# Induction of cell death by endogenous nerve growth factor through its p75 receptor

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**During development, neuronal survival is regulated by the limited availability of neurotrophins, which are proteins of the nerve growth factor (NGF) family. Activation of specific *trk* tyrosine kinase receptors by the neurotrophins blocks programmed cell death. The *trk*A-specific ligand NGF has also been shown to activate the non-tyrosine kinase receptor p75, a member of the tumour necrosis factor (TNF) receptor and Fas (APO-1/CD95) family. Here we report that, early in development, endogenous NGF causes the death of retinal neurons that express p75 but not *trk*A. These results indicate that, as with cells of the immune system, the death of neurons in the central nervous system can also be induced by ligands, and that the effect of NGF on cell fate depends on the type of receptor expressed by developing neurons.**

The elimination of neurons has long been recognized as an integral part of normal development. In vertebrates, this phenomenon is particularly well documented during the time corresponding to the innervation of target cells[1], and is easily monitored as net cell numbers decrease over time. During this period, programmed cell death can be prevented by the administration of exogenous neurotrophins, or increased by neutralizing the neurotrophins with antibodies or by deleting their genes[2]. Prevention of cell death involves tyrosine kinase receptors of the *trk* family, as the deletion of the genes coding for these receptors generates phenotypes similar to those resulting from ligand elimination[3]. In addition, cell death can also be observed in the nervous system before innervation of the target cells. Soon after leaving the cell cycle, many neurons die, as has been shown in the cerebral cortex[4], and in the worm *Caenorhabditis elegans*.[5] Here we consider the regulation of this early form of neuronal death.

In the absence of *trk*A, NGF can signal in non-transformed cells through the neurotrophin receptor p75, as shown by the nuclear translocation of the transcription activator NF-$\kappa$B[6]. To examine the relevance of p75 activation by NGF *in vivo*, we investigated the consequences of NGF removal on the development of the chick retina. Previous *in situ* hybridization studies[7] have demonstrated the presence of p75 messenger RNA as early as embryonic day 4 (E4), and staining of E6 retinal sections with antibodies to chick p75 confirmed the presence of this receptor mostly in the central retina, but also on scattered cells (data not shown). The *NGF* gene is expressed as early as E4, but the *trkA* gene is expressed only later in development (Fig. 1). Cell death is widespread in the dorsal part of the chick retina as early as E4 (ref. 8), before innervation of the tectum begins, and TUNEL staining, which detects DNA fragments on tissue sections, revealed a pattern corresponding to that of p75 expression (Fig. 2*e*). Double labelling with p75 antibodies showed that 95% of the TUNEL-positive cells were also p75-positive (94 cells counted). To test the possibility that endogenous NGF causes cell death, cells secreting an anti-NGF monoclonal antibody[9] were applied onto chick embryos at E2.5. When examined at E6, the retinae of embryos treated with anti-NGF antibodies showed a remarkable reduction in the number of TUNEL-positive cells when compared with those of control embryos (Fig. 2; compare *c* with *d*, and *e* with *f*). To quantify the reduction in cell death resulting from the administration of anti-NGF antibodies, soluble nucleosomes were measured in retinal extracts by using an immunoassay combining anti-DNA and anti-histone antibodies[10]. A marked reduction was observed at E6 (Table 1), and in three embryos, cell death was reduced by as much as 80%. Similar effects were observed with sheep anti-NGF polyclonal antibodies, but hybridoma cells producing an anti-NT-3 antibody had no

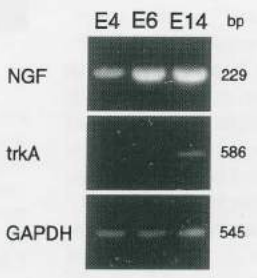


FIG. 1 *NGF* and *trkA* gene expression in the developing chick retina.

effect on developmental cell death (data not shown). NT-3 is also expressed in the early chick retina (like NGF), and is needed during retinogenesis[11], but preventing the generation of neurons dying at E6 by anti-NT-3 treatment might cancel out any possible increase in cell death caused by the anti-NT-3 antibody.

As p75 is also expressed on many cells that are not dying, we investigated whether the application of exogenous NGF would increase cell death. NGF was injected either directly into one eye at E5 (1 μl, 7.5 ng μl$^{-1}$, $n = 5$), using the contralateral eye as a control, or was applied systemically (2.5 μg NGF per embryo per day from E2.5 to E5.5, $n = 4$) using retinae from untreated embryos ($n = 5$) as a control. When soluble nucleosomes were quantified in E6 embryos, no increase in cell death could be observed. Thus expression of p75 alone is not sufficient to predict NGF-mediated cell death. Other conditions must presumably be met, such as the presence of cytoplasmic interactors mediating the effects of p75, and/or the activity of the still ill-defined cell-death program in p75-expressing cells. In embryos not treated with anti-NGF antibodies, p75 is likely to mediate cell death as no other receptors have been described for this neurotrophin, and no *trk*A is expressed during this early developmental period (Fig. 1). To demonstrate this point directly, we used anti-p75 antibodies that were raised against the extracellular domain of chick p75 and which have previously been shown to block the binding of NGF to this receptor[12]. Injecting these antibodies into the vitreal cavity of E4.5 embryos led to a substantial reduction in cell death, indicating that the death of retinal cells is mediated by a p75-dependent mechanism (Table 1).

