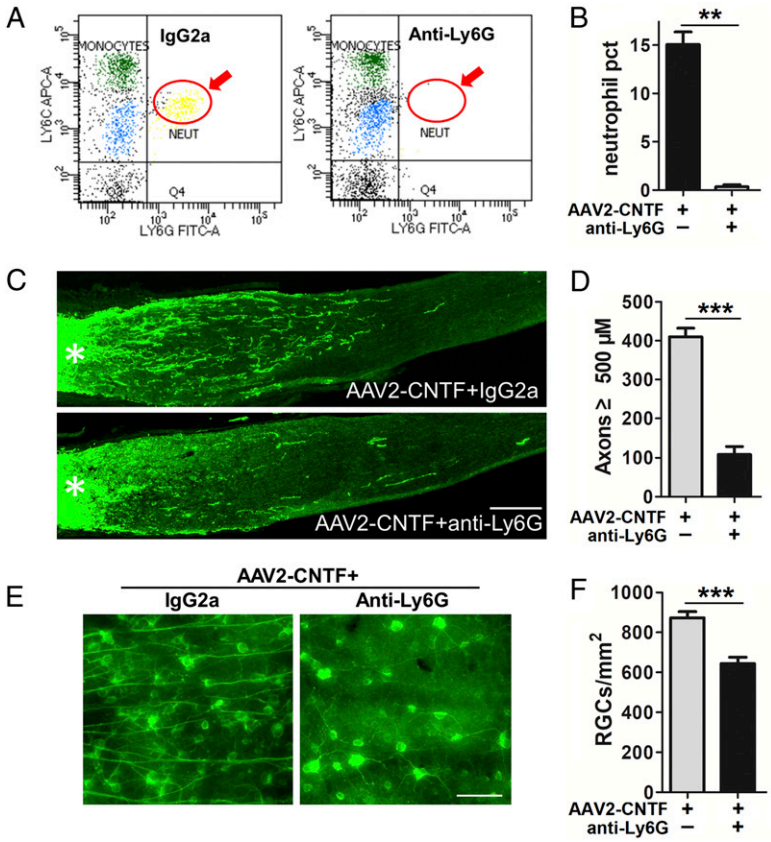

Fig. 4. Neutrophil depletion diminishes the effects of CNTF gene therapy. (*A* and *B*) Systemic administration of anti-Ly6G antibody eliminates neutrophils (NEUT) in peripheral blood. (*A*) Immune cells isolated from blood; stained with fluorescently conjugated antibodies to CD11b, Ly6G, and Ly6C; and analyzed by flow cytometry 2 wk after CNTF gene therapy with or without neutrophil depletion (systemic anti-Ly6G). (*B*) Quantitation of blood neutrophils. $^{**}P < 0.01$ (anti-Ly6G vs. IgG2a; $n = 3$ mice per group). (*C*–*F*) Neutrophil depletion suppresses the effects of CNTF gene therapy on optic nerve regeneration. (*C*) Regenerating axons visualized by CTB immunostaining (green). The asterisk indicates the injury site. (Scale bar, 150 µm.) (*D*) Quantitation of axon regeneration 0.5 mm past the injury site. Neutrophil depletion reduced the effects of CNTF gene therapy by 74%. $^{***}P < 0.001$ (IgG2a vs. anti-Ly6G; $n = 4$ to 10 nerves per group). (*E*) Retinal whole mounts immunostained for βIII tubulin (antibody TUJ1) (green) 2 wk after NC. (Scale bar, 60 µm.) (*F*) Quantitation of RGC survival. Neutrophil depletion reduced RGC survival by 21% (TUJ1$^+$ cells) (green in *E*). $^{***}P < 0.001$ (IgG2a vs. anti-Ly6G; $n = 8$ to 10 retinas per group). Bars show means ± SEM.

had little or no effect. Following intraocular AAV2-Sncg-Cas9 combined with AAV2-sgGPR75, GPR75 immunostaining decreased dramatically compared to control vector injected controls (*SI Appendix*, Fig. S5*B*) but with no effects on axon regeneration or RGC survival ($P = 0.35$ and $P = 0.27$, respectively; *SI Appendix*, Fig. S5 *C*–*F*).

Gain-of-function. We next tested whether CCL5 can mimic the effects of CNTF gene therapy in vivo. A single intraocular injection of rCCL5 (0.1 µg/µL) immediately after NC strongly increased axon regeneration and RGC survival (compared to controls injected with similar concentrations of BSA: $P < 0.001$; Fig. 8 *A* and *C*). In addition, because CNTF gene therapy would be expected to induce persistent elevation of CCL5, we examined whether multiple injections of CCL5 would enhance regeneration and neuroprotection even further. Three injections of rCCL5 (2 d before, the day of, and 3 d after NC) doubled the level of axon regeneration induced by a single injection ($P < 0.05$; Fig. 8 *A* and *C*). In terms of neuroprotection, a single intraocular injection of rCCL5 (1×) enhanced RGC survival by 28% ($P < 0.05$: rCCL5 vs. BSA; Fig. 8 *B* and *D*), whereas multiple injections of rCCL5 (3×) had no additional effects ($P = 1.00$: rCCL5 3× vs. rCCL5 1×; Fig. 8 *B* and *D*).

Prior studies have shown that elevation of cyclic adenosine monophosphate (cAMP) [using a nonhydrolyzable, membrane-permeable analog, e.g., (chlorophenylthio)adenosine–cAMP (CPT-cAMP)] strongly increases the effects of particular trophic factors on RGCs, in some cases, by inducing receptor translocation (8, 67, 68). However, combining rCCL5 with CPT-cAMP did not increase axon regeneration compared to rCCL5 alone (*SI Appendix*, Fig. S6). Finally, we investigated whether CCL5 alters established neuroprotective and proregenerative signaling pathways. A single intraocular injection of rCCL5 led to a 1.9- fold increase in immunostaining intensity for phosphorylated ribosomal protein S6, a marker of mTOR pathway activation ($P < 0.01$; Fig. 8 *E* and *F*), and a 1.4-fold increase in phosphorylated cAMP response element-binding protein (pCREB) ($P < 0.05$; Fig. 8 *G* and *H*) in RGCs. A single injection of rCCL5 reduced immunostaining intensity for phosphorylated extracellular signal-regulated kinase (pERK) by 58% ($P < 0.0001$; Fig. 8 *I* and *J*), and no differences were detected for phosphorylated protein kinase B (pAKT), phosphorylated signal transducer and activator of transcription 3 (pSTAT3), or phosphorylated cJUN (p-cJUN) (Fig. 8*K*).

These studies show that CNTF gene therapy strongly elevates expression of CCL5 in immune cells and retinal glia, that signaling through CCR5, the cognate receptor for CCL5, is required for most of the effects of CNTF gene therapy, and that rCCL5, particularly when provided over a 5-d period, induces robust regeneration. Combined with our findings that CNTF gene therapy induces regeneration through a mechanism involving neuroinflammation but not via direct action on RGCs, our results point to an indirect effect of CNTF gene therapy on RGC survival and axon regeneration that is mediated primarily by CCL5.

Discussion

CNTF is a leading therapeutic candidate for neurodegenerative conditions, with multiple ongoing or completed clinical trials for safety and efficacy in glaucoma and other ocular diseases. Multiple studies have demonstrated that CNTF gene therapy induces robust optic nerve regeneration in animal models (33–39), whereas the efficacy of CNTF per se has been unclear. Our results show that CNTF gene therapy improves RGC survival and promotes extensive optic nerve regeneration indirectly, largely through immune modulation and elevation of the chemokine CCL5. In loss-of-function studies, a pharmacological inhibitor of CCR5, the cognate receptor for CCL5, or deletion of CCR5 in RGCs, strongly suppressed the beneficial effects of CNTF gene therapy, whereas in gain-of-function studies, repeated injections of rCCL5 induced nearly as much optic nerve regeneration as CNTF gene therapy.

