CNTF-mediated protection of photoreceptors requires initial activation of the cytokine receptor gp130 in Müller glial cells

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Ciliary neurotrophic factor (CNTF) acts as a potent neuroprotective agent in multiple retinal degeneration animal models. Recently, CNTF has been evaluated in clinical trials for the inherited degenerative disease retinitis pigmentosa (RP) and for dry age-related macular degeneration (AMD). Despite its potential as a broad-spectrum therapeutic treatment for blinding diseases, the target cells of exogenous CNTF and its mechanism of action remain poorly understood. We have shown previously that constitutive expression of CNTF prevents photoreceptor death but alters the retinal transcriptome and suppresses visual function. Here, we use a lentivirus to deliver the same secreted human CNTF used in clinical trials to a mouse model of RP. We found that low levels of CNTF halt photoreceptor death, improve photoreceptor morphology, and correct opsin mislocalization. However, we did not detect corresponding improvement of retinal function as measured by the electroretinogram. Disruption of the cytokine receptor *gp130* gene in Müller glia reduces CNTF-dependent photoreceptor survival and prevents phosphorylation of STAT3 and ERK in Müller glia and the rest of the retina. Targeted deletion of *gp130* in rods also demolishes neuroprotection by CNTF and prevents further activation of Müller glia. Moreover, CNTF elevates the expression of LIF and endothelin 2, thus positively promoting Müller and photoreceptor interactions. We propose that exogenous CNTF initially targets Müller glia, and subsequently induces cytokines acting through gp130 in photoreceptors to promote neuronal survival. These results elucidate a cellular mechanism for exogenous CNTF-triggered neuroprotection and provide insight into the complex cellular responses induced by CNTF in diseased retinas.

Ciliary neurotrophic factor (CNTF) belongs to a small subfamily of cytokines and has long been recognized as a potent neuroprotective molecule in the vertebrate retina (1). Enhancement of photoreceptor survival by CNTF has been demonstrated in multiple animal models of retinal degeneration, ranging from zebrafish to canine (2). Interestingly, CNTF is effective in rescuing retinal degeneration due to various causes, including mutations in genes expressed by photoreceptors or the retinal pigment epithelium (RPE), as well as those induced by strong light, neurotoxins, or antibodies. In addition, CNTF prolongs the survival of retinal ganglion cells (3–5) and promotes axonal growth in optic nerve crush or transection models (6–8). Furthermore, CNTF may affect the physiology and survival of RPE cells (9, 10), which are critical in normal vision and retinal degenerative diseases. Based on its significant and broad neuroprotective effects in damaged retinas, a secreted form of human CNTF delivered from an encapsulated cell device has been tested in clinical trials and approved by the Food and Drug Administration (FDA) to treat retinitis pigmentosa (RP) and geographic atrophy (GA), a subset of age-related macular degeneration (AMD) (11–14). However, despite its clinical significance, the mechanisms of CNTF-elicited neuroprotection in diverse retinal pathological conditions remain unclear.

CNTF interacts with a tripartite receptor complex consisting of two single-pass membrane spanning receptor subunits (gp130 and LIFRβ) and an additional ligand-specific α receptor (CNTFRα) (15). Binding of CNTF to its receptor complex activates the Jak-STAT (16), the ERK (17), and the PI3K (18) pathways. In developing and mature rodent retinas, the CNTF family of cytokines stimulates Jak-STAT and ERK signaling (19–23). Previous studies have revealed that the dosage and duration of CNTF treatment critically affect the function of rescued neurons. Rodent retinal degeneration mutants treated with recombinant adeno-associated virus (rAAV) constitutively expressing CNTF show suppression of retinal function as measured by electroretinogram (ERG) despite the robust preservation of photoreceptors (24–26). Exposure of normal rabbit retinas with high-dose CNTF-secreting capsules similarly led to diminished cone ERGs (27). Molecular and cellular analyses have demonstrated that persistent CNTF-induced signaling alters the retinal transcriptome, including reduced expression of genes involved in phototransduction and severe suppression of cone opsins (25, 28). However, single intravitreal injection of CNTF results in transient but reversible shortening of the photoreceptor outer segments and perturbation of phototransduction genes (29).

To understand the mechanism of CNTF-triggered neuroprotection, we used a lentivirus to deliver human CNTF (hCNTF) to a transgenic mouse model carrying the *rds/peripherin P216L* mutation (30), which causes dominant RP in humans. We show that low levels of hCNTF not only prolong cell survival but also

Significance

The cytokine CNTF has been approved by the FDA as a neuroprotective treatment for major retinal degenerative diseases. However, the mechanism of CNTF-triggered protection and CNTF-responsive cells in the retina remains unknown. Using molecular genetic analyses in a retinal degeneration mouse model, we identify the Müller glial cell as the direct initial target of exogenous CNTF signals. We provide evidence that CNTF signals stimulate a Müller glia and photoreceptor intercellular signaling loop involved in neuronal survival. Nonetheless, despite the marked improvement in photoreceptor morphology and survival, CNTF treatment does not result in a corresponding improvement of visual function. These results provide insight into the mechanism of CNTF action in the diseased retinas relevant to its clinical applications.

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improve the morphology of photoreceptors harboring the *rds/P216L* mutation. In addition, using conditional gene deletions, we identified the target cell type for the exogenous hCNTF and the cell-specific requirements for the cytokine receptor gp130. We provide evidence that exogenous CNTF initially signals through gp130 in Müller glia, which subsequently triggers the production of neuroprotective signal(s) that require *gp130* in rod photoreceptors for prevention of cell death.

Results

Lentiviral-Mediated hCNTF Expression from RPE. We constructed a CNTF-expressing lentiviral vector (LV-hCNTF) that carries the cytomegalovirus (CMV) immediate-early promoter followed by a human CNTF cDNA encoding a secreted and modified CNTF identical to that used in the clinical trials (12) (Fig. 1*A*). The control lentivirus (LV-IG) encodes only the internal ribosomal entry site (IRES) and GFP downstream of the CMV promoter (31) (Fig. 1*A*). Western blots confirmed that hCNTF was indeed secreted into the culture media of HEK cells transfected with the LV-hCNTF plasmid (Fig. 1*B*). Immunolabeling showed that LV-hCNTF-transduced cells coexpressed CNTF and GFP, in contrast to LV-IG–transduced cells, which expressed only GFP (Fig. 1*C*). To measure the levels of CNTF production, ARPE19 cells infected by lentiviruses were analyzed by ELISA (Fig. 1*D*). Within a 24-h period, 170.0 ± 44.6 pg of hCNTF was produced from 1.5×10^5 cells, with infection rates at 90%.

To examine lentiviral-mediated CNTF expression in vivo, mature eyes were injected subretinally with LV-IG or LV-hCNTF. Immunolabeling detected GFP expression as early as 72 h after infection (Fig. S1 and Table S1). By day 7 after injection, GFP expression was localized to the RPE for both LV-IG– and LV-hCNTF–injected eyes, whereas low levels of widespread CNTF signals were detected in LV-hCNTF–injected eyes only (Fig. 1 *E* and *F*). ELISA analysis showed that 10 d after subretinal injection, LV-hCNTF–infected *rds/P216L* eyes contained 19.3 ± 5.0 pg/eye of hCNTF at postnatal day 35 (P35) (Fig. 1*D*), whereas control virus LV-IG–infected eyes did not have detectable hCNTF.

