Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 300 (2006) 583–598
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

# The CNTF/LIF signaling pathway regulates developmental programmed cell death and differentiation of rod precursor cells in the mouse retina *in vivo*

Jimmy Elliott [a,c], Michel Cayouette [a,b,*], Claude Gravel [c,d]

[a] *Institut de Recherches Cliniques de Montréal (IRCM), 110, avenue des Pins Ouest Montréal, Québec, Canada H2W 1R7*
[b] *Département de Médecine, Faculté de Médecine, Université de Montréal, Canada*
[c] *Laboratoire de Transfert de Gènes, Centre de Recherche Université Laval Robert-Giffard (CRULRG), 2601, de la Canardière, Beauport, Québec, Canada G1J 2G3*
[d] *Département de Psychiatrie, Faculté de Médecine, Université Laval, Cité Universitaire, Québec, Canada G1K 7P4*

Received for publication 3 February 2006; revised 31 August 2006; accepted 5 September 2006
Available online 9 September 2006

**Abstract**

Natural cell death is critical for normal development of the nervous system, but the extracellular regulators of developmental cell death remain poorly characterized. Here, we studied the role of the CNTF/LIF signaling pathway during mouse retinal development *in vivo*. We show that exposure to CNTF during neonatal retinal development *in vivo* retards rhodopsin expression and results in an important and specific deficit in photoreceptor cells. Detailed analysis revealed that exposure to CNTF during retinal development causes a sharp increase in cell death of postmitotic rod precursor cells. Importantly, we show that blocking the CNTF/LIF signaling pathway during mouse retinal development *in vivo* results in a significant reduction of naturally occurring cell death. Using retroviral lineage analysis, we demonstrate that exposure to CNTF causes a specific reduction of clones containing only rods without affecting other clone types, whereas blocking the CNTF/LIF receptor complex causes a specific increase of clones containing only rods. In addition, we show that stimulation of the CNTF/LIF pathway positively regulates the expression of the neuronal and endothelial nitric oxide synthase (NOS) genes, and blocking nitric oxide production by pre-treatment with a NOS inhibitor abolishes CNTF-induced cell death. Taken together, these results indicate that the CNTF/LIF signaling pathway acts via regulation of nitric oxide production to modulate developmental programmed cell death of postmitotic rod precursor cells.


*Keywords:* Retina; Cell death; Development; Cytokine; Cell fate; Photoreceptor; Nitric oxide

## Introduction

Through the processes of cell division, specification, differentiation, and programmed cell death, various cell types are generated in specific numbers to form the mature brain. Precise coordination of these processes is critical to the generation of a fully functional nervous system as the slightest imbalance could lead to abnormal development and disease. The retina is an ideal system to study these processes as it is easily accessible, it has a relatively simple anatomy, and it contains a manageable number of cell types that can all be identified with molecular markers. Pioneering cell lineage analyses using retroviral vectors or injection of tracers into individual retinal progenitor cells (RPCs) indicate that the earliest RPCs are multipotent and can give rise to all the different cell types in the retina (Holt et al., 1988; Turner and Cepko, 1987; Wetts and Fraser, 1988); astrocytes, by contrast, migrate into the retina from the optic nerve (Watanabe and Raff, 1988). These findings raised the important question of how multipotent RPCs choose between different fates and generate the correct proportion of retinal cell types.

During retinal development, naturally occurring programmed cell death is observed in successive waves during the first postnatal weeks (Young, 1984). As the cells become postmitotic and start to differentiate, apoptosis occurs in the ganglion

* Corresponding author. Cellular Neurobiology Research Unit, IRCM, 110, Avenue des Pins Ouest, Montreal, QC, Canada H2W 1R7. Fax: +1 514 987 5761.
*E-mail address:* michel.cayouette@ircm.qc.ca (M. Cayouette).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.09.002

cell layer and progresses to cells of the inner and then outer nuclear layer of the retina, peaking around postnatal day 7 in the latter (Young, 1984). Previous studies have suggested that naturally occurring cell death is significant and can even complicate the analysis of cell lineages in the retina (Voyvodic et al., 1995). Previous studies have shown that signaling through the low-affinity neurotrophin receptor p75 contributes to regulate naturally occurring cell death of ganglion cells in the retina (Ding et al., 2001; Frade and Barde, 1999; Frade et al., 1996; Harada et al., 2005), but it remains unclear whether other signaling pathways could act to regulate natural cell death in other cell populations of the developing retina.

Members of the ciliary neurotrophic factor (CNTF)/leukemia inhibitory factor (LIF) subfamily of cytokines have been extensively studied over the past years for their potential role as environmental signals regulating retinal development. *In vitro* studies have shown that CNTF can inhibit rhodopsin expression in newborn, postmitotic rod photoreceptor cells (Bhattacharya et al., 2004; Ezzeddine et al., 1997; Kirsch et al., 1996; Neophytou et al., 1997; Rhee et al., 2004). Furthermore, the observation that rhodopsin-positive cells are more numerous in retinal explant cultures from neonatal CNTF receptor α (CNTF-Rα) knockout mice (Ezzeddine et al., 1997) or in explants from wild-type mice after blockade of STAT signaling (Rhee et al., 2004) has been interpreted as further support that the CNTF signaling pathway is acting mainly on the differentiation of rod photoreceptors. However, if the CNTF pathway was only controlling rhodopsin expression, one would expect precocious expression of rhodopsin in the CNTF-Rα knockout retina, but not necessarily an increased number of rhodopsin-positive cells. Thus, the extra photoreceptors in the CNTF-Rα knockout mouse suggest that stimulation of the CNTF-Rα might also regulate cell death. Although CNTF is largely known for its anti-apoptotic activities on various neural cell populations including photoreceptors (Cayouette et al., 1998; Cayouette and Gravel, 1997; LaVail et al., 1992), previous results *in vitro* reported that CNTF can also act as a death-promoting factor on sympathetic neurons (Kessler et al., 1993).

In the nervous system, nitric oxide (NO) has been proposed to play a part in regulating various aspects of normal development, including cell proliferation, differentiation and death, as well as synaptogenesis (Gibbs, 2003; Hess et al., 1993; Roskams et al., 1994; Wang et al., 1995; Wu et al., 1994). In the retina, the enzymes that synthesize NO, the nitric oxide synthases (NOS), are expressed during development and their expression is maintained in adult (Goureau et al., 1997; Kim et al., 2000; Li et al., 2006; Patel et al., 1997; Sharma et al., 1997; Tsumamoto et al., 2002). Interestingly, light-induced photoreceptor degeneration requires NOS activity (Donovan et al., 2001), and inhibition of endogenous NOS in chick retinal cell cultures prevents the normal developmental cell death observed in the rod photoreceptor population (Goureau et al., 1999). These results suggest that NO may be involved in the regulation of naturally occurring cell death in the developing retina.

In this study, we tested the hypothesis that the CNTF/LIF signaling pathway might regulate programmed cell death of rod precursor cells *in vivo*. We show that stimulation of the CNTF/LIF signaling pathway during retinal development causes a specific and massive cell death of postmitotic rod photoreceptor precursors and that blocking the pathway with a CNTF receptor dominant-negative reduces naturally occurring cell death. In addition, we show that exposure to CNTF during retinal development significantly increases the expression of the neuronal and endothelial nitric oxide synthase (NOS), whereas blocking the CNTF/LIF pathway reduces the expression of both NOS isoforms. Consequently, we report that blocking NO production with the NOS inhibitor L-NAME can abolish CNTF-induced cell death and significantly reduce naturally occurring cell death in the developing retina. These results indicate that the CNTF/LIF signaling pathway regulates developmental rod photoreceptor cell death by controlling NO production during retinal development.

## Results

### *Exposure to CNTF during postnatal mouse retinal development in vivo leads to a specific photoreceptor cell deficit*

To examine the effects of a stimulation of the CNTF receptor complex on the postnatal development of photoreceptors in the murine retina *in vivo*, we first exposed the retina of mice from postnatal day 2 (P2) to increased levels of CNTF using a defective adenoviral vector (AdCNTF) encoding a secretable version of a *cntf* minigene, as described previously (Cayouette et al., 1998; Cayouette and Gravel, 1997). In all experiments, the contralateral eye was either left uninjected or injected with a defective adenoviral vector encoding *E. coli* β-galactosidase (AdLacZ) as control. The viral vectors were delivered into the vitreous using a transretinal approach, and most infected cells were found located in the iris epithelium, cornea, ciliary body, and pigmented epithelium, as previously shown (Cayouette et al., 1998; Cayouette and Gravel, 1997). Observations of retinal sections at P32 revealed that the photoreceptor layer of the AdCNTF-injected eye was remarkably thinner than that of the control retina and contained fewer photoreceptors (Figs. 1A, B), whereas retinas from non-injected eyes at P32 were not different from those of AdLacZ-injected eyes (Supplementary Fig. 1). In addition, the photoreceptors in the AdCNTF-injected eyes did not fully develop as judged by the absence of organized outer segments (Fig. 1B). The other cell layers (INL and GCL) appeared unaffected as we did not find any changes in their thickness.

In cultures of neonatal rat retina, it was previously reported that the decreased number of rhodopsin-positive ($rho^+$) cells following exposure to CNTF was accompanied by an increase in the number of cells expressing bipolar cell markers (Ezzeddine et al., 1997). To verify whether CNTF overexposure *in vivo* could respecify some retinal cells to adopt the bipolar cell fate, we counted the number of cells expressing the bipolar cell markers protein kinase C alpha (PKC), Islet-1 (Figs. 1C–F),

and RET-B1 (not shown) on retinal sections from AdCNTF-infected or control eyes. No statistically significant changes were found in the proportion of PKC-, Islet-1-, or RET-B1-positive cells in AdCNTF-treated retinas compared to controls (Figs. 1G, H, and not shown).

A recent study showed that CNTF can promote Müller glial cell genesis *in vitro* (Goureau et al., 2004). To determine the effect of CNTF exposure on Müller glial cell differentiation *in vivo*, we stained AdCNTF-injected and control retinas with GFAP, glutamine synthetase, and cyclin-D3, three markers of Müller glial cells. As expected (Wang et al., 2002), we found that CNTF exposure caused an important increase in GFAP expression in Müller cells (not shown). However, we did not detect any apparent changes in the number of glutamine synthetase and cyclin-D3-positive cells following CNTF exposure during retinal development *in vivo* (Supplementary Fig. 2). Interestingly, the position of the Müller cell bodies appeared slightly disorganized in some areas (Supplementary Fig. 2), most likely due to the important loss of photoreceptor cells. Thus, it appears that exposure to CNTF *in vivo* stimulates GFAP expression, as previously reported, but does not significantly affect the number of Müller glial cells that develop. We also stained CNTF-exposed retinas with amacrine cell markers and did not detect any changes in the number of amacrine cells (not shown). Consistent with these observations, we found that total INL cell densities were not significantly affected in CNTF-exposed retinas (75.7±8.7 cells/100 μm in controls, 69.0±4.4 cells/100 μm in AdCNTF retinas; $P=0.3221$). Taken together, these results indicate that overexposure to CNTF causes a specific deficit in photoreceptor cells and does not affect generation of other retinal cell types in the mouse retina *in vivo*.