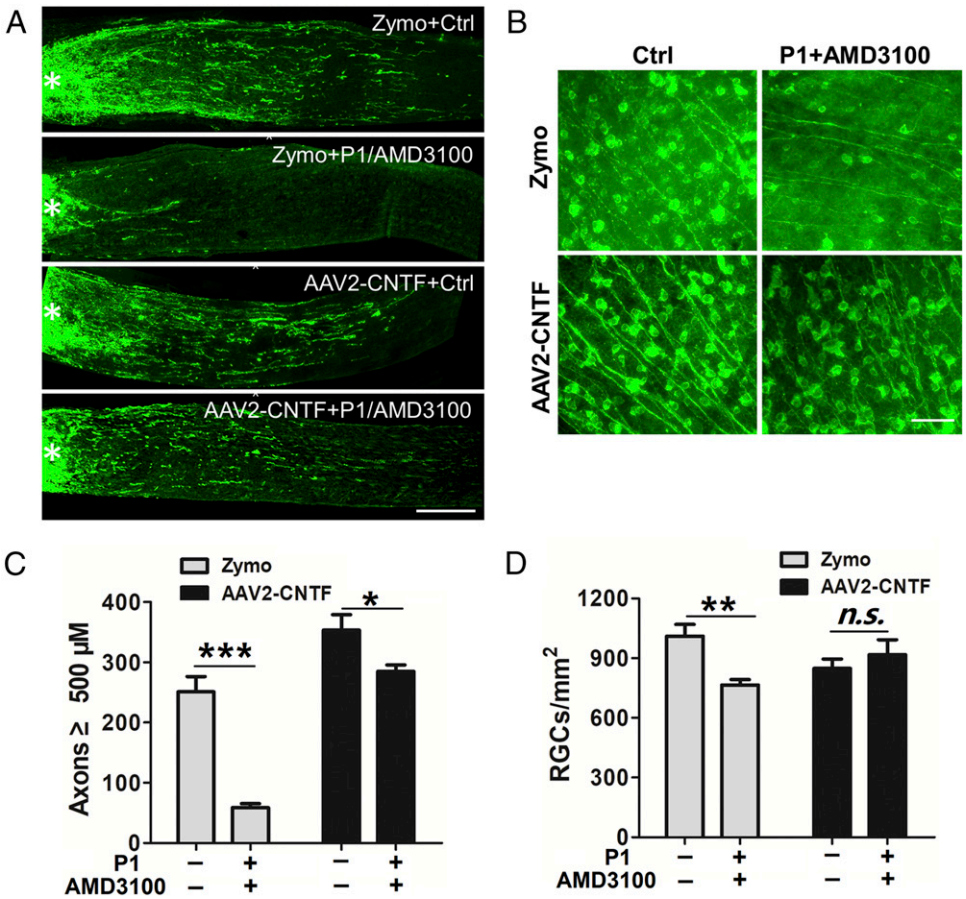

Fig. 5. Inhibition of Ocm and SDF1 has minor effect on CNTF gene therapy-induced axon regeneration. (*A–D*) Effects of blocking Ocm (Peptide P1) and SDF1 (AMD3100) on axon regeneration and RGC survival induced by zymosan (Zymo) (upper two images in *A* and *B*; positive control) and CNTF gene therapy (lower two images in *A* and *B*). (*A*) Regenerating axons visualized by CTB immunostaining (green). The asterisk indicates the injury site. (Scale bar, 150 μm.) (*B*) Whole-mounted retinas immunostained with antibody TUJ1$^+$ (green) to visualize βIII tubulin-positive RGCs. (Scale bar, 60 μm.) (*C*) Quantitation of regeneration. Intraocular injection of AMD3100 plus P1 eliminated zymosan-induced axon regeneration. ***$P < 0.001$ (zymosan + control vs. zymosan + P1/AMD3100; $n = 8$ nerves per group). The same inhibitors decreased CNTF gene therapy-induced axon regeneration by 19%. *$P < 0.05$ (CNTF gene therapy + control vs. CNTF gene therapy + P1/AMD3100; $n = 8$ nerves per group). (*D*) Quantitation of RGC survival. Antagonists to Ocm and SDF1 decreased RGC survival by 24% (**$P < 0.01$; zymosan + control vs. zymosan + P1/AMD3100; $n = 8$ retinas per group) but did not alter the neuroprotective effects of CNTF gene therapy ($P = 0.441$; $n = 8$ retinas/group). Bars show means ± SEM.

In contrast to CNTF gene therapy, rCNTF at concentrations up to three orders of magnitude above the half-maximal effective concentration produced little effect even with repeated injections. Earlier studies reported that rCNTF substantially elevates SOCS1 and SOCS3 mRNA and protein levels in RGCs, an effect that is diminished by JAK inhibition or CPT-cAMP (45) and that is absent when CNTF levels are elevated by gene therapy (69). SOCS3 is a strongly negative regulator of optic nerve regeneration, as demonstrated by studies showing that SOCS3 overexpression negatively impacts regeneration induced by rCNTF in the peripheral nerve grafting paradigm (69). Thus, an absence of SOCS3 elevation could potentially be one factor in the greater regeneration seen with CNTF gene therapy compared to rCNTF. However, we found that the robust regeneration induced by CNTF gene therapy was unaffected by deleting the obligatory subunit of the CNTF receptor, CNTFR-α, in RGCs, providing further evidence that, in our model, CNTF does not directly activate downstream signaling pathways required for axon regeneration in RGCs. On the other hand, ligands to related receptors (e.g., LIF, CT1, IL6) can activate the same signaling pathways as CNTF and may contribute to the considerable baseline regeneration seen in the peripheral nerve (PN) graft paradigm and to the augmented regeneration seen after SOCS3 deletion in mice (5). Because SOCS3 interferes with the docking of multiple SH2 domain proteins to Jak proteins, SOCS3 deletion can enhance signaling not only via STAT proteins but also via the MAPK or PI3 kinase pathways (5), consistent with results showing that the effects of rCNTF in the PN grafting paradigm are suppressed by blockers of the MAPK and PI3K/Akt pathways (5). In addition, CNTF can increase expression of related family members, e.g., LIF (45, 70) and, as shown here, CCL5, Ocm, and SDF-1, which may contribute to the positive effects of rCNTF reported by others. Interestingly, although SOCS3 deletion combined with rCNTF induces extensive optic nerve regeneration, this combination does not activate STAT phosphorylation in RGCs (5), further supporting the possibility that the effects of CNTF and SOCS deletion may be mediated through a pathway other than through Jak-STAT signaling in RGCs. Finally, intravitreally grafted neural stem cells (NSCs) genetically modified to secrete CNTF and/or GDNF effectively attenuate RGC loss in adult mice (71, 72), an effect that might be augmented by other trophic factors secreted by NSCs (e.g., NGF, BDNF) (73–75) and by factors induced by CNTF in other cells.

CNTF belongs to the interleukin 6 (IL-6) family of cytokines, which play key roles in immune homeostasis and inflammation (76). As a chemoattractant (42–44), continuous expression of CNTF may amplify the inflammation induced by intravitreal virus injections (41), a hypothesis that is supported by the effects of CNTF gene therapy seen here on both systemic and local inflammatory responses. CNTF gene therapy greatly increased circulating monocytes and CCL5 expression in immune cells and retinal Müller cells. CCL5, also referred to as RANTES, is a 68-amino acid protein that can act through several G protein-coupled receptors (GPCRs), including CCR1, CCR3, CCR5, and GPR75, to direct the migration and recruitment of T cells, monocytes/macrophages, and eosinophils at injury sites (77–79). CCL5 and its high-affinity receptors are constitutively expressed in the inner retina, are differentially induced by stressors, and are associated with retinal degenerative disease, although the relationship of this observation to the present work is unknown (80, 81). Single-cell sequencing studies show CCR5 to be expressed normally in 18 RGC subtypes and to be expressed in 16 additional subtypes after optic nerve injury (including several types of intrinsically sensitive, alpha, and direction-sensitive RGCs) (16). CCL5 deficiency leads to a disorganization of RGC dendrite and amacrine cell