Effects of hCNTF on Photoreceptor Survival and Morphology. We next examined whether CNTF secreted from viral infected RPE could protect photoreceptors from degeneration in *rds/P216L* mutant mice (30). At P25, the *rds/P216L* retina displayed 20–30% fewer nuclei in the outer nuclear layer (ONL) with shorter inner and outer segments compared with the WT retina (Fig. 2 *A* and *B*). By P52, only two to three rows of photoreceptor nuclei with severely deteriorated outer segments remained in the mutant retinas (Fig. 2 *C* and *G*). Strikingly, *rds/P216L* retinas infected with LV-hCNTF at P25 maintained the thickness of the ONL by P52 (Fig. 2 *B* and *E*). Morphometric quantification confirmed a pan-retinal rescue of the ONL in LV-hCNTF–injected eyes compared with LV-IG–injected eyes (Fig. 2*J*). Moreover, both histological and transmission electron microscopy (TEM) analyses revealed a significant lengthening of both the inner and outer segments in retinas expressing hCNTF (Fig. 2 *E*, *I*, and *K*). Interestingly, injection of the control LV-IG virus also resulted in a detectable but statistically nonsignificant enhancement of the inner and outer segments (Fig. 2 *C*, *D*, *G*, *H*, and *K*). The improvement of photoreceptor morphology was also observed in mutant retinas analyzed at P108 (Fig. S2*A*). These results demonstrate that low levels of exogenous CNTF protect photo-

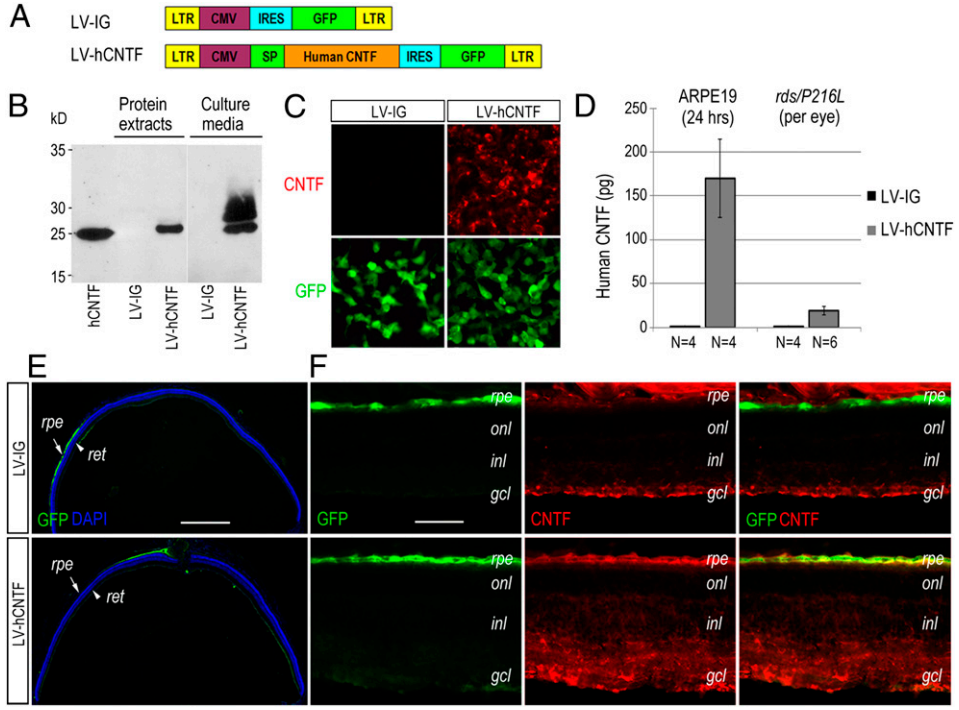

Fig. 1. Lentivirus-mediated expression of human CNTF in vitro and in vivo. (*A*) Schematics of lentiviruses encoding the CMV promoter followed by IRES-GFP (LV-IG) or human *CNTF* with a signal peptide (SP) and IRES-GFP (LV-hCNTF). (*B*) Western blot of cell extracts or culture media from HEK 293T cells transfected with LV-IG or LV-hCNTF probed for CNTF. The left lane was loaded with 100 ng of recombinant human CNTF. (*C*) Immunolabeling of 293T cells infected with LV-IG or LV-hCNTF for 48 h against GFP (green) and CNTF (red). (*D*) ELISA for hCNTF output from infected ARPE-19 cells (1.5×10^5 cells, between 48 and 72 h after infection) and hCNTF levels in *rds/P216L* eyes 10 d after subretinal injection with LV-IG or LV-hCNTF at P25. (*E* and *F*) Immunolabeling for GFP and CNTF of *rds/P216L* eyes 5 d after LV-IG or LV-hCNTF injections at P27. [Scale bars, (*E*) 500 and (*F*) 50 μm.] *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *onl*, outer nuclear layer; *rpe*, retinal pigment epithelium.