### *The CNTF/LIF signaling pathway regulates rhodopsin expression in vivo*

There is strong evidence indicating that the CNTF/LIF signaling pathway regulates the onset of rhodopsin expression in culture (Ezzeddine et al., 1997; Kirsch et al., 1998; Neophytou et al., 1997; Shuster and Farber, 1986). To determine the *in vivo* influence of the CNTF/LIF signaling pathway on the onset of opsin expression, we injected 250 ng of rat recombinant CNTF (rrCNTF) into one eye and recombinant β-Gal (rβ-Gal), or nothing, into the contralateral eye of P2 mice and analyzed the expression of rhodopsin at P3 or P9 by immunofluorescence. While numerous rho$^+$ cells are detected in the uninjected and rβ-Gal-injected retinas at P3 (Fig. 2A), the

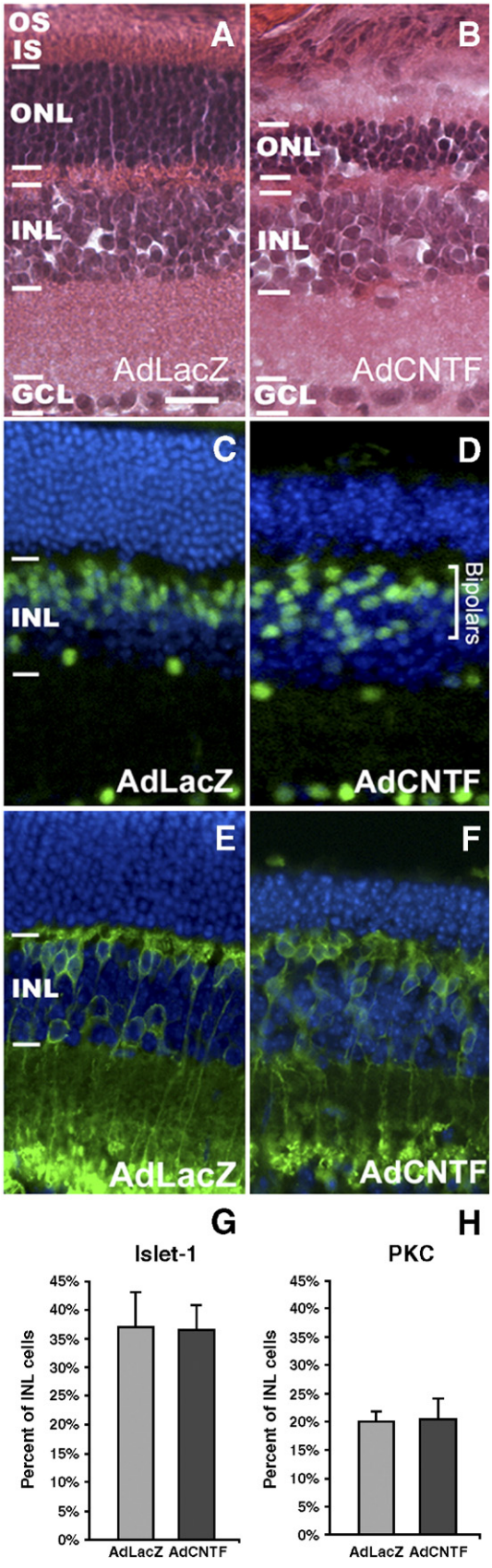


Fig. 1. Exposure to CNTF during retinal development *in vivo* leads to photoreceptor deficit in the mature retina, but does not affect bipolar cell production. Hematoxylin–eosin staining of retinal sections from eyes of a P32 mouse injected at P2 with AdLacZ into the left eye (A) and AdCNTF into the right eye (B). (A) In the control retina, the number of photoreceptor rows (ONL column height) has reached its adult value of 11–12 rows. In contrast, the retina exposed to the AdCNTF vector shows an important reduction in ONL column height. (C–E) P21 retinal sections from eyes injected with AdLacZ (C, E) or AdCNTF (D, F) at P2 show similar number of bipolar cells, as revealed by immunostaining for Islet-1 (C, D; bipolar cells are pointed by the bracket; other positive cells are subsets of amacrine cells and ganglion cells) and PKC-alpha (E, F). (G, H) The retinas exposed to CNTF have a similar number of Islet-1 (G) and PKC-positive cells (H) than controls. Scale bar=25 μm.

retina exposed to CNTF shows virtually no rho$^+$ cells (Fig. 2B), confirming that stimulation of the CNTF/LIF signaling pathway can block rhodopsin expression *in vivo*. Interestingly, observation of CNTF-injected retinas at P9 did not reveal any obvious changes in rhodopsin expression (not shown), suggesting that the blockade of rhodopsin expression requires sustained exposure to CNTF. Consistent with this hypothesis, retinas injected with the AdCNTF vector at P2 showed a marked reduction in rhodopsin expression up to 30 days after injection (Figs. 2C, D).

To determine whether an endogenous ligand of the CNTF/LIF signaling pathway is acting *in vivo* to block rhodopsin expression, as suggested by an *in vitro* study (Neophytou et al., 1997), we have constructed an adenoviral vector encoding the well-characterized dominant-negative ligand LIF05 (Hudson et al., 1996). First, we tested whether cells infected with this viral vector (AdLIF05) secreted the dominant-negative protein and can block signaling through the CNTF/LIF receptor complex. As shown in Fig. 2E, the supernatant collected from AdLIF05-infected Vero cells, but not that of control-infected or non-infected cells, can efficiently block the phosphorylation of STAT-3 in the neuroblastoma cell line IMR-32 exposed to CNTF. These results show that the LIF05 secreted from AdLIF05-infected cells can block the signaling through the CNTF receptor complex. We then injected the AdLIF05 or the AdLacZ vector into the vitreous at P2, or left the eyes uninjected, and counted the number of rho$^+$ cells at P3 and P7 (Figs. 2F, G). We found a significantly higher proportion of rho$^+$ cells in the retinas exposed to the LIF05 protein than in controls at both time points (Figs. 2F, G). Taken together, these results suggest that an endogenous ligand of the CNTF/LIF receptor complex is present in the retina during normal postnatal development and retards the expression of rhodopsin in some photoreceptor precursor cells.

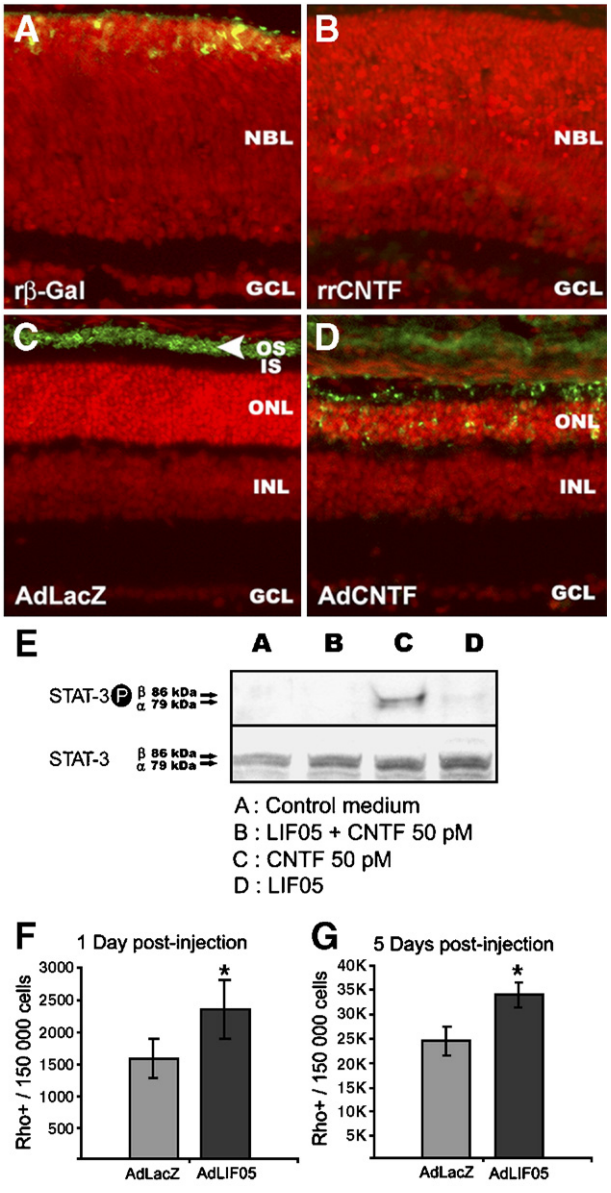


Fig. 2. The CNTF/LIF signaling pathway regulates the timing of rhodopsin expression *in vivo*. (A, B) Rhodopsin immunostaining on retinal sections from P3 (A, B) and P32 (C, D) mice injected into the vitreous at P2 with rβ-Gal (A), rrCNTF (B), AdLacZ (C), or AdCNTF (D). At P3, rhodopsin-positive cells are detected in the control retina (A), whereas essentially no rhodopsin-positive cells are detected in the rrCNTF-injected eyes (B). At P32, control retinas injected with AdLacZ at P2 show rhodopsin concentrated exclusively in the outer segments (OS) of photoreceptors (arrowhead). In contrast, eyes injected with AdCNTF at P2 display only a few rhodopsin-positive cell bodies in the ONL, and no rhodopsin-positive OS are detected (D). (E) Western blot analysis of STAT-3 phosphorylated isoforms α and β (STAT-3P) in IMR-32 neuroblastoma cell line. STAT-3 phosphorylation indicates CNTF/LIF signaling pathway activation. Supernatant from non-infected (lane A), AdLIF05-infected (lane B and D), or AdLacZ-infected (lane C) Vero cells was used to grow IMR-32 cells. When rrCNTF is added to the medium of supernatant from AdLIF05-infected cells, STAT-3 is not phosphorylated (lane B). In contrast, when rrCNTF is added to medium of supernatant from AdLacZ-infected cells, STAT-3 is phosphorylated, as expected (lane C). Control medium from non-infected cells (lane A) and the medium from AdLIF05-infected cells only (lane D) does not induce STAT-3 phosphorylation. Total STAT-3 was used at loading control. (F, G) Number of rhodopsin-positive cells 1 day (F) or 5 days (G) following intravitreal injection of the AdLacZ control vector into the left eye and the AdLIF05 vector into the right eye at P2. The AdLIF05-injected eyes show significantly more rhodopsin-positive cells at both time points (*$P<0.05$, Student's paired *t*-test).

### *Overexposure to CNTF during postnatal retinal development causes a dramatic increase in cell death*

Because the photoreceptor deficit caused by overexposure to CNTF during the early postnatal period is not compensated by a concomitant increase in the number of other retinal cell types, we have investigated the possibility that the photoreceptor deficit could result from an increase in cell death. For this purpose, the AdCNTF vector or AdLacZ control was injected into the vitreous of P2 mice, and the retinas were then collected and analyzed at P5, P15, P21 or P32. The presence of apoptotic cells was detected using terminal dUTP nick-end labeling (TUNEL). As shown in Fig 3A, we observed a dramatic increase in the number of apoptotic cells (TUNEL$^+$) at P5, 3 days after AdCNTF injection. At P15, the number of apoptotic cells was back to normal. Interestingly, a slight increase in apoptotic cells was again observed in the CNTF-treated retinas

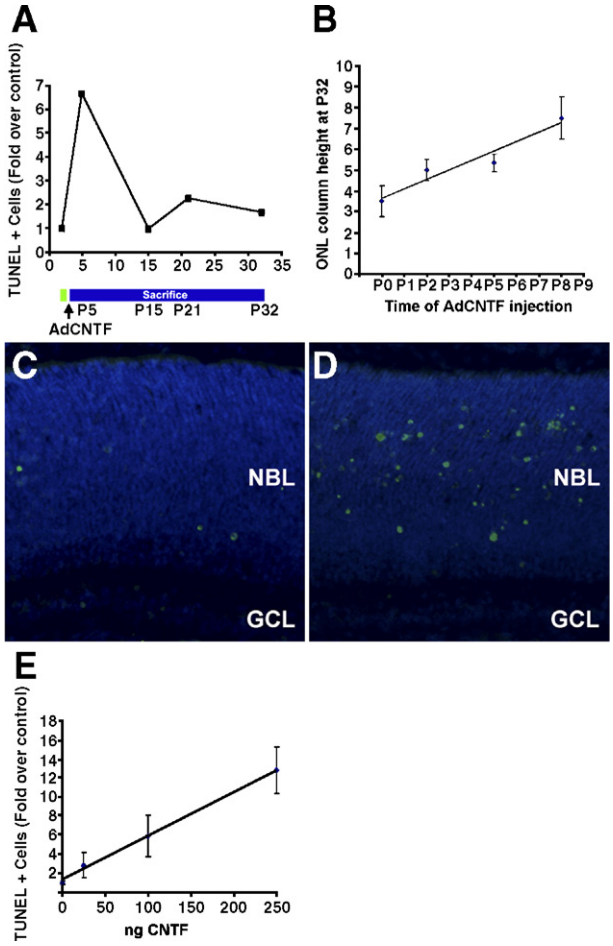


Fig. 3. Overexposure to CNTF during retinal development *in vivo* leads to increased retinal cell death. (A) Time course of apoptotic cell death during retinal development following intravitreal injection of AdCNTF at P2. Animals were sacrificed at P5, P15, P21, and P32 and retinal sections processed for TUNEL. Note that P5 retinas already contain about 7 times more TUNEL-positive cells than controls. The number of apoptotic is back to that of controls at P15, and then a second, less important, wave of apoptotic cells is observed between P15 and P32. (B) Quantitative analysis of ONL column height, indicative of photoreceptor layer thickness, in P32 retinas injected at P0, P2, P5, or P8 with the AdCNTF vector. The ONL column height at P32 in eyes injected with AdCNTF during postnatal development is correlated to the time of AdCNTF injection ($R^2=0.9263$). (C, D) Detection of apoptotic cells by TUNEL on P3 retinal sections from eyes injected at P2 with rβ-Gal (C) or rrCNTF (D). The sections are counterstained with Hoechst to reveal the cell nuclei (blue). The number of apoptotic cells in the neuroblast layer (NBL) of retinas exposed to CNTF is sharply increased (D), whereas apoptosis in the ganglion cell layer (GCL) is not affected. (E) A dose–response curve shows that the proportion of apoptotic cells increases as the concentration of injected rrCNTF increases.

at P21 (Fig. 3A). However, this second wave of cell death does not appear to contribute much to the overall loss of photoreceptor cells since retinas from AdCNTF-injected were already reduced to only 4–5 rows of photoreceptor nuclei at P15 (compared to 11–12 rows in controls), and less than one row of photoreceptors was further lost between P15 and P32. These results indicate that overexposing the early postnatal retina to CNTF results in an important apoptotic loss of cells and that the deficit in photoreceptors seen in adults following AdCNTF injection at P2 is essentially complete by P15.

