At Least Two Mechanisms Are Involved in the Death of Retinal Ganglion Cells Following Target Ablation in Neonatal Rats

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Removal of the superior colliculus (SC) in neonatal Wistar rats results in a rapid loss of retinal ganglion cells (RGCs). There is an early twofold increase in RGC death 4–8 hr postlesion (PL) followed by a later 10–11-fold increase in pyknosis about 24 hr PL. We have now used neurotrophic factors (BDNF, NT-4/5, NT-3, NGF, LIF), glutamate receptor antagonists (MK-801, DNGX, CNQX), an antioxidant (N-acetylcysteine), and an NOS inhibitor (L-NAME) to determine whether the early and late phases of lesion-induced RGC death involve similar or different mechanisms. Normal and pyknotic nuclei of tectally projecting RGCs were visualized by injecting the left SC of 2–3 d old rats with diamidino yellow (DY). Two days later the injection site was removed. In most rats, right eyes were injected with factors immediately after the SC ablation. Rats were perfused either 6 or 24 hr PL. In the latter group a second intravitreal injection of the appropriate factor was sometimes made 12 hr PL. NT-4/5 and BDNF significantly decreased RGC pyknosis 6 and 24 hr PL, whereas NT-3 was only protective 6 hr PL. LIF slightly reduced RGC death 24 hr PL, but NGF had no influence on RGC survival at either time point. NT-4/5 also reduced the rate of naturally occurring RGC death. MK-801, DNGX, CNQX, N-acetylcysteine, and L-NAME all prevented the early lesion-induced increase in RGC death but had no significant effect on RGC death measured 24 hr PL; none of these factors significantly reduced the rate of naturally occurring RGC death. Cycloheximide, shown previously to reduce RGC pyknosis 24 hr PL, did not prevent RGC death 6 hr PL. The data indicate that there are at least two mechanisms involved in RGC death after neonatal target ablation. The early increase is related to excitotoxic effects mediated by glutamate receptors and involves NOS and the production of free radicals. We found no evidence that RGC death measured 24 hr PL is dependent on these processes, but the later death does require protein synthesis and, most likely, the activation of an endogenous suicide program. NT-4/5 and BDNF protected RGCs from both types of lesion-induced death.

Key words: retinal ganglion cells, NT-4/5, brain-derived neurotrophic factor (BDNF), NT-3, NGF, leukemia inhibitory factor (LIF), glutamate receptor antagonists, excitotoxicity, nitric oxide synthase, free radicals, apoptosis

A major goal of neuroscience research is to elucidate the mechanisms underlying cell death in the CNS and PNS. Much of this work is directed toward identifying the factors that regulate cell survival during normal CNS and PNS development (reviewed in Oppenheim, 1991; Johnson and Deckworth, 1993; Davies, 1994). Other research is aimed at finding ways of promoting cell viability after acute traumatic insults or in chronic neurodegenerative disease in the adult nervous system (for recent reviews, see Mattson et al., 1993; Hefni, 1994; Lindsay et al., 1994; Lindvall et al., 1994; Mattson and Scheff, 1994). These studies are complementary: understanding the cause of neuronal death in one situation may shed light on the processes involved in neuronal death in other conditions.

In many parts of the developing mammalian visual system, as in other systems in the CNS, there is a wave of naturally occurring (or programmed) neuronal death (Finlay and Pallas, 1989). In the rat retina there is an initial overproduction of retinal ganglion cells (RGCs) followed by elimination of about 50% of the population. Loss of RGCs occurs just before birth and in the first few postnatal days (Dreher et al., 1983; Perry et al., 1983). It is generally accepted that developing neurons compete with each other for limited amounts of target-derived neurotrophic factors; experimental enlargement of targets reduces cell death whereas axonal damage or target ablation has the opposite effect (Oppenheim, 1991; Snider et al., 1992; Sohal, 1992). Consistent with this, fewer RGCs survive into adulthood after axotomy or removal of central target sites in neonatal rats (Perry and Cowey, 1979; Carpenter et al., 1986; Horsburgh and Setton, 1987).

Recently we described the time course and extent of death of identified retinotectally projecting ganglion cells following neonatal ablation of the rat superior colliculus (SC; Harvey and Robertson, 1992). Removal of this central target at P4 (day of birth = P0) resulted in the rapid loss of about 50% of the normal RGC population. There was an early increase in the number of dying (pyknotic) RGCs 4–8 hr postlesion (PL); the rate at this time was about 2%, twice the naturally occurring RGC death rate. This early increase in cell death was followed by a much larger increase in death that peaked at about 24 hr PL (Harvey and Robertson, 1992). The proportion of pyknotic to normal RGCs was 8–11.5% at this time (Harvey and Robertson, 1992; Harvey et al., 1994). Harvey and Robertson (1992) proposed that the early increase in RGC death might be caused by different mechanisms to those involved in the later wave of cell death.

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Further studies revealed that intravitreal injection of cycloheximide (CHX), but not ganglioside GM1, substantially reduced RGC pyknosis 24 hr PL (Harvey et al., 1994). These data were consistent with the proposal that RGC death 16–50 hr after target ablation was an active process requiring protein synthesis and, possibly, activation of some form of endogenous suicide program within the neurons (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990; Johnson and Deckworth, 1993). Recent studies in the rat retina by Rabacchi et al. (1994) are consistent with this view. However, the cause of the rapid increase in RGC death in the first few hours after SC removal remained undetermined.