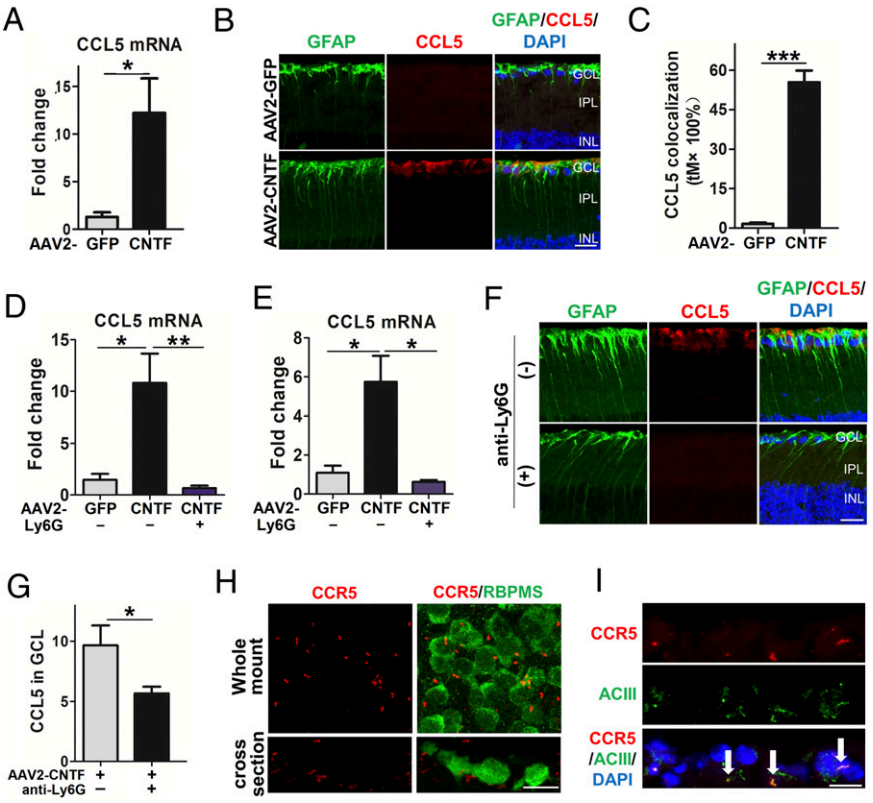

Fig. 6. CNTF gene therapy increases CCL5 expression in retinal glia and immune cells. (*A*) CNTF gene therapy increased CCL5 mRNA levels in the retina 9.5-fold. $*P < 0.05$ (AAV2-CNTF vs. AAV2-GFP; $n = 4$ retinas per group). (*B* and *C*) CNTF gene therapy enhanced CCL5 expression in GFAP$^+$ cells. (*B*) Retinal cross-sections from mice receiving intraocular AAV2-GFP or AAV2-CNTF stained for CCL5 (red), glia (GFAP) (green), and nuclei (DAPI) (blue). CCL5 was elevated in the innermost retina (ganglion cell layer [GCL]). (Scale bar, 30 μm.) (*C*) Quantitation of colocalization. CNTF gene therapy increased localization of CCL5 in GFAP-positive cells 32-fold. $***P < 0.001$ (AAV2-CNTF vs. AAV2-GFP; $n = 4$ to 5 retinas per group). (*D*) Neutrophil depletion blocked the effects of CNTF gene therapy on retinal CCL5 expression. $*P < 0.05$ (IgG2a: AAV2-CNTF vs. AAV2-GFP); $**P < 0.01$ (AAV2-CNTF: anti-Ly6g vs. IgG2a; $n = 4$ retinas per group). (*E*) CNTF gene therapy elevated CCL5 mRNA 4.3-fold in circulating immune cells. $*P < 0.05$ (IgG2a: AAV2-CNTF vs. AAV2-GFP; $n = 3$ mice per group). Neutrophil depletion eliminated the effects of CNTF gene therapy on CCL5 expression in blood-borne immune cells. $*P < 0.05$ (AAV2-CNTF: anti-Ly6G vs. IgG2a; $n = 3$ mice per group). (*F* and *G*) Neutrophil depletion blocked the effects of CNTF gene therapy on CCL5 expression in the retina. (*F*) Retinal cross-sections from mice with CNTF gene therapy, with or without neutrophil depletion, stained for CCL5 (red), GFAP (green), and nuclei (DAPI) (blue). (Scale bar, 30 μm.) (*G*) Quantitation. Neutrophil depletion suppressed CCL5 expression in the inner retina. $*P < 0.05$ (AAV2-CNTF vs. AAV2-GFP; $n = 9$ retinas per group). (*H*) Naïve retinal whole mounts (upper set) or cross-sections (lower set) immunostained to visualize the CCL5 receptor CCR5 (red) and RGCs (anti-RBPMS) (green). Most CCR5 immunostaining extends from RGC cell bodies. (Scale bar, 10 μm.) (*I*) Naïve retina cross-sections double-immunostained for CCR5 (red) and ACIII, a marker for primary cilia (green). Arrows indicate colocalization between CCR5 and ACIII. (Scale bar, 7 μm.) Bars show means ± SEM. INL, inner nuclear layer; IPL, inner plexiform layer.

morphology, suggesting that CCL5 could act as a normal modulator (82) of retinal development (83). Changes in RGC dendritic architecture have also been observed after long-term CNTF gene therapy (84), although the relationship of this finding to CCL5 is unknown. Although initially identified as a T cell-secreted chemokine, CCL5 can be expressed by multiple immune cells and glia, including macrophages, eosinophils, microglia, and astrocytes (85–87). Other studies suggest that multiple molecules shared by the immune system and the CNS might play essential roles in glia–neuron communication (88, 89).

Neutrophil depletion eliminated the effects of CNTF gene therapy on CCL5 expression in circulating immune cells and, unexpectedly, in retinal Müller cells as well. The mechanisms that underlie the role of inflammation in the elevation of retinal CCL5 in response to CNTF gene therapy remain to be investigated. Another issue not studied here are possible effects of species and strain. Mouse strains exhibit differences in their inflammatory response to spinal cord injury (90), and genetic background in both mice and rats can influence intrinsic immune responses in the eye, along with RGC and photoreceptor vulnerability after optic NC (ONC) (91–94). Further work will be needed to explore whether strain differences influence the outcome of CNTF gene therapy, particularly regarding the role of inflammation and CCL5. In any event, comparing our results with those of our previous studies and others suggests that CCL5 may be a more potent monotherapy for optic nerve regeneration than the other trophic factors or chemokines studied to date, including Ocm, CNTF, BDNF, SDF1, and IGF1 (5, 8, 10, 17, 32, 58, 95).

Although CCL5 can bind to and initiate signaling via CCR1, CCR3, CCR5, or GPR75 (60–63), our transcriptome data from FACS-isolated RGCs show that, among these receptors, adult RGCs only express CCR5 and GPR75 at detectable levels. CCR5 has been studied extensively as a coreceptor for HIV and a prominent receptor in microglia (96, 97). Our immunostaining results verified that both CCR5 and GPR75 are expressed in RGCs, although in different cellular compartments. Whereas GPR75 is expressed on RGC somata, CCR5 is located on primary cilia, microtubule-based organelles that process multiple molecular signaling cues and, in dividing cells, regulate cell cycle (98, 99). The stabilization of primary cilia was recently shown to rescue injured adult RGCs from apoptosis by reducing abortive cell-cycle reentry (99).

CCR5 is an emerging therapeutic target for improving outcome after stroke and traumatic brain injury (100). Either a CCR5 antagonist (maraviroc) or mutation of CCR5 (CCR5-Δ32) improves recovery after neurological impairments (100). In contrast, our results show that global deletion of CCR5, or a CCR antagonist, or RGC-selective CCR5 deletion all have deleterious effects in the context of CNTF gene therapy-induced optic nerve regeneration. The apparent discrepancy between the neuroreparative effects of CCL5 signaling in our studies and the beneficial effect of blocking CCL5 signaling after stroke remains unexplored, including the possibility that the latter effects may rely on suppressing deleterious effects of microglial activation.