These results indicate that the binding of NGF to p75 accounts for much of the early cell death during retinal development. The application of NGF has previously been reported to increase cell death in the isthmo-optic nucleus[13], and the death of motor neurons in the facial nucleus after sectioning of the facial nerve in new-born rats[14]; in both cases, cell death was prevented by BDNF. Given our results, it is possible that in these previous experiments the death induced by NGF might have resulted from the activation of p75, which is known to be expressed both by neurons of the isthmo-optic nucleus and by axotomized motor neurons[7,15]. Alternatively, exogenous NGF might prevent binding of endogenous ligands to p75 thought to help in the detection of low

TABLE 1 Cell death in E6 retina after antibody treatments

| Control | Anti-NGF | Anti-p75 |
|---|---|---|
| 1.01 ± 0.11 ($n = 9$)† | 0.47 ± 0.17* ($n = 12$) | – |
| 1.13 ± 0.34 ($n = 5$)‡ | – | 0.47 ± 0.16* ($n = 11$) |

Soluble nucleosomes were quantified in retinae from control and antibody-treated embryos. For each determination, the absorbance obtained in controls was normalized to a value of 1; $n$, total number of embryos used in 3 separate experiments with either anti-NGF or with anti-p75 antibodies.

* $P < 0.001$ (Student's $t$-test).

† Controls were injected with PBS.

‡ Controls were injected with an irrelevant antiserum.

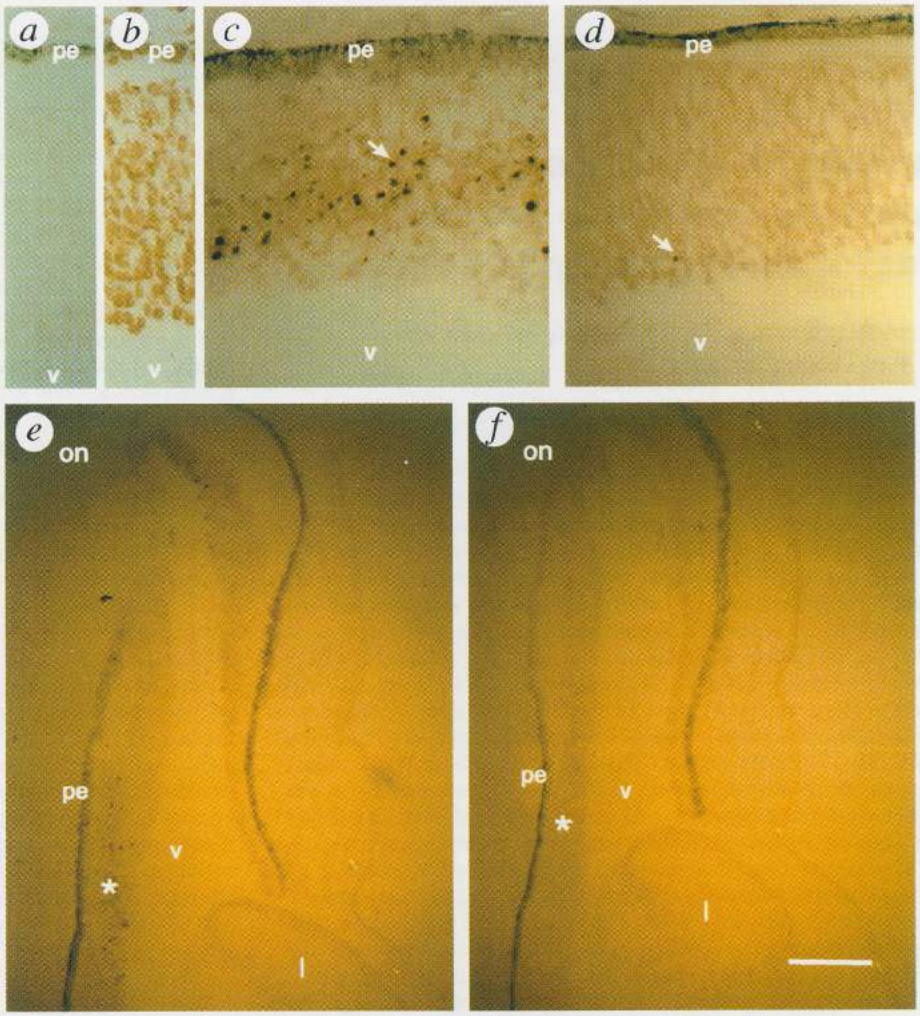


FIG. 2 NGF-antibodies reduce cell death in the E6 chick retina, as assessed by the terminal transferase reaction. *a*, Control TUNEL staining without incubation with terminal transferase. *b*, TUNEL staining on adjacent section after DNAse treatment. *c*, TUNEL staining of control, untreated embryos. Note abundant labelled nuclei (arrow). *d*, The same retinal region as *c*, from an anti-NGF-treated embryo. Note the dramatic reduction in the number of stained cells. *e*, Control retina at a lower magnification showing numerous stained nuclei in the central retina. The region with the asterisk corresponds to that shown in *c*. *f*, Anti-NGF-treated retina. The region by the asterisk corresponds to that shown in *d*. Abbreviations: l, lens; on, optic nerve; pe, pigment epithelium; v, vitreous body. Scale bar, 25 μm (*a*–*d*), 150 μm (*e*, *f*). Similar results were obtained in four separate experiments.