We found no deficit in photoreceptors at P32 or later following intravitreal injection of AdCNTF at P12 or later suggesting that overexposure to CNTF is particularly detrimental before P12, when photoreceptor production and differentiation are taking place. Indeed, histological analysis of retinas of P32 animals that received the AdCNTF vector in one eye at either P0, P2, P5, or P8 revealed a strong correlation ($R^2=0.9263$) between the thickness of the photoreceptor layer at P32 and the age of the animal at the time of injection (Fig. 3B), indicating that the earlier the retina is exposed to CNTF, the larger the deficit in photoreceptors.

To better evaluate the time course of the apoptotic response, P2 mice were injected into the vitreous of one eye with 250 ng of rat recombinant CNTF (rrCNTF), the other eye being left uninjected, or injected with 250 ng of recombinant *E. coli* β-galactosidase (rβ-Gal) as control. Eyes were collected at various intervals post-injection (p.i), and retinal sections processed for TUNEL. At 10.5 h p.i., the proportion of $TUNEL^+$ cells was similar in the retina of rrCNTF-injected eyes and that of controls (not shown). However, at 20 h p.i., the eyes injected with the CNTF protein showed many more apoptotic retinal cells than the controls (Figs. 3C, D), and the content of cytoplasmic histone-associated DNA fragments was more than 11 times higher in CNTF-treated retinas than in the control retinas, as revealed by ELISA ($P<0.0007$; not shown). The majority of these $TUNEL^+$ cells were located in the neuroblastic layer (Figs. 3C, D). As shown in Fig. 3E, a dose–response curve revealed that the proportion of apoptotic cells is increasing with increasing doses of injected rrCNTF. Interestingly, 9 days after injection of 250 ng rrCNTF, we did not detect any difference in the number of $TUNEL^+$ cells, or in the photoreceptor layer thickness, between the retinas of rrCNTF-injected and control-injected eyes (not shown). This indicates that a single injection of rrCNTF induces a rapid but transient wave of apoptotic cell death in the retina and that cells dying during this transient period are either replaced or not sufficiently numerous to cause a histologically detectable reduction in ONL thickness.

### *Stimulation of the CNTF/LIF signaling pathway during retinogenesis induces postmitotic photoreceptor precursor cell death*

To determine whether the apoptotic cells observed following CNTF administration at P2 were proliferating or postmitotic, we injected 250 ng of rrCNTF into the vitreous of P2 mice, collected and sectioned the retinas 20 h later, and processed the tissues for detection of both proliferating cell nuclear antigen (PCNA) and apoptosis using TUNEL. As shown in Fig. 4, we found that rrCNTF-injected eyes contained numerous PCNA-positive cells ($PCNA^+$) as well as many $TUNEL^+$ cells. Three-dimensional orthogonal plans (Figs. 4A–C) and colocalization analysis (Figs. 4D, E) revealed that $TUNEL^+$ cells observed in the rrCNTF-injected eye were largely PCNA-negative, although some $TUNEL^+$ cells were $PCNA^+$ (Fig. 4E), which most likely represent a basal level of cell death in proliferating progenitors, as this is also observed

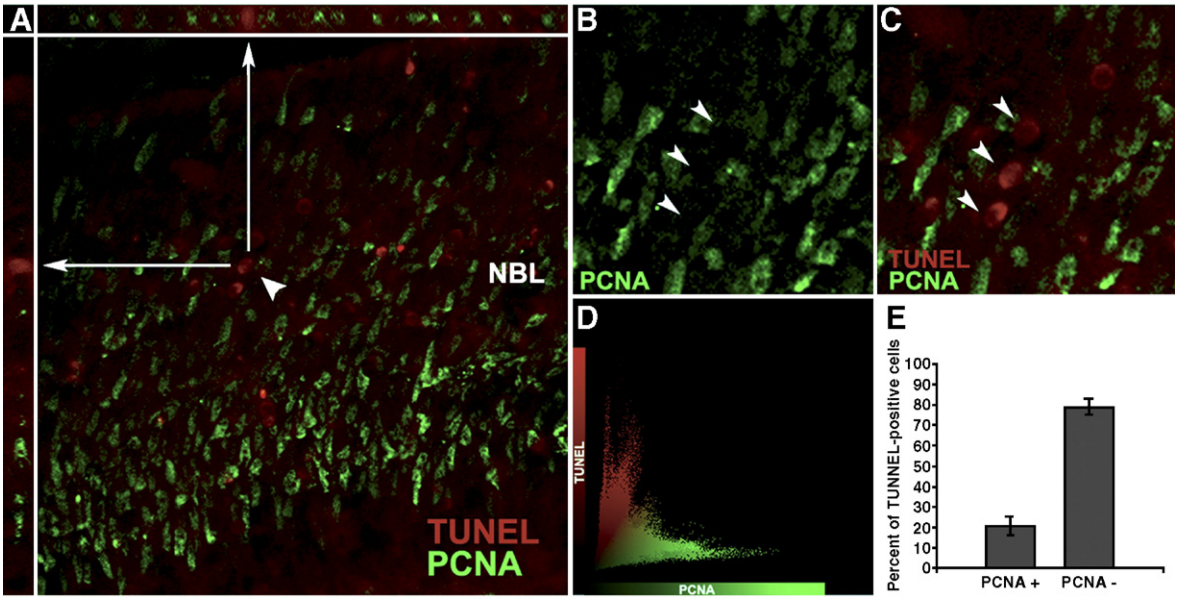


Fig. 4. CNTF overexposure during retinal development *in vivo* induces cell death of postmitotic cells. (A) Orthogonal representation of a *Z*-stack reconstruction of a double labeling for TUNEL and PCNA on P3 retinal sections of an eye injected with rrCNTF at P2. TUNEL-positive cells (red) are found mainly in the neuroblast layer (NBL) where proliferative (PCNA-positive, green) and some newly postmitotic cells are located. Examination of TUNEL-positive cells in three dimensions shows the absence of PCNA staining. An example of a TUNEL-positive cell is shown (arrowhead), and the arrows point to the *X*–*Z* and *Y*–*Z* view of that cell. (B, C) High magnification image showing $TUNEL^+/PCNA^-$ cells (arrowheads). (D) Scattered colocalization plot for TUNEL (red) and PCNA (green) shows a low colocalization factor (Pearson's coefficient 0.306±0.04). (E) Manual counting also indicates that most TUNEL-positive cells are not PCNA-positive.

in control retinas. In addition, similar experiments in which bromodeoxyuridine (BrdU) was injected 1 h before the intravitreal injection of rrCNTF revealed that very few cells were both $TUNEL^+$ and $BrdU^+$ (Supplementary Fig. 3). In both experiments, three-dimensional analysis was done to confirm the presence or absence of colocalization. Together with our finding that only photoreceptors are depleted following long-term CNTF overexposure, these results suggest that the large majority of the cells undergoing apoptosis in early postnatal retinas overexposed to CNTF are immature, postmitotic photoreceptor cells.

### *The pro-apoptotic effect of CNTF requires the stimulation of the CNTF receptor complex*

We next asked whether the increase in apoptotic cells seen in the developing retina following CNTF administration requires stimulation of the CNTF receptor complex. First, we injected intravitreally 250 ng of recombinant mouse leukemia inhibitory factor (rmLIF), a cytokine distantly related to CNTF with only 50% homology at the amino acid level, but signaling through the complex formed by the LIFRβ and gp130 receptor subunits, the two signaling β components of the tripartite CNTF receptor complex (Bazan, 1991). At 20 h p.i., retinal sections were analyzed by TUNEL. As seen following rrCNTF injection, intravitreal injection of rmLIF resulted in a massive amount of cell death in the ventricular zone (not shown). In contrast, retinal sections from eyes injected with 250 ng of recombinant murine interleukin-6 (rmIL-6), a cytokine also signaling through a gp130-associated receptor complex but not using the LIFRβ subunit, were indistinguishable from those of control rβ-Gal-injected eyes, showing no increase in $TUNEL^+$ cells 20 h p.i. (not shown).

To directly test the hypothesis that the pro-apoptotic effect of CNTF requires the stimulation of the CNTF receptor complex and to determine the specificity of this effect, we first injected one eye of P1 mice with either the AdLIF05 vector or the control AdLacZ vector, whereas the contralateral eye remained uninjected. Twenty-four hours later, the animals received a second injection in both eyes with 100 ng of rrCNTF. To confirm the success of the rrCNTF injection, fluorescent micro beads were mixed to the protein suspension. All the eyes injected with AdLIF05 only, as well as those injected with AdLIF05 followed by rrCNTF, did not show any increase in the number of apoptotic cells, compared to eyes injected with AdLacZ only (Figs. 5A–D). In contrast, eyes injected with the AdLacZ vector that were subsequently injected with rrCNTF showed the expected increase in apoptotic cells (Figs. 5E, F). In all the eyes injected with the rrCNTF protein, micro beads were found in the vitreous, confirming the success of the injection. These results show that the increase in cell death observed following CNTF administration can be blocked by pre-exposure to a dominant-negative ligand of the CNTF/LIF receptor complex, confirming that the pro-apoptotic effect of CNTF is mediated by signaling through the LIFRβ–gp130 complex.

### *Stimulation of the CNTF/LIF signaling pathway contributes to normal programmed cell death in the developing retina*

Programmed cell death occurs in many cell populations during normal retinal development, including a specific

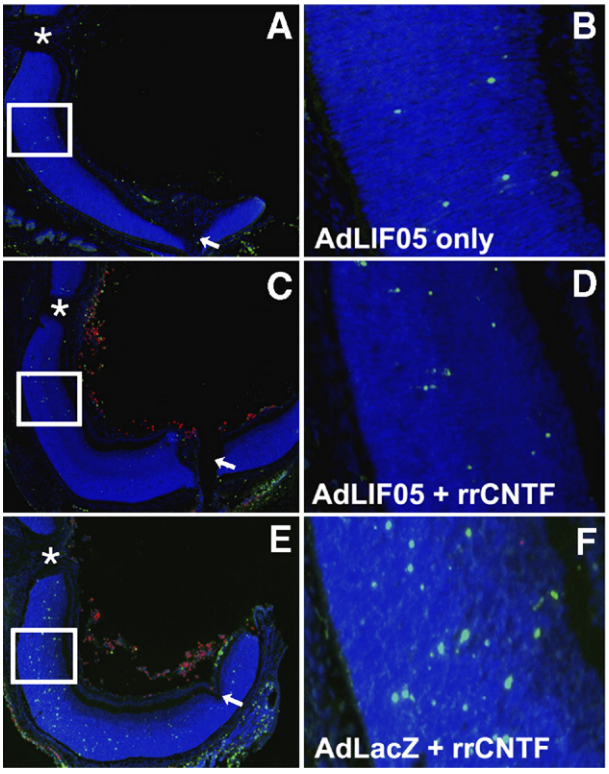


Fig. 5. CNTF-induced retinal cell death is abolished by prior exposure to the LIF05 dominant-negative ligand. Eyes were injected at P1 with AdLIF05 (A, B and C, D) or AdLacZ (E, F), and 24 h later injected with 100 ng of rrCNTF mixed with red fluorospheres (C, D, and E, F) or left uninjected (A, B). Detection of the fluorospheres confirms the success of the rrCNTF injection. (red in A, C, E). As expected, the injection of AdLIF05 does not increase cell death (A, B). However, prior exposure to AdLIF05 completely abolishes the ability of rrCNTF to induce cell death (C, D). As expected, injection of rrCNTF increases apoptosis in a retina previously exposed to the control AdLacZ vector (E, F). The optic nerve head is shown by an asterisk in panels A, C, and E, whereas the injection site is shown by an arrow. White boxes indicate blown-up regions shown in panels B, D, and F.