Exogenous application of neurotrophins and other growth factors can protect a diverse range of neuronal types in both the developing and mature CNS and PNS (Johnson and Deckworth, 1993; Mattson et al., 1993; Davies, 1994; Hefi, 1994; Lindsay et al., 1994; Lindvall et al., 1994; Mattson and Scheff, 1994). In studies in the visual system, we recently showed that intravitreal injection of the neurotrophin NT-4/5 significantly reduced naturally occurring RGC death in neonatal rats (Cui and Harvey, 1994b). Others have reported that survival of adult RGCs after axotomy or ischemia can be promoted by a variety of trophic factors, applied either intraocularly or to the cut end of the optic nerve. These factors include brain-derived neurotrophic factor (BDNF; Mey and Thanos, 1993; Mansour-Robaey et al., 1994; Unoki and LaVail, 1994), NT-4/5 (Crispen et al., 1994), nerve growth factor (NGF; Carmignoto et al., 1989; Siliprandi et al., 1993), ciliary neurotrophic factor (CNTF; Mey and Thanos, 1993; Unoki and LaVail, 1994), and acidic and basic fibroblast growth factors (aFGF and bFGF; Sievers et al., 1987; Unoki and LaVail, 1994). It is not known whether these (or other) neurotrophic factors have a protective effect on developing RGCs isolated in vivo from their central targets. We therefore carried out SC ablation experiments in neonatal rats (Harvey and Robertson, 1992; Harvey et al., 1994) and then injected a variety of neurotrophic factors into the eyes to determine if they enhanced RGC survival after target removal. Factors that were used were NT-4/5, BDNF; NGF; neurotrophin NT-1, and leukemia inhibitory factor (LIF). The main objective of this study was to determine the relative effects of these neurotrophic factors at different times after SC ablation. To this end, the extent of RGC death in the presence of neurotrophins was analyzed at 6 hr as well as 24 hr PL (i.e., during the early and late phases of lesion-induced death).

In order to test whether any of the RGC death observed after neonatal tectal lesions could be attributed to excessive stimulation of glutamate receptors and subsequent excitotoxic effects (Facci et al., 1990; Abu El Asrar et al., 1992; Choi, 1992; Siliprandi et al., 1992; Aksike et al., 1994), eyes of some rats were injected with dizocilpine maleate (MK-801), a noncompetitive antagonist of N-methyl-D-aspartate (NMDA)-type glutamate receptors (Wong et al., 1986). Eyes in other rats were injected with either 5,7-dinitroquinoxaline-2,3-dione (DNQX) or 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), competitive inhibitors of non-NMDA glutamate channels (Honoré et al., 1988). As in the neurotrophic factor studies, the impact of these drugs on RGC death was assessed 6 and 24 hr PL.

There is evidence that glutamate neurotoxicity is, at least in part, mediated by nitric oxide (NO) and free radicals (Dawson et al., 1991; Lipton et al., 1993; Borson et al., 1994; Mayer and Noble, 1994; Dawson and Dawson, 1995). In some systems it has also been shown that antioxidants prevent or delay apoptosis (Ferrari et al., 1995; Greenlund et al., 1995). We therefore examined whether RGC survival 6 or 24 hr PL was enhanced after intravitreal injection of either N-nitro-l-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase (NOS; Moncada et al., 1991), or N-acetyl-l-cysteine (NAC), an antioxidant that scavenges free radicals and also raises intracellular glutathione levels (Meister, 1988; Mayer and Noble, 1994; Ferrari et al., 1993).

Finally, we showed previously that blockade of protein synthesis enhanced RGC survival 24 hr PL (Harvey et al., 1994; cf. Rabacchi et al., 1994). To help determine whether the intracellular mechanisms regulating cell survival during the early and late phases of RGC death were the same or different, we repeated the intravitreal CHX injections after SC ablation, but assessed their effectiveness 6 hr PL.

Preliminary accounts of some of these data have been presented in abstract form (Cui and Harvey, 1994a, 1995).

**Materials and Methods**

The method used has been published in detail elsewhere (Harvey and Robertson; Harvey et al., 1994). Brieﬂy, RGCs in the right eye were retrogradely labeled by injection of the fluorescent dye diamidino yellow (DY) into the middle of the left SC containing the DY injection site. In some NT-4/5 treated animals the second intravitreal injection was made immediately after SC removal (0 hr). For rats surviving 24 hr PL a second injection was sometimes made 12 hr later (see Table 2). In some NT-4/5 treated animals the second intravitreal injection was made immediately after the lesion (0 hr). Most animals were perfused either 6 or 24 hr after SC ablation. The exception was the NT-4/5-treated group, in which a number of animals were perfused 16, 32, 72, or 48 hr PL.

MK-801 (Merck, Sharp and Dohme), DNQX (Tocris Cookson), and CNQX (Sigma) were all used at 100 nm concentrations. NAC (Sigma) was used at concentrations ranging from 1 to 50 nm, and L-NAME (Sigma) was used at a concentration of 0.1 mg/ml. All other chemicals were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, Mo.) or equivalent suppliers. The main objective of this study was to determine the relative effects of these neurotrophic factors at different times after SC ablation. To this end, the extent of RGC death in the presence of neurotrophins was analyzed at 6 hr as well as 24 hr PL (i.e., during the early and late phases of lesion-induced death).
tioned rats using only sterile saline or the BDNF, NT-4/5, or NT-3 buffers.

Counts of DY-labeled RGCs in retinal whole-mounts. For fixation, rats were deeply anesthetized (Nembutal, i.p.) and perfused with 6% formaldehyde in 0.1 M phosphate buffer (pH 7.4). Right eyes were removed and whole-mounts of the retinas were prepared. About 7% of retinas were not counted due to faint DY labeling, excessive autofluorescence, or if retinas showed signs of abnormal shrinkage (Harvey and Robertson, 1992). In the remaining retinas (290 in this study) the area containing the brightest labeling (usually about 10% or 2–3 mm² of the total retinal area) was photographed using a 40× oil immersion objective (Harvey and Robertson, 1992; Cui and Harvey, 1994b; Harvey et al., 1994). Within the region of DY label, fields were randomly chosen for photography and counts of normal and frankly pyknotic DY-labeled RGCs were then made from the projected color images (Harvey and Robertson, 1992; Harvey et al., 1994). Fifteen fields (0.714 mm²) were photographed in each 6 hr PL retina and 10 fields (0.476 mm²) in each 24 hr PL retina. The data were obtained from 48 different litters. In no case was an individual litter used for only one experimental group; each litter contained rats injected with different substances, surviving either 6 or 24 hr PL. In addition, there was almost always at least one lesion only or lesion plus sham eye-injected animal in each litter. When counting RGCs from each litter, the color slides were assigned a number and then randomly sorted so that the observer (Q.C.) did not know the origin of each retinal photomicrograph.

