

# Rapid Onset of Neuronal Death Induced by Blockade of Either Axoplasmic Transport or Action Potentials in Afferent Fibers during Brain Development

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**We have investigated how neurons in the optic tecta of embryonic day 16 chick embryos depend for survival on their afferents from the retina. To distinguish between activity-mediated effects and other, “trophic,” ones, we compared the effects on the tectal neurons of blocking intraocular axoplasmic transport (with colchicine) or action potentials (by means of TTX). Both interventions rapidly induced the appearance of dying (pyknotic) neurons in the tectum, with major increases in their number occurring within 13 hr post-colchicine and within 9 hr post-TTX. Following both drugs, the dying neurons were morphologically similar, and in both cases the cell death depended on protein synthesis. However, the effects of colchicine and of TTX could be dissociated, since the most superficial tectal neurons became pyknotic only in response to colchicine, and, with a sufficiently short survival time (9 hr), the deep cells of the stratum griseum centrale became pyknotic only in response to TTX. We hence argue that the survival of the tectal neurons depends on their ongoing maintenance by substances released from retinotectal axon terminals, the release being activity dependent in the case of the deep neurons but independent of activity in the case of the superficial ones.**

Neuronal death is a major phenomenon in the development of the nervous system, occurring particularly at the time when connections are being formed (Oppenheim, 1991). Although the best-established influence on neuronal survival is the receipt of trophic maintenance from the axonal periphery (Barde, 1989; Oppenheim, 1991; Thoenen, 1991), the receipt of afferents is also believed to be important since their elimination generally leads to an increase in neuronal death during the natural cell death period (Okado and Oppenheim, 1984; Clarke, 1985; Furber et al., 1987).

The means by which the afferents exert their survival-promoting effects are, however, unclear. Studies on the more general question of how afferents affect postsynaptic cells indicate that long-term effects (lasting days or weeks) are mediated either by the activity-mediated release from the nerve terminal of the

transmitter(s) (including neuromodulators) and conceivably of other co-released molecules, or else by the activity-independent release of various molecules that we shall call “trophic.” This has been most clearly shown in the case of the neuromuscular junction of adult vertebrates (e.g., Purves and Lichtman, 1985; Westgaard and Lømo, 1988; Laufer and Changeux, 1989; McMahan and Wallace, 1989), but there is evidence for a similar dual action of activity-mediated and “trophic” effects in the CNS (for reviews, see Purves, 1988; Clarke, 1991).

Most studies of the afferent control of neuronal survival fail to distinguish between activity-mediated effects and other, “trophic,” ones. However, in the avian ciliary ganglion there is evidence for both kinds of effect, since chronic blockade of ganglionic transmission during the period of naturally occurring ganglionic neuronal death causes a moderate decrease in neuronal survival (Wright, 1981; Meriney et al., 1987; Maderdrut et al., 1988), and total deafferentation causes a substantially larger decrease (Furber et al., 1987). In contrast, complete blockade of action potentials or partial blockade of axoplasmic transport in optic nerve fibers is reported to have no effect on neuron numbers in the rat lateral geniculate nucleus (Matthews et al., 1982; Riccio and Matthews, 1987).

We here address this question by evaluating the effects on the chick embryo's optic tectum of intraocular blockade of action potentials or of axoplasmic transport. Our observations were focused on the stratum griseum et fibrosum superficiale (SGFS) and on the stratum griseum centrale (SGC); the neurons in both these layers receive powerful inputs from the contralateral retina (Hunt and Brecha, 1984). In chick embryos of the age used here, occasional recordings from retinal ganglion cells have revealed spontaneous activity, albeit low (G. Rager, personal communication); the same is found in the retinas of immature mammals of an equivalent age (Maffei and Galli-Resta, 1990; Meister et al., 1991).

## Materials and Methods

Fertile White Leghorn eggs were incubated at 38°C and 60% humidity. At death, their developmental stages were estimated according to the criteria of Hamburger and Hamilton (1951).

Fifteen embryos were given a unilateral intraocular injection of colchicine (225 ng in 4.5  $\mu$ l saline) after 16.0 embryonic days of incubation (E16.0). This dose had previously been shown to block axoplasmic transport in the retinotectal axons (P. F. Blaser and P. G. H. Clarke, unpublished observations; see also Gremo and Marchisio, 1975; Clarke, 1982). The embryos were left to survive for 9 hr ( $n = 4$ ), 13 hr ( $n = 4$ ), 17 hr ( $n = 5$ ), or 20 hr ( $n = 2$ ) and were then killed with an overdose of chloral hydrate.

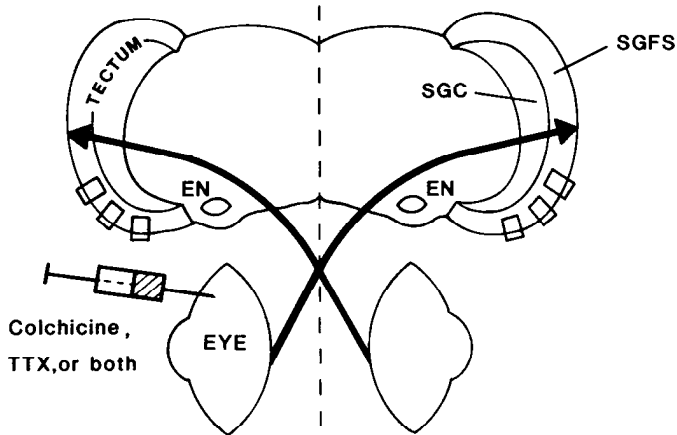
The influence of retinal electrical activity on the survival of tectal neurons was tested by injecting tetrodotoxin (TTX; 0.15  $\mu$ g in 2.5  $\mu$ l

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**Figure 1.** The experimental design showing the totally crossed retinotectal projections (arrows). Drugs were injected into one eye at E15.5 or E16.0; pyknotic cells were counted in both optic tecta after various survival times. The rectangles show the sampled areas in the SGFS. Ventral is down. EN, ectomammillary nucleus.