In conclusion, our results show that the striking effects of CNTF gene therapy after optic nerve injury are mediated through immune modulation and up-regulation of CCL5, a chemokine shown here to be a potent agent for optic nerve regeneration and RGC survival. These findings provide insights for understanding the mechanisms of action of CNTF gene therapy and guiding clinical trials. Our results also raise

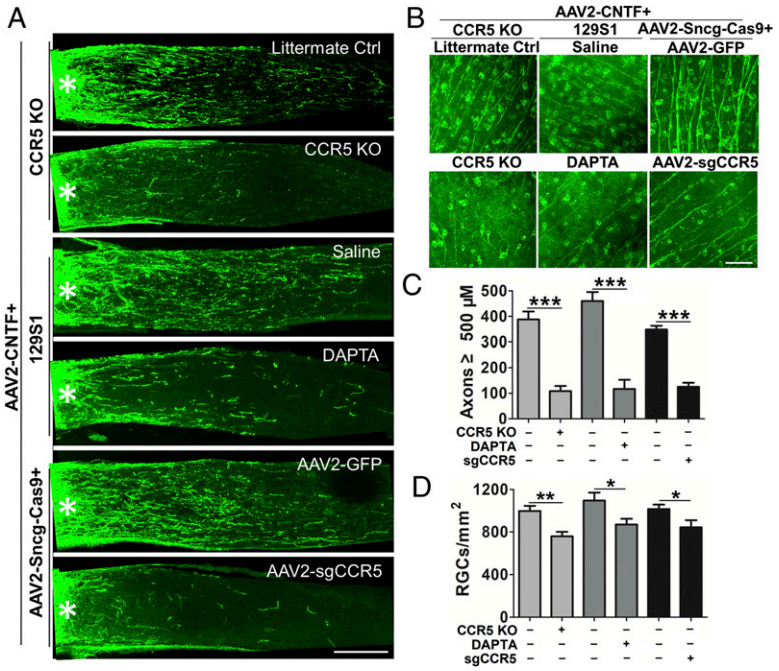

Fig. 7. CCR5 is required for the major effects of CNTF gene therapy. (*A–D*) CCR5 KO or the CCR5 antagonist DAPTA diminishes most effects of CNTF gene therapy. (*A*) Regenerating axons visualized by CTB immunostaining (green). Treatments are as indicated. The asterisk indicates the injury site. (Scale bar, 150 μm.) (*B*) Whole-mounted retinas immunostained with antibody TUJ1$^+$ (green). (Scale bar, 60 μm.) (*C* and *D*) Quantitation. (*C*) CCR5 KO reduced the effects of CNTF gene therapy on axon regeneration by 72%. ***$P < 0.001$ (NC + CNTF gene therapy: littermate control vs. CCR5 KO; upper pair). CCR5 antagonist DAPTA diminished the effects of CNTF gene therapy by 75% in wild-type 129S1 mice. ***$P < 0.001$ (NC + CNTF gene therapy: saline vs. DAPTA; middle set). CRISPR-mediated KO of CCR5 in RGCs diminished the effects of CNTF gene therapy on optic nerve regeneration by 64% in wild-type 129S1 mice. ***$P < 0.001$ (NC + CNTF gene therapy + AAV2-Sncg-Cas9: AAV2-GFP vs. AAV2-sgCCR5; $n = 8$ nerves per group; lower set). (*D*) RGC survival is decreased by 24% following CCR5 KO (**$P < 0.01$; NC + CNTF gene therapy: littermate control vs. CCR5 KO), by 20% in wild-type 129S1 mice treated with DAPTA (*$P < 0.05$; NC + CNTF gene therapy: saline vs. DAPTA), and by 17% in wild-type 129S1 mice with CRISPR-mediated deletion of the CCR5 gene in RGCs (*$P <$ 0.05; NC + CNTF gene therapy + AAV2-Sncg-Cas9: AAV2-GFP vs. AAV2-sgCCR5; $n = 8$ retinas per group). Bars show means ± SEM.

the possibility that other widely used gene therapies could act in part in an indirect manner via unanticipated indirect mechanisms.

Materials and Methods

ONC and Intraocular Injections. Experiments were performed at the Boston Children's Hospital with approval from the Institutional Animal Care and Use Committee. The experiments used adult male and female 129S1 wild-type mice, CCR2 conditional KO mice [B6.129(Cg)-Ccr2tm2.1Ifc/J; catalog no. 017586; The Jackson Laboratory], and CCR5 conditional KO mice (101) (B6.129P2-Ccr5tm1Kuz/J; catalog no. 005427; The Jackson Laboratory).

Surgeries for optic nerve injury and intraocular injections in mice 6 to 8 wk of age were performed under general anesthesia as described previously (8, 10). Reagents that were injected intraocularly include recombinant rat CNTF protein (0.03 to 0.5 μg/μL; 3 μL per eye; Alomone Labs); recombinant mouse CCL5 (0.1 μg/μL; 3 μL per eye; ThermoFisher Scientific); zymosan (12.5 μg/μL; sterilized before use; Sigma); recombinant rat Ocm (30 ng/μL; 3 μL per eye); the cAMP analog CPT-cAMP (50 μM; Sigma); AMD3100, a highly specific CXCR4 antagonist (100 μM; half-maximal inhibitory concentration [IC_{50}] = 0.02 to 0.13 μM; Sigma); the Ocm peptide antagonist P1 (10) (2.3 μg/μL; 3 μL per eye); AAVs expressing green fluorescent protein (AAV2-GFP), AAV2 expressing Cas9 driven by the RGC-selective promoter γ-synuclein (Sncg) (AAV2-Sncg-Cas9), and AAV2 expressing CNTF (all from Boston Children's Hospital Viral Vector Core); AAV2 expressing shRNA targeting CNTF receptor-α (AAV2-sh-CNTFRα; Vector Biolabs); AAV2 expressing a small guide RNA targeting CCR5 (AAV2-sgCCR5; Vigene Biosciences); and AAV2 expressing a small guide RNA targeting GPR75 (AAV2-sgGPR75; Vigene Biosciences). Viral vectors were injected 2 to 4 wk before ONC, whereas other reagents were introduced immediately after ONC or were injected repeatedly as noted, all in a volume of 3 μL. A highly specific CCR5 antagonist, DAPTA (10 μg/kg; IC_{50} = 0.06 nM; Selleckchem) was injected intraperitoneally daily. Cholera subunit B fragment (CTB) (Sigma) was injected intraocularly 2 d before mice were perfused to trace regenerating axons.

Immunodepletion of Neutrophils, Separation and Staining of Blood Immune Cells, and Flow Cytometry. To deplete neutrophils systemically, an anti-mouse Ly6G IgG (BE0075-1; Bio X Cell) or isotype-matched IgG2a (BE0085; Bio X Cell) was injected twice retroorbitally (100 μg; 3 d before and once after ONC) and twice intraperitoneally (200 μg immediately and 7 d after ONC) depending on experimental design using a modified protocol (56).

For separation of blood immune cells, 0.1 to 1 mL of peripheral blood was drawn from the mouse heart and gently mixed with ethylenediaminetetraacetic acid (EDTA) (0.5 M; Sigma). After centrifugation (1,000 rpm; 10 min), the pellet was mixed with RBC lysis buffer (150 mM NH_4Cl, 0.1 mM Na_2EDTA, 10 mM $KHCO_3$) for 5 to 10 min at 37 °C. Blood immune cells were then washed with phosphate-buffered saline (PBS) two times and examined by immunohistochemistry, FACS, or qPCR.

For flow cytometry, following RBC lysis, dissociated cells were incubated with blocking reagent and mouse myeloid-derived suppressor cell (MDSC) Flow Cocktail 2, composed of differentially labeled monoclonal antibodies (mAbs) to CD11b, Ly-6C, and Ly-6G (phycoerythrin [PE]-conjugated anti-CD11b, fluorescein isothiocyanate [FITC]-conjugated anti-Ly6G, and antigen-presenting cell [APC]-conjugated anti-Ly6C; no. 147003; BioLegend) on ice for 30 min. After washing with PBS three times and staining with DAPI, cells were applied to a BD FACSAria III Flow Cytometer and sorted based on the criteria of $CD11b^+Ly6G^{high}Ly6C^{intermediate}$ (neutrophils) or $CD11b^+Ly6G^{low}Ly6C^{high+intermediate}$ (monocytes).