About 2000–4500 RGCs were counted per retina. As in previous studies (Harvey et al., 1992; Harvey et al., 1994), most counts of normal and pyknotic DY-labeled RGCs were made within 1–2 mm of the optic nerve head, the majority in nasal retina. This is important because at P4/P5, RGC densities are generally uniform across the rat retina apart from a localized region of slightly lower density close to the superior margin (McCall et al., 1987). Thus density measurements were not significantly affected by interanimal variations in the location of RGC label in the retina. Assessment of cell density was subject to a small amount of interretinal variation due to differences in tissue shrinkage during fixation; however, pyknotic values were calculated as a proportion of total RGC number in each retina and were therefore not affected by such individual variations (Cui and Harvey, 1994b). For each experimental group the mean and standard deviation of the percentage of pyknotic RGCs and the density of normal DY-labeled RGCs per square millimeter were determined (Tables 1–3).

For statistical analysis of the 6 hr and 24 hr PL results, data were analyzed using one-factor analysis of variance using two-tailed Dunnett’s t tests (Dunnett, 1955). This rigorous procedure compares all treatment groups with a single control group; note that for numbers of treatments greater than 2, critical values in the Dunnett tables are larger than corresponding values using the Student’s t statistic. For the four data sets (rates of pyknosis and normal RGC densities, 6 and 24 hr PL), Dunnett’s test was used to analyze differences between experimental groups (including sham eye injection groups) and the respective lesion-only control group. In some cases, Bonferroni’s test was used to analyze differences within particular treatment groups (e.g., to compare between different doses of NGF 24 hr PL). We also used Dunnett’s procedure to compare the rates of pyknosis in the 6 hr lesion groups with the naturally occurring rate of RGC death in 4–5 d old rats (data from Cui and Harvey, 1994b). Values of p > 0.05 indicated nonsignificant differences between groups.

Results

RGC death 6 hr postlesion

Examples of DY-labeled retinas 6 hr PL are shown in Figure 1.

Note that the number of pyknotic RGCs (arrows) is greatest in the lesion-only retina (Fig. 1A, is somewhat less in an eye injected with MK-801 (Fig. 1B) and is least in an NT-4/5-treated retina (Fig. 1C). The 6 hr PL data are summarized in Table 1 and Figure 2A. In total, retinas from 114 rats were analyzed in this group. The mean rate of pyknosis in retinas from lesion-only rats was 1.88%, which was significantly higher (p < 0.01) than the rate of pyknosis in lesioned rats of the same age (1.04%; data from Cui and Harvey, 1994b). The proportion of dying DY-labeled RGCs in rats that received sham eye injections of 1 µl of saline, BDNF buffer, or NT-3 buffer averaged 1.81%, 2.19%, and 2.06%, respectively (mean for all sham eye-injections = 1.96%; see Fig. 2A). The rates of pyknosis for each of these three groups were not significantly different from each other or from lesion-only animals.

Cycloheximide. Intraocular injection of CHX resulted in a mean pyknotic rate 6 hr PL of 2.15%, not significantly different from the rate in lesion-only controls (Table 1, Fig. 2A).

Glutamate antagonists. The rates of pyknosis in retinas of MK-801-, DNQX-, and CNQX-treated animals were 0.93%, 0.94%, and 1.0%, respectively (Table 1, Fig. 2A). All values...
were significantly less than the proportion of dying RGCs in lesion-only rats (p < 0.01 for MK-801 and DNQX; p < 0.05 for CNQX). The average pyknotic rate in rats injected with a mixture of MK-801 and DNQX (Table 1, Fig. 2A) was also less than lesion-only rats but was not significantly different from the rates achieved using either glutamate receptor antagonist alone. Importantly, application of MK-801, DNQX, or CNQX reduced pyknotic rates to a level that was not significantly different from lesion-only rats but was not significantly different from the naturally occurring RGC death (Fig. 3A).

*Statistical analyses and significance levels are discussed in the text.*

| Number of animals (µg/µl) | Pyknosis (%): Mean and SD | Normal density (RGC/mm²): Mean and SD |
|---------------------------|---------------------------|--------------------------------------|
| 1.88                      | 2.19                      | 5142                                 |
| 0.5                       | 100 nm                    | 4884                                 |
| 1.81                      | 2.06                      | 4470                                 |
| 0.51                      | 0.72                      | 5817                                 |
| 2.15                      | 0.93                      | 5677                                 |
| 0.47                      | 0.33                      | 5706                                 |
| 0.94                      |                            |                                      |
| 1.24                      | 1.0                       | 5946                                 |
| 0.26                      | 0.21                      | 6061                                 |
| 0.26                      | 0.24                      | 4964                                 |
| 0.33                      | 0.23                      | 5350                                 |
| 0.33                      | 0.23                      | 5820                                 |
|                            | 0.25                      | 5395                                 |
|                            | 0.25                      | 5508                                 |
|                            | 0.75                      | 5632                                 |
|                            | 0.83                      |                                      |
| 1.05                      |                            |                                      |
|                            | 0.94                      |                                      |
|                            |                            |                                      |
| 1.86                      |                            |                                      |

### Table 1. Experimental conditions and results after SC lesion

- **Lesion only:** buffer, Saline, CHX, MK801, DNQX, MK801 + DNQX, L-NAME, NAC, NT-4/5, BDNF NT-3, NGF