population of photoreceptor precursor cells called inner rods that is located in the outermost part of the INL (Young, 1984). For this cell type, the peak of apoptosis is observed around P7, when these dying cells account for more than 50% of the total number of apoptotic cells seen at this age (Young, 1984). To test whether a blockade of the CNTF/LIF signaling pathway *in vivo* could reduce the normal programmed cell death of photoreceptor precursor cells (or inner rods) observed during retinal development, eyes were injected at P2 with AdLIF05, or with AdLacZ, or were left uninjected, eyes were collected at P7, and retinal sections were processed for TUNEL. We found that retinas exposed to the dominant-negative ligand LIF05 displayed a reduced number of apoptotic cells in the outer portion of the ventricular zone (Figs. 6A, B) compared to uninjected or AdLacZ-injected controls. This region contains mostly postmitotic rod precursor cells or inner rods (Young, 1984). Cell death in other retinal layers was unaffected (see Fig. 6B). To obtain a quantitative estimate of this reduction, retinas were dissociated upon collection, and the cell suspension plated on slides, stained by TUNEL, and the proportion of TUNEL$^+$ cells counted. Overall, we found that exposure to the LIF05 protein *in vivo* reduces apoptosis of developing retinal cells by about 65% over that of control retinas (Fig. 6C; $P=0.0016$, $N=7$). A similar decrease in the number of TUNEL$^+$ cells was found when counting cells on retinal sections. Thus, blocking the CNTF/LIF signaling pathway during retinal development *in vivo* with the LIF05

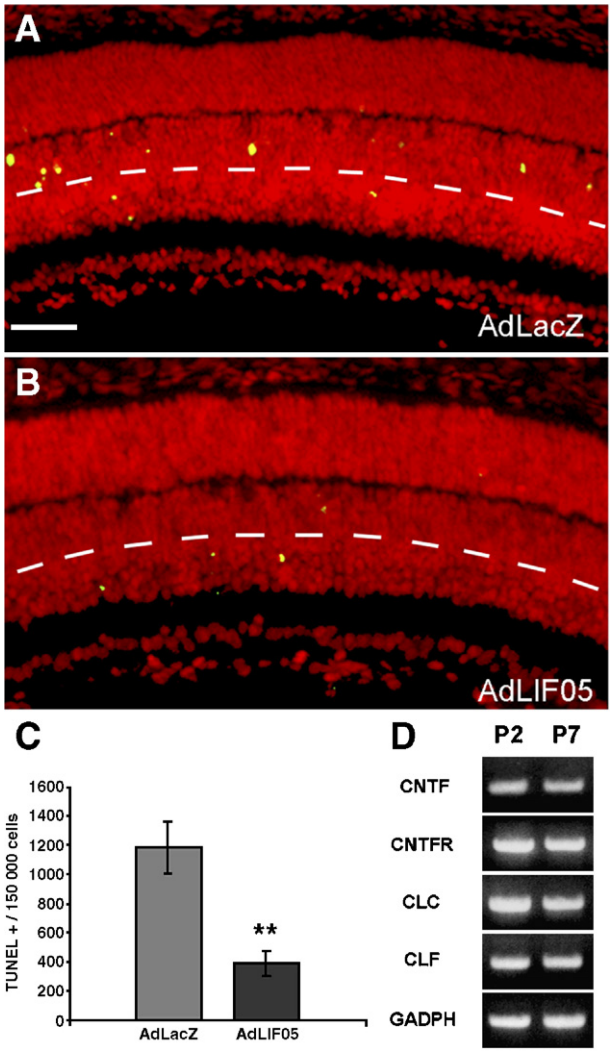


Fig. 6. Blocking the CNTF/LIF signaling pathway during retinal development *in vivo* reduces naturally occurring programmed cell death. Terminal dUTP nick-end labeling (TUNEL) on retinal sections from eyes injected at P2 with AdLacZ (A) or AdLIF05 (B) and analyzed at P7. The sections are stained with propidium iodide to reveal the cell nuclei (red). (A) Retinal sections from animals injected with AdLacZ at P2 (or left uninjected; not shown) display apoptotic cells that are mostly located in the outermost part of the INL corresponding to the inner rods region (above the broken white line). (B) In AdLIF05-injected eyes, very few apoptotic cells are observed in the outer INL, whereas normal apoptosis is still observed in the inner part of the INL (below the broken white line). (C) Quantification of total retinal cell death at P7 after injection of AdLIF05 or control vector at P2 revealed a more than 60% decrease in the number of apoptotic cells in AdLIF05-injected eyes relative to the controls (**$P<0.01$). Retinal cell death in uninjected eyes was not significantly different from AdLacZ-injected eyes (not shown). (D) RT-PCR shows that CNTF, the receptor CTNFRα, as well as the CNTF/LIF signaling pathway ligands cardiotrophin-like cytokine (CLC) and cytokine-like factor (CLF) are expressed in the retina at P2 and P7.

protein reduces the normal programmed cell death seen in P7 retinas, most likely that of postmitotic photoreceptor precursor cells.

These results suggest that one or more endogenous ligand(s) acting through the CNTF/LIF receptor complex could be responsible for a significant fraction of cell death observed at this age. Endogenous expression of LIF was previously reported in the developing retina (Neophytou et al., 1997), but whether other ligands of the CNTF/LIF receptor complex are expressed remained unknown. Thus, to determine whether such ligands are expressed in the developing retina, we conducted an RT-PCR analysis using primers specific for various members of the CNTF cytokine family on mRNA extracts from P2 and P7 retinas. We found that, in addition to CNTF, mRNAs encoding both cytokine-like factor (CLF) and cadiotrophin-like cytokine (CLC) are present at these time points (Fig. 6D). Furthermore, intravitreal injection of recombinant CLC/CLF at P2 produced the same pro-apoptotic effects as recombinant CNTF (not shown), consistent with a potential role of one or more members of this family in the developmental cell death of photoreceptor precursors.

*Stimulation of the CNTF/LIF signaling pathway selectively reduces the generation of photoreceptor-only clones without affecting the generation of other types of clones*

Although increasing CNTF concentration in the retina early after birth causes a profound deficit in rod photoreceptors, we found no changes in the thickness or cell density of the INL (Fig. 1), and immunostaining for bipolar cell markers (Fig. 2), or Müller (Supplementary Fig. 2), and amacrine cell markers (not shown) did not reveal any changes. This suggested to us that the subset of cells (largely postmitotic and rhodopsin-negative) that undergo apoptosis specifically gives rise to photoreceptors. To directly investigate this possibility, we used a replication-defective murine retroviral vector encoding human placental alkaline phosphatase (pCLE-AP) (Gaiano et al., 2000) to study the influence of CNTF on cell lineage *in vivo*.

P1 mice were first injected into the subretinal space of both eyes with the retroviral vector so as to genetically tag some proliferating progenitor cells. Three days later, one of the two eyes was injected into the vitreous with either 250 ng of rrCNTF or 250 ng rβ-Gal as control (Fig. 7A). Recombinant

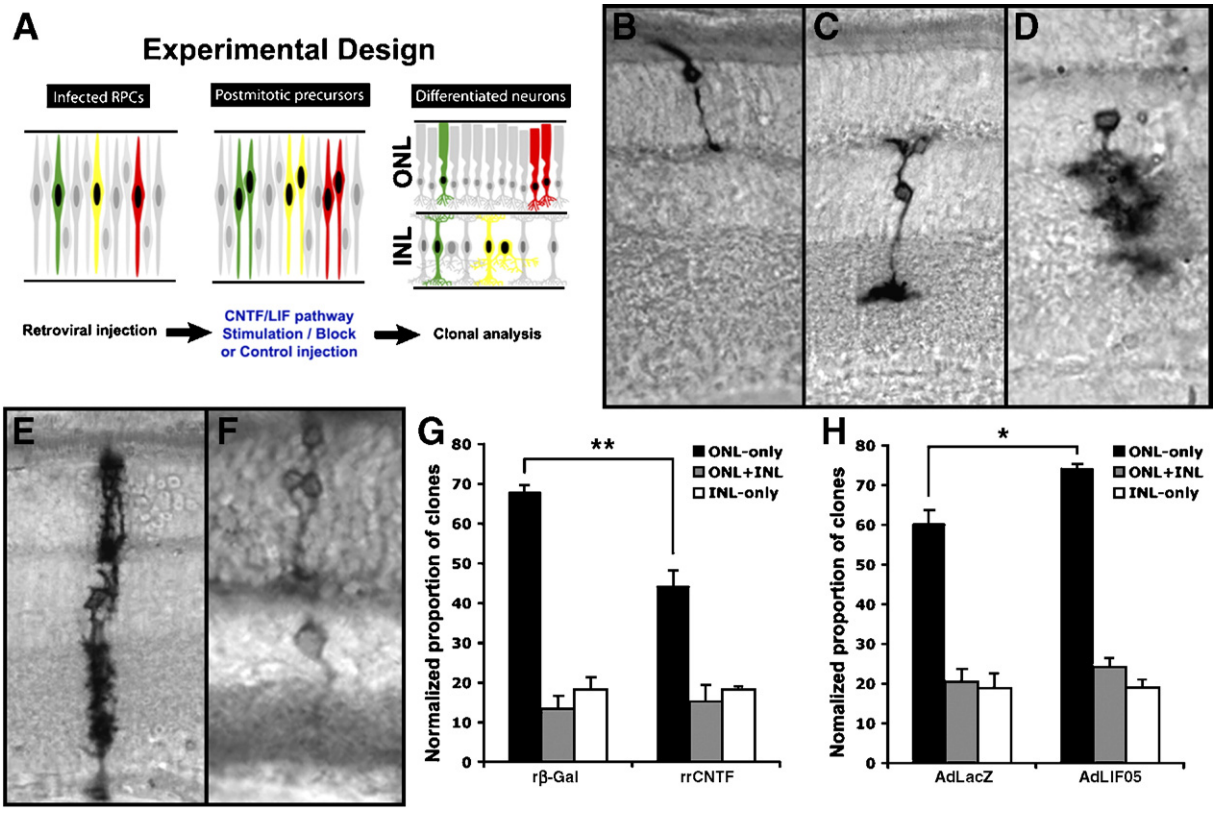


Fig. 7. The development of photoreceptor-only clones is specifically affected by manipulation of the CNTF/LIF signaling pathway during retinal development *in vivo*. (A) Schematic representation of the experimental design. Retinal progenitors are infected at P1 with a retroviral vector (left panel; colored cells), then 3 days later the eyes are injected with rrCNTF, the dominant-negative LIF05, or with control solutions (middle panel), and at P21 the clones are analyzed (right panel). (B–F) Examples of clones containing a photoreceptor (B), a bipolar cell (C), an amacrine cell (D), a Müller cell (E), or three photoreceptors and a bipolar cell (F). (G, H) Quantification of clone types by retroviral lineage analysis in rrCNTF-injected eyes (G) or AdLIF05-injected eyes (H). Clones were classified as containing only photoreceptors (ONL-only), a mixture of photoreceptors and cells located in the INL (ONL+INL), or only cells located in the INL (INL-only). The proportions of each clone types are normalized against INL-only clones, which do not die (see text), to avoid misrepresentation of proportions caused by dying cells. A significant and specific reduction of the proportion of ONL-only clones is observed in the eyes injected with rrCNTF (F; $P=0.0022$), whereas a significant and specific increase in ONL-only clones is observed in the eyes injected with the AdLIF05 vector (G; $P=0.0217$). Each condition represents clones computed from at least 3 different animals. The number of clones analyzed was: 510 clones for AdLacZ, 993 clones for AdLIF05, 322 clones for rβ-Gal, 531 clones for rrCNTF.

CNTF injection was chosen over AdCNTF because such a procedure causes only a transient increase in cell death peaking 20 h after injection, allowing us to rule out any effects due to the delayed cell death observed between P15 and P32 following AdCNTF injection (Fig. 4A). Retinas were collected at P21, and the composition of alkaline-phosphatase-positive clone population was analyzed. Because progenitor cells are labeled with the retroviral vector before CNTF exposure, any of the genetically labeled daughter cells undergoing apoptosis following CNTF administration will be missing from the clonal population at the time of analysis. Thus, even though rrCNTF injection causes only a transient increase in cell death, it would permanently affect clonal composition.