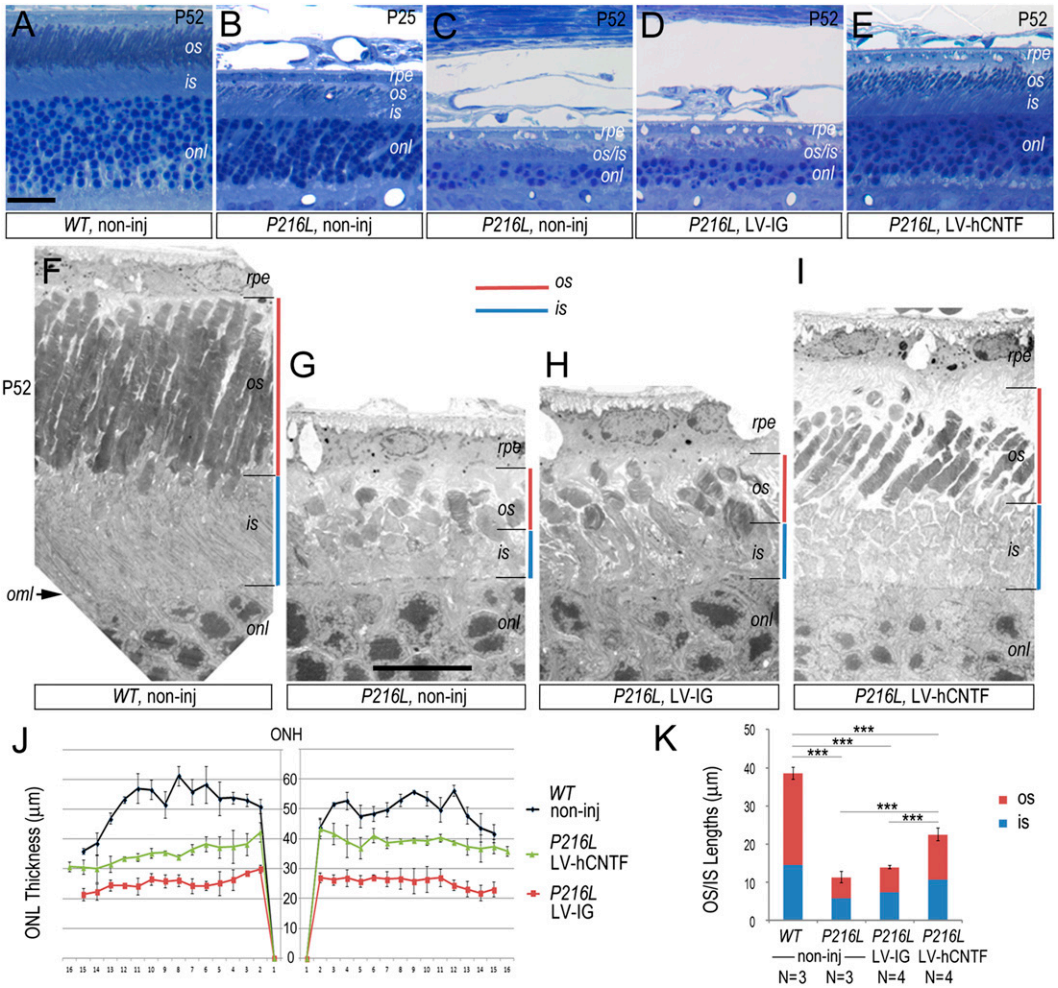

Fig. 2. Lentiviral-mediated CNTF expression prevents degeneration and improves the morphology of mutant photoreceptors. (*A–E*) Histological staining by toluidine blue shows degeneration caused by the *rds/P216L* mutation and the protective effect of LV-hCNTF. Viral infections were performed at P25 and eyes analyzed by P52. (*F–I*) TEM analysis reveals improved photoreceptor morphology of LV-hCNTF infected *rds/P216L* retinas. (*J*) Quantification of the ONL thickness at P45 from the optic nerve head (ONH) to the ciliary margin for the WT and *rds/P216L* mutant injected with LV-IG or LV-hCNTF at P25 ($n = 3$ for all). (*K*) Quantification of IS/OS lengths at P45 for WT and *rds/P216L* mutant photoreceptors with or without lentivirus infections. Statistically significant sample pairs are indicated. ***$P < 0.001$. [Scale bars, (*A–E*) 50 and (*F–I*) 10 µm.] *is*, inner segment; *olm*, outer limiting membrane; *onl*, outer nuclear layer; *os*, outer segment; *rpe*, retinal pigment epithelium.

receptors from degeneration and improve the morphology of photoreceptors carrying the *rds/P216L* mutation.

Effects of CNTF on Opsin Localization, Photoreceptor Gene Expression, and ERG. Previous studies showed that constitutive production of hCNTF from a strong promoter encoded by rAAV suppressed cone opsin expression and the ERG (25, 28). Therefore, we examined whether lentivirus-mediated hCNTF expression from RPE had similar effects. Immunocytochemistry showed that rhodopsin was mislocalized to the cell somata in the ONL of the LV-IG–injected *rds/P216L* retinas (Fig. 3 *B* and *C*). Similarly, M-opsin was mislocalized in the cone inner segments, somata, and the synaptic layer of the mutant retinas (Fig. 3 *E* and *F*). In contrast, LV-hCNTF–transduced *rds/P216L* mutant eyes not only retained rhodopsin and M-opsin expression, but also showed a surprising correction of opsin mislocalization found in the mutant (Fig. 3 *D* and *G*). Furthermore, labeling of whole mount retinas revealed an overall increase in peanut agglutinin (PNA) signals and elongation of cone outer segments under the influence of hCNTF (Fig. S2 *B* and *C*). However, we still observed a local reduction of M-opsin expression in the temporal and ventral quadrant near the injection site (Fig. 3 *I–P*). Furthermore, real-time PCR analyses showed that compared with LV-IG–injected controls, LV-hCNTF significantly reduced expression of cone-specific genes *M-opsin, S-opsin* and *Gnat2*, whereas no significant CNTF effects were found for rod-specific genes *Rhodopsin* and *Gnat1,* as well as genes expressed by both rod and cone cells, including *Crx, Nrl,* and *Nr2e3* in the *rds* retinas (Fig. 3*Q* and Tables S2 and S3).

We performed ERG assays to determine how LV-hCNTF affected retinal function. Representative ERG waveforms obtained from noninjected WT and *rds/P216L*, as well as virus-injected, mice are shown in Fig. S3*A*. Under scotopic conditions, LV-IG– and LV-hCNTF–injected eyes showed dramatic and highly significant reductions (up to 40–50%) in response amplitude compared with noninjected control eyes at both the level of the a- (Fig. 4 *B* and *D* and Tables S4 and S7) and b- (Fig. 4 *A* and *D* and Tables S5 and S7) waves. However, differences in a- or b-wave amplitudes between LV-IG– and LV-hCNTF–injected eyes were not statistically significant, although there was a trend in the direction of weaker signals in LV-hCNTF–injected eyes compared with the LV-IG–injected control eyes (Fig. 4 *A*, *B*, and *D* and Tables S4, S5, and S7). Correspondingly, b-wave amplitudes under photopic conditions were also reduced in LV-IG– and LV-hCNTF–injected eyes compared with noninjected *rds/P216L* eyes