- **Lesion BDNF NT-3:** buffer, Saline, CHX, MK801, DNQX, MK801 + DNQX, L-NAME, NAC, NT-4/5, BDNF NT-3, NGF

- **MK801 + DNQX:** L-NAME, NAC, NT-4/5, BDNF NT-3, NGF

**Normal density (RGC/mm²):**

- **Mean:** 5142, 4884, 4470, 5817, 5677, 5706, 5946, 6061, 4964, 5350, 5820, 5395, 5508, 5632

**Statistical analyses and significance levels are discussed in the text.*

Examples of DY-labeling in retinas 24 hr PL are shown in Figure 3. Rates of pyknosis were high in retinas of lesion-only rats (Fig. 3A), and in DNQX (Fig. 3B), NT-3 (Fig. 3D), and NGF (Fig. 3E) injected eyes. Rates of pyknosis were lower and the densities of normal DY-labeled RGCs were higher in retinas from eyes injected with BDNF (Fig. 3C) or NT-4/5 (Fig. 3F). Summary of most of the data are shown in Tables 2 and 3 (see also Figs. 2B, 4). A total of 158 animals was used in this part of the study. In lesion-only animals, the proportion of dying DY-labeled RGCs was 11.68%. Sham eye injections using 1 µl of BDNF buffer or sterile saline resulted in similar rates of pyknosis (10.7% and 12.5%, respectively). These rates were not significantly different from lesion-only controls.

**Cycloheximide.** Four animals received two intraocular injections of CHX, one immediately after the lesion and the second 12 hr later. This treatment regime was the same as that described recently (Harvey et al., 1994) and was undertaken to check the efficacy of the CHX used in the 6 hr PL group (Table 1, Fig. 2A). The rate of pyknosis 24 hr PL was 3.12% (Table 2, Fig. 2B), markedly similar to the 3.23% reported in an earlier article (Harvey et al., 1994) and significantly less (p < 0.01) than that seen in lesion-only animals. In accord with this reduction in RGC pyknosis, the mean density of normal DY-labeled RGCs was higher in CIUX-injected rats compared with lesion-only controls (p < 0.01; Table 2, Fig. 4).

**Glutamate antagonists.** The efficacy of MK-801 and DNQX in reducing RGC death 24 hr PL was tested in 14 rats. Either one or two intraocular injections were made in each animal. Neither of the glutamate antagonists was effective in reducing RGC death at this time and the two injection paradigm was no more effective than the single injection approach (Table 2, Fig. 2B). Pooling the data, the rate of pyknosis in rats treated with MK-801 was 11.64% and that in DNQX-injected animals was 11.7%. Densities of normal DY-labeled RGCs in MK-801 and DNQX-treated retinas (one and two injection data pooled together) were higher than the average RGC density found in lesion-only rats (Table 2), perhaps reflecting the early neuronal protection rendered by these drugs in the first few hours after target removal; however, the differences were not significant.
Figure 2. The mean percentage of DY-labeled RGCs that were pyknotic 6 hr PL (A) and 24 hr PL (B). Standard deviations are also shown. The column labeled Buffer or Saline in A and B includes all animals that received sham eye injections (Tables 1, 2). The dashed line in A and B shows the percentage of pyknotic RGCs in P4 rats in the absence of SC lesions (1.04%), that is, the naturally occurring cell death rate (data from Cui and Harvey, 1994b). In A, the data for different doses of NGF have been pooled together. In B, the MK-801, DNQX, NT-4/5, BDNF, and NT-3 data are pooled from the single (0 hr) and double (0+12 hr) eye injection groups (Tables 2, 3). The 24 hr PL data for different doses of NGF have also been pooled together. Note that MK-801, DNQX, CNQX, L-NAME, NAC, and NT-3 prevented the lesion-induced increase in RGC death 6 hr after SC ablation but were ineffective 24 hr PL. CHX had no effect on pyknotic rates 6 hr PL but did reduce RGC death measured 24 hr PL. NT-4/5 and BDNF significantly reduced RGC pyknosis at both time points.
L-NAME and NAC. Unlike their effectiveness 6 hr PL, intraocular injection of these factors at similar concentrations did not significantly reduce pyknosis or increase normal RGC density 24 hr PL (Table 2, Fig. 2B). Note, however, that the mean rate of pyknosis after L-NAME treatment was less than in lesion-only rats (Fig. 2B) and only just failed to reach significance using Dunnett's procedure. In four additional animals, injection of a higher concentration (50 μM) of NAC also failed to be neuroprotective 24 hr PL (mean rate of pyknosis = 12.14%).

Neurotrophic factors: effects of BDNF, NT-3, NGF, and LIF. In all animals treated with these factors, one injection of the respective neurotrophic molecule was made immediately after the SC lesion. In some instances a second injection was made 12 hr later (Table 2). After a single intravitreal injection of BDNF, the rate of dying DY-labeled RGCs was 3.0% compared with 3.71% after two injections; these two values were not significantly different from each other. Pooling the BDNF data gave a mean rate of pyknosis of 3.3%, considerably lower than the rate in lesion-only animals (p < 0.01; Fig. 2B).

After one or two intracocular injections of NT-3, the proportion of dying DY-labeled RGCs averaged 11.71% and 9.57%, respectively (Table 2), values that were not significantly different
from each other. Combining the NT-3 data together gave a mean rate of pyknosis of 10.64%, which was not significantly different from the lesion-only control group. The lack of effect of NT-3 at 24 hr PL (Fig. 2B) contrasted with the more clearcut survival-promoting influence of the neurotrophin 6 hr after tectal ablation (Fig. 2A).