To stain blood-derived immune cells, following RBC lysis, cells from 0.1 mL of blood were suspended with 500 μL of DMEM; 100 μL of cells were seeded onto a poly-L-lysine–coated glass coverslip for 1 h at 37 °C in a humidified atmosphere of 5% CO_2 and then fixed with 4% paraformaldehyde (PFA) for 10 min. Fixed cells were stained with anti-Gr1(MCA2387; Bio-Rad) and anti-F4/80 (MCA497RT; Bio-Rad) antibodies. Cell numbers were counted manually based on the criteria of $Gr1^{high}F4/80^{negative}$ (neutrophils) or $Ly6G^{low}F4/80^{high}$ (macrophages).

qRT-PCR Analysis for Retinas and Whole Eyes. Retinas or whole eyes or blood immune cells were isolated from mice after 2 wk of AAV2-CNTF or control vector injections. RNA was extracted with an RNeasy kit (Qiagen), and cDNA synthesis was performed using the cDNA Synthesis kit (Bio-Rad). Real-time PCR was carried out with iQSYBR Green Supermix Kit (Bio-Rad) and the following primers: CNTF-forward (F): TCTGTAGCCGCTCTATCTGG; CNTF-reverse (R): GGTACACCATCCACTGAGTCAA; CCL3-F: AGTCAGGAAAATGACACCTGGC; CCL3-R: AACATTCCTGCCACCTGCATA; CCL4-F: TTTGGTCAGGAATACCACGACT; CCL4-R: GAGGAGCCACTTCAGGAGAG; CCL5-F: AGTCGATCTCCCACAGCCTCT; CCL5-R: CAGGGTCAGAATCAAGAAACC; CCL6-F: AAAGATGATGCCCGGCTTGA; CCL6-R: TTGCTTGAGAAGGAGGGCAG; SDF1-F: ATGGACGCCAAGGTCGTCGCCGT; SDF1-R: TCGGGTCAATGCACACTTGTC; Ocm-F: CCAAGACCCAGACACCTTTGA; Ocm-R: GGCTGGCAGACATCTTGGAG; 18S-F: CGGCTACCACATCCAAGGAA; and 18S-R: GCTGGAATTACCGCGGCT.

qRT-PCR results are based on at least four replicates. The relative expression in each sample was first normalized by the level of 18S RNA and then by the value of the control group.

RNA Scope. An RNAscope probe targeting CNTFRα was synthesized by ACD Biosystems (catalog no. 457981-C3). RNAscope was carried out with the RNAscope Fluorescent Multiplex Reagent Kit (320293; ACD Biosystems) according to the ACD Biosystems protocol. An ACD 3-plex negative control

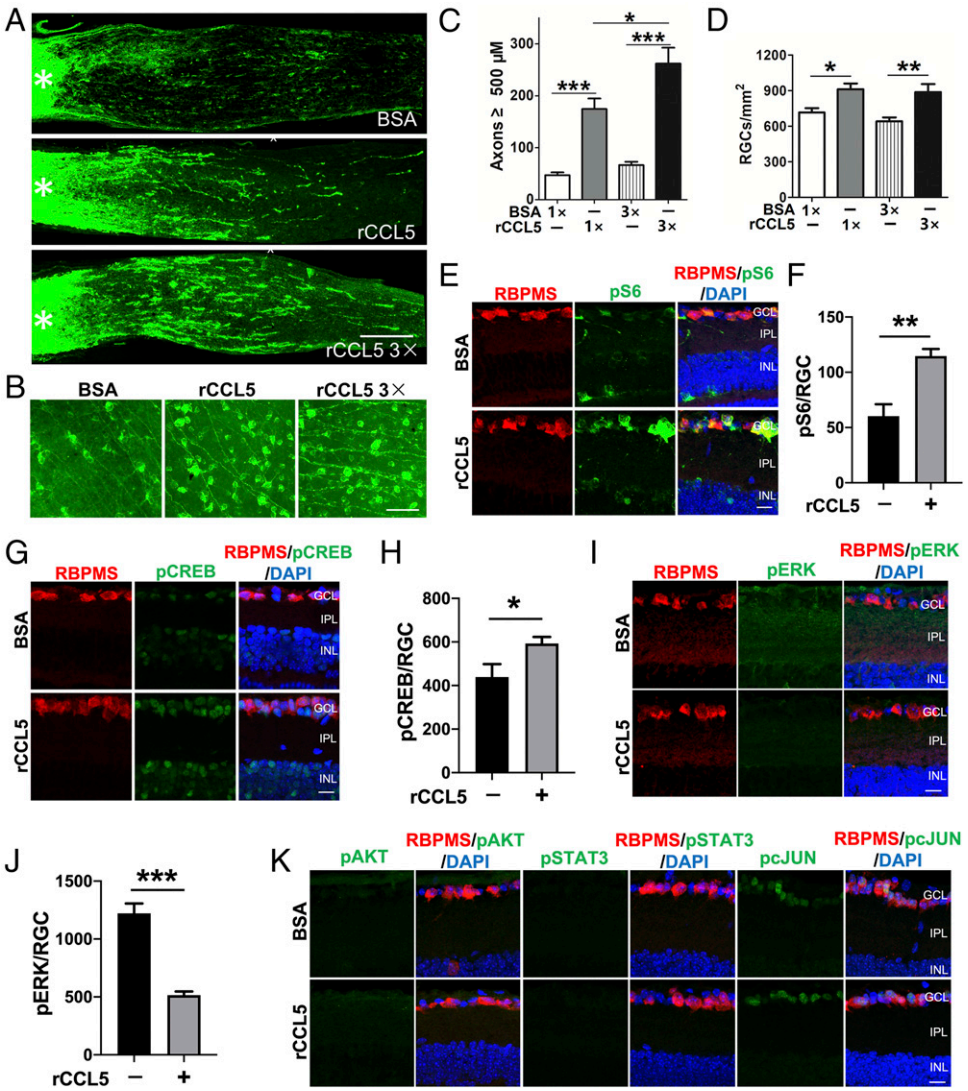

Fig. 8. CCL5 induces optic nerve regeneration in vivo. (*A–D*) rCCL5 stimulates optic nerve regeneration and RGC survival. (*A*) Axon regeneration was induced by the indicated treatments and visualized by CTB immunostaining (green). The asterisk indicates the injury site. (Scale bar, 150 μm.) (*B*) Whole-mounted retinas immunostained with antibody TUJ1$^+$ (green) to visualize surviving RGCs. (Scale bar, 60 μm.) (*C*) Quantitation of regeneration. A single intraocular injection of rCCL5 (0.1 μg/μL) immediately after NC increased regeneration (***$P < 0.001$; rCCL5 vs. BSA; $n =$ 9 to 11 nerves per group), and three injections of rCCL5 (2 d before, the day of, and 3 d after NC) doubled levels of regeneration induced by a single injection (***$P < 0.001$: rCCL5 3× vs. BSA 3×; *$P < 0.05$: rCCL5 1× vs. rCCL5 3×; $n = 9$ to 11 nerves per group). (*D*) Quantitation of RGC survival. Intraocular rCCL5 (1×) enhanced RGC survival (**$P < 0.05$; rCCL5 vs. BSA; $n = 9$ to 12 retinas per group), while multiple injections had no additional effect ($P = 1.00$; rCCL5 3× vs. rCCL5 1×; $n = 9$ to 12 retinas per group). (*E* and *F*) rCCL5 increases ribosomal protein S6 phosphorylation (pS6). (*E*) Retinal cross-sections from mice with intraocular injections of BSA or rCCL5 stained to visualize pS6 (green), RBPMS-positive RGCs (red), and cell nuclei (DAPI) (blue). (Scale bar, 13 μm.) (*F*) Quantitation. rCCL5 increased pS6 levels in RGCs. **$P < 0.01$ (rCCL5 vs. BSA; $n = 4$ retinas per group). (*G*) As in *E* but immunostained to visualize pCREB (green), RBPMS-positive RGCs (red), and cell nuclei (DAPI) (blue). (Scale bar, 13 μm.) (*H*) Quantitation. rCCL5 increased pCREB levels in RGCs. *$P < 0.05$ (rCCL5 vs. BSA; $n = 4$ retinas per group). (*I*) As in *E* but stained to visualize pERK (green), RBPMS-positive RGCs (red), and cell nuclei (DAPI) (blue). (Scale bar, 13 μm.) (*J*) Quantitation. rCCL5 decreased pERK levels in RGCs by 58%. ***$P < 0.001$ (rCCL5 vs. BSA; $n = 4$ retinas per group). (*K*) Retinal cross-sections from mice with intraocular injections of BSA or rCCL5 stained to visualize pAKT, pSTAT3, p-cJUN (green), RBPMS-positive RGCs (red), and cell nuclei (DAPI) (blue). (Scale bar, 13 μm.) Bars show means ± SEM. GCL, ganglion cell layer; INL, inner nuclear layer; IPL, inner plexiform layer.

probe was applied on one retinal section per slide to exclude nonspecific signals.