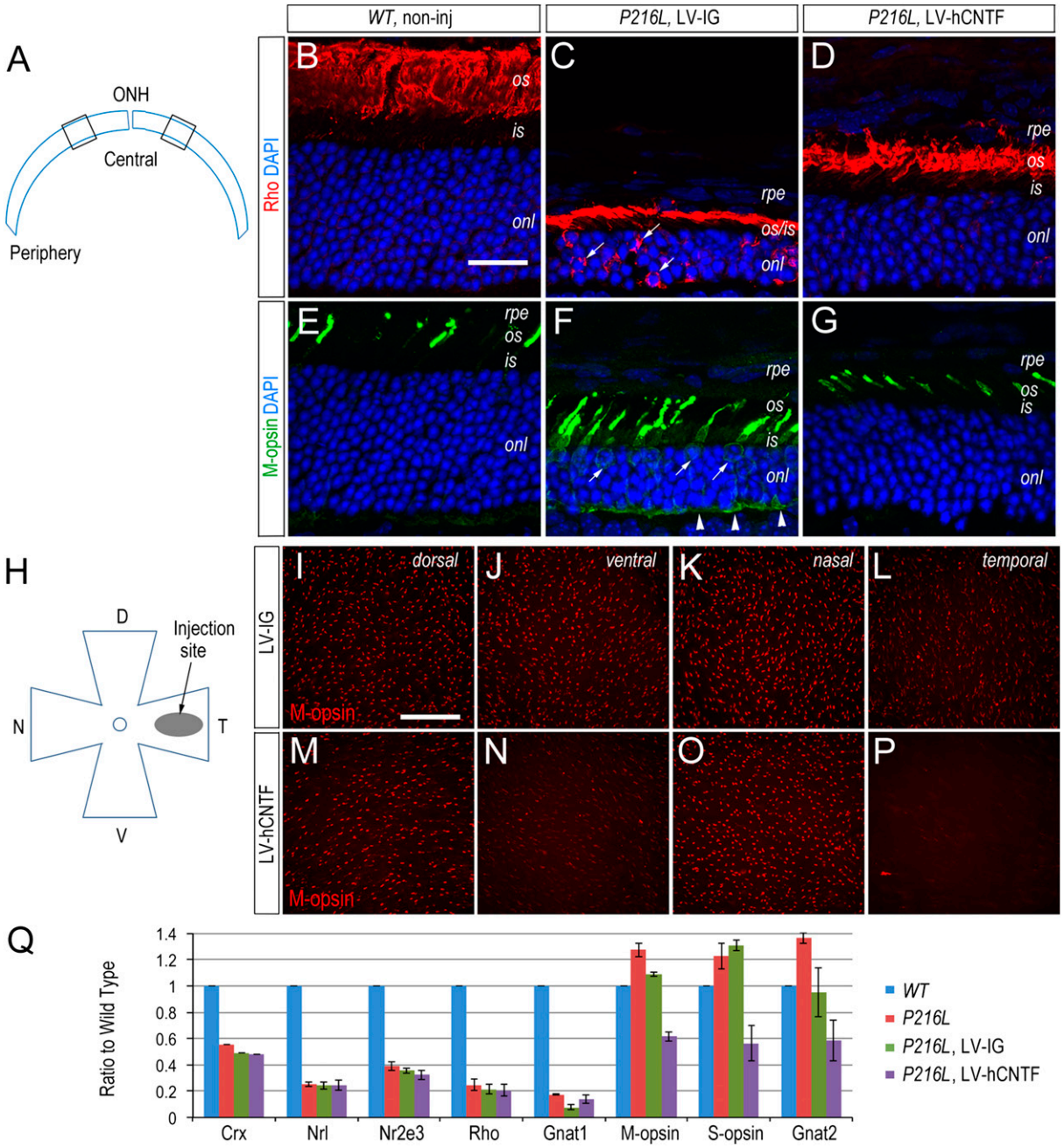

Fig. 3. Exogenous CNTF corrects mislocalization of opsins and affects photoreceptor gene expression. (*A*) A diagram shows where images were obtained from an adult retinal section. (*B*–*G*) Confocal micrographs of immunolabeled P45 retinas for rhodopsin (*B*–*D*) or M-opsin (*E*–*G*) merged with DAPI images. Viral injections for *rds* retinas were performed at P25. Arrows and arrowheads indicate mislocalized opsins in cell soma and synapses, respectively. (*H*) A diagram of a flat mount adult retina showing the injection site. (*I*–*P*) Images of flat mount *rds/P216L* retinas injected at P25 with LV-IG or LV-hCNTF and immunolabeled for M-opsin at P45. (*Q*) Quantification of photoreceptor transcripts by real-time PCR at P52 ($n = 3$ for all). For statistics, see Table S3. [Scale bars, (*B*–*G*) 20 and (*I*–*P*) 100 μm.] *is*, inner segment; *onl*, outer nuclear layer; *os*, outer segment; *rpe*, retinal pigment epithelium.

(Fig. 4*C* and Table S6). However, only at the brightest stimulus intensities did the difference between noninjected *rds/P216L* eyes and the LV-hCNTF–injected eyes reach statistical significance (Fig. 4*D* and Tables S4–S7), which may reflect the suppression by hCNTF on cone gene expression (Fig. 3*Q* and Table S3). Because injection of the control LV-IG virus or the vehicle resulted in similar ERG reductions (Fig. S3*B* and Tables S8–S10), these findings suggest that the reduced response amplitudes in injected eyes were largely due to the traumatic effects of the subretinal injection itself and not from the effects of CNTF. Nonetheless, hCNTF treatment did not result in an improved ERG response as one might have expected from the marked improvement of photoreceptor morphology.

Effects of *gp130* Deletion in Müller Cells on CNTF-Mediated Neuroprotection and Signaling. Intravitreal injection of recombinant CNTF resulted in rapid STAT3 phosphorylation in Müller glia in the *rds/P216L* retina (Fig. 5 *A* and *B*), suggesting that Müller cells could be direct targets for exogenous CNTF. To test this hypothesis, we ablated the prerequisite cytokine receptor

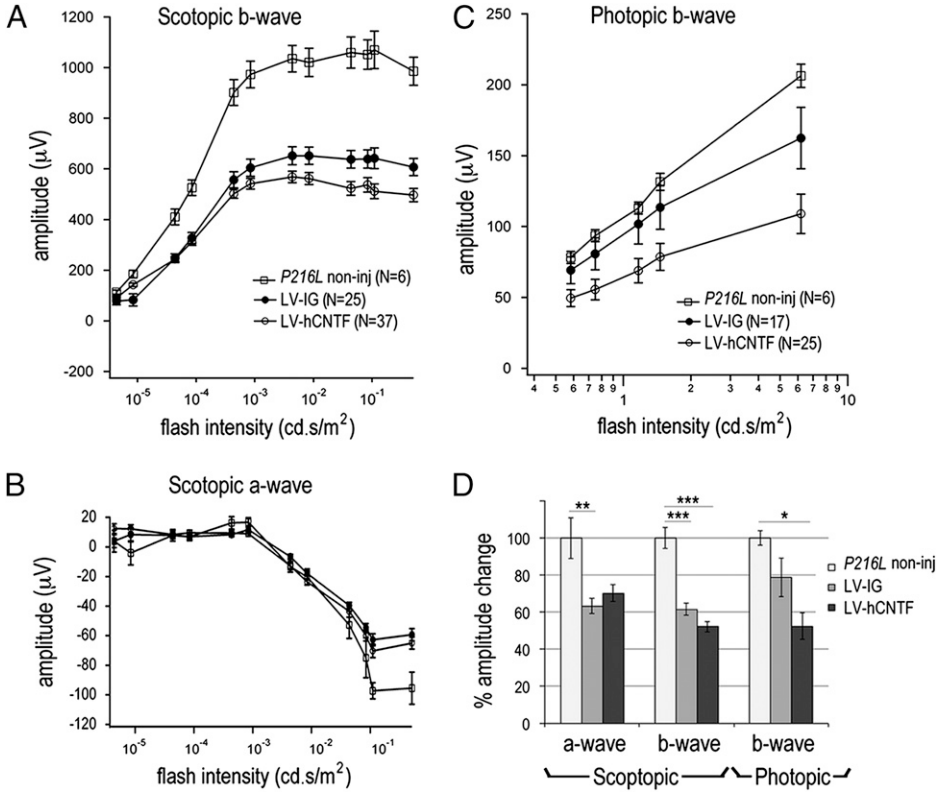

Fig. 4. ERG analyses of lentiviral transduced *rds/P216L* mutant retinas. (*A* and *B*) Scotopic b-wave (*A*) and a-wave (*B*) amplitudes at P45 are shown for noninjected *rds/P216L* eyes ($n = 6$), and *rds/P216L* eyes injected at P25 with LV-IG ($n = 25$) or LV-hCNTF ($n = 37$). (*C*) Photopic b-wave amplitudes recorded at P45 are shown for noninjected *rds/P216L* eyes ($n = 6$), and *rds/P216L* mutants injected at P25 with LV-IG ($n = 17$) or LV-hCNTF ($n = 25$). (*D*) Changes of ERG responses relative to noninjected *rds/P216L* mutants at the highest scotopic or photopic stimuli. $^*P < 0.02$; $^{**}P < 0.01$; $^{***}P < 0.001$. Detailed statistics for ERG are shown in Tables S4–S7.

gp130 (32) in Müller cells using a *GFAP-Cre* driver mouse (33). When crossed with ROSA.EYFP reporter mice (34), YFP and the Müller marker CycD3 were coexpressed (Fig. 5*C*). FACS analysis showed that $4.3 \pm 0.3\%$ of retinal cells expressed glutamine synthetase (GS), a Müller glial marker, and $59.3 \pm 0.5\%$ of the GS-positive cells also expressed YFP.

We delivered LV-hCNTF subretinally into the *rds/P216L* mice with the genotype of *gp130*$^{fl/+}$*;GFAP-Cre*, which served as controls, or *gp130* Müller conditional KO (cKO) mutant with the genotypes of *gp130*$^{fl/fl}$*;GFAP-Cre (gp130 Müller cKO)*. ROSA. YFP indicated extensive Cre activity in Müller cells in both *gp130*$^{+/-}$ and *gp130 Müller cKO* retinas (Fig. 5 *D*, *E*, *H*, and *I*). Morphometric quantification detected significant reduction of ONL thickness in *gp130 Müller cKO* retinas compared with the *gp130* heterozygous controls (Fig. 5*L*). Immunolabeling for the major CNTF downstream signaling events, the phosphorylation of STAT3 and ERK, detected broad distribution of phospho-STAT3 (pSTAT3) and phospho-ERK (pERK) in the LV-hCNTF–injected *gp130* heterozygous retinas (Fig. 5 *F* and *G*). However, in *gp130 Müller cKO* retinas, CNTF-induced STAT3 and ERK activation was severely dampened (Fig. 5 *J* and *K*). Furthermore, Western blots showed that recombinant CNTF-induced phosphorylation of STAT3 was attenuated in *gp130 Müller cKO* retinas compared with *gp130* heterozygous *rds/P216L* eyes (Fig. 5*M*). Together, these results indicate that a functional gp130 receptor is required in Müller glia for CNTF-mediated neuroprotection of photoreceptors, and deletion of *gp130* in Müller glia prevents activation of CNTF-triggered signaling pathways in the rest of the retina.

