Unscheduled re-entry into the cell cycle induced by NGF precedes cell death in nascent retinal neurones

José María Frade
Instituto Cajal de Neurobiología, CSIC, Avenida Doctor Arce 37, Madrid E28002, Spain
e-mail: isrf303@fresno.csic.es
Accepted 12 January; published on WWW 7 March 2000

SUMMARY

During their early postmitotic life, a proportion of the nascent retinal ganglion cells (RGCs) are induced to die as a result of the interaction of nerve growth factor (NGF) with the neurotrophin receptor p75. To analyse the mechanisms by which NGF promotes apoptosis, an in vitro culture system consisting of dissociated E5 retinal cells was established. In this system, NGF-induced apoptosis was only observed in the presence of insulin and neurotrophin-3, conditions that favour the birth of RGCs and other neurones expressing the glycoprotein G4. The pro-apoptotic effect of NGF on the G4-positive neurones was evident after 10 hours in vitro and was preceded by a significant upregulation of cyclin B2, but not cyclin D1, and the presence of mitotic nuclei in these cells. Brain-derived neurotrophic factor prevented both the increase of cyclin B2 expression in the G4-positive neurones and the NGF-induced cell death. Finally, pharmacologically blocking cell-cycle progression using the cyclin-dependent kinase inhibitor roscovitine prevented NGF-induced cell death in a dose-dependent manner. These results strongly suggest that the apoptotic signalling initiated by NGF requires a driving stimulus manifested by the neuronal birth and is preceded by the unscheduled re-entry of postmitotic neurones into the cell cycle.

Key words: Neurotrophin receptor p75, Nerve growth factor, Neuronal apoptosis, Cell cycle, Cyclin B2
neuronal cells. This fine control is reflected in studies of the retinoblastoma gene product, which has been shown in a number of studies to arrest the cell cycle in late G1 phase (Goodrich et al., 1991). Retinoblastoma-null mutant mouse embryos present massive cell death in the CNS just outside the ventricular area where postmitotic, migrating neurones can be found (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). The observation of mitotic figures in the same regions as the dying cells led Lee et al. (1992) to hypothesise that once a neuroblast ended its normal period of cell division no further re-entry into the cell cycle is allowed and, if the control of this process breaks down the cell then dies. Along the same lines, Herrup and Busser (1995) have shown that the induction of multiple cell-cycle events precedes target-related neuronal death in the CNS and ElShamy et al. (1998) associated the death of dorsal root ganglion neurones with exit from the cell cycle in neurotrophin-3 (NT3) null-mutant mice. These cells, in the absence of NT3, fail to arrest the cell cycle and die in S phase. In vitro support for the association between ectopic cell division and cell death in the nervous system has been provided by Freeman et al. (1994), who showed that the death of cultured sympathetic neurones, triggered by NGF withdrawal, leads to the accumulation of cyclin D1 (a G1 phase marker known to activate cyclin-dependent kinase 4 (CDK4)/CDK6). Moreover, Kranenburg et al. (1996) have shown that the artificial elevation of cyclin D1 levels is sufficient to induce apoptosis in neural and non-neural cell types. In addition, the use of CDK inhibitors or dominant-negative forms of CDK4/CDK6 promotes the survival of NGF-deprived sympathetic neurones (Park et al., 1996, 1997a). Finally, another cell-cycle progression marker that has been shown to be upregulated during dopamine-triggered neuronal apoptosis is cyclin B2 (Shirvan et al., 1997, 1998). Cyclin B2 is directly involved in the transition from the G2 phase to mitosis, activating CDK1/CDC2.

In the embryonic chick retina, a peak of apoptosis mediated by NGF affects some p75NGFR-positive cells. This peak correlates with the active period of retinal ganglion cell (RGC) neurogenesis (Frade et al., 1996c; Frade and Barde, 1998). Interestingly, when BDNF was administered in ovo during the period immediately prior to the peak of cell death, a significant reduction in the amount of apoptosis was observed (Frade et al., 1997), resulting in an increase in the number of postmitotic RGCs. In addition, the number of nascent, premigratory RGCs was not altered by BDNF treatment. These results strongly suggest that the dying retinal cells, rescued by BDNF, were postmitotic neurones in the process of migration to the vitreal portion of the retina.