CNTFRα Colocalization Studies. All retinas to be compared were immunostained, and images were taken at the same time. Colocalization analyses were carried out by Image J software using four whole-mounted retinas per group. Mander's value (tM) was used to represent the extent of colocalization (13).

Quantitation of RGC Survival and Axon Regeneration. RGC survival and axon regeneration were quantified as described previously (57). Mice were perfused transcardially with saline and 4% PFA. Eyes and optic nerves were dissected and postfixed in 4% PFA for 1 h at room temperature (RT). Whole retinas were dissected and immunostained for βIII-tubulin (ab18207; Abcam) to distinguish RGCs from other cells in the retina (102). RGC survival was quantified in 8 to 16 predesignated fields in each retina, as described previously (6). Nerves were cryostat-sectioned longitudinally at 14 μm after transferring to 30% sucrose at 4 °C overnight. Sections were immunostained to detect CTB (GWB-7B96E4; Genway Biotech) in regenerating axons. Axons were quantified in four to eight sections per case at prespecified distances from the injury site, as described (6).

Preparation and Staining of Retinal Sections. To prepare retinal sections, eyes were collected and postfixed in 4% PFA for 1 h, transferred to 30% sucrose overnight at 4 °C, and frozen-sectioned at 14 μm. Sections were incubated with primary antibodies at 4 °C overnight after blocking with appropriate sera for 1 h at RT. After washing three times, sections were incubated with the appropriate fluorescent secondary antibody and DAPI and then mounted. Primary antibodies that were used included an anti-CNTFRα polyclonal antibody (PA5-77379; ThermoFisher Scientific), anti-SOCS3 mAb (MA1-19373; ThermoFisher Scientific), anti-CCL5 mAb (sc-373984; Santa Cruz Biotechnology), anti-CCR5 mAb (sc-17833; Santa Cruz Biotechnology), anti-GPR75 polyclonal antibody (SAB4500182; Sigma), and anti-adenylyl cyclase 3 polyclonal antibody (PA5-35382; ThermoFisher Scientific). Images were taken by a Nikon E800 microscope or a Zeiss LSM700 or Zeiss LSM710 confocal microscope.

Statistical Analyses. Values are presented as means ± SEM. Statistical significance was evaluated with one-way ANOVA, followed by Bonferroni post hoc tests or unpaired two-tailed Student's *t* tests if comparing two groups using SPSS software version 19.0 (IBM).

Data Availability. The transcriptome dataset has been deposited in the GEO database (accession no. GSE142881).