Requirement of gp130 in Rod Photoreceptors for Neuroprotection. Although CNTF initially up-regulated pSTAT3 in Müller glia, long-term CNTF expression via LV-hCNTF led to increased pSTAT3 in the entire retina including the ONL (Fig. 5*F*). In addition, the *rds/P216L* retina exposed to CNTF for 24 h also showed pSTAT3 distribution in the ONL (Fig. 6 *A* and *B*). To determine whether neuroprotection by CNTF required gp130 function in rod photoreceptors, we analyzed CNTF effects in the *rds/P216L* retina with *gp130* deletion in rods using a transgenic line with the rhodopsin promoter driving Cre (Rho-iCre) (35). In both the *gp130*$^{fl/+}$*;Rho-iCre* and *gp130*$^{fl/fl}$*;Rho-iCre* (*gp130 Rod cKO*) retinas injected with LV-hCNTF, the ROSA YFP reporter showed robust ONL expression (Fig. 6 *C* and *D*). However, CNTF-dependent photoreceptor rescue was severely diminished in the *gp130 Rod cKO rds/P216L* retinas but remained intact in the *gp130* heterozygous controls (Fig. 6 *C*, *D*, *F*, and *G*). Quantification further confirmed the significant reduction of ONL thickness in *gp130 Rod cKO* retinas compared with the heterozygous control mice injected with LV-hCNTF (Fig. 6*K*). Moreover, in contrast to the inner retina and RPE, the *gp130 Rod cKO* retina did not show elevated pSTAT3 in ONL rod cells in response to hCNTF (Fig. 6 *E*–*G*, *E′*–*G′*, and *E″*–*G″*). These results indicate that the gp130 receptor is required in rod cells for STAT3 activation and for CNTF-dependent neuronal survival.

In the control *rds* retina, colabeling by pERK and GS detected a further activation of Müller glia by hCNTF as revealed by more robust Müller cell processes in the ONL, inner nuclear layer (INL), and in the ganglion cell layer (Fig. 6 *H* and *I*). Interestingly, in *gp130 Rod cKO* retinas, the GS signals labeling Müller glia were substantially reduced in the ONL and INL (Fig. 6 *I* and *J*), suggesting that *gp130* is required in rod cells to sustain

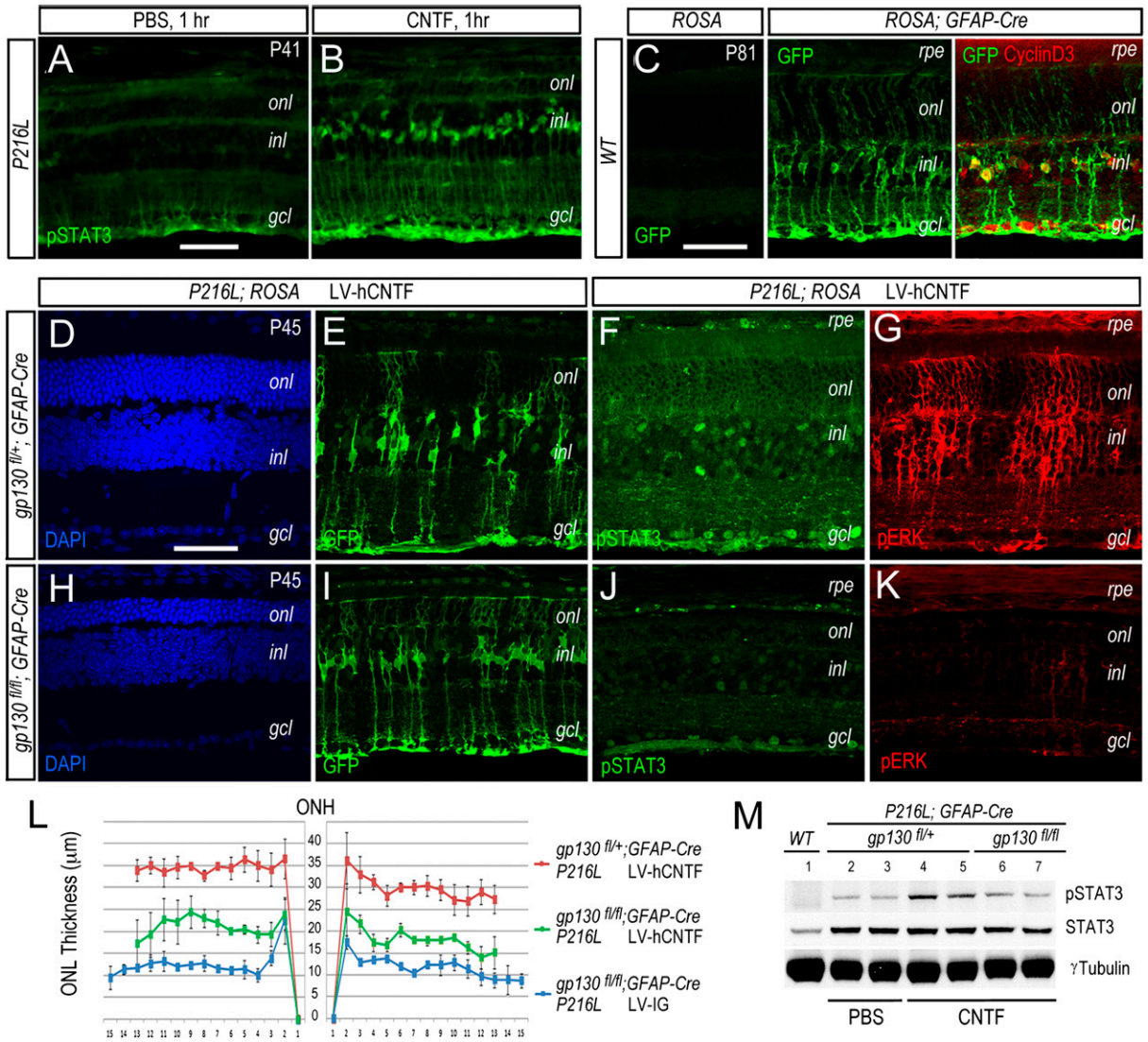

Fig. 5. Deletion of *gp130* in Müller glia reduces neuroprotection and blocks activation of STAT and ERK by CNTF. (*A* and *B*) Immunolabeling for pSTAT3 in *rds/P216L* mutant retinas 1 h after PBS (*A*) or CNTF (*B*) injection at P41. (*C*) Immunolabeling of *ROSA-YFP* retinas for GFP and Cyclin D3 at P81 in the presence or absence of *GFAP-Cre*. (*D*–*K*) Confocal images of *P216L; ROSA* retinas injected with LV-hCNTF at P25 and labeled at P45 for DAPI (*D* and *H*), GFP (*E* and *I*), pSTAT3 (*F* and *J*), and pERK (*G* and *K*). (*L*) Quantification of ONL thickness for the control *rds/P216L* eyes injected with LV-hCNTF ($n = 3$) and *gp130* Müller cKO eyes injected with LV-IG ($n = 2$) or LV-hCNTF ($n = 3$). (*M*) Western blot of retinal extracts from *rds/P216L* eyes that were *gp130* Müller cKO or $gp130^{+/-}$ injected with PBS or CNTF for 1 h and probed for pSTAT3, STAT3, and γ Tubulin. Except for noninjected WT, extracts from two individual injected eyes are shown. [Scale bars, (*A*, *C*, and *D*), 50 μm.] *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *onl*, outer nuclear layer; *rpe*, retinal pigment epithelium.