We have investigated whether the cell death initiated by p75NGFR upon binding to NGF is preceded by the upregulation of components required for cell-cycle progression in newborn, postmitotic neurones. To test this hypothesis, an in vitro system consisting of dissociated E5 retinal cells cultured on laminin-1 in the presence of 1 μM insulin and 0.1 nM NT3 was used. These conditions induce the birth of G4-positive neurones (de la Rosa et al., 1994; Frade et al., 1996a,b), a subset of retinal cells that includes RGCs and some amacrine neurones, and are known to express p75NGFR (Von Bartheld et al., 1991). This model system has enabled us to show that exposure to NGF induced apoptosis in retinal cells only in the presence of insulin and NT3. This was preceded by the increase of cyclin B2 and the presence of mitotic figures in G4-positive neurones. The re-entry into the cell cycle appears to be involved in the induction of apoptosis since BDNF diminished the presence of cyclin B2 in G4-positive neurones and then rescued cell death. Moreover, the pharmacological blockage of the G2/M transition was able to prevent NGF-induced cell death.

**MATERIALS AND METHODS**

**Chick embryos**

Fertilised eggs from White Leghorn hens were obtained from a local supplier and were incubated at 38.5°C in an atmosphere of 70% humidity. The embryos were staged according to Hamburger and Hamilton (1951).

**Antibodies**

The mAb G4 labels a glycoprotein located on neurites of a subset of differentiated neurones (Rathjen et al., 1987). It stains 10-12% of differentiated neurones in dissociated cultures from the E9 chick retina. These neurones include retinal ganglion cells and a population of neurones in the inner nuclear layer (Frade et al., 1996b). The monoclonal antibody 27/21 (used here at 120 ng/ml) specifically blocks the biological activity of NGF, as reported elsewhere (Korsching and Thoenen, 1987). The rabbit polyclonal antisera #9651, made against the extracellular domain of mouse p75NGFR (amino acids 43-161) as a GST-2T fusion protein (Huber and Chao, 1995), was a generous gift from M. V. Chao (New York, USA). The antibody blocks binding of 125I-NGF to rat or mouse p75NGFR in affinity crosslinking assays (1:100 dilution). The rabbit polyclonal antisera R18 raised against chick cyclin B2 has been described elsewhere (Gallant and Nigg, 1992) and was a generous gift of E. A. Nigg (Geneva, Switzerland). The affinity-purified rabbit polyclonal antibody H-295 (Santa Cruz Biotecnology) was raised against a recombinant protein corresponding to amino acids 1-295, representing the full-length cyclin D1 of human origin.

**Preparation of retinal cells and cell cultures**

Retinas from E5 embryos were dissected free from pigment epithelium and dissociated as described previously (Rodriguez-Tébar et al., 1989; de la Rosa et al., 1994). Dissociated cells were suspended in culture medium and plated (20,000 cells/cm²) on 10-mm round glass coverslips into four-well dishes (Greiner). The coverslips were previously coated with polylornithine (Sigma)/laminin-1 (Gibco BRL) (Collins, 1978). Cells were cultured in 50% DMEM/50% F12 HAM (Sigma) with the N2 supplements (Bottenstein and Sato, 1979) lacking insulin where indicated. Cultures were maintained at 37°C in a water-saturated atmosphere containing 5% CO₂. Where mentioned, cultures were supplemented with recombinant β-NGF, BDNF, NT3 (a generous gift of Y.-A. Barde; Martinsried, Germany) or 7S NGF (Boehringer Mannheim). 7S NGF (Varon et al., 1967) is an α₂ β₂ γ₂ complex in which the α-NGF dimer is associated with two α-NGF and two γ-NGF subunits, which belong to the glandular kallikrein family of serine proteinases. The γ-NGF subunit is an active serine proteinase capable of processing the precursor form of β-NGF, whereas α-NGF is an inactive serine proteinase. β-NGF and 7S NGF were used at the concentrations mentioned in the text and both showed equivalent results, therefore both will be called NGF hereafter. BDNF and NT3 were used at 0.1 nM. Roscovitine (Calbiochem) was prepared at 10 mM in dimethylsulfoxide (DMSO) and then diluted in culture medium to the final concentrations of 1-50 μM. At this range of concentration, roscovitine is a known specific inhibitor of CDK1/CDK2, CDK2 and CDK5 (Meijer et al., 1997; De Azavedo et al., 1997).