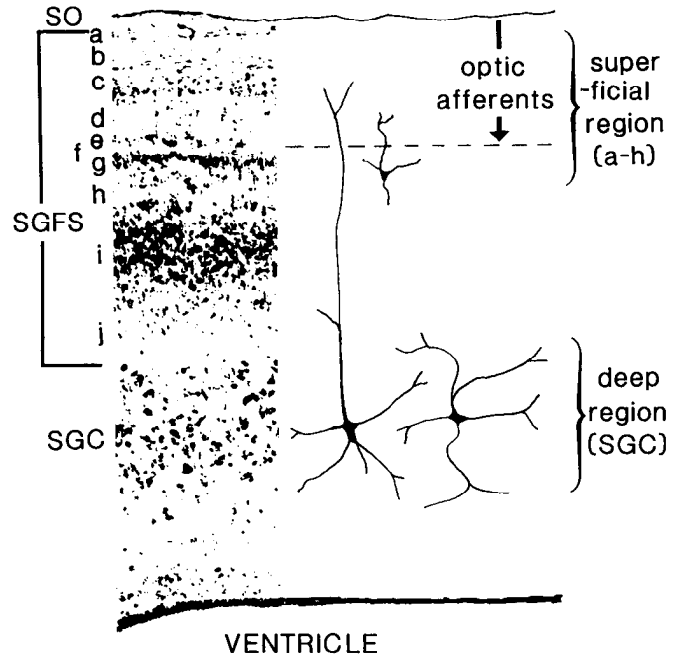
saline) into one eye of 14 embryos at E15.5 or E16.0. This dose is known to block the arrival of action potentials in the optic tectum for at least 2 d (Péquignot and Clarke, 1991). The embryos were kept in the incubator for 5 hr ( $n = 3$ ), 9 hr ( $n = 3$ ), 13 hr ( $n = 3$ ), 17 hr ( $n = 3$ ), 24 hr ( $n = 2$ ), or 30 hr ( $n = 2$ ) and then killed.

Double injections of TTX and colchicine were performed on 14 embryos: at E15.5, TTX (as above) was injected into one eye; 15 min later, colchicine (as above) was injected into the same eye. The embryos were kept in the incubator for 13 hr ( $n = 6$ ), 17 hr ( $n = 3$ ), or 20 hr ( $n = 3$ ) and then killed.

The ability of cycloheximide, a blocker of protein synthesis, to prevent the occurrence of pyknosis in the optic tectum was tested as follows. Four embryos were injected intraocularly with colchicine and another four with TTX at E16.0, as described above. Starting at 8.5 hr post-colchicine or 4 hr post-TTX, cycloheximide (100  $\mu\text{g}$  in 100  $\mu\text{l}$  saline) was pipetted onto the chorioallantoic membrane every 3 hr, and the embryos were killed 9 hr after the first administration of cycloheximide. This treatment was shown to inhibit protein synthesis profoundly in the brain, the incorporation of  $^3\text{H}$ -leucine into tetrachloroacetic acid-precipitable material being reduced by 90–95% at the time of death (M. Catsicas and P. G. H. Clarke, unpublished observations).

All brains were fixed by immersion in Carnoy's fixative and processed for paraffin embedding; 12  $\mu\text{m}$  coronal sections were cut throughout the optic tectum and stained with cresyl violet.

In each embryo, pyknotic cells (and in a few cases, healthy neurons) were counted in both optic tecta at the level of the ectomammillary nucleus, that is, halfway along the rostrocaudal extent of the tectum (Fig. 1). The zone for counting was restricted to the ventral half of each tectum, since the period of naturally occurring neuronal death ends there before the period under study (E16–E17), whereas it continues longer in the more dorsal parts (M. Catsicas, unpublished observations). Neurons were counted in a superficial region and in a deep one. In the superficial region, in each of five sections sampled at one in four, pyknotic cells were counted in three bands, 350  $\mu\text{m}$  wide, stretching from the pial surface to the border between layers 2h and 2i of the SGFS (Cowan et al., 1961) (Fig. 2), which correspond to layers 9 and 10 of Ramón y Cajal (1911). In chick embryos of the age used here, as in adult birds, all the retinotectal afferents terminate superficially to this border, being restricted to layers 2a–2f of the SGFS (Cowan et al., 1961; Crossland et al., 1975; Repérant and Angaut, 1977), and in the SGFS the cell depletion caused by induced anophthalmia is more pronounced superficially (Kelly and Cowan, 1972; Crossland et al., 1975). In the deep region, in each of three sections spaced at one in four, pyknotic cells were counted throughout the ventral half of the SGC. Many of the SGC neurons have long ascending dendrites (Hunt and Brecha, 1984); the total number of SGC neurons is reduced by about 40% following induced anophthalmia (Kelly and Cowan, 1972) (see Fig. 2). Since the retinotectal fibers are entirely crossed in chicks and in late chick embryos, the tectum ipsilateral to the injection(s) was considered to be a



**Figure 2.** Illustration of the tectal layers (left) in an E16 chick embryo (based on LaVail and Cowan, 1971), showing the two regions (superficial and deep) subjected to pyknotic counts. The drawings of neurons on the right illustrate the fact that most neurons in the superficial region have dendrites extending into the layers (a–f) receiving optic afferents, as do some, but less, of the cells in the deep region (Hardy et al., 1985).

control: the level of pyknosis there in each of the experimental situations was comparable to that in the tecta of untreated embryos of the same developmental stage (M. Catsicas, unpublished observations).