Three different doses of NGF were used (0.5, 1, and 2.0 μg), all injected immediately after the SC lesion (Table 2). None of these regimes was effective in reducing RGC death 24 hr PL. Pooling the NGF data, the mean pyknotic rate was 10.15% (Fig. 3B), which was not significantly different from lesion-only animals. Five animals lesioned at P4 received two intravitreal injections of LIF at 0 and 12 hr PL. The proportion of dying RGCs in these animals averaged 7.19%, which was less than in lesion-only rats (p < 0.05) but clearly not as dramatic a reduction as that seen after BDNF treatment.

The densities of normal DY-labeled RGCs 24 hr PL were also assessed in these four treatment groups (Table 2). Densities in BDNF-treated retinas were higher than in lesion-only rats (p < 0.01). The density of RGCs in those rats that received two BDNF eye injections was particularly high (5208/mm²). Normal RGC density in NT-3 and LIF-injected eyes were both higher (p < 0.01 for NT-3, p < 0.05 for LIF) than lesion-only densities but similar to densities in rats that received sham eye injections of BDNF buffer (Table 2). The average density of normal DY-labeled RGCs in NGF-treated retinas was not significantly different from the lesion-only group. The greater density of normal RGCs in NT-3 versus NGF-injected eyes may reflect the early protective effect of NT-3 in the first few hours after target ablation.

Table 2. Experimental conditions and results 24 hr after SC lesion (excluding NT-4/5 data)

| Lesion | Dose (μg/kg) | Time applied | Pyknotosis (%) | Mean | SD | Normal density (0-100 mm²) | Mean | SD | Statistical analysis and significance levels are discussed in the text. |
|--------|--------------|--------------|----------------|------|----|-----------------------------|------|----|-----------------------------|
| BDNF   | 0.5, 1, 2    | 0, 12        | 10.15%         | 3.71 | 0.96| 368                         | 0.85 | 1.69| Statistical analysis and significance levels are discussed in the text. |
| LIF    | 0, 12        | 0            | 7.19%          | 1.44 | 0.23| 276                         | 3.66 | 4.68| Statistical analysis and significance levels are discussed in the text. |

We were intrigued by the fact that in the retinas of both BDNF- and NT-4/5–injected rats examined 24 hr PL, pyknotic RGC rates were greatly reduced from the levels seen in lesion-only and sham eye-injected retinas; however, unlike the 6 hr PL group, rates were not reduced below the levels of naturally occurring cell death. The lowest rate in an individual 24 hr PL animal injected with BDNF was 1.48% and a rate of 1.19% was seen in one rat twice injected with NT-4/5. In a previous study (Harvey and Robertson, 1992) it was shown that the beginning of the massive wave of RGC death following SC ablation began about 12 hr PL and a pyknotic rate of about 3–4% was seen 14–16 hr after target loss. We hypothesized that the rate of about 3% seen 24 hr after BDNF or NT-4/5 treatment might reflect a loss of efficacy of the trophic molecules in the eye about 8–10 hr after intravitreal injection; 14–16 hr later (24 hr PL) we were therefore seeing the early onset of a wave of RGC death, merely delayed by the application at 0 hr PL of neurotrophic molecules. Thus in rats that received a second injection at 12 hr PL, this second injection was too late to prevent many target-deprived RGCs from initiating a death program during the period (from
To test this hypothesis, we carried out two further experiments. In one series of studies, rats received NT-4/5 injections immediately after SC ablation and a second injection was made either 5 hr, 8 hr, or 20 hr PL. Pyknotic rates in these animals averaged 1.81%, 2.97%, and 4.59%, respectively (Table 3). The rate of pyknosis in the group injected at 0 and 5 hr was significantly different from the rate in the 0 and 20 hr group (p < 0.05, Bonferroni test), supporting the proposal that early application of the second injection sustained NT-4/5 levels in the 12 hr following target removal and thus reduced the amount of RGC death measured 24 hr PL. Indeed, the lowest pyknotic rate in an individual animal from the 0 and 5 hr injected group was 0.53%, well below the level of naturally occurring RGC death in P4/P5 rats. Normal DY-labeled RGC density was also high in this treatment group (Table 3).

In a second experiment, rats received intraocular injections of NT-4/5 at 0 and 12 hr after SC removal and the retinas were examined at different times after the target injury (16, 32, 38, or 48 hr PL). The mean proportion of pyknotic RGCs in the four groups was 0.64% (n = 4), 8.09% (n = 6), 9.51% (n = 5), and 6.0% (n = 3), respectively. With this injection regime, RGC pyknosis averaged 2.3% in rats perfused 24 hr PL (Table 3). For comparison, without exogenous trophic support the rate of cell death 16 hr PL was about 4% (Harvey and Robertson, 1992), it was 11.56% at 24 hr PL (present results), and 5.9% and 2.6% after 28 hr and 50 hr, respectively (Harvey and Robertson, 1992). These comparisons confirm that intravitreal injection of NT-4/5 at the time of the lesion substantially reduced RGC death, but the neurotrophin was protective for only a limited period of time. After application of NT-4/5, the rate of RGC death was low until at least 16 hr PL, was slightly increased at 24 hr and peaked around 32–38 hr PL. In contrast, in the absence of exogenous support the peak of RGC death occurred at about 24 hr PL.
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Table 3. Application of NT-4/5 at various postlesion times and the results 24 hr after SC removal

| Number of animals | 4 | 5 | 6 | 5 | 11 | 5 | 11 | 11 |
|-------------------|---|---|---|---|----|---|----|----|
| Dose (µg/ml)      | 0.29 | 0.29 × 2 | 0.29 × 2 | 0.29 × 2 | 0.29 × 2 | 0.29 | 0.29 | 0.29 |
| Time applied (hours postlesion) | 0 | 0, 12 | 0, 5 | 0, 8 | 0, 20 | 12 | 16 | 20 |
| Pyknosis (%)      | Mean | 2.86 | 2.3 | 1.81 | 2.97 | 4.59 | 2.05 | 2.02 | 1.84 | 3.48 |
|                   | SD   | 1.05 | 1.07 | 1.04 | 0.99 | 1.64 | 1.05 | 0.58 | 1.14 | 2.16 |
| Normal density (RGCs/mm²) | Mean | 4780 | 4633 | 5925 | 4697 | 5543 | 4952 | 5479 | 4614 | 3925 |
|                   | SD   | 356 | 491 | 944 | 681 | 739 | 272 | 515 | 787 | 486 |

Statistical analyses and significance levels are discussed in the text.