**Quantification of apoptosis and mitotic numbers**

Cell counting was carried out on a Zeiss Axioptph microscope with
phase contrast and epifluorescence illumination. At least 5% of the total number of cells was observed per culture and each experimental point was the average ± s.e.m. of at least three cultures. To characterise apoptosis by morphological criteria, DNA was labelled with 1 μg/ml bisbenzimide (Sigma) in 4% paraformaldehyde-fixed cells and the number of pyknotic nuclei was established. Apoptotic cells were also detected by using terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) (in situ cell death detection kit, POD; Boehringer Mannheim), following the manufacturer’s instructions. TUNEL yielded similar results to those obtained by morphological criteria (data not shown). Mitotic nuclei were identified by bisbenzimide labelling. Due to the low percentage of mitotic nuclei present in the cultures, at least half of the total cells were observed and, when possible, over 50 mitotic nuclei were counted per culture.

**Cell staining**

For immunocytochemistry, cultured cells were incubated for 20 minutes at room temperature in a 400-fold dilution of ascites fluid containing G4 mAb in Krebs-Ringer-Hepes (KRH) buffer. Cells were then subjected to two successive incubations of 20 minutes each in a 400-fold dilution in KRH of biotinylated anti-mouse Ig antibody (Jackson) and a 400-fold dilution of streptavidin-Alexa 568 (Molecular Probes). Washing steps with KRH were performed after each single incubation. Finally, cells were fixed for 20 minutes in 4% paraformaldehyde. For double staining with G4 mAb and an additional rabbit polyclonal antiserum, the previous protocol was extended as follows. Fixed cells were washed in PBS and permeabilised in PBS/0.5% Triton X-100 (PBTx) for 15 minutes. Then, they were incubated in PBTx containing the respective antisera (1:600, anti-cyclin B2; 2 μg/ml, anti-cyclin D1) and, finally, incubated in a 4000- or 400-fold dilution of anti rabbit coupled to Alexa 488 (Molecular Probes) in PBTx respectively. Washes with PBTx were intercalated among the incubations with antibodies. Cells were then mounted in PBS/glycerol.

**Neurogenesis assay**

Neuron differentiation in vitro was studied as previously described (Frade et al., 1996a). Briefly, culture medium contained 0.5 μCi/ml [3H]methyl thymidine (25 Ci/mmol; Amersham International). After 20 hours incubation, retinal cells were labelled with G4 mAb and exposed for thymidine autoradiography for 2-3 days. Cells that were G4-positive/thymidine-positive were those that had proliferated as neuroepithelial cells and converted to neurons in vitro.

**Fig. 1.** Induction of cell death by NGF. E5 retinal cells were cultured on laminin-1 in a chemically defined medium containing 1 μM insulin and 0.1 nM NT3 for 18 hours in the presence or absence of NGF. Apoptosis was analysed by staining with bisbenzimide (A,B). (C,D) The bright field corresponding to A and B, respectively. Note how pyknotic nuclei (arrows) are easily observed in cultures in the presence of NGF, and the presence of a mitotic nucleus (arrowhead). The percentage of pyknotic nuclei was quantified in cells cultured with 1.5 nM NGF (E). Basal levels of cell death were observed in control cultures. This value was significantly increased in the presence of 1.5 nM NGF (−), an effect that was reversed by anti-p75NTR polyclonal antiserum #9651 (Huber and Chao, 1995) (α-p75) used at 1:300 dilution (left) or 1:1000 (right), or the anti-NGF mAb 27/21 (Korsching and Thoenen, 1987) (α-NGF) used at 120 ng/ml. The effect of NGF was saturating at 1.5 nM, as observed in (F). The apoptotic effect of 1.5 nM NGF was delayed in time and significant levels of apoptosis were not observed before 10 hours in vitro (G). Values are means ± s.e.m. (n=4). (*P<0.05; **P<0.005; Student’s t-test). Bar, 30 μm.
Image analysis
Quantification of cyclin expression levels was performed as follows. Staining of cyclin D1 and cyclin B2 was all performed on one occasion to eliminate effects of different batches of reagents. Fields showing cyclin/G4 double immunolabelled neurones were randomly selected and digitalised using confocal microscopy (Leica). The cyclin B2 or cyclin D1 signal per each G4-positive cell was then quantified using the Q500 MC QWin software (Leica, version V01.01). Final values were obtained by multiplying the intensity of signal by the total area and then the average was normalised to 1 in
controls. Final values were distributed into arbitrary intervals and the frequency of these values in each interval was plotted as a function of density.