As expected (Turner and Cepko, 1987; Turner et al., 1990), three types of labeled clones were obtained in both control and rrCNTF-treated retinas: clones containing cells located only in the ONL (ONL-only clones), clones containing cells located only in the INL (INL-only clones), and clones containing cells located in both the INL and ONL (ONL + INL clones) (Figs. 7B–F). Quantitative analysis of the relative proportions of the various clones containing photoreceptors in the experimental retinas revealed a specific and significant reduction of the proportion of ONL-only clones in the rrCNTF-treated retinas, whereas the relative proportion of ONL + INL clones was found similar to that of controls (Fig. 7G). This suggests that the majority of photoreceptor precursors that died following overexposure to CNTF were born from progenitors generating only photoreceptors, and not from progenitors generating both INL and ONL cells.

These results raise the possibility that the photoreceptor precursors dying from normal programmed cell death resulting from stimulation of the CNTF/LIF receptor complex are born from progenitors generating only photoreceptors. If this hypothesis were true, one would expect that blockade of the CNTF/LIF receptor complex would result in an increased proportion of ONL-only clones. To test this hypothesis, eyes of P1 mice were injected with the retrovirus and a day later were injected into the vitreous with either the AdLIF05 or the AdLacZ vector. After computation of over a thousand clones from 4 retinas, statistical analysis confirmed a significant relative increase of ONL-only clones in eyes injected with AdLIF05, while the relative proportion of ONL + INL clones remained unchanged (Fig. 7H). The ONL-only clones observed in LIF05-treated eyes ranged from 1 to 5 cells, as in the controls, and the distribution of clone size was unchanged. These results are consistent with the hypothesis that photoreceptor precursors born from progenitors generating only photoreceptors, and not those born from progenitors generating both photoreceptors and INL cells, are affected by programmed cell death involving signaling through the CNTF/LIF receptor complex.

### *The CNTF/LIF signaling pathway regulates retinal cell death by controlling nitric oxide production*

Because previous evidence suggests that increased NO production can trigger the death of photoreceptors (Donovan et al., 2001; Goureau et al., 1999; Ju et al., 2001) and that NO synthases can be regulated by cytokines via JAK/STAT signaling (Aktan, 2004; Dell'Albani et al., 2003), we wanted to test whether stimulation of the CNTF/LIF signaling pathway might regulate cell death by controlling NO production.

If increased signaling through the CNTF/LIF signaling pathway causes apoptosis of photoreceptor precursors *in vivo* by stimulating the production of NO, then increasing NO production in the developing retina by other means should also increase cell death of the precursors, and reducing NO production should decrease it. To test this hypothesis, we first injected NOC-18, a NO donor, into the vitreous of P2 animals and then counted the number of $TUNEL^+$ cells at P3. We found that NOC-18 dramatically increased the number of apoptotic cells compared to controls (Fig. 8A), and the location of the apoptotic cells was not different from that of apoptotic cells observed following rrCNTF injections. In contrast, intraperitoneal injections of L-NAME, an inhibitor of NOS, significantly reduced the number of $TUNEL^+$ cells detected in the retina compared to controls (Fig. 8B). Together, these results show that increasing NO production during retinal development causes an increase in cell death, similar to results obtained when stimulating the CNTF/LIF pathway, whereas blocking NO production with a NOS inhibitor significantly reduces natural cell death, similar to results obtained when blocking the CNTF/LIF pathway.

If exposure to CNTF during retinal development causes cell death by increasing production of NO, the prediction is that blocking NOS would be sufficient to abolish the pro-apoptotic effects of CNTF overexposure during retinal development. Consistent with this hypothesis, we found that co-injection of L-NAME together with the CNTF protein in the vitreous of P2 mice can significantly abolish the CNTF-induced cell death seen at P3 compared to injection of CNTF alone (Fig. 8C). These results indicate that NOS activity is necessary for CNTF-mediated developmental cell death.

Finally, we tested whether stimulating or blocking the CNTF/LIF signaling pathway could regulate the expression of NOS. Analysis of mRNA extracted at P3 from retinas treated with rrCNTF at P2 reveals that stimulation of the CNTF/LIF signaling pathway causes a significant increase in the expression of mRNA encoding both nNOS and eNOS isoform expression at P3 (Fig. 8E), whereas iNOS isoform expression did not change (not shown). Conversely, blocking the CNTF/LIF signaling pathway by injecting AdLIF05 at P2 resulted in a significant reduction in the expression of both nNOS and eNOS at P7 (Fig. 8F). Taken together, these results suggest that stimulation of the CNTF/LIF signaling pathway increases the expression of NOS in the developing retina, which in turn increases the production of NO and leads to photoreceptor precursor cell death.

## Discussion

In this study, we have stimulated or blocked the CNTF/LIF signaling pathway at different stages of postnatal mouse retinal development to study the role of this pathway in retinal cell

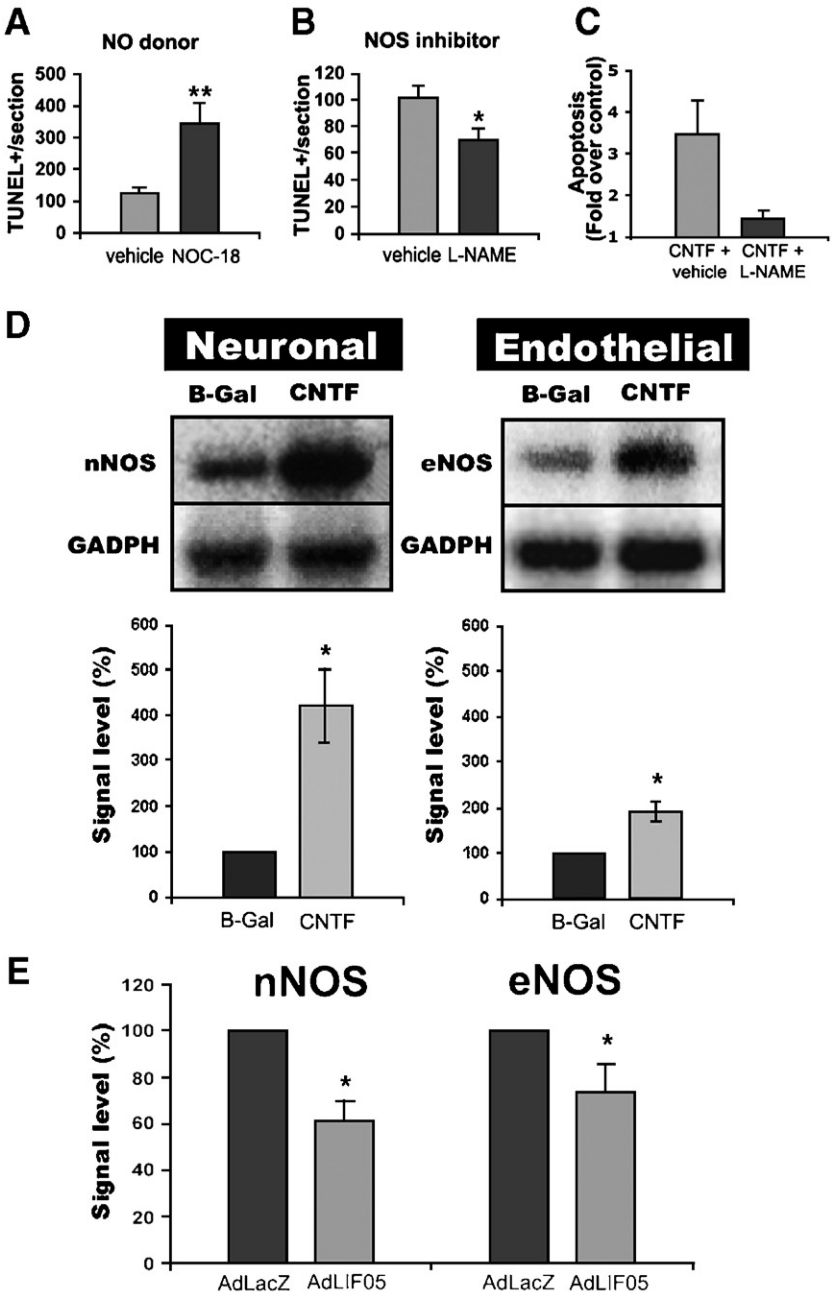


Fig. 8. CNTF/LIF signaling pathway causes apoptosis by regulating NO production through neuronal (n) and endothelial (e) nitric oxide synthase (NOS) expression. (A) Intravitreal injection of a NO donor (NOC-18) at P2 sharply increases cell death as detected by TUNEL at P3. (B) In contrast, intraperitoneal injections of a NOS inhibitor (L-NAME) at P6 significantly decrease naturally occurring cell death in the retina. (C) Co-injection of L-NAME and rrCNTF abolishes CNTF-induced cell death. Uninjected eyes were not significantly different from vehicle-injected (not shown). Semi-quantitative analysis of nNOS and eNOS isoforms mRNA levels in P3 (D) and P7 (E) retinas from eyes injected with rβ-Gal or rrCNTF (D) and AdLacZ or AdLIF05 (E). Signal intensity was analyzed with a phosphoimager, and the relative expression of nNOS and eNOS was normalized against GADPH.

death and differentiation *in vivo*. We found that the CNTF/LIF signaling pathway participates in the regulation of opsin expression *in vivo* and in the programmed cell death of a sub-population of postmitotic precursor cells committed to the rod photoreceptor lineage via stimulation of NO production. We propose that the CNTF/LIF signaling pathway acts during development to fine-tune the numerical balance of photoreceptors.

### *Regulation of photoreceptor cell differentiation by the CNTF/LIF signaling pathway*

Previous *in vitro* studies have shown that stimulation of the CNTF/LIF signaling pathway in rodents reduces the number of cells expressing rhodopsin (Ezzeddine et al., 1997; Neophytou et al., 1997; Ozawa et al., 2004). In addition, retinal explants from CNTFRα−/− newborn mice show a slight increase in the

number of rhodopsin-positive cells after 10 days in culture (Ezzeddine et al., 1997), and blockade of STAT signaling in explants from wild-type newborn mice also results in more rhodopsin-positive cells over controls (Rhee et al., 2004). In an *in vitro* assay, Neophytou et al. provided evidence that stimulation of the CNTF pathway acts by regulating the onset of rhodopsin expression rather than influencing cell fate decisions in dissociated cultures (Neophytou et al., 1997). In agreement with these results, we found that overexposure to CNTF during retinal development *in vivo* reduces the expression of rhodopsin, whereas blocking the stimulation of the CNTF/LIF pathway with the dominant-negative LIF05 accelerates the appearance of rhodopsin-positive cells. Since previous studies have shown that rods can delay their final differentiation for several days after they become postmitotic (Morrow et al., 1998), our *in vivo* results suggest that the CNTF/LIF signaling pathway may play an important part in this process.

In a previous study, when cultures of newborn rat retinal cells were overexposed to CNTF, the number of cells expressing markers of bipolar cells was found to increase (Ezzeddine et al., 1997). Exposure of rat retinal explants to high concentrations of CNTF also resulted in an increased number of cells expressing bipolar cell markers, but also revealed that a significant number of these cells were located in the photoreceptor layer and had photoreceptor cell morphology, suggesting that exposure to high concentrations of CNTF may induce aberrant bipolar cell marker expression in some photoreceptor cells (Schulz-Key et al., 2002). In the present study, although we observed a reduction of rhodopsin-positive cells and delayed expression of rhodopsin after overexposure to CNTF *in vivo*, we did not find evidence for increased number of cells expressing bipolar cell markers and did not observe any cells in the photoreceptor layer expressing bipolar cell markers. The nature of the discrepancy between our *in vivo* findings in the mouse retina and these previous *in vitro* results in rat retina remains unclear. In addition to possible species-specific differences, it is possible that the concentrations achieved in the eye following injection of rrCNTF or AdCNTF vectors are not as high as those used in these studies.