## Results

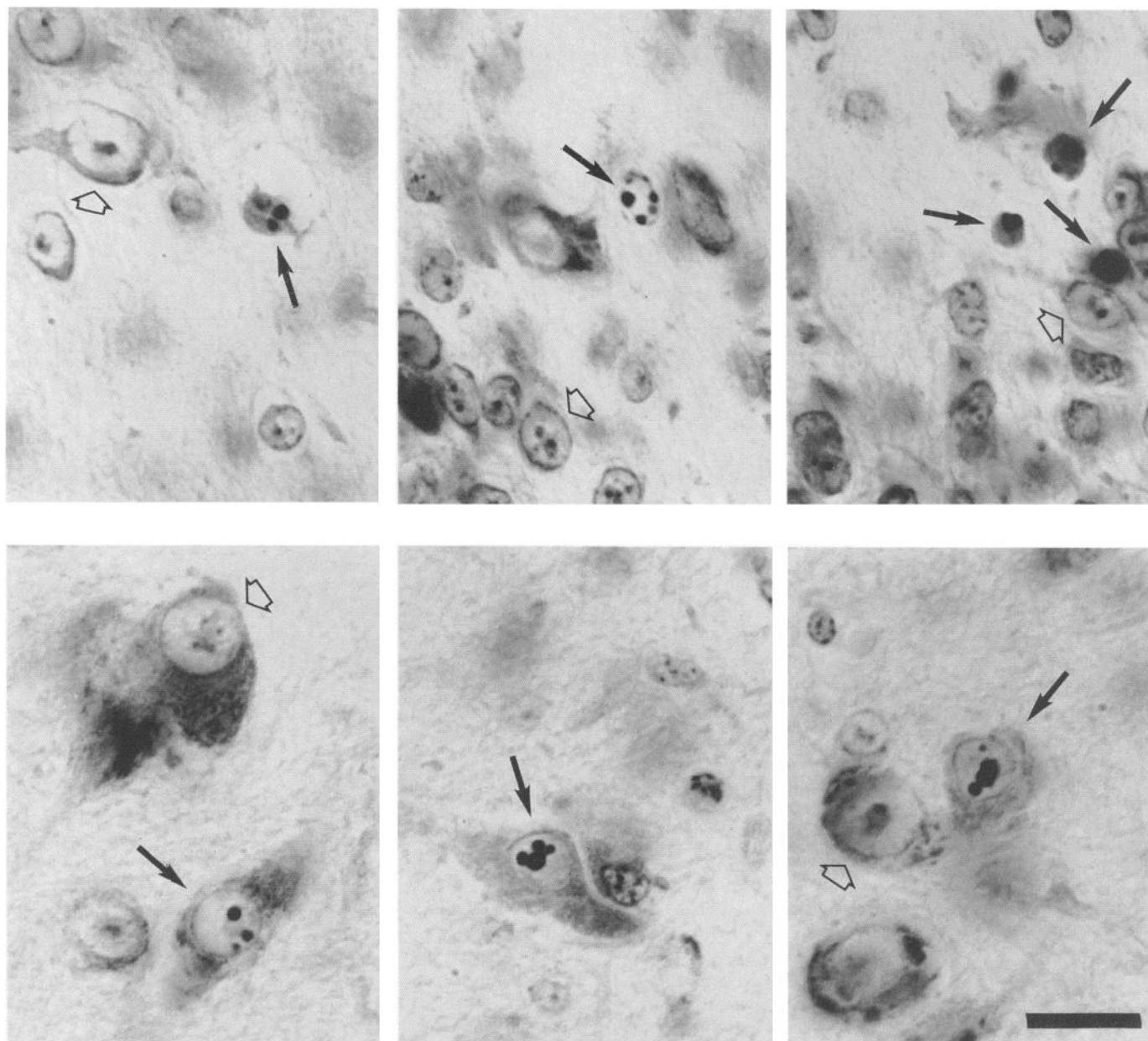
### Colchicine injections

To elucidate the dependency of the tectal neurons on trophic support from their optic afferents, we compared the numbers of pyknotic (hence, dying) cells in the tecta contralateral, and ipsilateral, to the colchicine-injected eye. Although the term “pyknotic cell” literally means “condensed cell,” in current usage it refers to a cell whose nucleus contains heavily stained material. This can take the form of one or several heavily stained balls, larger than the nucleoli, in an otherwise unstained nucleus (e.g., Hamburger, 1975), or else the whole nucleus may be darkly and uniformly stained, as well as shrunken (e.g., Sengelaub et al., 1985). In our Nissl-stained sections, both kinds of pyknotic cell occurred, and could be readily identified (Fig. 3).

Pilot experiments showed that the level of pyknosis increased greatly in the optic tectum contralateral to the colchicine injection compared to the low levels in the ipsilateral (control) tectum. We sought to determine when this difference first occurred.

**SGFS.** As early as 9 hr post-colchicine, there was a slight but significant ( $P < 0.01$ ,  $t$  test) difference between the two tecta, and at 13 hr the counts were already almost fivefold higher contralaterally ( $13.3 \pm 2.2$  per 1000  $\mu\text{m}^2$ , approximately 2.4% of the number of healthy neurons) than ipsilaterally ( $2.9 \pm 0.9$  per 1000  $\mu\text{m}^2$ , 0.5% of the healthy neurons); this difference was highly significant ( $P < 0.01$ ,  $t$  test). At 17 hr and 20 hr post-colchicine, the numbers had not substantially changed, having already reached a plateau by 13 hr (Fig. 4).