RESULTS
NGF induces cell death in cultured E5 retinal cells
During the main onset of neurogenesis (E5-E7) a number of retinal neurones that have left the cell cycle and are migrating to their neuronal layer are induced to die by NGF in vivo (Frade et al., 1996c, 1997). To gain an insight into the mechanism used by NGF to induce apoptosis, precursor cells isolated from E5 retinas were cultured on laminin-1 in a chemically defined medium containing 1 μM insulin and 0.1 nM NT3 as described previously (Frade et al., 1996a,b). Insulin used in the micromolar range is likely to be acting on insulin-like growth factor (IGF)-I receptors, thus mimicking the differentiative effect of IGF-I on the E5 retinal cells, as previously described (Frade et al., 1996b). After 18 hours in the absence of NGF, control cultures showed spontaneous cell death in about 7% of the total cells, as detected by the evaluation of pyknotic nuclei using TUNEL (not shown) or bisbenzimide (Fig. 1). When 1.5 nM NGF was added, the proportion of dying cells was significantly increased to around 14% (Fig. 1). This cell death-promoting effect was abolished by treatment with an anti-NGF mAb or an anti-p75NTR polyclonal antiserum (Fig. 1E), indicating that NGF specifically induced cell death. The half-maximal induction of cell death was obtained at about 0.15 nM NGF and the maximal effect was reached with 1.5 nM NGF (Fig. 1F). The induction of apoptosis by NGF was not apparent before 10 hours and the effect was maximal after 14 hours (Fig. 1G). This delay of several hours in the apoptotic response to NGF suggests that an indirect mechanism is necessary for the transduction of the p75NTR-mediated signal, such as the expression of chemical mediators by the cultured cells (see below).

The absence of neurogenesis-promoting conditions abolishes cell death in cultured E5 retinal cells
The removal of insulin and NT3 from the culture medium used in this study results in the abolition of the genesis of G4-positive neurones (de la Rosa et al., 1994; Frade et al., 1996b). Fig. 2A shows the effect of different combinations of these factors on neurogenesis, measured as described in Materials and Methods. In the absence of insulin, a block of the genesis of G4-positive neurones occurred, even in the presence of NT3. Insulin itself had a small amount of neurogenic activity, which was highly enhanced by NT3. This profile correlated with the levels of apoptosis induced by NGF in the presence of such combinations of factors (Fig. 2B), suggesting that NGF might execute its killing action on precursor cells committed to become G4-positive neurones. To test this idea the percentage of G4-positive neurones remaining in NT3/insulin-containing cultures in the presence or absence of NGF was quantified after 18 hours. This analysis showed a significant decrease in the proportion of these neurones in NGF-treated cultures (Fig. 3A), suggesting that G4-positive neurones are targets of NGF. Cells with pyknotic nuclei harbouring the G4 antigen on their membrane were not observed in the NGF-treated cultures, probably due to the loss of this particular marker from the cell membrane in apoptotic cells. Nevertheless, dying cells expressing cytoplasmic G4 antigen were frequently observed in cultures treated with NGF, suggesting that G4 was somehow translocated from the cell membrane during the apoptotic process (Fig. 4).
NGF induces unscheduled cell-cycle events before the onset of cell death in vitro

In an attempt to understand the molecular basis underlying the proapoptotic effect of NGF on retinal cells, we postulated that this factor could trigger cell death in G4-positive postmitotic neurones by inducing them to re-enter the cell cycle. Indeed, an emerging view considers the process of neuronal apoptosis as a consequence of unscheduled and unsynchronised induction of cell-cycle mediators (see Introduction). As a first approximation to establish a relationship between NGF-induced cell death and re-entry into the cell cycle, the percentage of mitotic figures present in control versus NGF-treated cultures was estimated at a time point when cell death is just starting (10 hours) (Fig. 1G). The proportion of cells showing colocalisation of mitotic figures with the neuronal marker G4 was dramatically increased in the presence of NGF (Table 1). This result suggested that the upregulation of cyclin B2 represented an early step involved in the induction of cell death. Indeed, an early landmark during neuronal death initiated under different physiological and experimental situations (Freeman, 1994; Kranenburg et al., 1996; Shirvan et al., 1997; 1998). Cultures in the presence and absence of 1.5 nM NGF were immunostained with an anti-G4 mAb, and then with specific antisera against these two cyclins. A basal expression of both molecules was seen in most of the G4-positive cells even in control cultures (Fig. 5). Due to the fluctuating expression of these molecules during the cell cycle it was impossible to establish a baseline below which a cell could be unambiguously classified as cyclin-negative. In an attempt to solve this problem, the levels of both cyclins expressed by the G4-positive cells were quantified in both control and NGF-treated cultures by means of image analysis (see Materials and Methods). The mean intensity of cyclin D1 expressed by the G4-positive neurones was unchanged upon treatment with NGF (Table 1). On the contrary, the mean intensity of cyclin B2 expressed by G4-positive neurones was slightly, but significantly, increased in the presence of NGF (Table 1). This result was confirmed by a clear increase in the frequency of G4-positive neurones expressing high levels of cyclin B2 (Fig. 6). The time point at which the study was performed (10 hours) preceded the period of maximum cell death, suggesting that the upregulation of cyclin B2 might be part of the early events that cause apoptosis induced by NGF in this culture system.