The graph in Figure 4 summarizes much of the 24 hr PL data; each point shows the mean % of pyknotic RGCs (y-axis) and the mean density per mm² of normal DY-labeled RGCs (x-axis) for nine of the experimental groups (one and two injection data pooled for each group). For each group, the oval areas denote the standard deviations (pyknosis in the y-dimension, normal RGC density in the x-dimension). In this figure, the NT-4/5 group includes all animals perfused 24 hr PL (n = 20) that received an initial injection at time zero and a second injection 5, 8, or 12 hr later (Table 3). It is important to note that decreased rates of pyknosis in CHX-, BDNF-, and NT-4/5-injected rats were consistently correlated with an increase in the number of normal DY-labeled RGCs per unit area. This correlation confirmed that the three treatments suppressed RGC death for most of the 24 hr period following SC removal. It also indicates that the low rates of pyknosis seen in these treatment groups were not due to a change in the time taken to remove and clear pyknotic nuclei (Harvey and Robertson, 1992).

Delayed application of NT-4/5. In in vitro studies it has been shown that, after trophic factor withdrawal, cells can still be rescued by delayed application of neurotrophic support (Edwards et al., 1991; Deckworth and Johnson, 1993). To test this in our in vivo model, the left SC of rats was injected with DY at P2, lesioned at P4 and NT-4/5 was injected into the vitreous of the right eye 12, 16, or 20 hr PL (Table 3). The rates of pyknosis in these three groups averaged 2.05%, 2.02%, and 1.84%, respectively. Clearly, application of an appropriate neurotrophic molecule as late as 20 hr PL was still effective in significantly reducing RGC death when measured 24 hr after target ablation.

The mean pyknotic rate in rats receiving one NT-4/5 injection 20 hr PL was less (p < 0.01, Bonferroni test) than the rate seen in rats twice injected with NT-4/5, at 0 and 20 hr PL (Table 3). The data seemed to indicate that the 20 hr injection was less effective if the eye had been injected before. Was this due to previous exposure to NT-4/5 or was it due to less specific effects of the right eye 12, 16, or 20 hr PL (Table 3). The rates of pyknosis in these three groups averaged 2.05%, 2.02%, and 1.84%, respectively. Clearly, application of an appropriate neurotrophic molecule as late as 20 hr PL was still effective in significantly reducing RGC death when measured 24 hr after target ablation.

The mean pyknotic rate in rats receiving one NT-4/5 injection 20 hr PL was less (p < 0.01, Bonferroni test) than the rate seen in rats twice injected with NT-4/5, at 0 and 20 hr PL (Table 3). The data seemed to indicate that the 20 hr injection was less effective if the eye had been injected before. Was this due to previous exposure to NT-4/5 or was it due to less specific effects related to prior physical damage of the eyeball? In an attempt to answer this question, we carried out a further experiment in which rats received a sham eye injection of NT-4/5 buffer at time zero followed by an injection of NT-4/5 at 20 hr PL (Table 3). There was an enormous range in this group; individual pyknotic values varied from 0.93% to 8.31% (mean = 3.48%). Because of this variability, the mean rate of pyknosis for this group was not significantly different from the means for the 20 hr PL delay group or the rats injected with NT-4/5 at 0 and 20 hr PL.

Discussion

The main aim of this study was to examine the influence of (1) neurotrophic factors, (2) glutamate receptor antagonists, and (3) inhibitors of NOS and free-radical formation on the viability of tectally projecting RGCs at various times after SC ablation in neonatal rats. We were particularly interested to determine the relative effectiveness of these different factors during the early and late phases of lesion-induced RGC death (i.e., 6 hr and 24 hr PL).

Two distinct mechanisms of RGC death after neonatal target removal

The observation that MK-801, DNQX, CNQX, L-NAMe, and NAC all protected RGCs 6 hr but not 24 hr PL, whereas CHX was effective 24 hr but not 6 hr PL (cf. Harvey et al., 1994), strongly suggests that at least two distinct mechanisms are involved in RGC death following neonatal target ablation. The early phase of RGC death does not require protein synthesis but is dependent on glutamate receptor activation and appears to involve NOS and the production of free radicals. In contrast, the late phase is an active process requiring protein synthesis and, possibly, activation of some form of intrinsic suicide program (Martin et al., 1988; Oppenheim et al., 1990; Johnson and Deckworth, 1993). In accord with this, DNA fragmentation typical of apoptotic cell death has recently been described in neonatal (Rabacchi et al., 1994) and adult (Garcia-Valenzuela et al., 1994) RGCs following optic nerve lesion.