**SGC.** At 9 hr post-colchicine, there was no difference between



**Figure 3.** Photomicrographs of Nissl-stained sections showing several examples of pyknotic cells (solid arrows) in the SGFS (top row) and in the SGC (bottom row). Some healthy neurons are indicated by open arrows. Note the Nissl-stained material in the perikarya and dendrites of the pyknotic cells in the SGC, showing them to be neurons. Scale bar, 10  $\mu$ m.

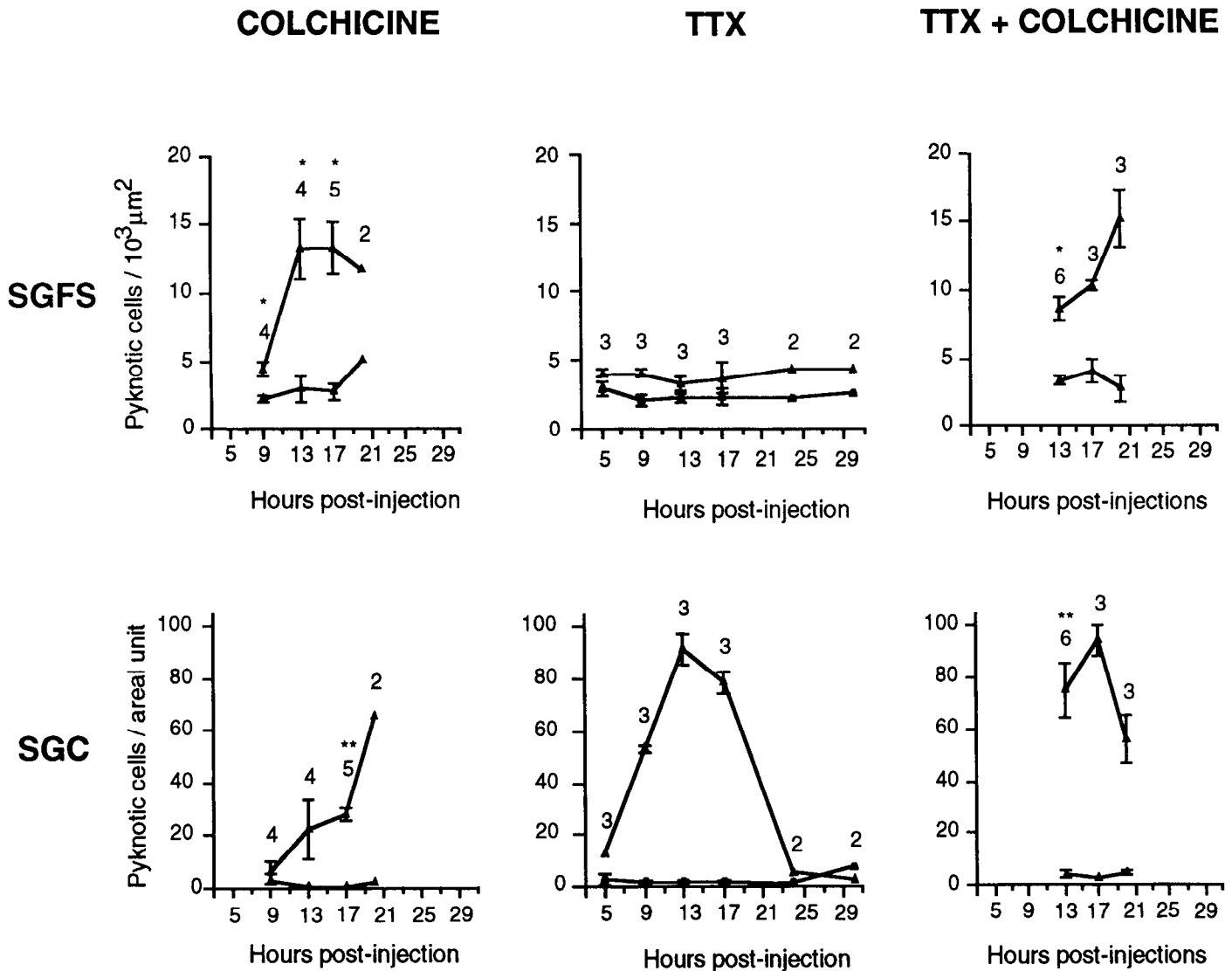
the two sides. After 13 hr, the number of pyknotic cells was much higher in the contralateral tectum ( $22.7 \pm 11.4$ ) than in the ipsilateral one ( $1.2 \pm 0.2$ ), and comparable to that after 17 hr ( $28.1 \pm 2.3$  contralaterally vs  $0.9 \pm 0.2$  ipsilaterally;  $P < 0.001$ ,  $t$  test). At 20 hr post-colchicine, the counts were even higher in the contralateral tectum ( $65.7$ , 2 embryos), suggesting that, unlike in the SGFS, the pyknosis induced in the SGC did not reach a plateau during the period tested (Fig. 4).

#### TTX injections

To evaluate the dependency of the tectal neurons on activity-mediated support from their optic afferents, we injected TTX intraocularly and quantified pyknosis in both optic tecta at various survival times (Fig. 4).