BDNF reverses unscheduled cell-cycle events and rescues neurones induced to die by NGF

BDNF has been shown to rescue cell death in chick retinas in vivo (Frade et al., 1997). In vitro, 0.1 nM BDNF was able to prevent the apoptotic effect of 1.5 nM NGF on retinal cells cultured for 18 hours (Fig. 7). Importantly, 0.1 nM BDNF decreased the mean intensity of cyclin B2 expressed by the G4-positive neurones cultured in presence of NGF for 10 hours (Table 1). This result suggested that the upregulation of cyclin B2 represented an early step involved in the induction of cell death by NGF. Nevertheless, the crucial experiment to support this assumption would be the analysis of the blockage of cell-cycle progression in terms of cell death.

Blockage of cell-cycle progression inhibits the proapoptotic effect of NGF

To analyse whether the cell-cycle-related changes induced by...
NGF in G4-positive neurones might be involved in the induction of cell death by this neurotrophin, cell-cycle progression was pharmacologically blocked by the CDK-inhibitor roscovitine and the consequences of this blockage in terms of apoptosis were studied. Roscovitine, added to NGF-treated cultures for 18 hours at concentrations that specifically affect CDK1/CDC2, CDK2 and CDK5 (de Azevedo et al., 1997; Meijer et al., 1997), was able to reduce the proportion of apoptotic cells in a dose-dependent manner (Table 2). Therefore, the simultaneous blockage of cell death and cell proliferation by roscovitine supported the hypothesis that the pro-apoptotic action of NGF implied, at least in part, the re-entry into the cell cycle of postmitotic neurones. Roscovitine executed its anti-apoptotic effect presumably through the inhibition of the cyclin B2-dependent kinase (CDK1/CDC2), although additional effects via blockage of alternative kinases (see Maas et al., 1998) cannot be ruled out. Roscovitine used at 50 μM induced an apparent drop of viability, as previously shown by Maas et al. (1998). These authors suggested that this effect was probably due to solvent toxicity since this CDK inhibitor was prediluted in DMSO before the final dilution in medium. To test this hypothesis, E5 retinal cells were cultured in the presence of NGF with DMSO at the same concentration as used with 50 μM roscovitine. Such a treatment did not augment the levels of pyknotic cells (Table 2), suggesting that the deleterious effect of 50 μM roscovitine was due to the CDK inhibitor and not to the solvent. Indeed, 50 μM roscovitine was able to induce pyknotic nuclei in control cultures (Table 2), indicating that at high concentrations, roscovitine could have pleiotropic effects that compromise cellular survival.

**DISCUSSION**

In this article we show that NGF is capable of inducing apoptosis of postmitotic neurones that express the neuronal marker G4. This induction of apoptosis is accompanied by an increase of cyclin B2, which is constitutively expressed at low levels by these cells, and re-entry into the cell cycle. BDNF prevents NGF-induced cell death and simultaneously decreases the level of cyclin B2 in G4-positive cells treated with NGF. Finally, blocking cell-cycle progression by means of the cyclin-dependent kinase inhibitor roscovitine prevents NGF-induced cell death.