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1. T. L. Dickendesher *et al*., NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans. *Nat. Neurosci.* **15**, 703–712 (2012).
2. Y. Shen *et al*., PTPsigma is a receptor for chondroitin sulfate proteoglycan, an inhibitor of neural regeneration. *Science* **326**, 592–596 (2009).
3. K. K. Park *et al*., Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway. *Science* **322**, 963–966 (2008).
4. D. L. Moore *et al*., KLF family members regulate intrinsic axon regeneration ability. *Science* **326**, 298–301 (2009).
5. P. D. Smith *et al*., SOCS3 deletion promotes optic nerve regeneration in vivo. *Neuron* **64**, 617–623 (2009).
6. S. Leon, Y. Yin, J. Nguyen, N. Irwin, L. I. Benowitz, Lens injury stimulates axon regeneration in the mature rat optic nerve. *J. Neurosci.* **20**, 4615–4626 (2000).
7. Y. Yin *et al*., Macrophage-derived factors stimulate optic nerve regeneration. *J. Neurosci.* **23**, 2284–2293 (2003).
8. Y. Yin *et al*., Oncomodulin is a macrophage-derived signal for axon regeneration in retinal ganglion cells. *Nat. Neurosci.* **9**, 843–852 (2006).
9. T. Kurimoto *et al*., Long-distance axon regeneration in the mature optic nerve: Contributions of oncomodulin, cAMP, and pten gene deletion. *J. Neurosci.* **30**, 15654–15663 (2010).
10. Y. Yin *et al*., Oncomodulin links inflammation to optic nerve regeneration. *Proc. Natl. Acad. Sci. U.S.A.* **106**, 19587–19592 (2009).
11. S. Li *et al*., Promoting axon regeneration in the adult CNS by modulation of the melanopsin/GPCR signaling. *Proc. Natl. Acad. Sci. U.S.A.* **113**, 1937–1942 (2016).
12. J. H. Lim *et al*., Neural activity promotes long-distance, target-specific regeneration of adult retinal axons. *Nat. Neurosci.* **19**, 1073–1084 (2016).
13. Y. Li *et al*., Mobile zinc increases rapidly in the retina after optic nerve injury and regulates ganglion cell survival and optic nerve regeneration. *Proc. Natl. Acad. Sci. U.S.A.* **114**, E209–E218 (2017).
14. E. F. Trakhtenberg *et al*., Zinc chelation and Klf9 knockdown cooperatively promote axon regeneration after optic nerve injury. *Exp. Neurol.* **300**, 22–29 (2018).
15. Y. Zhang *et al*., Elevating growth factor responsiveness and axon regeneration by modulating presynaptic inputs. *Neuron* **103**, 39.e5–51.e5 (2019).
16. N. M. Tran *et al*., Single-cell profiles of retinal ganglion cells differing in resilience to injury reveal neuroprotective genes. *Neuron* **104**, 1039.e12–1055.e12 (2019).
17. A. Heskamp *et al*., CXCL12/SDF-1 facilitates optic nerve regeneration. *Neurobiol. Dis.* **55**, 76–86 (2013).
18. M. W. Norsworthy *et al*., Sox11 expression promotes regeneration of some retinal ganglion cell types but kills others. *Neuron* **94**, 1112.e4–1120.e4 (2017).
19. R. Cartoni *et al*., The mammalian-specific protein Armcx1 regulates mitochondrial transport during axon regeneration. *Neuron* **92**, 1294–1307 (2016).
20. A. Müller, T. G. Hauk, M. Leibinger, R. Marienfeld, D. Fischer, Exogenous CNTF stimulates axon regeneration of retinal ganglion cells partially via endogenous CNTF. *Mol. Cell. Neurosci.* **41**, 233–246 (2009).
21. A. Ohlmann, E. R. Tamm, Norrin: Molecular and functional properties of an angiogenic and neuroprotective growth factor. *Prog. Retin. Eye Res.* **31**, 243–257 (2012).
22. E. Y. Chew *et al*.; Macular Telangiectasia Type 2-Phase 2 CNTF Research Group, Effect of ciliary neurotrophic factor on retinal neurodegeneration in patients with macular telangiectasia type 2: A randomized clinical trial. *Ophthalmology* **126**, 540–549 (2019).
23. D. G. Birch, L. D. Bennett, J. L. Duncan, R. G. Weleber, M. E. Pennesi, Long-term follow-up of patients with retinitis pigmentosa receiving intraocular ciliary neurotrophic factor implants. *Am. J. Ophthalmol.* **170**, 10–14 (2016).
24. S. P. Squinto *et al*., Identification of functional receptors for ciliary neurotrophic factor on neuronal cell lines and primary neurons. *Neuron* **5**, 757–766 (1990).
25. T. G. Boulton, N. Stahl, G. D. Yancopoulos, Ciliary neurotrophic factor/leukemia inhibitory factor/interleukin 6/oncostatin M family of cytokines induces tyrosine phosphorylation of a common set of proteins overlapping those induced by other cytokines and growth factors. *J. Biol. Chem.* **269**, 11648–11655 (1994).
26. S. Davis *et al*., The receptor for ciliary neurotrophic factor. *Science* **253**, 59–63 (1991).
27. S. Davis *et al*., Released form of CNTF receptor alpha component as a soluble mediator of CNTF responses. *Science* **259**, 1736–1739 (1993).
28. W. M. Peterson, Q. Wang, R. Tzekova, S. J. Wiegand, Ciliary neurotrophic factor and stress stimuli activate the Jak-STAT pathway in retinal neurons and glia. *J. Neurosci.* **20**, 4081–4090 (2000).
29. S. A. Jo, E. Wang, L. I. Benowitz, Ciliary neurotrophic factor is an axogenesis factor for retinal ganglion cells. *Neuroscience* **89**, 579–591 (1999).
30. A. Müller, T. G. Hauk, D. Fischer, Astrocyte-derived CNTF switches mature RGCs to a regenerative state following inflammatory stimulation. *Brain* **130**, 3308–3320 (2007).
31. M. Leibinger *et al*., Neuroprotective and axon growth-promoting effects following inflammatory stimulation on mature retinal ganglion cells in mice depend on ciliary neurotrophic factor and leukemia inhibitory factor. *J. Neurosci.* **29**, 14334–14341 (2009).
32. V. Pernet, A. Di Polo, Synergistic action of brain-derived neurotrophic factor and lens injury promotes retinal ganglion cell survival, but leads to optic nerve dystrophy in vivo. *Brain* **129**, 1014–1026 (2006).
33. E. R. Bray *et al*., Thrombospondin-1 mediates axon regeneration in retinal ganglion cells. *Neuron* **103**, 642–657.e7 (2019).
34. S. G. Leaver *et al*., AAV-mediated expression of CNTF promotes long-term survival and regeneration of adult rat retinal ganglion cells. *Gene Ther.* **13**, 1328–1341 (2006).
35. V. Pernet *et al*., Long-distance axonal regeneration induced by CNTF gene transfer is impaired by axonal misguidance in the injured adult optic nerve. *Neurobiol. Dis.* **51**, 202–213 (2013).
36. B. J. Yungher, M. Ribeiro, K. K. Park, Regenerative responses and axon pathfinding of retinal ganglion cells in chronically injured mice. *Invest. Ophthalmol. Vis. Sci.* **58**, 1743–1750 (2017).
37. B. J. Yungher, X. Luo, Y. Salgueiro, M. G. Blackmore, K. K. Park, Viral vector-based improvement of optic nerve regeneration: Characterization of individual axons' growth patterns and synaptogenesis in a visual target. *Gene Ther.* **22**, 811–821 (2015).
38. X. Luo *et al*., Enhanced transcriptional activity and mitochondrial localization of STAT3 Co-induce axon regrowth in the adult central nervous system. *Cell Rep.* **15**, 398–410 (2016).
39. F. Bei *et al*., Restoration of visual function by enhancing conduction in regenerated axons. *Cell* **164**, 219–232 (2016).
40. M. Hellström, A. R. Harvey, Retinal ganglion cell gene therapy and visual system repair. *Curr. Gene Ther.* **11**, 116–131 (2011).
41. Y. F. Liu *et al*., Longitudinal evaluation of immediate inflammatory responses after intravitreal AAV2 injection in rats by optical coherence tomography. *Exp. Eye Res.* **193**, 107955 (2020).
42. L. P. Cen *et al*., Chemotactic effect of ciliary neurotrophic factor on macrophages in retinal ganglion cell survival and axonal regeneration. *Invest. Ophthalmol. Vis. Sci.* **48**, 4257–4266 (2007).
43. H. Kobayashi, A. P. Mizisin, CNTFR alpha alone or in combination with CNTF promotes macrophage chemotaxis in vitro. *Neuropeptides* **34**, 338–347 (2000).
44. J. Vernerey, M. Macchi, K. Magalon, M. Cayre, P. Durbec, Ciliary neurotrophic factor controls progenitor migration during remyelination in the adult rodent brain. *J. Neurosci.* **33**, 3240–3250 (2013).
45. K. K. Park *et al*., Cytokine-induced SOCS expression is inhibited by cAMP analogue: Impact on regeneration in injured retina. *Mol. Cell. Neurosci.* **41**, 313–324 (2009).
46. C. Dallner, A. G. Woods, T. Deller, M. Kirsch, H. D. Hofmann, CNTF and CNTF receptor alpha are constitutively expressed by astrocytes in the mouse brain. *Glia* **37**, 374–378 (2002).
47. L. V. Blomster *et al*., Mobilisation of the splenic monocyte reservoir and peripheral CX_3CR1 deficiency adversely affects recovery from spinal cord injury. *Exp. Neurol.* **247**, 226–240 (2013).
48. J. C. Gensel *et al*., Macrophages promote axon regeneration with concurrent neurotoxicity. *J. Neurosci.* **29**, 3956–3968 (2009).
49. L. N. Saligan, H. S. Kim, A systematic review of the association between immunogenomic markers and cancer-related fatigue. *Brain Behav. Immun.* **26**, 830–848 (2012).
50. J. Shi *et al*., The relationship of platelet to lymphocyte ratio and neutrophil to monocyte ratio to radiographic grades of knee osteoarthritis. *Z. Rheumatol.* **77**, 533–537 (2018).
51. W. Fang *et al*., CCR2-dependent monocytes/macrophages exacerbate acute brain injury but promote functional recovery after ischemic stroke in mice. *Theranostics* **8**, 3530–3543 (2018).
52. A. Paré *et al*., IL-1β enables CNS access to $CCR2^{hi}$ monocytes and the generation of pathogenic cells through GM-CSF released by CNS endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.* **115**, E1194–E1203 (2018).
53. F. Mollinedo, Neutrophil degranulation, plasticity, and cancer metastasis. *Trends Immunol.* **40**, 228–242 (2019).
54. B. Uhl *et al*., Aged neutrophils contribute to the first line of defense in the acute inflammatory response. *Blood* **128**, 2327–2337 (2016).
55. O. Soehnlein, L. Lindbom, C. Weber, Mechanisms underlying neutrophil-mediated monocyte recruitment. *Blood* **114**, 4613–4623 (2009).
56. J. M. Daley, A. A. Thomay, M. D. Connolly, J. S. Reichner, J. E. Albina, Use of Ly6G-specific monoclonal antibody to deplete neutrophils in mice. *J. Leukoc. Biol.* **83**, 64–70 (2008).
57. T. Kurimoto *et al*., Neutrophils express oncomodulin and promote optic nerve regeneration. *J. Neurosci.* **33**, 14816–14824 (2013).
58. Y. Yin *et al*., "SDF1 is highly expressed in macrophages and contributes to inflammation-induced optic nerve regeneration" in *2018 Neuroscience Meeting Planner* (Society for Neuroscience, Washington, DC, 2018), program no. 115.18/B23.
59. J. A. Burger, A. Peled, CXCR4 antagonists: Targeting the microenvironment in leukemia and other cancers. *Leukemia* **23**, 43–52 (2009).
60. J. E. Pease, Tails of the unexpected–An atypical receptor for the chemokine RANTES/CCL5 expressed in brain. *Br. J. Pharmacol.* **149**, 460–462 (2006).
61. S. W. Choi *et al*., CCR1/CCL5 (RANTES) receptor-ligand interactions modulate allogeneic T-cell responses and graft-versus-host disease following stem-cell transplantation. *Blood* **110**, 3447–3455 (2007).
62. P. M. Murphy, International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol. Rev.* **54**, 227–229 (2002).
63. C. Blanpain *et al*., A chimeric MIP-1alpha/RANTES protein demonstrates the use of different regions of the RANTES protein to bind and activate its receptors. *J. Leukoc. Biol.* **69**, 977–985 (2001).
64. W. Xue *et al*., Ciliary neurotrophic factor induces genes associated with inflammation and gliosis in the retina: A gene profiling study of flow-sorted, Müller cells. *PLoS One* **6**, e20326 (2011).
65. G. A. Bishop, N. F. Berbari, J. Lewis, K. Mykytyn, Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. *J. Comp. Neurol.* **505**, 562–571 (2007).