**SGFS.** At all survival times (5–30 hr), TTX had little if any effect on the level of pyknosis in the SGFS (Fig. 4).

**SGC.** In contrast to its weak effect on the SGFS, TTX had a major and very rapid effect on the SGC (Fig. 4). At 5 hr post-TTX, the number of pyknotic cells contralaterally ( $12.9 \pm 0.3$ ) was already higher than ipsilaterally ( $3.2 \pm 2.1$ ); by 13 hr post-TTX, it had reached a peak value ( $91.7 \pm 6.1$  contralaterally compared to  $2.0 \pm 0.5$  ipsilaterally) corresponding to approximately 8.3% with respect to the healthy neurons. However, by 24 hr post-TTX, the level of pyknosis in the contralateral optic tectum had fallen to control levels. Comparison of the numbers of healthy neurons between the two tecta 24 hr or 30 hr post-TTX revealed in each case a loss of approximately 30% of the neurons in the contralateral SGC (data not shown).



**Figure 4.** Number of pyknotic (dying) cells at different survival times after intraocular injections of colchicine, TTX, or both, in the superficial layers (SGFS) and the deep layer (SGC) of the optic tectum. Pyknosis was quantified in the tectum contralateral (*upper curves*) and ipsilateral (*lower curves*) to the injection(s). Error bars represent SEs. Sample sizes are indicated for each time point. Statistical analyses were performed for sample sizes of four or more. \*,  $P < 0.01$ ; \*\*,  $P < 0.001$ ; *t* test. Comparisons are between contralateral and ipsilateral sides.

#### Double injections of TTX and colchicine

In view of the unprecedentedly early induction of pyknosis following the colchicine injections, we wondered whether this might be due to a colchicine-induced depression of the activity of the ganglion cells or to inactivation of their tectal synapses through the latter being deprived of nutrients (Perišić and Cuénod, 1972), or else, whether the colchicine excites the retinal ganglion cells, or their fibers, having thus an excitotoxic effect on the tectal neurons (Dasheiff and Ramirez, 1985). The answer to the first of these questions was already available from the above experiments involving a single injection of TTX (see Discussion). To answer the second question, we tested whether TTX would counteract the colchicine; we injected first TTX, then, 15 min later, colchicine, into the same eye and quantified pyknosis in the optic tectum (Fig. 4).

**SGFS.** Although at 13 hr postinjection the number of pyknotic cells in the contralateral tectum was somewhat lower ( $8.6 \pm 0.9$ ) than after colchicine alone ( $13.3 \pm 2.2$ ;  $P = 0.054$ , *t* test),

the overall effect of colchicine did not seem to be clearly modified by the concomitant injection of TTX, since after 17 hr there was no clear difference between the contralateral tectum in the two experiments, and since, at 20 hr postinjection, the number of pyknotic cells was even higher ( $15.3 \pm 2.1$ ) than after colchicine alone ( $11.8$ , 2 embryos) (Fig. 4). Taken together with the TTX-alone data, this suggests to us that the effect of intraocular colchicine on the SGFS is largely activity independent (see Discussion).

**SGC.** The level of pyknosis in the contralateral tectum was not clearly different from that after TTX alone, at any of the survival times tested (Fig. 4).

#### Systemic injections of cycloheximide

To investigate whether the pyknosis of the tectal neurons observed in the previous experiments was dependent on protein synthesis (see Discussion), we injected cycloheximide systemically after an intraocular injection of either colchicine or TTX (Fig. 5). Repeated doses of cycloheximide given from 8.5 hr

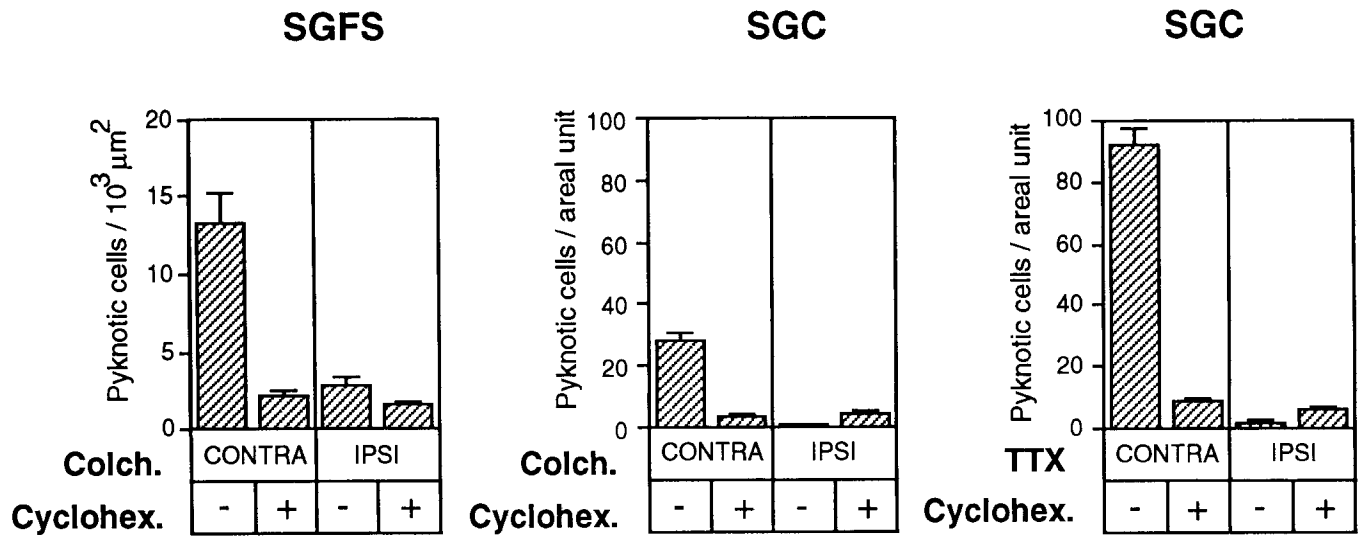


Figure 5. Effects of cycloheximide (*Cyclohex.*) on pyknosis induced by intraocular colchicine (*Colch.*) in SGFS and in SGC, or by TTX in SGC, contralateral (*CONTRA*) or ipsilateral (*IPSI*) to the injection. Each histogram is based on eight embryos (four with cycloheximide and four without). Error bars represent SEs.