**NGF induces cell death in postmitotic neurones**

During the early developmental stages of the chick retina, a number of RGC neurones that have left the cell cycle and started migrating towards their adult layer suffer apoptosis induced by NGF upon its interaction with p75NTR (Frade et al., 1996c, 1997). This conclusion has been corroborated in null mutant mice for ngf or p75NTR (Frade and Barde, 1999). In this article we have used an in vitro system to analyse apoptotic signals triggered by p75NTR upon binding to NGF. As such, dissociated cells from E5 chick retina were cultured under conditions that have previously been shown to induce the birth of G4-positive neurones (de la Rosa et al., 1994; Frade et al., 1996a,b). This neuronal subpopulation is composed of RGCs and neurones located at the vitreal region of the inner nuclear layer (Frade et al., 1996b), all of them known to express p75NTR (Von Bartheld et al., 1991). The neurogenesis-promoting conditions mentioned above are characterised by the presence of 1 μM insulin and 0.1 mM NT3 in the culture medium (de la Rosa et al., 1994; Frade et al., 1996b). When NGF was added to this assay an increase in cell death was observed. The induction of cell death was specific for NGF since a previously described mAb that inhibits the biological activity of NGF (Korsching and Thoenen, 1987) blocked the pro-apoptotic effect of this neurotrophin. In addition, the induction of cell death was dose dependent, reaching its maximum at 1.5 nM NGF, thus resembling the data obtained by Davey and Davies (1998) for the induction of apoptosis by NGF in neurones of the trigeminal mesencephalic nucleus. The time course over which NGF exhibits its effect indicated a delay of 10 hours in triggering cell death. This contrasts with the data obtained from mature oligodendrocytes where more rapid kinetics were found (Cassacia-Bonnefill et al., 1996). In this respect, our data resemble more closely those obtained by Davey and Davies (1998), who observed the effect of NGF after 48 hours in vitro. The delay observed in the induction of apoptosis could be explained by the involvement in the downstream signalling of the upregulation of cyclin B2 (see below). The pro-apoptotic effect of NGF was clearly mediated by p75NTR since a polyclonal antiserum generated against the extracellular domain of mouse p75NTR (Huber and Chao, 1995) was able to block the killing action of NGF.
Three lines of evidence led us to conclude that NGF was responsible for the death of postmitotic neurones in our system. First, neurogenesis-promoting conditions (de la Rosa et al., 1994; Frade et al., 1996a,b) were necessary for NGF to induce apoptosis. Thus, the removal of insulin and NT3 prevented the pro-apoptotic effect of NGF. Second, NGF reduced the proportion of G4-positive neurones concurrently with the induction of apoptosis. And, third, cells positive for the G4 antigen could be observed harbouring pyknotic nuclei in NGF-treated cultures. There are many examples in the literature where cells induced to die by NGF are postmitotic and express high levels of p75NTR, for instance, long-term cultures of mature postmitotic oligodendrocytes (Casaccia-Bonnefil et al., 1996), postnatal rat sympathetic neurones during the developmental period of naturally occurring cell death (Bamji et al., 1998) and neurones of the trigeminal mesencephalic nucleus of chick E10 embryos (Davey and Davies, 1998). Furthermore, NGF-induced cell death has also been described in the mouse spinal cord where p75NTR is also expressed by differentiated cells (Frade and Barde, 1999). All these data suggest that NGF could exert its pro-apoptotic effect mainly on postmitotic neural cells.

Cell-cycle re-entry as a common early feature of neuronal cell death

One of the main features of the nervous system is that their principal cellular constituents become postmitotic at a specific moment during their development and remain quiescent the rest of their adult life. Keeping this in mind, it is not unusual that any perturbation inducing the re-entry into the cell cycle in a neurone would result in a regulatory conflict that may be translated into the death of the cell. So far, many reports have shown that apoptosis in cells of neural origin operates through the upregulation of molecules involved in cell-cycle progression, frequently in a disorganized manner (Freeman et al., 1994; Herrup and Busser, 1995; Kranenburg et al., 1996; Shirvan et al., 1997; ElShamy et al., 1998). This can be prevented by agents that block cell-cycle progression (Park et al., 1997a,b; Markus et al., 1997; Maas et al., 1998). Therefore,

![Fig. 5. NGF increases the proportion of cells expressing cyclin B2, but not cyclin D1. E5 retinal cells were cultured for 10 hours in neurogenic conditions in the absence (A,C,E,G) or presence (B,D,F,H) of 1.5 nM NGF and then immunostained for the neuronal marker G4 (arrows in C,D,G,H). Cultures were then subjected to immunostaining using specific antisera directed against cyclin D1 (A,B) or cyclin B2 (E,F) (see Materials and Methods). The proportion of G4-positive cells that expressed high levels of cyclin B2 increased in cultures treated with NGF (for quantification, see Fig. 6). Cyclin D1 levels were not affected by this treatment. Bar, 30 μm.]