CNTF-triggered Müller activation. To examine whether CNTF altered expression of signaling molecules in the retina, we performed real-time PCR analyses 27 d after viral injection. As expected, glial fibrillary acidic protein (GFAP) mRNA was elevated in the *rds/P216L* retinas (Fig. 7*A* and Table S11). Compared with age-matched WT retinas, the noninjected *rds/P216L* retinas expressed higher levels of transcripts for CNTF and cardiotrophin-1 (CT-1), but without significant changes for leukemia inhibitory factor (LIF), cardiotrophin-like cytokine (CLC) and oncostatin M (OSM) (Fig. 7*A* and Table S11). In viral injected *rds* retinas, LV-hCNTF transduction further increased expression of endothelin 2 (EDN2) and brain-derived neurotrophic factor (BDNF), compared with LV-IG injection (Fig. 7*A* and Table S11). Interestingly, intravitreal injections of recombinant CNTF caused a rapid but transient increase of LIF transcripts, whereas a further increase of endogenous CNTF was detected 24 h later (Fig. 7*B* and Table S12).

Discussion

Müller glial cells have the same developmental origin as retinal neurons and share many transcripts expressed by retinal progenitor cells (36, 37). In the mature retina, Müller glia play important roles in maintaining retinal network homeostasis and integrity and undergo reactive gliosis in response to stress and damage (38, 39). In the zebrafish and chicken, Müller glia have been identified as a source of endogenous stem cells able to repair damaged retinas (40–42). It is also known that Müller glia in the mature retina produce multiple signaling molecules that could critically influence retinal neuronal activity, survival, and repair (22, 43–45). In this study, we provide genetic evidence that the protection of photoreceptors by exogenous hCNTF relies on initial signaling through the gp130 receptor in Müller glia. Conditional deletion of *gp130* in Müller glia significantly decreases CNTF-dependent protection (Fig. 7*C*). Importantly, CNTF-induced STAT3 and ERK activation is severely attenuated throughout

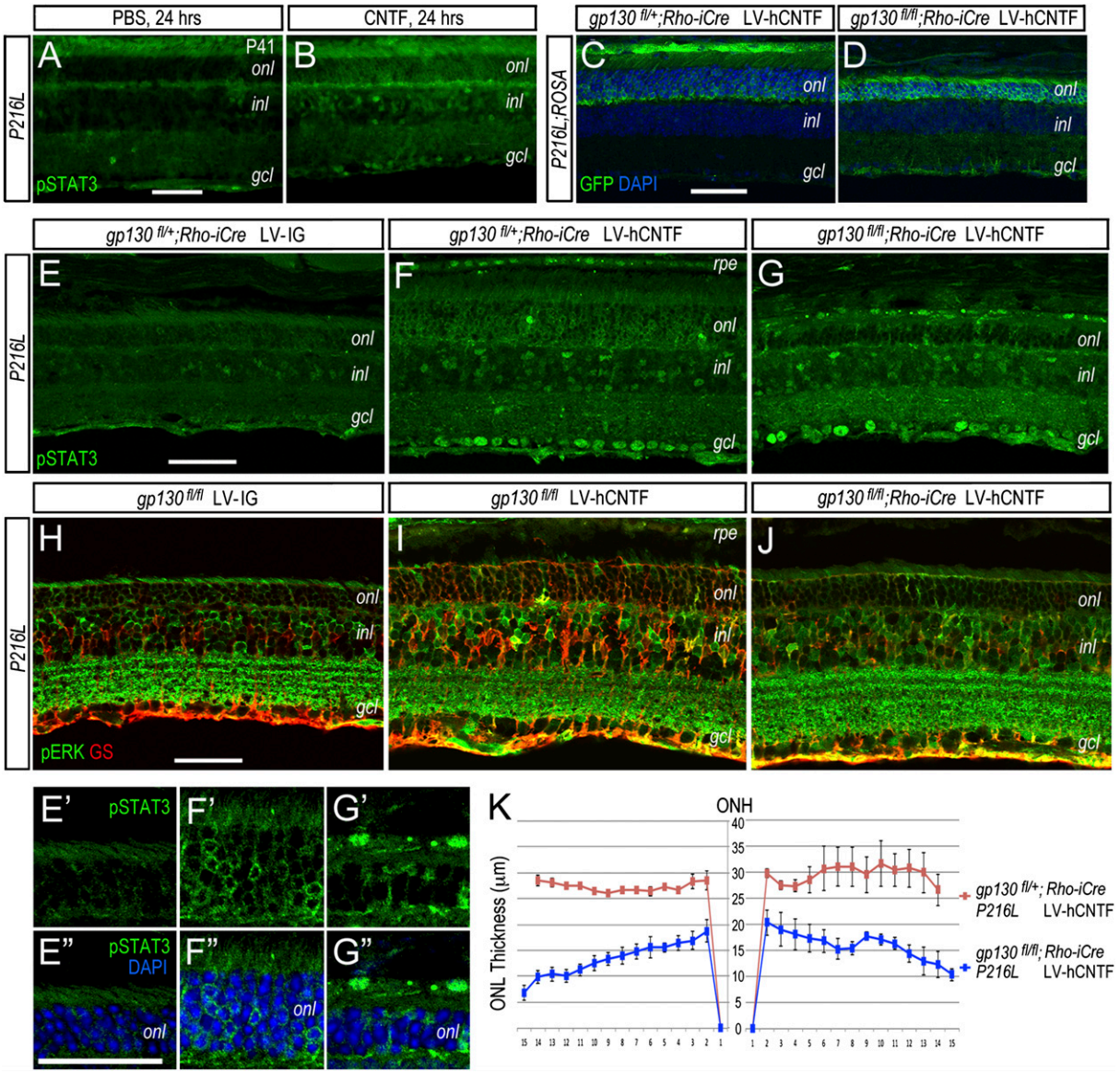

Fig. 6. Deletion of *gp130* in rod cells prevents CNTF-mediated rescue of mutant photoreceptors from degeneration. (*A* and *B*) Immunolabeling for pSTAT3 in *rds/P216L* mutant retinas 24 h after CNTF injection at P41. (*C* and *D*) Confocal images of *P216L;ROSA* control *gp130*$^{+/-}$ (*C*) and *gp130 Rod cKO* (*D*) retinas injected with LV-hCNTF at P25 and labeled for GFP and DAPI at P45. (*E–J*) Confocal images of control (*E*, *F*, *H*, and *I*) and *gp130 Rod cKO* (*G* and *J*) *P216L* retinas injected with LV-IG or LV-hCNTF and labeled for pSTAT3 (*E–G*) or pERK and GS (*H–J*). (*E′–G″*) show magnified images for pSTAT3 (*E′–G′*) and merged DAPI /pSTAT3 (*E″–G″*) labeling of the outer nuclear layer from *E–G*, respectively. (*K*) Quantification of ONL thickness for the control *gp130*$^{+/-}$ ($n = 6$) and *gp130 Rod cKO;P216L* ($n = 5$) retinas injected with LV-hCNTF. (Scale bars, 50 μm in all panels.) *gcl*, ganglion cell layer; *inl*, inner nuclear layer; *onl*, outer nuclear layer; *rpe*, retinal pigment epithelium.

the entire *gp130* Müller cKO retina, indicating that the main signaling events in Müller cells precede subsequent events in the rest of the retina. Because the *gp130* deletion may not have occurred in all Müller cells, the loss of subsequent signaling events in the retina suggests that a critical Müller activation threshold may exist for the exogenous CNTF to trigger a full protection response. Based on these results, we propose an "indirect model" as the cellular signaling mechanism for CNTF-mediated neuroprotection in the adult mouse retina (Fig. 7*D*). Incomplete ablation of *gp130* from all Müller cells may also account for the residual neuroprotective effects seen in the *gp130* Müller cKO retina. Alternatively, it is possible that residual signal transduction from retinal ganglion cells (RGCs), astrocytes, or the RPE can provide minor rescuing effects for rod cells.