It is widely held that the cytotoxicity associated with excessive stimulation of glutamate receptors is due to Ca²⁺ influx and a subsequent increase in intracellular Ca²⁺ concentration (Choi, 1992). It has further been proposed that these increased levels of neuronal Ca²⁺ result in activation of Ca²⁺-dependent enzymes, in particular NOS, which in turn leads to an increase in the intracellular concentration of nitric oxide (NO) and the formation of damaging free radicals (Dawson et al., 1991; Lipton et al., 1993; Mayer and Noble, 1994; Dawson and Dawson, 1995). Our 6 hr PL data are clearly consistent with this type of death mechanism in neonatal RGCs. NMDA receptors have usually been implicated in neuronal excitotoxicity; however, there is accumulating evidence that Ca²⁺ influx through non-NMDA channels can also lead to death (Bronson et al., 1994). In the retina, MK-801 has been shown to protect RGCs and other neurons from NMDA and glutamate-induced toxicity (Abu El Asrar et al., 1992; Stiprandi et al., 1992; cf. Akaeke et al., 1994), and from ischemic/hypoxic damage (Mosinger et al., 1991; Abu El
Asgar et al., 1997); however, Mosinger et al. (1991) reported that antagonism of both NMDA and non-NMDA receptors was required for optimal protection against ischemia. Our results clearly demonstrate that blockade of either NMDA or non-NMDA receptors is equally as effective in preventing the increase in RGC death 6 hr after target ablation. This is consistent with data showing that neonatal rat RGCs express a wide range of glutamate receptors that include NMDA and Ca2+ permeable non-NMDA channels (Müller et al., 1992; Rorig and Grantyn, 1993a,b; cf. Watanabe et al., 1994). Interestingly, the protective effects of MK and non-NMDA antagonists on RGCs 6 hr PL were not additive, suggesting the involvement of a shared intracellular pathway in this degenerative process.

MK-801, DNQX, CNXQ, L-NAM, and NAC all reduced the rate of RGC pyknosis 6 hr PL but only to the level of naturally occurring cell death. In other words, these treatments specifically prevented the increase in death associated with SC injury. Clearly, naturally occurring RGC death and the early phase of lesion-induced RGC death are caused by different mechanisms. The lack of effect of MK-801, DNQX, or CNXQ on ongoing naturally occurring RGC death is consistent with previous studies in normal (unlesioned) rats (Bunch and Fawcett, 1993; Cui and Harvey, 1994b), suggesting that during normal development NMDA and non-NMDA receptors do not play a significant role in regulating overall cell numbers in the postnatal rat retina. Note here that in other systems, use of MK-801 to block NMDA glutamate receptors can either decrease (O'Donoghue et al., 1993; Greensmith et al., 1994) or increase (Gould et al., 1994) naturally occurring cell death (cf. Yan et al., 1994). In the visual system, MK-801 treatment does, however, alter the pattern of RGC death in the developing eye, impairing the refinement of retinotectal topography (Bunch and Fawcett, 1993).

It has recently been reported that the antioxidant NAC prevents apoptosis in neural cells (Mayer and Noble, 1994; Ferrari et al., 1995; cf. Greenlund et al., 1995). We found no evidence for the involvement of NO or free radicals in naturally occurring RGC death in vivo; however, it should be borne in mind that extracellular application of antioxidants need not necessarily be effective since the critical event in apoptosis may be the generation of free radicals within an individual neuron that act intracellularly and at short range (Greenlund et al., 1995).

The morphology of dying RGCs

In this and previous studies (Harvey and Robertson, 1992; Cui and Harvey, 1994b; Harvey et al., 1994), nuclei of RGCs projecting to the SC were identified using the retrograde fluorescent tracer DY. We then used nuclear condensation and fragmentation of labeled cells (features normally thought of as being typical of apoptosis) as the basis for our cell death counts. As reviewed previously (Harvey and Robertson, 1992), this type of degeneration in the retina has been described many times and in many different species, and is regarded as being the major morphological indicator of RGC death, both in normal development and after neonatal injury. The effectiveness of glutamate receptor blockade in reducing pyknosis 6 hr PL suggests that, in the neonatal retina at least, excitotoxicity leads to a similar morphological end-point as protein synthesis-dependent apoptosis.

Classically, necrotic and apoptotic cell death can be distinguished both morphologically and pathophysiological (Wyllie, 1987; Choi, 1992; Schwartzman and Cidlowski, 1993), although in his review Choi (1992) described excitotoxic changes as involving swelling followed by "degeneration of intracellular organelles and nuclear pyknosis." Recent evidence provides support for the view that a clear distinction between necrosis and apoptosis cannot always be made. For example, in the cerebellum, pyknotic cells in the granule cell layer do not necessarily label for DNA fragmentation (Wood et al., 1993). It has also been shown that not all cells that undergo programmed cell death die via apoptosis and DNA cleavage is not always necessary (e.g., Schwartz et al., 1993). Excitotoxic injury can sometimes lead to so-called apoptotic changes—in that glutamate-induced death can be associated with DNA fragmentation and is prevented by transcription inhibitors or endonuclease inhibitors (Kure et al., 1991; Samples and Dubinsky, 1993; Mitchell et al., 1994). Others have commented that there is likely to be more than one trigger that activates a given intracellular cell death pathway (Vaux, 1993) and that it is sometimes difficult to make the "traditionally clear distinction between necrotic and apoptotic forms of cell death" (Rubin et al., 1994).

The protective effects of NT-4/5 and BDNF

NT-4/5 or BDNF treatment dramatically reduced the rate of RGC pyknosis measured 6 and 24 hr PL. These neurotrophins thus protected neonatal RGCs during both the excitotoxic and protein synthesis–dependent phases of cell death. Normal RGC density measured 24 hr PL was also much higher (Fig. 4), confirming that survival was enhanced throughout most of this period. Consistent with these observations, both BDNF and NT-4/5 enhance RGC survival in vitro (Johnson et al., 1986; Thanos et al., 1989; Cohen et al., 1994) and BDNF has been shown to delay RGC death in adult rats after optic nerve section (Mey and Thanos, 1993; Mansour-Robaey et al., 1994) or after ischemia (Unoki and LaVail, 1994).