| Table 1. Upregulation of cyclin B2 levels in the G4-positive neurones induced by NGF |
|-----------------|-----------------|-----------------|
| Treatment       | Cyclin D1       | Cyclin B2       |
| Control         | 1.00±0.05 (19)  | 1.00±0.08 (48)  |
| NGF             | 1.04±0.10 (14)  | 1.36±0.10 (43)* |
| NGF/BDNF        | n.d.            | 0.91±0.08 (49)  |

E5 retinal cells were cultured for 10 hours in neurogenic conditions and then the level of cyclin D1 and cyclin B2 expressed by the G4-positive neurones were quantified by means of image analysis (see Materials and Methods). Average values from individual data were normalised in the control situation. Values are means ± s.e.m. (n); (*P<0.01; Student’s t-test). n.d., not determined.
the emergent concept of unscheduled cell-cycle re-entry as a general cause of neuronal cell death is becoming accepted in the field. In the present report we have shown that the unscheduled induction of cell-cycle events also precedes the cell death caused by NGF in retinal postmitotic neurones. Thus, we demonstrated an increase of mitotic nuclei associated with G4-positive cells in NGF-treated cultures before NGF-induced apoptosis becomes apparent. Although not directly demonstrated, this effect was likely to be mediated by p75NTR since TrkA, the alternative known receptor of NGF, has not been detected in the retinal cells at this particular developmental stage (Frade et al., 1996c). The fact that p75NTR is known to be expressed only by postmitotic retinal neurones (von Bartheld et al., 1991) is consistent with the increase in mitotic nuclei taking place exclusively in these cells.

Among the different regulators of cell-cycle progression presumably being affected by NGF, the G2/M phase-specific cyclin B2 was found to be upregulated in the G4-positive neurones before the onset of cell death. Again, although not directly demonstrated, this effect was likely to be mediated by p75NTR since TrkA has not been detected in the E5 retinal cells (Frade et al., 1996c). The upregulation of cyclins is one early and critical step that occurs during apoptotic cell death of neural cells. Cyclin B2 has been shown to present a double wave of transcriptional upregulation when sympathetic neurones are induced to die by treatment with dopamine (Shirvan et al., 1997). This cyclin is a known activator of CDK1/CDC2, which is a rate-limiting step that occurs during apoptotic cell death in higher eukaryotic cells (Fotedar et al., 1995; Shimizu et al., 1995). The activity of CDK1/CDC2 can lead to apoptosis in higher eukaryotic cells (Fotedar et al., 1995; Shimizu et al., 1995). CDK1/CDC2 immunoreactivity has been detected in avian postmitotic RGCs (Espanel et al., 1997), thus making possible its interaction with cyclin B2 in those neurones that upregulate this cyclin. In the future, it will be necessary to analyse whether the rather modest increase of cyclin B2 levels induced by NGF (around 30%) is sufficient to induce a substantial rise in CDK1/CDC2 activity. Interestingly, cyclin D1, a known G1 marker, was also expressed in many cells including those positive for G4, but did not show any change in its protein levels after treatment with NGF. This was reminiscent of dopamine-
NGF-induced apoptosis and cell-cycle re-entry

triggered cell death in sympathetic neurones where cyclin D1, unlike cyclin B2, was not re-expressed (Shirvan et al., 1997).

The correlation between the cell-cycle events mentioned above and the cell death triggered by NGF in the G4-positive neurones suggest that this neurotrophin causes apoptosis, at least in part, through the unscheduled induction of cell-cycle events in postmitotic neurones. This assumption is further supported by the inhibition of NGF-dependent cell death by blocking cell-cycle progression using the purine analogue, roscovitine. This compound is a potent and selective inhibitor of CDK1/CDC2, CDK2 and CDK5 (de Azevedo et al., 1997; Meijer et al., 1997). Roscovitine was able to reverse the pro-apoptotic effect of NGF. Further support that cell-cycle events are related to NGF-induced cell death derive from the reduction in the proportion of mitotic nuclei and cyclin B2 upregulation in G4-positive neurones when BDNF is used to block NGF-induced apoptosis. Therefore, these results illustrate the importance of the aberrant re-entry into cell cycle as a crucial step in the induction of cell death by NGF. The question as to whether over-expression of cyclin B2 in retinal cells induces them to die remains to be studied, possibly providing further evidence that the expression of cyclins in neurones induces their death, previously demonstrated in different neuronal systems (see Introduction).