A recent study suggested that addition of CNTF to cell cultures of mouse retinal cells causes an increase in the number of Müller glial cells (Goureau et al., 2004). Here, although we find that exposure to CNTF during retinal development *in vivo* causes an increase in GFAP expression, as reported in this cell culture study (Goureau et al., 2004), we did not find evidence that CNTF exposure increased the number of Müller cells generated as the number of cells expressing markers of Müller glial cells did not appear different following exposure to CNTF (Supplementary Fig. 2) and the number of Müller cells generated in the lineage experiments was not changed after exposure to CNTF (clones contained 2.4±1.3% of Müller cells in CNTF-treated eyes, compared to 2.0±0.5% in rβ-Gal-injected eyes). Thus, at least in the mouse retina *in vivo*, stimulation of the CNTF/LIF signaling pathway appears to act by modulating the timing of rhodopsin expression, but does not induce cell fate changes.

### *Regulation of naturally occurring cell death in the developing retina by the CNTF/LIF signaling pathway*

Addition of CNTF or LIF to developing sympathetic neurons of the rat superior cervical ganglion in culture causes neuronal cell death in a dose-dependent manner, an effect mediated by the classic CNTF receptor (Kessler et al., 1993). Similarly, in our study, we showed that increasing the concentration of CNTF in the postnatal mouse retina *in vivo* during the period of rod cell generation and differentiation results in the dose-dependent apoptotic death of many postmitotic rhodopsin-negative rod precursor cells and that a sustained overexposure beginning shortly after birth leads to a profound deficit in photoreceptors in the mature retina, while other retinal cell types are numerically unaffected. It is likely that this pro-apoptotic effect of CNTF is mediated by signaling through the CNTF/LIF receptor complex, based on the following observations. (1) A similar pro-apoptotic effect is seen with LIF, a protein with relatively low sequence homology to CNTF but using the same transducing components (i.e. LIFRβ and gp130), whereas administration of purified murine IL-6, an interleukin of the same family that uses gp130 but not LIFRβ, has no effect on apoptosis. (2) Prior exposure to the LIF05 protein, a dominant-negative ligand of the CNTF/LIF signaling complex, abolishes the pro-apoptotic effect of CNTF and also reduces the number of photoreceptor precursors triggering naturally occurring cell death.

In the rhodopsin knockout mouse, the absence of rhodopsin results in a complete absence of rod outer segments and a progressive loss of photoreceptors (Humphries et al., 1997; Lem et al., 1999). Could the massive deficit in photoreceptors seen following stimulation of the CNTF/LIF receptor complex be only secondary to the blockade in rhodopsin expression? Our results strongly argue against this possibility. First, in the rhodopsin knockout mice, significant photoreceptor degeneration only begins around P15 (Humphries et al., 1997; Lem et al., 1999), whereas we have shown that immature retinas exposed to AdCNTF from P2 already have an important reduction of more than half the normal ONL thickness as early as P9. Second, a single injection of rrCNTF causes a dramatic increase in cell death as early as 20 h after injection; such a rapid response is unlikely to be the result of an indirect effect from a blockade of rhodopsin expression. Third, CNTF-induced apoptosis in the immature retina is observed at P1 following injection of rrCNTF at P0 (not shown), when we hardly detect any $rho^{+}$ cells at P1 in the untreated retinas. So, while we do indeed see a second wave of increased apoptosis between P15 and P21 following administration of the AdCNTF vector at P2 that could be the result of prolonged blockade of rhodopsin expression achieved by prolonged exposure to CNTF, this secondary wave contributes minimally to the photoreceptor deficit since most cell loss has already occurred by P9, many days before the beginning of photoreceptor loss in rhodopsin knockout mice.

An interesting observation in our study is that overexposure to CNTF leads to a specific cell death of postmitotic photoreceptor precursor cells. In cell lineage tracing experiments

using retroviral vectors, we found that the proportion of clones containing only photoreceptor cells was dramatically reduced following overexposure to CNTF, whereas that of other types of clones was unaffected. These results suggest that postmitotic precursors committed to the rod photoreceptor lineage are specifically responding to this increase in retinal CNTF by triggering a cell death program. Moreover, these results suggest that postmitotic rod precursors born from progenitors generating only ONL cells are different from those generated from progenitors giving rise to ONL+INL cells, at least relative to their response to environmental signals regulating cell survival. Interestingly, photoreceptor-only clones are the most numerous types of clones found in retinal lineage studies (Holt et al., 1988; Turner and Cepko, 1987; Turner et al., 1990; Wetts and Fraser, 1988), suggesting that a certain amount of cell death might be necessary in these clones to fine-tune the numerical balance of photoreceptors.

### *Mechanism of CNTF-induced developmental cell death*

In this report, we provide a potential mechanism by which stimulation of the CNTF/LIF pathway might lead to photoreceptor precursor cell death. We found that the CNTF/LIF pathway can positively regulate the expression of neuronal and endothelial NOS and that the pro-apoptotic effects of a stimulation of the CNTF/LIF pathway during retinal development can be blocked by a pan-NOS inhibitor. In addition, developmental retinal cell death is increased or decreased with a NO donor or a NOS inhibitor respectively. These results strongly suggest that stimulation of the CNTF/LIF signaling pathway during retinal development leads to an increase in NO production by stimulation of NOS expression, which in turn triggers developmental cell death of photoreceptor precursors. But which retinal cell type could respond to a CNTF ligand by increasing NO production? The prediction is that this cell type would require the presence of the CNTFRα. However, the presence of the CNTFRα protein and the responsiveness of rod photoreceptors or precursors to members of the CNTF family ligands are still controversial. Numerous studies have reported either the presence of the protein or the expression (Ju et al., 2000; Rhee and Yang, 2003; Schulz-Key et al., 2002; Valter et al., 2003) or absence thereof (Beltran et al., 2005; Harada et al., 2002; Kirsch et al., 1997; Wahlin et al., 2004) of the gene encoding this receptor subunit in developing and adult rodent photoreceptors. Nonetheless, recent results showed that stimulation of the CNTF signaling pathway induces a strong STAT3 activation in postmitotic immature rod photoreceptors within minutes after stimulation (Rhee et al., 2004), suggesting that overexposure to CNTF in our study might act directly on postmitotic immature photoreceptor cells. It is also possible, however, that the pro-apoptotic effects of CNTF are indirect. One possible candidate cell type for such indirect effects is the retinal ganglion cell (RGC). It is clear that RGCs express CNTFRα (Kirsch et al., 1997) and can respond to CNTF (Meyer-Franke et al., 1995). Interestingly, a recent study has shown that NOS is expressed in RGCs and that RGCs can produce NO (Tsumamoto et al., 2002). Together with data showing that NO can induce cell death of both mature and developing photoreceptors (Donovan et al., 2001; Goureau et al., 1999; Ju et al., 2001), these results suggest that a CNTF ligand might stimulate NOS expression in RGCs and the release of NO, which could act to induce cell death in adjacent developing photoreceptor precursor cells.

In retinal cell culture experiments, increased cell death was not reported following addition of CNTF. How can we reconcile these results with our findings *in vivo*? First, it is possible that increased cell death in culture went undetected as dying cells tend to detach from the culture plate and neighboring cells *in vitro*. Second, because our data suggest that the induction of cell death *in vivo* after exposure to CNTF might occur indirectly via RGCs, it is possible that these RGCs, or another cell type responsible for NO production following stimulation of the CNTF/LIF pathway, are either not present – or have died – under the culture conditions used. Third, as the effects of CNTF require NO production, it is likely that NO rapidly diffuses away from the culture medium *in vitro*, thereby preventing the induction of cell death.

What could be the downstream events that lead to cell death in postmitotic photoreceptor precursor cells following NO production? Two pro-apoptotic Bcl-2 family members, Bak and Bax, are apparently implicated in developmental photoreceptor apoptosis (Hahn et al., 2003). Indeed, it has been reported that the double knockout mouse (Bak−/−, Bax−/−) shows an almost complete block of natural cell death in the immature INL at P7 (Hahn et al., 2003). Interestingly, in the Bax−/− retina, overall cell death is reduced in the INL, but not in the inner rods population (photoreceptor precursors), suggesting that both Bak and Bax are required for normal photoreceptor cell death at P7 (Hahn et al., 2003). When we blocked the CNTF/LIF signaling pathway using LIF05, we found a reduced number of cell death in the inner rods population located in the outer INL, whereas cell death in the inner part of the INL was not affected. Consistent with previous results in the Bak and Bax knockouts, RT-PCR analysis on retinas injected with rrCNTF revealed an increase in Bak, but not Bax mRNA, compared to control (Supplementary Fig. 4). These observations suggest that blocking the CNTF receptor complex might regulate Bak signaling, which would explain the specific blockade of apoptosis in photoreceptor precursor cells, but not in other cell types.

### *Ligands mediating developmental retinal cell death*

As the CNTF protein does not have a leader signal sequence and is not normally secreted by cells *in vitro* (Stockli et al., 1989), it seems more likely that other ligands signaling through the CNTF/LIF pathway mediate the *in vivo* effects. One potential candidate is LIF, which has been shown to be expressed in the postnatal retina (Neophytou et al., 1997), and injection of rmLIF also increases developmental cell death (not shown). Other candidates include cardiotrophin-like cytokine (CLC), which, in association with cytokine-like factor (CLF) or with the soluble form of CNTFRα, can generate soluble composite cytokines which also bind and activate the CNTF

receptor complex (Elson et al., 2000; Lelievre et al., 2001; Plun-Favreau et al., 2001). In addition, a new cytokine recently discovered that it is closely related to CNTF, named Neuropoietin, is expressed during embryonic development of the nervous system including the retina and can also activate the CNTF/LIF signaling pathway (Derouet et al., 2004). Using RT-PCR, we confirmed in this study that CLC and CLF are expressed in the retina and intravitreal injection of CLC and CLF in neonatal mouse caused a sharp increase in cell death similar to that observed with injections of CNTF. Thus, we suggest that these secreted ligands, such as LIF, CLC, or CLF, might be the natural stimulators of the CNTF/LIF signaling pathway *in vivo*. Future studies should help determine directly whether one or more of these factors are involved in the regulation of the developmental cell death of rod photoreceptors.

### *Conclusions*

As photoreceptor cells are the major cell type in the retina, composing more than 70% of all the retinal cells, and that photoreceptor-only clones are the major type of clones observed in lineage experiments, it is likely that a certain amount of programmed cell death is required to fine-tune the numerical balance between photoreceptors and other retinal cell types. A detailed analysis of natural cell death in the developing retina suggested that programmed cell death has a profound effect on shaping retinal cell numbers (Voyvodic et al., 1995). Based on our findings, we propose that, in addition to the low-affinity neurotrophin receptor p75NTR, the CNTF/LIF signaling pathway plays a critical part in regulating this process.

## Materials and methods

### *Animals*

All animal work was carried in accordance with Université Laval and Canadian Institute of Health Research guidelines. C57/B16J and Balb/C were used for this study. Mice were injected at different postnatal day and were kept under cyclic light (12 h light/dark) until sacrifice.

### *Viral vectors and intraocular injections*

Injections were performed under stereomicroscopy using a glass capillary as previously described (Cayouette and Gravel, 1997). The volume of the injection was maintained between 0.5 and 1 μl, and the inoculum was delivered with a varistaltic pump at 0.5 μl/min. The titers of adenoviral vectors were: AdCNTF ($2.9\times10^7$ transducing units (TUs)), AdLacZ ($2\times10^7$ TUs), and AdLIF05 ($2.5\times10^8$ TUs). The procedures for construction and purification of adenoviral vectors have been described elsewhere (Cayouette and Gravel, 1997; Vilquin et al., 1995). Purified rat recombinant CNTF (rrCNTF, PeproTech), mouse recombinant leukemia inhibitory factor (mrLIF, PeproTech), rat interleukin-6 (IL-6, PeproTech) or recombinant *E. coli* β-Galactosidase (β-Gal, Life Technologies) was injected into the vitreous at different concentrations (see text) diluted in a physiological buffer. All animals injected with AdCNTF or rrCNTF into one eye received a control injection of vehicle, rβ-Gal, or AdLacZ into the contralateral eye, so that one eye could always serve as an internal control.

For lineage analysis, mice were injected at P1 with 1 μl of pCLE-AP (Gaiano et al., 1999), a replication-incompetent retrovirus encoding human placental alkaline phosphatase in both eyes. The retroviral vector was prepared as previously described (Cayouette et al., 2001). At P4, 250 ng rrCNTF was injected into the vitreous of the left eye and the right eye was injected with rβ-Gal as control. For AdLIF05 experiments, mice were injected into the vitreous with 0.5 μl of AdLIF05 or AdLacZ the day after retroviral vector injection. The eyes were collected at P21 and sectioned.