66. Z. Wang, T. Phan, D. R. Storm, The type 3 adenylyl cyclase is required for novel object learning and extinction of contextual memory: Role of cAMP signaling in primary cilia. *J. Neurosci.* **31**, 5557–5561 (2011).
67. A. Meyer-Franke, M. R. Kaplan, F. W. Pfrieger, B. A. Barres, Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. *Neuron* **15**, 805–819 (1995).
68. A. Meyer-Franke *et al*., Depolarization and cAMP elevation rapidly recruit TrkB to the plasma membrane of CNS neurons. *Neuron* **21**, 681–693 (1998).
69. M. Hellström *et al*., Negative impact of rAAV2 mediated expression of SOCS3 on the regeneration of adult retinal ganglion cell axons. *Mol. Cell. Neurosci.* **46**, 507–515 (2011).
70. C. J. LeVaillant *et al*., Significant changes in endogenous retinal gene expression assessed 1 year after a single intraocular injection of AAV-CNTF or AAV-BDNF. *Mol. Ther. Methods Clin. Dev.* **3**, 16078 (2016).
71. K. Flachsbarth *et al*., Pronounced synergistic neuroprotective effect of GDNF and CNTF on axotomized retinal ganglion cells in the adult mouse. *Exp. Eye Res.* **176**, 258–265 (2018).
72. K. Flachsbarth *et al*., Neural stem cell-based intraocular administration of ciliary neurotrophic factor attenuates the loss of axotomized ganglion cells in adult mice. *Invest. Ophthalmol. Vis. Sci.* **55**, 7029–7039 (2014).
73. S. W. Jeong *et al*., Human neural stem cell transplantation promotes functional recovery in rats with experimental intracerebral hemorrhage. *Stroke* **34**, 2258–2263 (2003).
74. P. Lu *et al*., Long-distance growth and connectivity of neural stem cells after severe spinal cord injury. *Cell* **150**, 1264–1273 (2012).
75. P. Lu, L. L. Jones, E. Y. Snyder, M. H. Tuszynski, Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Exp. Neurol.* **181**, 115–129 (2003).
76. S. A. Jones, B. J. Jenkins, Recent insights into targeting the IL-6 cytokine family in inflammatory diseases and cancer. *Nat. Rev. Immunol.* **18**, 773–789 (2018).
77. J. T. Liou, H. B. Yuan, C. C. Mao, Y. S. Lai, Y. J. Day, Absence of C-C motif chemokine ligand 5 in mice leads to decreased local macrophage recruitment and behavioral hypersensitivity in a murine neuropathic pain model. *Pain* **153**, 1283–1291 (2012).
78. C. Weber *et al*., Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells. *Blood* **97**, 1144–1146 (2001).
79. A. E. Proudfoot *et al*., Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines. *Proc. Natl. Acad. Sci. U.S.A.* **100**, 1885–1890 (2003).
80. D. S. Duncan *et al*., Constitutive and stress-induced expression of CCL5 machinery in rodent retina. *J. Clin. Cell. Immunol.* **8**, 506 (2017).
81. M. Krogh Nielsen *et al*., Chemokine profile and the alterations in CCR5-CCL5 axis in geographic atrophy secondary to age-related macular degeneration. *Invest. Ophthalmol. Vis. Sci.* **61**, 28 (2020).
82. D. Tripathy, L. Thirumangalakudi, P. Grammas, RANTES upregulation in the Alzheimer's disease brain: A possible neuroprotective role. *Neurobiol. Aging* **31**, 8–16 (2010).
83. D. S. Duncan *et al*., Ccl5 mediates proper wiring of feedforward and lateral inhibition pathways in the inner retina. *Front. Neurosci.* **12**, 702 (2018).
84. J. Rodger, E. S. Drummond, M. Hellström, D. Robertson, A. R. Harvey, Long-term gene therapy causes transgene-specific changes in the morphology of regenerating retinal ganglion cells. *PLoS One* **7**, e31061 (2012).
85. V. Avdoshina, F. Biggio, G. Palchik, L. A. Campbell, I. Mocchetti, Morphine induces the release of CCL5 from astrocytes: Potential neuroprotective mechanism against the HIV protein gp120. *Glia* **58**, 1630–1639 (2010).
86. A. B. Patel, I. Tsilioni, S. E. Leeman, T. C. Theoharides, Neurotensin stimulates sortilin and mTOR in human microglia inhibitable by methoxyluteolin, a potential therapeutic target for autism. *Proc. Natl. Acad. Sci. U.S.A.* **113**, E7049–E7058 (2016).
87. M. J. Stables *et al*., Transcriptomic analyses of murine resolution-phase macrophages. *Blood* **118**, e192–e208 (2011).
88. W. Rostène, P. Kitabgi, S. M. Parsadaniantz, Chemokines: A new class of neuromodulator? *Nat. Rev. Neurosci.* **8**, 895–903 (2007).
89. A. Pittaluga, CCL5-glutamate cross-talk in astrocyte-neuron communication in multiple sclerosis. *Front. Immunol.* **8**, 1079 (2017).
90. K. A. Kigerl, V. M. McGaughy, P. G. Popovich, Comparative analysis of lesion development and intraspinal inflammation in four strains of mice following spinal contusion injury. *J. Comp. Neurol.* **494**, 578–594 (2006).
91. Q. Cui, S. I. Hodgetts, Y. Hu, J. M. Luo, A. R. Harvey, Strain-specific differences in the effects of cyclosporin A and FK506 on the survival and regeneration of axotomized retinal ganglion cells in adult rats. *Neuroscience* **146**, 986–999 (2007).
92. J. M. Luo *et al*., Influence of macrophages and lymphocytes on the survival and axon regeneration of injured retinal ganglion cells in rats from different autoimmune backgrounds. *Eur. J. Neurosci.* **26**, 3475–3485 (2007).
93. H. Matsumoto *et al*., Strain difference in photoreceptor cell death after retinal detachment in mice. *Invest. Ophthalmol. Vis. Sci.* **55**, 4165–4174 (2014).
94. J. P. Templeton *et al*., Differential response of C57BL/6J mouse and DBA/2J mouse to optic nerve crush. *BMC Neurosci.* **10**, 90 (2009).
95. X. Duan *et al*., Subtype-specific regeneration of retinal ganglion cells following axotomy: Effects of osteopontin and mTOR signaling. *Neuron* **85**, 1244–1256 (2015).
96. M. Samson *et al*., Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**, 722–725 (1996).
97. J. He *et al*., CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385**, 645–649 (1997).
98. I. Sánchez, B. D. Dynlacht, Cilium assembly and disassembly. *Nat. Cell Biol.* **18**, 711–717 (2016).
99. B. K. A. Choi, P. M. D'Onofrio, A. P. Shabanzadeh, P. D. Koeberle, Stabilization of primary cilia reduces abortive cell cycle re-entry to protect injured adult CNS neurons from apoptosis. *PLoS One* **14**, e0220056 (2019).
100. M. T. Joy *et al*., CCR5 is a therapeutic target for recovery after stroke and traumatic brain injury. *Cell* **176**, 1143.e13–1157.e13 (2019).
101. W. A. Kuziel *et al*., CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. *Atherosclerosis* **167**, 25–32 (2003).
102. Q. Cui, H. K. Yip, R. C. Zhao, K. F. So, A. R. Harvey, Intraocular elevation of cyclic AMP potentiates ciliary neurotrophic factor-induced regeneration of adult rat retinal ganglion cell axons. *Mol. Cell. Neurosci.* **22**, 49–61 (2003).