Neurotrophins can protect neurons after various types of acute traumatic insult. For example, BDNF enhances neuronal survival following glutamate-induced neurotoxicity (Lindholm et al., 1993; Shihomaha et al., 1993; Cheng and Mattson, 1994), glucose deprivation (Cheng and Mattson, 1994), and ischemia (Unoki and LaVail, 1994). NT-4/5 also protects cells against energy deprivation and excitotoxicity (Cheng et al., 1994). The death of neurons resulting from excessive stimulation of glutamate receptors occurs even though, under other perhaps more physiological conditions, activation of NMDA-type receptors can induce the expression of neurotrophin mRNAs in the same neuronal populations (Zafra et al., 1991; Favarone et al., 1993; Hughes et al., 1993; cf. Hahm et al., 1988). The mechanisms by which exogenously applied neurotrophins protect neurons from glutamate neurotoxicity are not entirely understood, but there is evidence that they may act by stabilizing intracellular calcium levels, by limiting the formation of free radicals and/or by regulating the expression of the glutamate receptor proteins themselves (Choi, 1992; Mattson et al., 1993; Cheng and Mattson, 1994; Lindvall et al., 1994; Mattson and Scheff, 1994). In our in vivo system, the protective effects of NT-4/5 and BDNF 6 hr PL may well have involved Ca2+ and/or free radical homeostasis, given that both L-NAM and NAC also prevented the early lesion-induced increase in RGC death.

There is now considerable evidence that one action of neurotrophins is to suppress endogenous suicide programs within neurons; loss of trophic support from a target results in the activation of a cascade of intracellular molecular events that leads to apoptotic neuronal death (Martin et al., 1988; Oppenheim et al., 1990; Scott and Davies, 1990; Edwards et al., 1991; Martin et al., 1992; Johnson and Deckworth, 1993). The efficacy of NT-
NT-4/5 was still effective in promoting RGC survival when injected that showed that the time point at which deprived neurons could be rescued by readdition of NGF was much later than for CHX after critical levels of killer proteins (thanatins) have been reached. This may be achieved by inactivation of preexisting thanatins by a posttranslational mechanism or by a posttranslational modification of an alternate life-sustaining pathway within the cell (Edwards et al., 1991; Deckworth and Johnson, 1993).

**RGC receptors for neurotrophins and cytokines**

There has been a plethora of recent reviews on the structure of neurotrophins and cytokines, the nature of their receptors, and what is currently known about the mechanisms of signal transduction (Korsching, 1993; Barbacid, 1994; Chao, 1994; Heumann, 1994; Ip and Yancopoulos, 1994; Kaplan and Stephens, 1994; Lindsay et al., 1994). All neurotrophins bind with similar affinity (but with different kinetics) to the low-affinity glycoprotein receptor p75 and high-affinity binding is mediated by the Trk family of tyrosine protein kinase receptors. The three known Trk receptors display distinct but overlapping specificities for the neurotrophins. Cytokines such as LIF andCNTF bind to a different type of receptor and utilize different signaling mechanisms, but may act synergistically with the neurotrophins (Ip and Yancopoulos, 1994). RGCs are sensitive to cytokines—we have shown that LIF reduces RGC death 24 hr PL and others have reported that CNTF has a protective effect on adult RGCs (Mey and Thanos, 1993; Cohen et al., 1994; Unoki and LaVail, 1994)—but the nature of the cytokine receptors expressed by RGCs is not yet known.

RGCs express both high- and low-affinity neurotrophin receptors. In development, RGCs and their axons transiently express high levels of the p75 receptor (Yan and Johnson, 1988; Carmignoto et al., 1991; Takahashi et al., 1993; cf. Allendoerfer et al., 1994). RGCs have also been reported to express TrkA (Ernfors et al., 1992; Zanaletto et al., 1993; Rickman and Brecha, 1995), TrkB (Ernfors et al., 1992; Jelsma et al., 1993; Takahashi et al., 1993; Allendoerfer et al., 1994; Escandon et al., 1994; Rickman and Brecha, 1995), and TrkC (Ernfors et al., 1992; Allendoerfer et al., 1994; Escandon et al., 1994) receptors. Expression of trkB is relatively high, particularly during development (Ernfors et al., 1992; Jelsma et al., 1993; Takahashi et al., 1993; Allendoerfer et al., 1994). As visual pathways mature, the full-length TrkB receptor is still found on RGCs, but the optic nerve and central target tissues contain mostly truncated TrkB transcripts (Jelsma et al., 1993; Allendoerfer et al., 1994). Taken together, it is highly likely that TrkB receptors mediated all of the protective effects of NT-4/5 and BDNF on neonatal RGCs described in the present study. It has been reported that NGF promotes RGC survival in vivo (Carmignoto et al., 1989; Siliprandi et al., 1993); however, in our hands NGF had no protective influence on the survival of tectally projecting RGCs, either 6 or 24 hr after target loss. In accord with these data, NGF has not been found to significantly enhance the viability of fetal (Johnson et al., 1986) or adult (Thanos et al., 1989; Cohen et al., 1994) RGCs in vitro.

The NT-3 data were intriguing in that, after SC ablation, the neurotrophin enhanced RGC survival during the early excitotoxic phase but had no effect on the later, protein synthesis-dependent phase of cell death. NT-3 also had no significant impact on the level of naturally occurring RGC death. This limited action of NT-3 may have been mediated by TrkC receptors, by one of the other Trk receptors, or perhaps by the low-affinity p75 receptor. The latter seems unlikely, first because NGF, which also binds to p75, had no influence on RGC death, and second because NT-3 still rescued RGCs 6 hr PL even in the presence of antibodies (192-1g) to the p75 receptor (Cui and Harvey, unpublished observations). The lack of effect of NGF also argues against the involvement of TrkA receptors, suggesting that the neuroprotective influence of NT-3 on RGC survival 6 hr PL was most likely mediated by TrkC and/or perhaps by TrkB receptors.

Certainly, the contrasting efficacy of NT-4/5 and NT-3 in preventing naturally occurring RGC death and in promoting neonatal RGC survival 24 hr PL indicates that these neurotrophins were capable of activating different intracellular pathways within the neurons. Future molecular studies should help define the signaling mechanisms through which BDNF, NT-4/5, and NT-3 exert their different protective effects on neonatal RGCs, and may also show whether such mechanisms are involved in the maintenance of RGC viability in normal and axotomized adult retinas.

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