### *Histology and immunohistochemistry*

Animals were sacrificed and eyes were fixed by immersion in 4% paraformaldehyde, cryoprotected in sucrose 30% and both eyes frozen and embedded in the same block. Cryostat sections (12 μm) encompassing the 2 retinas were collected on poly-L-lysine coated slides. Some sections were stained with hematoxylin–eosin for histological analysis or processed for immunofluorescence. Sections were pre-incubated for 30 min in PBS, 0.2% Triton X-100, and 10% horse serum and then incubated overnight at 4°C with primary antibodies: anti-opsin antibody rho 1D4 (1:100; (Molday and MacKenzie, 1983)), anti-Islet-1 monoclonal antibody (1:100; from Dev. Studies Hybridoma bank), anti-protein kinase C (PKC) (1:100; Santa Cruz), or anti-RET-B1 antibody (1:20, (Barnstable, 1980)) diluted in PBS, 0.1% Triton X-100, and 5% horse serum. Bound antibodies were detected with goat anti-mouse Alexa 488 (1:500, Molecular Probes) in PBS or by incubation for 2 h in donkey anti-rabbit or anti-mouse biotinylated IgG antibody (1:500, Jackson Immunoresearch) followed by 1 h incubation in streptavidin–FITC (1:1000, Jackson Immunoresearch) in PBS. Sections were mounted in Mowiol containing 5 μg/ml propidium iodide, 6 μg/ml DAPI, or 1 μg/ml Hoechst and 25 units RNase A and observed under a fluorescence microscope (Nikon TE2000-U or Leica DM6000B).

For alkaline phosphatase detection, retinal sections were first immersed in PBS at 65°C for 1 h and then in a solution of 4-nitro blue tetrazolium chloride (NBT/BCIP, Roche) at 37°C until the desired level of staining was achieved (typically 1–2 h).

### *Dominant-negative LIF05 construction and functional assay*

Human LIF cDNA was altered using oligonucleotide-mediated mutagenesis so that the encoded protein has the following substitutions: A117E, D120R, I121K, G124N, S127L, Q25L, S28E, Q32A, and S36K. The mutated cDNA was then inserted into an E1-deleted Ad5 genome under the control of the cytomegalovirus immediate-early promoter. The resulting viral vector (AdLIF05) was tested for its capacity to confer production and secretion of the CNTF receptor dominant-negative ligand to infected cells. For this purpose, supernatants from AdLIF05-infected, AdLacZ-infected, or sham-infected Vero cells (ATCC number CCL-81) cultured in DMEM without serum were added in various amounts to the culture medium of the neuroblastoma cell line IMR-32 that expresses the tripartite CNTF receptor complex (Bartoe and Nathanson, 2002) (J.E. and C.G. unpublished). The culture medium was then supplemented with 50 pM rrCNTF for 20 min, the cells collected, and protein extracts prepared and analyzed for their content in total and phosphorylated STAT-3.

### *Western blots*

Cells were harvested, sonicated, and lysed in a Tris–EDTA buffer: 0.25 M Tris pH 7.6, 10 mM EDTA with Complete™ protease inhibitor cocktail (Roche). Eighty micrograms of protein samples was separated by electrophoresis on an 8% SDS-PAGE gels for 60 min and then transferred onto Immobilon membranes (Millipore, Bedford, MA) for 60 min at 100 mV in 10% methanol transfer buffer. The membranes were blocked with 5% milk in PBS, 0.1% Triton, pH 7.4. Immunoblotting with the rabbit anti-phospho-STAT3 (Cell Signaling Technology) or rabbit anti-STAT3 (1:1000, Upstate) was performed at 4°C overnight in PBS, 0.05% Triton with 1% milk. The primary antibody was detected with an HRP-conjugated donkey anti-rabbit (1: 10,000) (Jackson Immunoresearch) in PBS–Triton 0.05%. Detection of bound antibodies was visualized with ECL kit (Perkin Elmer Life Science).

### *TUNEL, PCNA labeling, and cell death ELISA*

To detect apoptotic cells, retinal sections were processed for terminal dUTP nick-end labeling (TUNEL), using the Apoptag® Plus kit (Serologicals Corp.) according to the recommendations of the manufacturer.

For the TUNEL-PCNA double labeling, TUNEL was performed before the PCNA detection. After TUNEL labeling, sections were incubated for an hour in urea 0.8 M at 65°C for antigen recovery. Sections were then rinsed in PBS and permeabilized for 30 min in PBS–Triton 0.2% containing 10% horse serum then incubated overnight at 4°C in the anti-PCNA monoclonal antibody (1:100, Zymed laboratories inc.) diluted in PBS–Triton 0.2% containing 5% horse serum. Primary antibodies were detected as described above, and slides were mounted with Mowiol, and 3 regions of retina per animal were analyzed by acquiring *Z*-stacks. Colocalization analysis and orthogonal plans were made using the Volocity®3.6 software (Improvision).

Quantification of cell death was done using the Cell Death Detection ELISA$^{PLUS}$ (Roche Biochemicals). Retinas were individually trypsinized, rinsed in PBS, and the cells resuspended in the supplied lysis buffer. The lysate was centrifuged at 20,000×*g* for 10 min, and the supernatant diluted 1:15 before processing for ELISA following the recommendations of the manufacturer. The amount of soluble nucleosomes in each sample was used as an estimate of apoptotic rate in the retina and was normalized with the total protein content. Retinas from control or rrCNTF-treated eyes with or without 800 ng of L-NAME were compared. Statistical comparisons were done using the Student's *t*-test.

### *RT-PCR*

Retinas were pooled by group of 3, and total RNA was extracted from each pool using the RNeasy kit (Qiagen). Four micrograms of RNA was reverse-transcribed in a volume of 20 μl and 1 μl of the cDNA mixture subjected to 30 rounds of PCR amplification using 200 nM of primers. Six microliters of cDNA was used for NOS, Bax, and Bak amplifications and 2 μl for GADPH. Primers used were:

mCNTF forward 5′-TGTCGACAAGTAAATCCACA-3′
mCNTF reverse 5′-GGAGCACCACATTAAATACC-3′
mCNTFR forward 5′-GTGAATTCGTCAAAGGTGAT-3′
mCNTFR reverse 5′-CTACTACCCCAATACCTACA-3′
mCLC forward 5′-CGAGCCTGACTTCAATCCTC-3′
mCLC reverse 5′-GAAACCATGTGCCTCCAAGT-3′
mCLF forward 5′-AGCAGTCAGGAGACAATCTG-3′
mCLF reverse 5′-GAGGACATCAGATCTTGCTG-3′.

For NOS, amplicons from RT-PCR were fractionated on 2% agarose gels and blotted onto nylon membranes. Southern blot analysis was performed with probes generated by PCR and end-labeled with [α-$^{32}$P] dCTP. The result was visualized and quantified with the Personal Molecular imager FX (Bio-Rad).

### *Retinal cell dissociation and quantitative analysis*

Retinas were dissected in PBS and dissociated as described (Cayouette et al., 2003) and resuspended in PBS. 150,000 cells in 200 μl were plated on slides using a cytospin (5 min at 700 rpm) and then fixed with 4% paraformaldehyde. The cells were immediately post-fixed in an ethanol:acetic acid (95:5) solution and processed for TUNEL and rhodopsin immunohistochemistry as described above. Individual retinal cytospins were observed under fluorescence microscope (Nikon Eclipse TE2000-U), and cells were counted in three regions using Metamorph® (Universal Imaging Corporation™).

### *Morphometric analysis*

The outer nuclear layer (ONL) column height (Michon et al., 1991) was measured by counting the number of rows of photoreceptor nuclei making up the ONL at 100–125 μm intervals from the ora serrata to the optic nerve head on five hematoxylin–eosin stained sections spaced 60 μm apart. The mean of these values was used as the estimate of ONL column height for each eye. Statistical comparisons were done using Student's *t*-test. For the PKC and Islet-1 immunolabeling, cells were counted (INL total cells and positive cells) on three half retina per animal.

## Acknowledgments

We thank Caroline Paquet and Ann Lorain for expert technical assistance, Drs. Robert Molday and Colin Barnstable for providing the Rho1D4 and Ret-B1 antibodies, and Martin Raff for comments on the manuscript. This work was supported by grants from the Canadian Institute of Health Research (M.C. and C.G.) and the Foundation Fighting Blindness-Canada (M.C.). M.C. is a W.K. Stell Scholar of the Foundation Fighting Blindness-Canada.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.09.002.

## References

Aktan, F., 2004. iNOS-mediated nitric oxide production and its regulation. Life Sci. 75, 639–653.

Barnstable, C.J., 1980. Monoclonal antibodies which recognize different cell types in the rat retina. Nature 286, 231–235.

Bartoe, J.L., Nathanson, N.M., 2002. Independent roles of SOCS-3 and SHP-2 in the regulation of neuronal gene expression by leukemia inhibitory factor. Brain Res. Mol. Brain Res. 107, 108–119.

Bazan, J.F., 1991. Neuropoietic cytokines in the hematopoietic fold. Neuron 7, 197–208.

Beltran, W.A., Rohrer, H., Aguirre, G.D., 2005. Immunolocalization of ciliary neurotrophic factor receptor alpha (CNTFRalpha) in mammalian photoreceptor cells. Mol. Vision 11, 232–244.

Bhattacharya, S., Dooley, C., Soto, F., Madson, J., Das, A.V., Ahmad, I., 2004. Involvement of Ath3 in CNTF-mediated differentiation of the late retinal progenitors. Mol. Cell. Neurosci. 27, 32–43.

Cayouette, M., Gravel, C., 1997. Adenovirus-mediated gene transfer of ciliary neurotrophic factor can prevent photoreceptor degeneration in the retinal degeneration (rd) mouse. Hum. Gene Ther. 8, 423–430.

Cayouette, M., Behn, D., Sendtner, M., Lachapelle, P., Gravel, C., 1998. Intraocular gene transfer of ciliary neurotrophic factor prevents death and increases responsiveness of rod photoreceptors in the retinal degeneration slow mouse. J. Neurosci. 18, 9282–9293.

Cayouette, M., Whitmore, A.V., Jeffery, G., Raff, M., 2001. Asymmetric segregation of Numb in retinal development and the influence of the pigmented epithelium. J. Neurosci. 21, 5643–5651.

Cayouette, M., Barres, B.A., Raff, M., 2003. Importance of intrinsic mechanisms in cell fate decisions in the developing rat retina. Neuron 40, 897–904.

Dell'Albani, P., Santangelo, R., Torrisi, L., Nicoletti, V.G., Giuffrida Stella, A.M., 2003. Role of the JAK/STAT signal transduction pathway in the regulation of gene expression in CNS. Neurochem. Res. 28, 53–64.

Derouet, D., Rousseau, F., Alfonsi, F., Froger, J., Hermann, J., Barbier, F., Perret, D., Diveu, C., Guillet, C., Preisser, L., Dumont, A., Barbado, M., Morel, A., deLapeyriere, O., Gascan, H., Chevalier, S., 2004. Neuropoietin, a new IL-6-related cytokine signaling through the ciliary neurotrophic factor receptor. Proc. Natl. Acad. Sci. U. S. A. 101, 4827–4832.

Ding, J., Hu, B., Tang, L.S., Yip, H.K., 2001. Study of the role of the low-affinity neurotrophin receptor p75 in naturally occurring cell death during development of the rat retina. Dev. Neurosci. 23, 390–398.

Donovan, M., Carmody, R.J., Cotter, T.G., 2001. Light-induced photoreceptor apoptosis in vivo requires neuronal nitric-oxide synthase and guanylate cyclase activity and is caspase-3-independent. J. Biol. Chem. 276, 23000–23008.