It is known that photoreceptors and Müller glia have close reciprocal interactions. Injured photoreceptor-derived endothelin2 (EDN2) acts on its receptor EDNRb expressed by Müller glia (46). Furthermore, EDN2 can induce Müller glia to produce LIF, which acts as a chief neuroprotective agent in the light-induced and inherited retinal degeneration models (22, 45). Using cell-specific *gp130* deletion, we also demonstrated that CNTF-dependent photoreceptor survival in the *rds/P216L* RP model relies on gp130 expressed by rod cells themselves. The *Rho-iCre* mediated *gp130* deletion abolishes activation of STAT3 in rod photoreceptors and results in a more pronounced reduction of CNTF rescuing effects compared with *gp130* deletion in Müller cells (Fig. 7*C*). These deficiencies observed in the rod *gp130* cKO retina indicate that the major survival factor(s) triggered by hCNTF belong to the same cytokine subfamily. Among

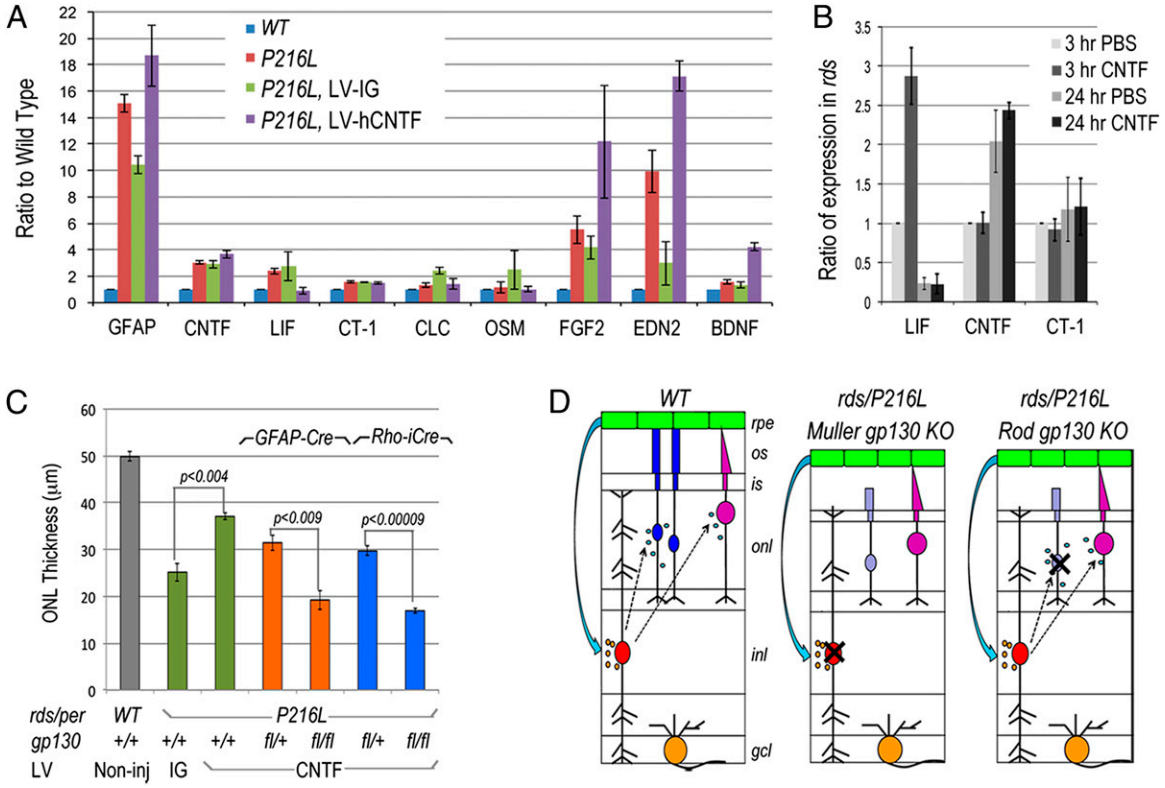

Fig. 7. Summary of CNTF triggered protection in the *rds/P216L* model of photoreceptor degeneration. (*A*) Real-time PCR quantification of GFAP and growth factor transcripts. *WT* and *P216L* are noninjected samples at P52; *P216L*, LV-IG and *P216L,* LV-hCNTF were injected with viruses at P25 and analyzed at P52. n = 3 for all. For statistics, see Table S11. (*B*) Real-time PCR quantification of transcript levels for LIF, CNTF, and CT-1 in *rds/P216L* retinas. Injections of CNTF or PBS were performed at P25, and RNAs were collected at 3 or 24 h after injections. n = 3 for all. For statistics, see Table S12. (*C*) Comparison of ONL thickness for WT and *rds/P216L* mutant retinas under the influence of hCNTF in *gp130*$^{+/+}$, *gp130 Müller or rod* heterozygous, and *gp130 Müller or Rod cKO* genetic backgrounds. Significant P values (Student t test) are indicated. (*D*) A proposed indirect model of exogenous CNTF-induced neuroprotection of rod photoreceptors in the *rds/peripherin* mutant retina. The secreted human CNTF derived from LV-transduced RPE initially activates signaling events in Müller glia through the gp130 cytokine receptor. This Muller glia response triggers the release of other signaling molecules, which subsequently activate gp130 expressed by rod photoreceptors to enhance cell survival. Loss of gp130 in Müller glia prevents the activation of the entire signaling cascade, whereas removal of gp130 from rod cells prevents rod photoreceptor rescue and diminishes sustained activation of Müller glia.

the likely candidates is LIF, because it similarly requires the gp130 receptor complex for signaling. Our data show that CNTF can cause a rapid and transient increase of LIF transcripts. The transient nature of LIF induction may reflect a negative feedback regulation. Interestingly, we observed lower levels of CNTF-triggered Müller cell activation when gp130 was deleted from rod cells. This result suggests a positive feedback loop whereby gp130-mediated cell signaling events in rod cells further strengthen the initial CNTF-dependent Müller cell activation. Our quantitative PCR (qPCR) results show that hCNTF significantly enhances EDN2 mRNA expression, thus supporting the participation of exogenous hCNTF in this positive feed-forward signaling loop between Müller glia and photoreceptors (22, 45, 46). In addition to promoting *EDN2* expression, hCNTF also stimulated expression of BDNF and possibly fibroblast growth factor 2 (FGF2), both of which can act as trophic factors on multiple cell types. Although exogenous CNTF does not directly signal to rod cells initially, it is possible that endogenous CNTF signals elevated in injured retinas (47–49) (Fig. 7 *A* and *B*) may synergize with other induced factors to promote survival. It is worth noting that in contrast to our findings here, it has been reported that light preconditioning-induced protection of photoreceptors requires gp130 in rod cells, but not in Müller glia (23, 50).