It is interesting that both cyclin D1 and cyclin B2 remained expressed at low levels in most G4-positive neurones in vitro. The expression of cell-cycle progression markers in mature neurones has been described previously. As such, CDK1/CDC2 has been shown to be expressed by postmitotic RGCs and to exhibit a temporal pattern of expression similar to cyclin A and cyclin B2 in the avian retina (Espanel et al., 1997), CDK5 is mainly expressed at adult stages in the mouse forebrain (Tsai et al., 1993), and has been found to be associated to apoptotic cell death during development and tissue remodelling (Zhang et al., 1997). The function of these markers in postmitotic neurones, apart from participating in the induction of apoptosis, remains unknown.

The unscheduled re-entry into the cell cycle induced by NGF in postmitotic cells could induce the activation of the p53 pathway. p53 is a known tumour suppressor gene product that is activated during situations of cellular stress, or after DNA damage in cycling cells, and it induces apoptosis when proliferative mechanisms are deregulated (for a review, see Jacks and Weinberg, 1996; Levine, 1997). p53 has been implicated in the signalling of sympathetic neurone death after NGF withdrawal or activation of p75NTR with BDNF (Aloyz et al., 1998). Cell death of sympathetic neurones due to NGF deprivation has also been associated with the upregulation of cyclin D1 (Freeman et al., 1994). Whether or not p53 might be involved in the induction of apoptosis by NGF in our system remains to be determined.

**BDNF as a factor that blocks NGF-induced cell death**

BDNF has been described as an anti-apoptotic agent that counterbalances the proapoptotic effect of p75NTR induced by NGF (Davey and Davies, 1998; Yoon et al., 1998). This same conclusion was obtained from the analysis of cell death taking place in the chick retina after pharmacological treatment with BDNF in vivo (Frade et al., 1997). In vitro, both the upregulation of cyclin B2 and the subsequent death of retinal cells induced by NGF were reversed by the addition of BDNF, demonstrating that the same population of retinal cells induced to die by NGF can be rescued by BDNF. The effect of BDNF was probably mediated by TrkB, which is expressed by E5 retinal cells (Frade et al., 1997), since the concentration of BDNF used in this study was saturating for this receptor (Rodríguez-Tébar and Barde, 1988).

In other paradigms of p75NTR-induced cell death, neurotrophins other than BDNF have been shown to prevent p75NTR-dependent neuronal death, as is the case for NGF in the death of sympathetic neurones induced by BDNF (Bamji et al., 1998). In addition, cell death is blocked by NT3 during the neurogenesis phase of sensory neurones (ElShamy et al., 1998). The authors of the latter work found that, in the absence of NT3, a number of proliferation progression factors were upregulated in sensory neurones, a fact that triggered cell death. Whether or not NT3 prevents p75NTR-induced cell death in this system is uncertain.

In conclusion, we propose a mechanism downstream of p75NTR that is involved in the apoptotic signalling induced by NGF upon interaction with this receptor. Evidence has been presented to suggest that the unscheduled re-entry into the cell cycle of postmitotic neurones is responsible, at least in part, for the apoptotic effect of NGF. This phenomenon could be blocked by BDNF, which would maintain the postmitotic state of the neurones during the critical temporal window when the cell decides to enter into the G0 phase. Therefore, a model could be postulated whereby ‘neurotrophic’ agents, acting through Trk receptors, would be required at certain critical developmental time points to prevent the re-entry into the cell cycle of quiescent neural cells induced by the binding of ‘proapoptotic’ agents to p75NTR.

The author is grateful to Y.-A. Barde and A. Rodríguez-Tébar for constructive criticisms and valuable comments on this article, and M. V. Chao and E. A. Nigg for the generous gift of antisera against p75NTR and cyclin B2, respectively. I am also grateful to Dr M. Sefton for grammatical corrections of the manuscript. This work was supported by the European Union, Biotechnology Programme (PL 960024) and Ministerio de Educación y Ciencia (PM95-0025).

**REFERENCES**