Elson, G.C., Lelievre, E., Guillet, C., Chevalier, S., Plun-Favreau, H., Froger, J., Suard, I., de Coignac, A.B., Delneste, Y., Bonnefoy, J.Y., Gauchat, J.F., Gascan, H., 2000. CLF associates with CLC to form a functional

heteromeric ligand for the CNTF receptor complex. Nat. Neurosci. 3, 867–872.
Ezzeddine, Z.D., Yang, X., DeChiara, T., Yancopoulos, G., Cepko, C.L., 1997. Postmitotic cells fated to become rod photoreceptors can be respecified by CNTF treatment of the retina. Development 124, 1055–1067.
Frade, J.M., Barde, Y.A., 1999. Genetic evidence for cell death mediated by nerve growth factor and the neurotrophin receptor p75 in the developing mouse retina and spinal cord. Development 126, 683–690.
Frade, J.M., Rodriguez-Tebar, A., Barde, Y.A., 1996. Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 383, 166–168.
Gaiano, N., Kohtz, J.D., Turnbull, D.H., Fishell, G., 1999. A method for rapid gain-of-function studies in the mouse embryonic nervous system. Nat. Neurosci. 2, 812–819.
Gaiano, N., Nye, J.S., Fishell, G., 2000. Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26, 395–404.
Gibbs, S.M., 2003. Regulation of neuronal proliferation and differentiation by nitric oxide. Mol. Neurobiol. 27, 107–120.
Goureau, O., Regnier-Ricard, F., Jonet, L., Jeanny, J.C., Courtois, Y., Chany-Fournier, F., 1997. Developmental expression of nitric oxide synthase isoform I and III in chick retina. J. Neurosci. Res. 50, 104–113.
Goureau, O., Regnier-Ricard, F., Desire, L., Courtois, Y., 1999. Role of nitric oxide in photoreceptor survival in embryonic chick retinal cell culture. J. Neurosci. Res. 55, 423–431.
Goureau, O., Rhee, K.D., Yang, X.J., 2004. Ciliary neurotrophic factor promotes muller glia differentiation from the postnatal retinal progenitor pool. Dev. Neurosci. 26, 359–370.
Hahn, P., Lindsten, T., Ying, G.S., Bennett, J., Milam, A.H., Thompson, C.B., Dunaief, J.L., 2003. Proapoptotic bcl-2 family members, Bax and Bak, are essential for developmental photoreceptor apoptosis. Invest. Ophthalmol. Vis. Sci. 44, 3598–3605.
Harada, T., Harada, C., Kohsaka, S., Wada, E., Yoshida, K., Ohno, S., Mamada, H., Tanaka, K., Parada, L.F., Wada, K., 2002. Microglia–Muller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. J. Neurosci. 22, 9228–9236.
Harada, C., Harada, T., Nakamura, K., Sakai, Y., Tanaka, K., Parada, L.F., 2005. Effect of p75(NTR) on the regulation of naturally occurring cell death and retinal ganglion cell number in the mouse eye. Dev. Biol.
Hess, D.T., Patterson, S.I., Smith, D.S., Skene, J.H., 1993. Neuronal growth cone collapse and inhibition of protein fatty acylation by nitric oxide. Nature 366, 562–565.
Holt, C.E., Bertsch, T.W., Ellis, H.M., Harris, W.A., 1988. Cellular determination in the *Xenopus* retina is independent of lineage and birth date. Neuron 1, 15–26.
Hudson, K.R., Vernallis, A.B., Heath, J.K., 1996. Characterization of the receptor binding sites of human leukemia inhibitory factor and creation of antagonists. J. Biol. Chem. 271, 11971–11978.
Humphries, M.M., Rancourt, D., Farrar, G.J., Kenna, P., Hazel, M., Bush, R.A., Sieving, P.A., Sheils, D.M., McNally, N., Creighton, P., Erven, A., Boros, A., Gulya, K., Capecchi, M.R., Humphries, P., 1997. Retinopathy induced in mice by targeted disruption of the rhodopsin gene. Nat. Genet. 15, 216–219.
Ju, W.K., Lee, M.Y., Hofmann, H.D., Kirsch, M., Oh, S.J., Chung, J.W., Chun, M.H., 2000. Increased expression of ciliary neurotrophic factor receptor alpha mRNA in the ischemic rat retina. Neurosci. Lett. 283, 133–136.
Ju, W.K., Chung, I.W., Kim, K.Y., Gwon, J.S., Lee, M.Y., Oh, S.J., Chun, M.H., 2001. Sodium nitroprusside selectively induces apoptotic cell death in the outer retina of the rat. NeuroReport 12, 4075–4079.
Kessler, J.A., Ludlam, W.H., Freidin, M.M., Hall, D.H., Michaelson, M.D., Spray, D.C., Dougherty, M., Batter, D.K., 1993. Cytokine-induced programmed death of cultured sympathetic neurons. Neuron 11, 1123–1132.
Kim, K.Y., Ju, W.K., Oh, S.J., Chun, M.H., 2000. The immunocytochemical localization of neuronal nitric oxide synthase in the developing rat retina. Exp. Brain Res. 133, 419–424.
Kirsch, M., Fuhrmann, S., Wiese, A., Hofmann, H.D., 1996. CNTF exerts opposite effects on in vitro development of rat and chick photoreceptors. NeuroReport 7, 697–700.
Kirsch, M., Lee, M.Y., Meyer, V., Wiese, A., Hofmann, H.D., 1997. Evidence for multiple, local functions of ciliary neurotrophic factor (CNTF) in retinal development: expression of CNTF and its receptors and in vitro effects on target cells. J. Neurochem. 68, 979–990.
Kirsch, M., Schulz-Key, S., Wiese, A., Fuhrmann, S., Hofmann, H., 1998. Ciliary neurotrophic factor blocks rod photoreceptor differentiation from postmitotic precursor cells in vitro. Cell Tissue Res. 291, 207–216.
LaVail, M.M., Unoki, K., Yasumura, D., Matthes, M.T., Yancopoulos, G.D., Steinberg, R.H., 1992. Multiple growth factors, cytokines, and neurotrophins rescue photoreceptors from the damaging effects of constant light. Proc. Natl. Acad. Sci. U. S. A. 89, 11249–11253.
Lelievre, E., Plun-Favreau, H., Chevalier, S., Froger, J., Guillet, C., Elson, G.C., Gauchat, J.F., Gascan, H., 2001. Signaling pathways recruited by the cardiotrophin-like cytokine/cytokine-like factor-1 composite cytokine: specific requirement of the membrane-bound form of ciliary neurotrophic factor receptor alpha component. J. Biol. Chem. 276, 22476–22484.
Lem, J., Krasnoperova, N.V., Calvert, P.D., Kosaras, B., Cameron, D.A., Nicolo, M., Makino, C.L., Sidman, R.L., 1999. Morphological, physiological, and biochemical changes in rhodopsin knockout mice. Proc. Natl. Acad. Sci. USA 96, 736–741.
Li, S., Tay, D., Shu, S., Bao, X., Wu, Y., Wang, X., Yip, H.K., 2006. Endothelial nitric oxide synthase is expressed in amacrine cells of developing human retinas. Invest. Ophthalmol. Visual Sci. 47, 2141–2149.
Meyer-Franke, A., Kaplan, M.R., Pfrieger, F.W., Barres, B.A., 1995. Characterization of the signaling interactions that promote the survival and growth of developing retinal ganglion cells in culture. Neuron 15, 805–819.
Michon, J.J., Li, Z.L., Shioura, N., Anderson, R.J., Tso, M.O., 1991. A comparative study of methods of photoreceptor morphometry. Invest. Ophthalmol. Visual Sci. 32, 280–284.
Molday, R.S., MacKenzie, D., 1983. Monoclonal antibodies to rhodopsin: characterization, cross-reactivity, and application as structural probes. Biochemistry 22, 653–660.
Morrow, E.M., Belliveau, M.J., Cepko, C.L., 1998. Two phases of rod photoreceptor differentiation during rat retinal development. J. Neurosci. 18, 3738–3748.
Neophytou, C., Vernallis, A.B., Smith, A., Raff, M.C., 1997. Muller-cell-derived leukaemia inhibitory factor arrests rod photoreceptor differentiation at a postmitotic pre-rod stage of development. Development 124, 2345–2354.
Ozawa, Y., Nakao, K., Shimazaki, T., Takeda, J., Akira, S., Ishihara, K., Hirano, T., Oguchi, Y., Okano, H., 2004. Downregulation of STAT3 activation is required for presumptive rod photoreceptor cells to differentiate in the postnatal retina. Mol. Cell. Neurosci. 26, 258–270.
Patel, J.I., Gentleman, S.M., Jen, L.S., Garey, L.J., 1997. Nitric oxide synthase in developing retinas and after optic tract section. Brain Res. 761, 156–160.
Plun-Favreau, H., Elson, G., Chabbert, M., Froger, J., deLapeyriere, O., Lelievre, E., Guillet, C., Hermann, J., Gauchat, J.F., Gascan, H., Chevalier, S., 2001. The ciliary neurotrophic factor receptor alpha component induces the secretion of and is required for functional responses to cardiotrophin-like cytokine. EMBO J. 20, 1692–1703.
Rhee, K.D., Yang, X.J., 2003. Expression of cytokine signal transduction components in the postnatal mouse retina. Mol. Vision 9, 715–722.
Rhee, K.D., Goureau, O., Chen, S., Yang, X.J., 2004. Cytokine-induced activation of signal transducer and activator of transcription in photoreceptor precursors regulates rod differentiation in the developing mouse retina. J. Neurosci. 24, 9779–9788.
Roskams, A.J., Bredt, D.S., Dawson, T.M., Ronnett, G.V., 1994. Nitric oxide mediates the formation of synaptic connections in developing and regenerating olfactory receptor neurons. Neuron 13, 289–299.
Schulz-Key, S., Hofmann, H.D., Beisenherz-Huss, C., Barbisch, C., Kirsch, M., 2002. Ciliary neurotrophic factor as a transient negative regulator of rod development in rat retina. Invest. Ophthalmol. Visual Sci. 43, 3099–3108.
Sharma, R.K., Perez, M.T., Ehinger, B., 1997. Immunocytochemical localisation of neuronal nitric oxide synthase in developing and transplanted rabbit retinas. Histochem. Cell. Biol. 107, 449–458.
Shuster, T.A., Farber, D.B., 1986. Rhodopsin phosphorylation in developing normal and degenerative mouse retinas. Invest. Ophthalmol. Visual Sci. 27, 264–268.

Stockli, K.A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Gotz, R., Lindholm, D., Thoenen, H., 1989. Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature 342, 920–923.

Tsumamoto, Y., Yamashita, K., Takumida, M., Okada, K., Mukai, S., Shinya, M., Yamashita, H., Mishima, H.K., 2002. In situ localization of nitric oxide synthase and direct evidence of NO production in rat retinal ganglion cells. Brain Res. 933, 118–129.

Turner, D.L., Cepko, C.L., 1987. A common progenitor for neurons and glia persists in rat retina late in development. Nature 328, 131–136.

Turner, D.L., Snyder, E.Y., Cepko, C.L., 1990. Lineage-independent determination of cell type in the embryonic mouse retina. Neuron 4, 833–845.

Valter, K., Bisti, S., Stone, J., 2003. Location of CNTFRalpha on outer segments: evidence of the site of action of CNTF in rat retina. Brain Res. 985, 169–175.

Vilquin, J.T., Guerette, B., Kinoshita, I., Roy, B., Goulet, M., Gravel, C., Roy, R., Tremblay, J.P., 1995. FK506 immunosuppression to control the immune reactions triggered by first-generation adenovirus-mediated gene transfer. Hum. Gene Ther. 6, 1391–1401.

Voyvodic, J.T., Burne, J.F., Raff, M.C., 1995. Quantification of normal cell death in the rat retina: implications for clone composition in cell lineage analysis. Eur. J. Neurosci. 7, 2469–2478.

Wahlin, K.J., Lim, L., Grice, E.A., Campochiaro, P.A., Zack, D.J., Adler, R., 2004. A method for analysis of gene expression in isolated mouse photoreceptor and Muller cells. Mol. Vision 10, 366–375.

Wang, T., Xie, Z., Lu, B., 1995. Nitric oxide mediates activity-dependent synaptic suppression at developing neuromuscular synapses. Nature 374, 262–266.

Wang, Y., Smith, S.B., Ogilvie, J.M., McCool, D.J., Sarthy, V., 2002. Ciliary neurotrophic factor induces glial fibrillary acidic protein in retinal Muller cells through the JAK/STAT signal transduction pathway. Curr. Eye Res. 24, 305–312.

Watanabe, T., Raff, M.C., 1988. Retinal astrocytes are immigrants from the optic nerve. Nature 332, 834–837.

Wetts, R., Fraser, S.E., 1988. Multipotent precursors can give rise to all major cell types of the frog retina. Science 239, 1142–1145.

Wu, H.H., Williams, C.V., McLoon, S.C., 1994. Involvement of nitric oxide in the elimination of a transient retinotectal projection in development. Science 265, 1593–1596.

Young, R.W., 1984. Cell death during differentiation of the retina in the mouse. J. Comp. Neurol. 229, 362–373.