levels of neurotrophin by the *trk* receptors. Previous *in vitro* studies[16,17] have suggested a role for p75 in mediating cell death, but reached the conclusion that NGF blocked cell death, whereas we found that NGF caused cell death *in vivo*.

Thus, NGF seems to be unique endogenous ligand needed both to cause and prevent cell death during normal development. *In vitro*, ligand-mediated elimination of nerve cells is already well documented in the developing nervous system. In particular, the bone morphogenetic protein 4 (Bmp-4) has been shown to induce neural crest depletion in rhombomeres 3 and 5 (ref. 18). Ligand-mediated cell death might be part of a morphogenetic process, and/or contribute to the elimination of unwanted cellular phenotypes. It will be interesting to examine whether NGF participates in the elimination of the neurons that form transient structures, such as the cortical subplate. This transient cell population is involved in the formation of ocular dominance columns, and expresses p75 before its elimination[19,20]. In the central retina, ligand-mediated cell death might create space for the axons of the retinal ganglion cells converging towards the central retina to form the optic nerve. Indeed, the distribution of dying cells, as observed on reconstruction of retinal section, seems to be related to the development of the optic fibres[8]. □

## Methods

**Embryos.** Fertilized eggs form white-leghorn hens were incubated at 38.5 °C in 70% humidity, and were staged according to published methods[21].

**Reverse transcription–polymerase chain reaction (RT–PCR).** Total RNA from E4, E6 and E14 retinae was extracted using TRIZOL reagent (Gibco BRL), and 500 μg total RNA was used for cDNA synthesis using SuperScript II Rnase H⁻ reverse transcriptase (Gibco BRL), and 16 μg cDNA used for PCR amplification (*NGF* primers, bp 595–614 and 804–823, ref. 22; *trkA* primers, 1443–1462 and 1743–1762, ref. 23; glyceraldehyde-3-phosphate dehydrogenase, 659–678 and 1186–1205, ref. 24). *NGF* and *trkA* mRNAs were amplified for 33 cycles (59 °C for 30 s/72 °C for 1 min/95 °C for 30 s), and GAPDH mRNA for 30 cycles (65 °C for 30 s/72 °C for 1 min/95 °C for 30 s). Portions of the reactions were fractionated in 1% agarose gels. Amplification reactions in the absence of reverse transcription did not lead to the detection of any reaction product (not shown).

**TUNEL staining.** E6 chicken embryo heads were fixed in 4% paraformaldehyde/PBS overnight at 4 °C, incubated in 100 mM sodium phosphate, pH 7.3, containing 30% sucrose. Cryopreserved retinal sections (8 μm) were stained with the *in situ* cell-death detection kit (Boehringer Manheim) following the manufacturer's instructions.

**Quantification of cell death.** Cell death was quantified by an enzyme-linked immunosorbent assay (ELISA) (Boehringer Mannheim) using a combination of antibodies recognizing histones and DNA, allowing the quantification of soluble nucleosomes in cell lysates[10]. Retinae of control and treated embryos were homogenized in 200 μl PBS containing 1 mM PMSF and centrifuged at 20,000*g* for 10 min. A portion of supernatant was used to quantify proteins by standard methods, and the rest was diluted 1:10 in the supplied buffer and processed. Absorbance values ranged between 0.1 and 0.7, and were normalized with respect to the values obtained with control retinae.

**Embryo treatment with hybridoma cells.** Hybridoma cells secreting anti-NGF or anti-NT-3 antibodies (both IgG1) were grown *in ovo* following the procedure previously described for quail embryos[9,25]. Eggs were incubated for 2.5 days (until stage 14–15 of development), and a suspension of $2 \times 10^6$ hybridoma cells in 50 μl PBS was applied onto the embryos that were killed after 3.5 days. In the anti-NGF-treated embryos, $44 \pm 9$ μg anti-NGF immunoglobulin per g protein was measured in the retinae.

**Eye injections of anti-p75.** In the anti-p75 experiments, 1 μl of the CHEX antisera (diluted 1:10 in PBS) was injected into the vitreal space of E4.5 chicken eyes using a Microinjector 5242 (Eppendorf). After 12 h, $7 \pm 1.7$ μg immunoglobulin per g protein was detected in the eye, and nucleosomes quantified and compared with the levels determined in the contralateral, control eye. Embryos treated with an irrelevant antisera diluted 1:10 in PBS were also used as controls.

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