post-colchicine onward reduced the level of pyknosis in the contralateral tectum more than sixfold ( $P < 0.01$ ,  $t$  test) in the SGFS, and approximately eightfold ( $P < 0.01$ ,  $t$  test) in the SGC. Cycloheximide administered from 4 hr post-TTX onward reduced the level of pyknosis by approximately 10-fold ( $P < 0.01$ ,  $t$  test) in the SGC of the contralateral tectum (Fig. 5). We did not count the pyknotic cells in the SGFS since TTX had so little effect there.

## Discussion

Our principal claim is that the blockade of electrical activity or of axoplasmic transport in optic axons induces significant numbers of neurons to die in the optic tectum where the axons terminate, within the surprisingly short time spans of 9 or 13 hr, respectively (Fig. 4). Before this can be accepted, we need to resolve three problems of interpretation.

### Three problems of interpretation

(1) *Evaluation of diffusion of the injected agents.* Although substances (including TTX; Péquignot and Clarke, 1991) injected intraocularly in chick embryos are believed ultimately to diffuse systemically, reaching the brain parenchyme, the present effects cannot be accounted for by such diffusion since they were unilateral. Directed diffusion of TTX or colchicine along the optic nerve and tract to the contralateral optic tectum can likewise be discounted for the following four reasons. (1) In 15-d-old rabbits, it has been shown that intraocularly injected <sup>3</sup>H-colchicine diffuses no farther than the part of the optic nerve closest to the eye (Matthieu et al., 1981). (2) In chick embryos, if significant amounts of colchicine diffused as far as the chiasm, this should block anterograde transport in both retinotectal pathways and retrograde transport in both isthmo-optic pathways, and should provoke the degeneration of both isthmo-optic nuclei; none of these predictions is fulfilled even when the injected dose is more than 10 times greater than in the present experiments (P. G. H. Clarke, unpublished observations; see also Clarke, 1982; S. Catsicas and Clarke, 1987). (3) It is known that intraocularly injected tracer substances do not thus diffuse in

chick embryos: HRP does not diffuse from the eye along the optic nerve to the chiasm (Clarke and Cowan, 1976), and intraocularly injected tritiated amino acids do not diffuse to the tectum, since they fail to label glia among the optic axons in the optic tract and in the stratum opticum of the tectum (P. G. H. Clarke and P. F. Blaser, unpublished observations), and can lead (through incorporation in proteins and anterograde transport) to spatially specific, low background labeling in the tectum (Crossland et al., 1974). (4) Intraocularly injected TTX, in the present dose and in the same age of chick embryo, does not detectably affect slow potentials evoked in the ipsilateral tectum following electrical stimulation of the contralateral optic nervehead (Péquignot and Clarke, 1991).

(2) *Multistage retrograde effects?* The intraocular injections will have affected not only retinofugal fibers, but also retinopetal ones originating from neurons in and around the contralateral isthmo-optic nucleus. These are in turn innervated by an uncrossed projection from tectal neurons at the border between layers 2h and 2i of the SGFS, which is at the lower limit of the region subjected to counts. However, our results cannot have been contaminated by multistage retrograde effects via the isthmo-optic nucleus, because these would occur too late. Intraocular injections of colchicine or TTX in chick embryos take at least 24 hr to produce noticeable effects in the isthmo-optic nucleus (Péquignot and Clarke, 1991; Blaser and Clarke, 1992), which appeared normal in all of the present embryos.

(3) *Validity of pyknotic cell counts.* Our use of counts of pyknotic cells might be criticized on four grounds. First, not all dying cells exhibit pyknosis (Clarke, 1990), so we might miss dying cells. This is true, but hardly invalidates the present *positive* findings. Second, it might be suggested that the pyknotic cells were glia, rather than neurons. Their neuronal identity was, however, supported by their containing substantial Nissl material occasionally in the SGFS and frequently in the SGC (Fig. 3, bottom row, middle and left panels), and in the latter the size and shape of the pyknotic cells confirmed that they were neurons (Fig. 3, bottom row). Third, in the absence of data concerning the time required for clearing away dead cells, pyknotic counts give only a very rough idea of the total numbers of cells that

die. Nevertheless, our counts of healthy neurons in the SGC indicate that 30–35% of its neurons died within 24 hr of TTX injection, and studies on many neural systems report that percentages of pyknotic cells similar to those provoked by colchicine in the present study (2–3%) indicate a substantial rate of neuronal loss, for example, 30–60% of the neurons in a few days (e.g., Oppenheim and Chu-Wang, 1983). Our purpose, moreover, was not to evaluate the extent of cell death, but to demonstrate that it occurred and to determine its time of onset. Fourth, experimental procedures may increase the clearance time of naturally dying cells, giving the false impression that cell death has increased, but this can hardly have affected our results, because the level of naturally occurring pyknosis was essentially zero. Since pyknotic counts are a measure, albeit crude, of the *rate* of cell death, they are a sensitive indicator of its onset; time derivatives are sharper than their integrals. The alternative method of counting all the neurons would not yield onset times unless one interpolated from counts at different postinjection survival times—a cumbersome procedure.