Despite promoting neuronal survival, prolonged exposure to high levels of CNTF can cause detrimental effects, including suppression of both cone opsin expression and ERG in the *rds/P216L* model (25, 28). This phenomenon raises the question whether lower-dose CNTF treatments can avoid these harmful effects. Although it is difficult to directly compare the levels of CNTF produced in the current study with the doses produced by encapsulated cells in the human trials, we detected hCNTF levels at around 20 pg/eye in the LV-hCNTF–infected mouse eyes. Under these conditions, we observed a remarkable improvement in photoreceptor morphology represented by the lengthening of both inner and outer segments. This observation is reminiscent of the cone outer segment regeneration promoted by CNTF in a rat rhodopsin mutant (51). Furthermore, we also observed a correction of mislocalized rod and cone opsins in CNTF-treated retinas carrying the dominant *P216L* mutation, suggesting that CNTF is able to enhance the WT peripherin function and to partially overcome the dominant mutant effect in this mouse model. Although the exact protective mechanism of CNTF remains obscure, the benefits of low exogenous CNTF on preventing further degeneration and promoting photoreceptor structural recovery have clear clinical implications. In the light-damaged zebrafish retina, activated ERK but not STAT3 is required for neuroprotection by exogenous CNTF (52). Future investigations are necessary to determine the specific downstream signaling modalities required for low-level CNTF-dependent cell survival in the mammalian retina.

Paradoxically, the remarkable improvement of photoreceptor morphology and survival are not accompanied by any significant

enhancement in retinal function as indicated by the ERG. The reason for this finding is not understood but likely involves CNTF-induced alteration of transcription, which ultimately leads to the suppression of visual function. As we have shown previously (28), exposure to high levels of CNTF down-regulates a number of photoreceptor specific genes, particularly those associated with phototransduction. Consistent with our findings that cone cells are more sensitive to CNTF signaling, we also detected mild suppression on cone opsin and *Gnat2* expression even under low CNTF expression conditions, which may contribute to the mild suppression in photopic b-wave amplitudes. Intriguingly, in high-dose CNTF–treated eyes, the nuclear morphology of rod photoreceptors loses the stereotypical organization and resembles those of cone cells with less heterochromatin and more euchromatin (25), indicating altered chromatin organization. Accumulating evidence indicates that mature retinal neurons are plastic and can undergo fate reprogramming. For example, ablating the *Nrl* gene in mature rod photoreceptors results in partial transformation of rod cells toward a cone cell fate, accompanied by altered rod nuclear morphology and enhanced cell survival (53). It is possible that Jak-STAT signaling directly influences the chromatin structure of photoreceptor cells in a cell autonomous fashion. However, because exogenous CNTF induces the release of a myriad of growth factors in the retina, it remains to be determined which signaling pathway contributes to the epigenetic changes observed in CNTF-treated retinas. Furthermore, intravitreal injection of LIF causes a down-regulation of RPE65 protein, the key isomerase required in the RPE to restore the 11-*cis*-retinal chromophore, leading to a slower regeneration of rhodopsin (10). We also observed CNTF-induced STAT3 phosphorylation in RPE cells (Figs. 5 and 6). Because inhibition of RPE65 activity is known to prevent light-induced degeneration (54, 55), CNTF-like cytokine-induced modulation of the visual cycle could in part account for the suppression of ERG and enhanced survival of photoreceptors. Therefore, current data indicate that CNTF treatment of mature retinas causes complex molecular and cellular responses in multiple cell types, some of which may profoundly influence neuronal characteristics and retinal function.

Together, the results presented in this study demonstrate that exogenous CNTF initially targets Müller glia to trigger a cascade of signaling events leading to photoreceptor survival. Future investigations focusing on the downstream signaling events induced by CNTF and the mechanisms involved in its beneficial and detrimental impacts on cellular physiology will enhance our ability to develop more effective neuroprotective therapies in the mammalian retina.

Materials and Methods

Animals. The *rds/peripherin P216L* transgenic mice (30) were maintained on a WT CD1 background. Mice with a floxed allele of *gp130* (32) on a mixed background were crossed with transgenic mouse lines *GFAP-Cre* (33) or *Rho-iCre75* (35) on the C57BL/6J background to generate *gp130* deletions in Müller glia or rod photoreceptors, respectively. In some experiments, the *rds/P216L* transgene and/or the *ROSA.EYFP* Cre reporter (34) were also present in the *gp130* retinal mutant background. Genotyping primers are summarized in *SI Materials and Methods*. All animal procedures followed National Institutes of Health guidelines and were approved by the Animal Research Committee at University of California–Los Angeles.

Lentiviral Vector Construction, Production, and Injection. LV-IG (31) encodes the CMV promoter followed by the IRES-GFP cassette. LV-hCNTF was constructed by inserting the cDNA of a secreted form of human CNTF with S166D and G167H substitutions (25) between the CMV promoter and IRES-GFP cassette. The production, titer determination, and subretinal injection of lentiviruses were performed as previously described (56). Lentiviral stocks with titers of 1×10^7 CFU/mL were used for in vivo subretinal injections, unless otherwise specified. For in vivo injections, 1 μg of recombinant human CNTF (PeproTech) in 1 μL volume per eye was injected intravitreously.

FACS, Immunohistochemistry, Histology, and TEM. FACS analysis was performed as described in ref. 57 and in *SI Materials and Methods*. For immunofluorescent labeling, cells or tissues were fixed with 4% (wt/vol) paraformaldehyde in PBS and processed as described previously (28). Whole mount retinas were incubated overnight with primary and secondary antibodies at 4 °C, followed by extensive washes. Fluorescent images were captured using a Spot II camera and Nikon E800 or Olympus FluoView 1000 confocal microscopes. Antibodies used are summarized in Table S1. For 1-μm semithin sections and TEM, eyes were fixed in 2% (wt/vol) formaldehyde and 2.5% (wt/vol) glutaraldehyde in 0.1 M sodium phosphate buffer and processed as described previously (58).

Morphometrics. A minimum of three sections from each eye was used to acquire digital images of the retina. The ONL thickness as demarcated by DAPI-positive photoreceptor nuclei was determined at 15 equal spatial intervals from the optic nerve head to the ciliary margin. The lengths of inner and outer segments of photoreceptors were measured using 1-μm semithin sections stained with toluidine blue and ImagePro Plus software (Media Cybernetics).

Western Blot, ELISA, and Real-Time PCR. Western blots of cell lysates, medium, or retinal extracts were processed as described previously (21, 28). The amounts of viral produced hCNTF in vitro and in vivo were determined with a human CNTF Quantikine ELISA kit (R&D Systems) as described in *SI Materials and Methods*. Real-time PCRs were performed as described in ref. 28 and *SI Materials and Methods*, and primers used for qPCR are shown in Table S2.

Electroretinogram. Detailed procedures for ERG are described in *SI Materials and Methods* and ref. 59.

Statistics. One-way ANOVA was performed for the comparison with multiple subgroups, and pairwise comparisons within ANOVA were conducted with Tukey corrections if the ANOVA was statistically significant. Values of $P < 0.02$ were considered statistically significant. For the pairwise analysis presented in Fig. 7*C*, the Student *t* test was used and values of $P < 0.01$ were considered statistically significant.

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