#### *Rapid initiation of pyknosis*

Although it is well known that deafferentation causes developing neurons to die, there was little previous evidence to suggest that the induction of cell death would be rapid. In most cases, the earliest effects of deafferentation have simply not been investigated (for review, see Oppenheim, 1991), or an arbitrary delay has been introduced by eliminating the afferents several days before their time of synapse formation (e.g., Bondy et al., 1978; Okado and Oppenheim, 1984; Clarke, 1985). The minority of experiments that did address the timing mostly indicated that cell death would occur several days after deafferentation (for review, see Cowan, 1970). The most rapid induction of cell death by deafferentation occurred not in development but in *adult* rats, where destruction of the olfactory bulb provoked the transneuronal degeneration of olfactory cortical neurons beginning 12–16 hr later (Heimer and Kalil, 1978). In development, the most rapid such effect was found by Ostrach and Mathers (1979) in an experimental situation strikingly close to our own. They observed that destruction of the retina in E14–E18 chick embryos led to fiber degeneration in certain secondary visual centers within as little as 24 hr. Arguing that the secondary centers in question received projections from the SGC, they attributed the fiber degeneration to the early death of afferent-deprived neurons in SGC, but did not attempt to observe directly the occurrence of dying neurons in the tectum. Our present observations go beyond those of Ostrach and Mathers (1979) in that we document the cell death directly, showing it to be induced even earlier (within 9 or 13 hr) and in more retinal layers (SGFS as well as SGC). Moreover, we have been able to dissociate the survival-promoting effect of electrical activity from other trophic effects, showing that the blockade of *either* induces cell death rapidly in the tectum.

#### *Activity-independent survival promotion in the SGFS*

The fact that our colchicine injections led to considerable pyknosis in the SGFS whereas our TTX injections had little effect there suggests that the survival-promoting influence of the optic axons is largely independent of electrical activity. This is surprising in view of the very early onset of pyknosis following the colchicine injections, so it is essential to consider all possible ways in which the colchicine might have modified activity.

A *decreased* stimulation of the tectal cells may have occurred if the colchicine depressed synaptic transmission in retinotectal

synapses (Perišić and Cuénod, 1972) or reduced the spontaneous activity of the retinal ganglion cells. This cannot easily explain the present results given that the TTX injections induced little if any pyknosis in the SGFS.

Alternatively, the colchicine may have *increased* the activity of retinal ganglion cells, perhaps greatly, since colchicine is known sometimes to induce epileptiform activity and seizures when injected into certain brain regions of various mammals (Dasheiff and Ramirez, 1985). But again, this cannot explain the effectiveness of the colchicine, since the latter injected in the presence of TTX was still effective in inducing pyknosis in the SGFS (although, admittedly, the effects at 13 and 17 hr post-colchicine seem to have been slightly reduced).

We conclude that the survival-promoting influence of the optic axons on the SGFS is indeed largely activity independent.

#### *Survival-promoting effects of electrical activity in the SGC*

In contrast to the SGFS, the SGC exhibited considerable pyknosis following the TTX injections, suggesting that here survival is supported by electrical activity in optic axons. However, other interpretations must be considered. The binding of TTX to (most) voltage-dependent sodium channels is exquisitely specific, and there is little doubt that the only initial effect of TTX is the blockade of action potentials combined with little or no changes in membrane resting potential (for discussion in the context of the chick retina, see Péquignot and Clarke, 1992). However, as a result of this specific electrical effect, intraocular injections of TTX lead, secondarily, to a reduction in the incorporation of sugars into glycoproteins in retinal ganglion cells, causing diminished anterograde transport of glycoproteins in the optic axons, although the transport of nonglycosylated proteins is unaffected (Edwards and Grafstein, 1984; Riccio and Matthews, 1985).

However, we can be certain that the effect of our TTX injections on the SGC was not mediated by such a secondary modulation of anterograde transport, because TTX provoked considerable pyknosis as little as 9 hr postinjection, when the effect on the SGC of a colchicine injection was still minimal. Moreover, at 13 hr or 17 hr survival times, the pyknosis occurring in the SGC after an injection of TTX alone or in combination with colchicine was much more abundant than after an injection of colchicine alone (Fig. 4). We conclude that the activity-dependent release, from the optic axons, of transmitters or of other co-released molecules, promotes (directly or indirectly) neuronal survival in the SGC.

Whether the slower effects on the SGC of the colchicine injections were due to an activity-*independent* mechanism is uncertain, since intraocular TTX (injected alone or with colchicine) affected the SGC and did not therefore provide an adequate control for the hypothetical effects of colchicine-induced activity.

#### *Specificity of the effects of TTX*

Why the TTX-induced pyknosis should be specific to the SGC is unclear. The invulnerability of the SGFS neurons might be attributed to their being excited by nonoptic afferents, but this fails to explain why the SGC neurons are not similarly protected; the proportion of brain-derived afferents as opposed to optic ones is probably higher to the SGC than to the SGFS (Hunt and Brecha, 1984). Alternatively, the invulnerability of the SGFS neurons might be attributed to their electrical properties being less mature, since they are generated about 2 d later than the neurons in the SGC (LaVail and Cowan, 1971). However, elec-



trical stimulation of the optic nerve evokes responses from superficial tectal neurons as early as E12, and at E16 these seem to be at least as responsive as the ones in the deeper layers (Rager, 1976).

The fact that the TTX-induced pyknosis in the SGC fell to control levels by 24 hr post-TTX, and that the reduction in total numbers of SGC neurons remained at about 30% between 24 and 30 hr post-TTX, suggests that only a subpopulation of the SGC neurons was vulnerable to TTX and that all its members were eliminated within the first 24 hr. Why there should be such specificity among the SGC neurons is unknown, but it should be remembered that only a subpopulation of the SGC neurons is excited monosynaptically by optic nerve fibers (Hardy et al., 1985). Although the survival-promoting activity of optic nerve action potentials may be mediated in part polysynaptically, it seems plausible that the monosynaptically activated neurons might be the most vulnerable to abrupt elimination of their optic activation.

#### *Prevention of pyknosis by cycloheximide: implications*

Following injections of either TTX or colchicine, the numbers of pyknotic cells were reduced by the subsequent systemic administration of cycloheximide, indicating that in both cases the processes leading to pyknosis were active, depending on protein synthesis (Fig. 5). It seems probable that the cycloheximide prevented cell death, or delayed it for many hours, although we cannot rule out the possibility that it may have switched the cell death to another, non-pyknotic kind. Dependence on protein synthesis has been found in several instances of neuronal death (e.g., Martin et al., 1988; Oppenheim et al., 1990) and in many cases of non-neuronal cell death (Tata, 1966), and has been reported in at least one previous case of neuronal death provoked by deafferentation, in the olfactory system of adult rats (López-Mascaraque and Price, 1990).

However, certain kinds of cell death do not depend on protein synthesis (Lockshin and Beaulaton, 1974), and this distinction is of interest in the present context since it helps us to rule out two hypotheses concerning the activity-mediated neuronal death in the SGC.

Superficially, this resembles a well-studied case of neuronal death—that which occurs in the second-order auditory nucleus “magnocellularis” of chicks following ipsilateral cochlear removal. This operation causes an immediate cessation of action potentials in the afferents to nucleus magnocellularis, and all the observed consequences in the magnocellularis neurons can be reproduced by blocking conduction in the afferent fibers. The ultimate consequence is the death of some 25% of the neurons, a few days after cochlear removal, and the shrinkage of the others. Those destined to die exhibit an early and complete cessation of protein synthesis, owing to the disruption of ribosomes, the latter being detectable as little as 1.5 hr after cochlear removal (Born and Rubel, 1988; Hyson and Rubel, 1989; Rubel et al., 1991). Despite the similarities with our SGC cell death—notably the dependence on activity, and the rapid effect on about 25% of the neurons coupled with the invulnerability of the others—there are two crucial differences: the SGC cell death involves pyknosis whereas the nucleus magnocellularis cell death does not, and the SGC cell death is prevented by cycloheximide whereas the nucleus magnocellularis cell death seems to be *accompanied* by a loss of protein synthesis (Rubel et al., 1991) and is not prevented by cycloheximide (Garden et al., 1991).

The cycloheximide blockade of cell death in the SGC also contradicts another possible interpretation. It might be argued that this cell death resulted from reduced excitation of inhibitory neurons in the SGC, leading to disinhibition of other SGC neurons and hence their death by acute excitotoxic mechanisms, but this kind of cell death is too rapid to depend on *de novo* protein synthesis and, in the retina at least, has been shown not to be blocked by cycloheximide (M. Catsicas, unpublished observations), so this interpretation would seem implausible.

#### *Survival-promoting molecules*

In the light of the above discussion, the most straightforward interpretation of our results is that the retinotectal axon terminals normally liberate molecules promoting the survival of the tectal neurons.

In the SGC, where action potentials promote neuronal survival, this is probably mediated by the transmitter(s), or perhaps by other co-released molecules. As mentioned above, our results do not rule out the possibility that activity-independent release may also play a role in the SGC.

In the upper SGFS, where the survival promotion is almost entirely independent of action potentials, the effect is probably mediated by an anterograde trophic molecule—carried presumably by the fast phase of axoplasmic transport, in view of the rapidity of the effect. It will be interesting to determine what kind of molecule is involved. Our preliminary experiments indicate that the almost total blockade of retinal protein synthesis at E15 leads to no increase in pyknosis in the tectum 25 hr, 28 hr, or 60 hr later. This suggests that the trophic molecule either is not a protein, or else is a protein that is held in the cell body for long periods before being exported for fast transport. In the retinocollicular pathway of adult rodents, a proportion of the glycoproteins that undergo fast transport has been shown to wait in the cell body for a week or more before export into the axon (Specht and Grafstein, 1977; Goodrum and Morell, 1982).

#### *General implications*

To evaluate the implications of the present observations, it will be necessary to find out whether such rapid anterograde effects are restricted to a critical period of development, as is suggested by the experiments of Ostrach and Mathers (1979), or whether they occur even in the adult (Heimer and Kalil, 1978), and the occurrence of similar phenomena needs to be investigated in other systems.

However, whatever the results of these future investigations, the possibility of rapid anterograde effects such as ours can no longer be ignored. We would emphasize that the *transience* of the tectal pyknosis following the TTX injections may have caused it to be missed in other situations. In numerous experiments involving intraocular injections of TTX followed by the inspection of visual centers (e.g., Kuppermann and Kasamatsu, 1983; Riccio and Matthews, 1987), the animals were always allowed to survive several days, at the very least, after the injection(s).

Finally, our results with TTX emphasize the absolute need of some neurons to be stimulated every few hours. This casts new light on the importance of spontaneous activity, serves as a warning that activity-depressing drugs may in some cases kill neurons, and suggests a possible reason why sleep is punctuated every hour or two by periods of “rapid eye movement sleep” involving increased brain activity.

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